The Calcitonin Gene-related Peptide Family

Debbie L. Hay • Ian M. Dickerson
Editors

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Form, Function and Future Perspectives

Springer

Editors
Debbie L. Hay
University of Auckland
Private Bag 90219
Auckland 1142
New Zealand
dl.hay@auckland.ac.nz

Ian M. Dickerson
Department of Neurobiology and Anatomy
University of Rochester
601 Elmwood Avenue, Box 603
Rochester, NY, 14642, USA

ISBN 978-90-481-2908-9 e-ISBN 978-90-481-2909-6
DOI 10.1007/978-90-481-2909-6
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009929391

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

In 1925, J.B. Collip (1925) reported that extracts of parathyroid gland contained an activity that raised calcium levels in the blood of parathyroidectomized animals, and suggested that this was due to a hormone produced in the parathyroid gland. The story of parathyroid hormone discovery was indicative of ever-increasing sophistication in sample preparation and protein isolation techniques. This paper resolved earlier controversies over the function of the parathyroid glands and control of blood calcium. The year 1961 was a banner year for parathyroid research, in which the peptides parathyroid hormone and calcitonin were purified, and in which it was suggested that calcitonin could lower blood calcium (Copp and Cameron 1961). In 1982 it was discovered that in neurons the primary RNA transcript for calcitonin could be alternatively-spliced to give calcitonin gene-reated peptide (CGRP), and shortly thereafter amylin (previously named islet amyloid polypeptide, IAPP) was identified and shown to have homology to CGRP. Since then α and β CGRP have been delineated and adrenomedullin and intermedin discovered, and this family of homologous peptides has emerged. This family of peptide hormones has a diverse and constantly expanding range of important physiologic functions, including regulation of blood calcium, vascular tension, feeding behavior and pain recognition. This peptide family is unique in that the five current members bind to two common G protein-coupled receptors, calcitonin receptor (CTR) and calcitonin-like receptor (CLR), with pharmacologic specificity controlled by three accessory proteins named receptor activity modifying protein (RAMP1,2,3) and signaling at AM and CGRP receptors regulated by a fourth accessory protein named CGRP-receptor component protein (RCP). Recent genetic advances developing mice lacking these individual proteins has provided surprising new information on an increasingly broad physiologic role for this peptide family in vivo.

Despite these important physiologic functions, therapeutic strategies targeting this family of peptides have been limited. This has partly been due to the difficulty identifying the multi-protein receptor complexes, and partly due to the peptide nature of these hormones and the inherent instability associated with small proteins. Recent advances identifying the receptors for this peptide family and the subsequent development of small molecule non-peptide CGRP receptor antagonists have provided promising new reagents with which the physiologic and pathophysiologic roles of this peptide family can be investigated and remedied. In November 2007

researchers from Japan, the United States, Europe, Australia and New Zealand gathered in San Diego, California for the "Sixth International Symposia on the CGRP family: CGRP, Adrenomdullin, Amylin, Intermedin and Calcitonin." This book represents some of the highlights from that meeting, and gives an indication of the possibilities for basic and translational research as we go forward.

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Contents

Chapter 1
Molecular and Functional Evolution of the Adrenomedullin Family in Vertebrates: What Do Fish Studies Tell Us?

Yoshio Takei, Maho Ogoshi, Marty K. S. Wong, and Shigenori Nobata

Abstract Adrenomedullin (AM) comprises a unique family of five paralogous peptides (AM1, 2, 3, 4 and 5) in teleost fish, of which AM1 is an ortholog of mammalian AM, and AM1/4 and AM2/3 were produced at the teleost-specific whole genome duplication. Therefore, CGRP, amylin, AM1, AM2 and AM5 existed when ray-finned fish and lobe-finned fish (leading to tetrapods) were diverged. Based on this finding, we discovered novel AM2 and AM5 in mammals. In addition, comparative genomic analyses based on fish studies delineated an evolutionary history of the CGRP family of peptides in vertebrates. As a first chapter of this volume, we initially propose an idea of how the CGRP family, including multiple AM peptides, have been organized during the course of vertebrate evolution. We will also show how comparative fish studies can contribute to general and clinical endocrinology by providing new insights into the molecule and function of the CGRP family throughout vertebrate species.

Keywords Molecular evolution • comparative genomics • body fluid regulation • cardiovascular regulation • vertebrate phylogeny • evolution from aquatic to terrestrial habitat

Y. Takei (✉) and S. Nobata
Laboratory of Physiology, Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo, 164-8639, Japan
e-mail: takei@ori.u-tokyo.ac.jp

M. Ogoshi
Department of Biological Sciences, Tokyo Institute of Technology, Kanagawa, 226-8501, Japan

M.K.S. Wong
HADEEP, Ocean Research Institute, The University of Tokyo, Tokyo, 164-8639, Japan

D.L. Hay and I.M. Dickerson (eds.), *The Calcitonin Gene-related Peptide Family: Form, Function and Future Perspectives*, DOI 10.1007/978-90-481-2909-6_1,

Abbreviations

1R	first-round whole genome duplication
2R	second-round whole genome duplication
3R	third-round whole genome duplication
AM	adrenomedullin
ANP	atrial natriuretic peptide
CGRP	calcitonin gene-related peptide
CLR	calcitonin receptor-like receptor
CRSP	calcitonin receptor-stimulating peptide
CT	calcitonin
CTR	calcitonin receptor
EST	expressed sequence tag
GFR	glomerular filtration rate
GH	growth hormone
MSH	melanocyte stimulating hormone
Myr	million years
RAMP	receptor activity-modifying protein
RCP	receptor component protein
RT-PCR	reverse-transcription polymerase chain reaction

1.1 Introduction

Vertebrates first emerged in brackish waters as a result of evolution from chordate stock (Carroll 1988), and are thought to have first entered inland fresh waters before expansion of their habitats to the sea and onto the land (Romer and Grove 1935). This evolutionary experience of low osmotic pressure environments may account for, at least in part, why most extant vertebrates, including lampreys, bony fishes and tetrapods (mammals, birds, reptiles and amphibians), have tonicity of extracellular fluids approximately one third that of seawater irrespective of present environmental conditions (Marshall and Grosell 2005). Exceptions are marine hagfish that have ion concentrations of extracellular fluids almost identical to seawater, and marine cartilaginous fish (sharks, rays and chimeras) and a marine lobe-finned bony fish (coelacanth) that accumulate urea in extracellular fluids to increase their osmolality to a seawater level. Therefore, these rather ancient species do not lose water from the body surfaces by osmosis even in the marine environment. Probably because of such ability, cartilaginous fishes and lobe-finned bony fish seem to have entered the sea in the early Devonian period of the Paleozoic era more than 450 million years (Myr) ago (Romer and Grove 1935). On the other hand, invasion into the sea of the ray-finned fish was much delayed and it was later in the Jurassic period of the Mesozoic era. They entered the sea with low plasma osmotic pressure because they acquired an ability to extrude excess ions by concentrating them above a seawater level. During the course of such expansion of habitats, vertebrates have developed characteristic mechanisms for body fluid regulation to adapt to diverse osmotic environments. It has become more and more evident that the endocrine system plays a central role in such homeostatic regulation (McCormick 2001; Bentley 2002).

1.1.1 Body Fluid Regulation in Tetrapods

As the vertebrate body contains 65% to 85% water relative to body weight, tetrapods must retain water to adapt to the desiccative terrestrial environments (Takei 2000; Bentley 2002). After abandoning life in the water, they developed mechanisms to retain water in the body. In addition, the land is generally an ion-deficient environment, so terrestrial vertebrates also have developed mechanisms to retain ions; this is especially the case in granivores and herbivores whose diet contains little Na^+ and Cl^-. Thus ion retention is as important as water retention for terrestrial animals. The major ions in the extracellular fluid are Na^+ and Cl^-, which mostly move in parallel in the transport epithelia. As these monovalent ions are of primary importance for body fluid regulation, the unspecified ions mentioned in this chapter can be read to mean Na^+ and Cl^- unless otherwise specified. Further, water is often transported in parallel with the transport of ions across the osmoregulatory epithelia, as ion transporters and aquaporin water channels are generally co-localized on the epithelial cells of terrestrial animals.

Water is lost by respiration, evaporation from the body surfaces and renal excretion, but the former two routes are hardly controllable as they are indispensable for life on the land to acquire oxygen and to regulate body temperature respectively (Bentley 2002). The major regulatory site for water and ion (both mono- and divalent) in terrestrial vertebrates is the kidney where glomerular filtration rate (GFR) and reabsorption of water and ions at renal tubules are elaborately manipulated. Tubular reabsorption is a major determinant of volume and composition of urine in terrestrial animals, which contrasts to the greater importance of GFR in aquatic fishes (Brown et al. 1993). Evolution of the function and morphology of the vertebrate kidney has attracted the attention of investigators for many years, with many reviews being published since the early seminal work of Homer Smith (1932). Extensive studies using mammalian kidney have revealed that final urine volume and concentration are determined by the recruitment of vesicular aquaporin-2 to the apical membrane of epithelial cells of the collecting duct, and that urine Na^+ concentration is also regulated principally by the Na^+,K^+-ATPase and Na^+ channels located in the distal nephron (Bentley 2002). Two hormones are known as major kidney-based, body fluid-regulating hormones in terrestrial animals: antidiuretic hormone (vasopressin/vasotocin) and the Na^+-sparing hormone aldosterone, the lack of which causes severe symptoms that make it difficult to survive on land without continuous supply of water and/or Na^+ (White 2004; Fujiwara and Bichet 2005). Other important regulators for body fluid balance are the oral intake of water and ions and subsequent absorption by the intestine (Takei 2000; Bentley 2002). In terrestrial animals, especially herbivores and granivores, almost all gains of water and ions are derived from the intestinal lumen. Accordingly, thirst and salt appetite that motivate oral intake of water and ions are major regulators for the gain in the whole-body regulation. Angiotensin II is the most potent dipsogenic hormone thus far known, and it also induces sodium appetite cooperatively with aldosterone (Kobarashi and Takei, 1996; Fitzsimons 1998). It appears that retention of both water and ions are the keys to survival of tetrapods in the terrestrial environment.

1.1.2 Body Fluid Regulation in Fish

The fish mentioned in this chapter, unless otherwise stated, will be teleost species that maintain their plasma ion concentrations lower than seawater as do tetrapods. Body fluid regulation has reverse requirements for fish living in fresh water and in seawater. In freshwater, fish are challenged by the hypervolemia that results from water influx across the gills according to the existing osmotic gradient. They also face hyponatremia that results from ion loss at the gills driven by the concentration gradient between plasma and environment. To cope with hypervolemia, fresh water fish excrete a large volume of dilute urine. However, as their kidney has limited ability for ion reabsorption by the renal tubules, significant amounts of ions are lost in urine (Brown et al. 1993). Therefore, the most important factor required for survival in hypotonic fresh water is an ability to take up Na^+ and Cl^- from the ion-deficient environment. This is achieved by active absorption at the gills energized by Na^+,K^+-ATPase and H^+-ATPase, and from food by the intestine in carnivorous species (Marshall and Grosell 2005). Prolactin has long been known as the most important fresh water-adapting hormone in fish, which acts by decreasing osmotic permeability of water at the gills and other transport epithelia. However, we expect that yet unknown hormones promote Na^+ and Cl^- uptake from fresh water media.

In contrast to fresh water fish, seawater fish must cope with hypovolemia and hypernatremia. To this end, they actively excrete excess monovalent ions by the mitochondria-rich, chloride cells of the gills via Na^+,K^+-ATPase-driven transport processes (Evans et al. 2005), and excess divalent ions by the active secretion at proximal tubules of the kidney (Beyenbach 1995). To cope with hypovolemia, they drink large volumes of seawater and absorb almost all of the ingested water by monovalent ion-coupled uptake at the intestine (Loretz 1995). Therefore, marine fish can maintain water balance by drinking surrounding seawater and extruding excess ions from the body even though they are in a dehydrating environment. The marine fish situation emphasizes the importance of ion extrusion for seawater adaptation. The human body loses water after drinking seawater as mammals have no chloride cells (or salt gland) that specifically excrete concentrated Na^+ and Cl^- and their kidney is unable to concentrate these ions to the level higher than seawater (Schimidt-Nielsen 1997). Collectively, the most important mechanism for body fluid regulation in seawater fish is ion-extrusion but not water-retention as observed in terrestrial animals. This difference appears to originate from the fact that seawater fish have easier access to water and can drink whenever necessary. An additional important difference is that water and ions are regulated in the same direction in terrestrial tetrapods but in the opposite direction in aquatic fishes. In fishes, cortisol and growth hormone (GH) are important long-acting, seawater-adapting hormones that re-organize the osmoregulatory epithelia to a seawater type (McCormick 2001). Atrial natriuretic peptide (ANP) is the most potent fast-acting hormone thus far known that promotes seawater adaptation in fish (Takei and Hirose 2002).

1.1.3 Difference in Gravitational Influence on Fish and Tetrapod

Another important difference between the aquatic and terrestrial environments is the influence of gravity. Terrestrial animals circulate blood throughout the body under the influence of gravitational forces imparted on the body. To this end, they have a powerful heart to pump relatively large volumes of blood to targets including those located above the level of the heart. Thus they usually have higher arterial pressure. This is particularly prominent in endotherms such as mammals and birds in which the heart is continuously supplied with oxygen-rich arterial blood by the coronary system to support constant hard work. By contrast, as the high specific gravity of water (relative to air) almost nullifies the gravitational force in aquatic environments, fish can circulate blood with lesser power at lower arterial pressure. Fish heart also has a coronary system but loosely packed cardiac myocytes can take up oxygen directly from intra-cardiac blood. Because of such obvious differences in cardiac performance and arterial pressure, cardiotropic and hypertensive hormones, such as angiotensin II, vasopressin and endothelin, play critical roles in terrestrial animals, while hypotensive hormones such as natriuretic peptides and CGRP peptides play more important roles in teleost fish (Takei et al. 2007).

We are comparative endocrinologists seeking new hormones essential for seawater adaptation in fish (Takei 2008). During the course of this study, we found that ion-extruding and/or vasodepressor hormones, such as natriuretic peptides (Inoue et al. 2003) and guanylins (Yuge et al. 2003), were diversified and developed a unique hormone family in teleost fish. The receptors for such hormones were also diversified in teleost fish (Takei and Hirose 2002; Yuge et al. 2006). The dominance of hormones promoting ion extrusion, rather than those promoting ion acquisition, in teleost fish may be accounted for by the fact that they have diversified and prospered after they entered the sea with the ability to extrude excess ions from the body. In fact, teleost fish are a thriving group of vertebrates, whose species number and biomass exceed the sum of other vertebrate taxa. The genes duplicated at the third-round whole genome duplication (3R) in teleost fishes may have acquired new functions in ion extrusion and maintenance of low arterial pressure and, being thus advantageous, many of them are still retained in fish. The third such example is the adrenomedullin (AM) peptides. AM was previously thought to be a member of the calcitonin gene-related peptide (CGRP) family, but it is now evident that multiple AM peptides create an independent group in the CGRP family in vertebrates. In this chapter, we introduced our recent comparative approach that provides new insights into the evolution of structure and function of the CGRP family across vertebrate species including mammals with emphasis on the AM peptides.

1.2 Identification of a New AM (Sub)Family

AM was first isolated from the pheochromocytoma cells of adrenal medullary origin (Kitamura et al. 1993). AM is a multifunctional peptide that possesses a spectrum of actions related to various aspects of homeostasis (López and Martínez, 2002). Among

the actions, inhibition of thirst and sodium appetite, stimulation of GH release, and natriuretic effect attracted our attention in relation to seawater adaptation, as these actions are similar to those of ANP, which is now known as an important hormone for seawater adaptation in teleost fish (Tsukada and Takei 2006).

1.2.1 AM Peptides in Teleost Fish

An extensive search for AM in the genome database of tiger pufferfish (*Takifugu rubripes*) resulted in identification of five AM-like peptides, which are named AM1, 2, 3, 4 and 5 because they are all shown to be paralogs by the molecular phylogenetic analysis (Ogoshi et al. 2003, 2006). Five AMs are identifiable in the database of all teleost species thus far examined including green pufferfish (*Tetraodon nigroviridus*), zebrafish (*Danio rerio*), and medaka (*Oryzias latipes*) (Takei et al. 2004a). Comparative genomic analysis showed that AM1 is an ortholog of mammalian AM (thus AM(1) is used for mammalian AM hereafter), and that AM1/AM4 and AM2/AM3 were duplicated at the 3R that occurred early in the teleost lineage ca. 350 Myr ago (Vandepoele et al. 2004). The counterpart of duplicated AM5 seems to have disappeared after the 3R. It is intriguing to note that the sequence identity of duplicate paralogs of teleost AMs differs greatly among peptide species; AM1 and AM4 have only 30–40% identity, the counterpart of AM5 may have changed into a pseudogene, but AM2 and AM3 still retain more than 80% identity after the 3R. The sequence identity of each AM within teleost species is also highly variable; 62–75% for AM1, 87–98% for AM2, 75–95% for AM3, 38–55% for AM4 and 73–81% for AM5 between different species of teleost fish. Such large variations in sequence identity that may be derived from the difference in selection pressure imply differences in their relative physiological importance in teleost fish.

Molecular phylogenetic analyses revealed that the five teleost AMs are clustered with mammalian AM independently of CGRP and amylin, supporting the kin relationship among AM peptides (Ogoshi et al. 2003). RT-PCR analyses showed that AM1 and AM4 gene transcripts are distributed ubiquitously in various tissues of pufferfish as mammalian AM, but AM2 and AM3 genes were expressed almost exclusively in the brain. We cloned AM1, AM2, AM3 and AM5 in the eel and examined the tissue distribution of their transcripts (Nobata et al. 2008). In this species, AM2 and AM3 are more widely distributed in different tissues than AM1, suggesting species difference in the expressing tissues. A cDNA coding for AM4 was not cloned in the eel because of high variability among species. The AM5 gene was expressed in hematopoietic and immune-related tissues such as spleen, head kidney (equivalent to bone marrow) and gills of teleost fish.

1.2.2 AM Peptides in Tetrapods

Comparative genomic analyses of the teleost AM family strongly suggest that AM1, AM2 and AM5 existed when lobe-finned fish that evolved to tetrapods

diverged from ray-finned fish in the bony fish lineage (Ogoshi et al. 2006). Since mammalian AM is an ortholog of teleost AM1, there is a possibility that AM2 and AM5 still exist in tetrapods including mammals. Therefore, we sought the orthologs of teleost AM2 and AM5 in the anticipated region of mammalian chromosomes using a newly developed search program and identified AM2 in the human, rat and mouse (Takei et al. 2004b) (Fig. 1.1). AM2 was also discovered by Hsu and his colleagues and named intermedin (Roh et al. 2004). Accordingly, the new peptide should be designated as AM2/intermedin to avoid confusion (Takei 2006), although the name 'intermedin' was used previously for melanophore-stimulating hormone (MSH) (Johnsson and Hoegberg 1952). In this chapter, we use the name AM2 to emphasize its origin (see below). AM2 exists in all mammalian species thus far examined (Fig. 1.1).

Fig. 1.1 Adrenomedullin 2 and 5 mature sequences thus far known in mammals. Amino acid residues of each peptide that are conserved in more than half of the species are *shaded*. *A single nucleotide insertion (hedgehog) or two nucleotide deletion (human and chimp) occurs at the amino acid residues surrounded by a *square*, but the sequence except for the mutation is still highly conserved. *Bracket* shows disulfide bond

We also identified an AM-like sequence in the genome database of mammals and amphibians, and determined it as an ortholog of AM5 by synteny analyses (Ogoshi et al. 2006). We confirmed that the new AM5 gene is expressed as mRNA and the synthetic mature peptide exhibits cardiovascular actions in mammals (Takei et al. 2008). The AM5 gene was identified in primates (tupai, rhesus monkey, chimpanzee, and human), carnivores (dog and cat), and ungulates (pig, ox, sheep and horse), but not in rodents (rat and mouse) (Fig. 1.1). In primates, a two-nucleotide deletion occurred in the coding region of the AM5 gene in the human and chimp, so that its mRNA registered in the EST database may be translated as a different protein. Since the nucleotide deletion is absent in the rhesus monkey, the deletion should have occurred after divergence of Old World monkeys and anthropoids in the primate lineage ca. 23 Myr ago (Glazko and Nei 2003). It will be interesting to examine for the presence of the AM5 gene in other anthropoid species such as orangutan and gorilla. Based on these results, it is now generally accepted that three types of AM peptides, AM(1), AM2 and AM5, form an independent (sub)family in the CGRP family in mammals as in teleost fishes (Fig. 1.2). In other tetrapods, AM(1) has been identified in birds and amphibians, AM2 in amphibians, and AM5 in reptiles and amphibians.

1.3 Evolutionary History of the CGRP Family

Until recently, it was thought that the CGRP family consisted of two CGRPs, AM, AM2/intermedin, amylin and calcitonin (CT) receptor-stimulating peptide (CRSP) (Muff et al. 2004). However, we now know that an additional AM peptide, AM5, is a CGRP family member. The next step is to determine how the CGRP family members have arisen and have been added and deleted during vertebrate evolution, and what is the ancestral molecule of the CGRP family. It is apparent that CGRP, AM1, AM2, AM5 and amylin existed when ray-finned fish and lobe-finned fish diverged 450–500 Myr ago (Ogoshi et al. 2006). Although we have no information at present on the CGRP family of molecules in the lobe-finned fishes (lungfish and coelacanth, as living representatives of ancient groups near to those that evolved to tetrapods), we can trace the evolutionary history of diversification of family members in ray-finned fish and tetrapods using comparative genomic analyses of currently available genome data.

1.3.1 Diversification of CGRP Peptides in Mammals

Two CGRP genes (α-CGRP and β-CGRP) and a single AM(1), AM2 and amylin gene have been identified in primates (human and chimp) and rodents (rat and mouse), while the AM5 gene has been mutated to a different gene (anthropoids) or might be silenced (rodents) as mentioned above. In more ancient primates (tupai and rhesus monkey), the AM5 gene appears to be functional, and is transcribed and translated to AM5 protein. The two CGRP genes are produced by tandem duplication

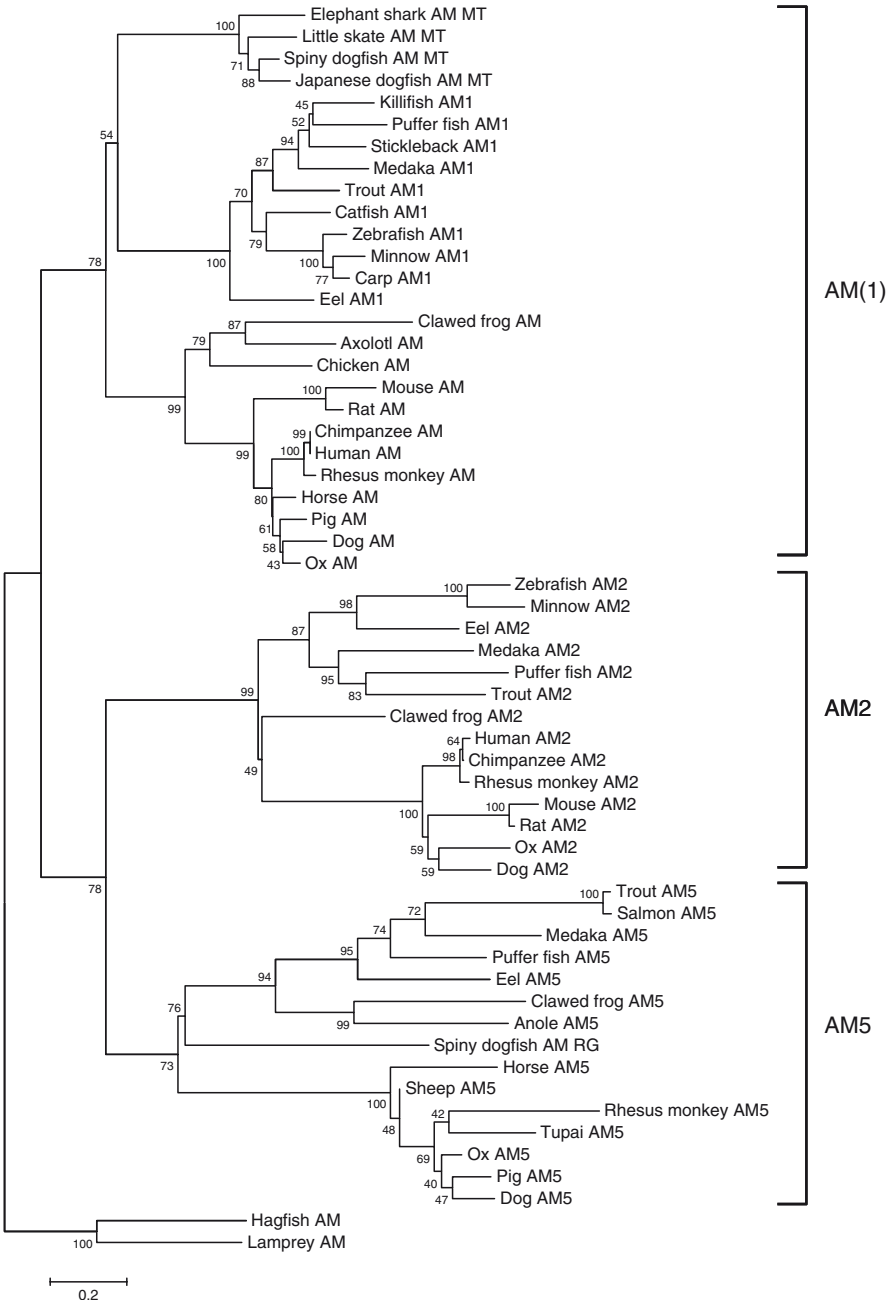

Fig. 1.2 Molecular phylogenetic analysis of adrenomedullin (AM) peptide precursors (including signal peptides) thus far identified using a neighbor-joining method. Teleost AM3 and AM4 duplicated from AM2 and AM1, respectively, at the third round of whole genome duplication (see Fig. 1.3) are not included in the analysis. Hagfish and lamprey are used as outgroup. MT, multiple tissue type; RG, rectal gland type. *Numbers* at each node are bootstrap values

as they exist in close proximity on the same chromosome (Amara et al. 1985). CT mRNA is produced from the α-CGRP gene by alterative splicing but not from the β-CGRP gene. Therefore, only one CT is present in the primates and rodents. In ungulates and carnivores, a single CGRP, AM(1), AM2, AM5 and amylin gene exist in all species thus far examined. In addition, multiple CRSP genes are also identified in these species (Katafuchi et al. 2003). Recent genomic analyses showed that the CRSP genes are products of tandem duplication of the CGRP gene as they are localized in the vicinity of the CGRP gene on the same chromosome in the pig, horse and dog (Ogoshi et al. 2006; Rezaeian et al. 2008; Osaki et al. 2008). Further, some of the CRSP genes can produce a second CT by alternative splicing in the dog (Osaki et al. 2008). Therefore, the CGRP gene is multiplied frequently by tandem duplication and the duplicated genes are still retained in mammals, probably because they are assigned to a new function. In fact, CRSP peptides have high affinity to CT receptor alone without forming complex with receptor activity-modifying protein (RAMP) (Katafuchi et al. 2003; see below).

1.3.2 Diversification of CGRP Peptides in Teleost Fish

Two CGRP genes, duplicated AM1/AM4 and AM2/AM3 genes, and a single AM5 and amylin gene have been identified in teleost fish (Ogoshi et al. 2006). The two CGRP genes are the product of 3R and thus named CGRP1 and CGRP2 to distinguish from mammalian α-CGRP and β-CGRP that are generated by tandem duplication. Two different CTs are transcribed from the teleost CGRP genes by alternative splicing. Teleost CTs have much greater hypocalcemic activity than homologous CT in mammals, partly because of their longer half-life in mammalian blood (Hirsch and Baruch 2002). Similar to the AM5 gene, one of the amylin genes duplicated at the 3R is not detectable at the expected region of the chromosome in medaka and pufferfish, probably because the duplicated counterpart is subjected to change into a pseudogene.

Recently, the process of re-organization of teleost chromosomes after the 3R has been suggested based on the study of the green pufferfish (Jaillon et al. 2004) and medaka (Kasahara et al. 2007) genomes. According to the medaka analysis, there were 13 proto-chromosomes before 3R. After duplication, some of the chromosomes are fused or further separated, and finally re-organized into 24 chromosomes. We showed that the AM1 and CGRP genes are localized close to each other on proto-chromosome E, the AM2 and amylin genes on proto-chromosome F, and AM5 on an unidentified but different proto-chromosome (Fig. 1.3). Judging from the intimate relationship between AM1/CGRP and AM2/amylin on the same chromosome, these two coupled genes were likely to have been generated at the second-round whole genome duplication (2R) that is thought to have occurred at the transition from the jawless agnathans to jawed gnathostomes ca. 550–600 Myr ago (Vandepoele et al. 2004; Yamanoue et al. 2006) or by a block duplication independent of the whole

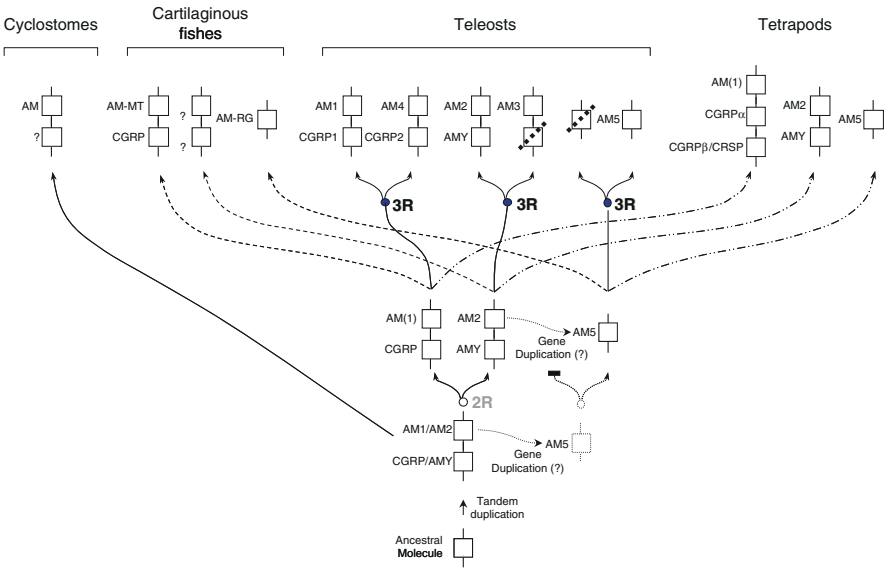

Fig. 1.3 A hypothetical schema depicting a process of diversification of the CGRP family peptides during vertebrate evolution. *Dotted line* and ? show 'still unknown' or 'still undetermined'. AM, adrenomedullin; AMY, amylin; CGRP, calcitonin gene-related peptide; CRSP, calcitonin receptor-stimulating peptide, MT, multiple tissue type; RG, rectal gland type; 2R and 3R, second- and third-round whole genome duplication

Human CGRP	ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF–NH2
Takifugu CGRP	ACNTATCVTHRLADFLSRSGGMGNSNFVPTNVGAKAF–NH2
Human Amylin	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY–NH2
Takifugu Amylin	KCNTATCVTQRLADFLVRSSNTIGTVYAPTNVGSTTY–NH2
Human AM(1)	FGCRFGTCTVQKLAHQIYQF–TDKDKDNVAPRSKISPQGY–NH2
Takifugu AM1	NGCSLGTCTVHDLAFRLHQL–GFQYKIDIAPVDKISPQGY–NH2
Human AM2	VGCVLGTCQVQNLSHRLWQLMGPAGRQDSAPVDPSSPHSY–NH2
Takifugu AM2	VACVLGTCQVQNLSHRLYQLIGQSGKEDSSPMNPHSPHSY–NH2

Fig. 1.4 Amino acid sequence comparison of CGRP family peptides of human and tiger pufferfish (*Takifugu*) at the mature portion. Sequences are highly conserved between CGRP and amylin or between AM1 and AM2. *Bracket* shows disulfide bond. Amino acid residues conserved in more than half of the peptides in each group are *shaded*

genome event. The counterparts of duplicated genes appear to be AM1/AM2 and CGRP/amylin as inferred by the molecular phylogenetic analysis, particularly by the similarity of the mature sequence (Fig. 1.4). The AM5 gene was apparently produced from the AM2 gene by an autonomous gene duplication. Furthermore, the AM1 and CGRP gene or the AM2 and amylin gene were generated by tandem duplication that

should have occurred quite a long time ago. In this connection, it needs to be determined which member of the CGRP family is most akin to an ancestral molecule of the CGRP family. This can be assessed by identifying the CGRP family molecules in the phylogenetically more ancient species, such as cartilaginous fish and jawless cyclostomes (hagfish and lamprey). The extant species of such ancient groups have undergone their own evolution after divergence long ago in the Paleozoic era, but it is possible that they still retain the ancient nature of the molecule.

1.3.3 Ancient Molecule of the CGRP Family

We identified two AM-like molecules in cartilaginous fishes (elasmobranchs and holocephalans) (Wong and Takei, 2009). Molecular phylogenetic analyses showed that one of the AM genes that is expressed ubiquitously in almost all tissues examined, named multiple tissue type (AM-MT), is clustered with the AM1 gene of other species, while the other gene that is expressed in the rectal gland (salt gland of marine cartilaginous fish), named AM-RG, is clustered with the AM2/AM5 gene, being closer to the AM5 gene of other species (Fig. 1.2). Interestingly, the inferred mature sequences of both AM-MT and AM-RG exhibited higher similarities to that of AM2, although phylogenetic analysis of precursors grouped elasmobranch AM-MT and AM-RG with AM1 and AM5 (or AM2) of other species, respectively. Therefore, duplication of the AM1 and AM2 genes may have occurred before the divergence of bony fish and cartilaginous fish. We could mine the CGRP sequence from the genome database of elephantfish that belongs to another group of cartilaginous fishes (holocephalan, unpublished data), so that tandem duplication of AM1 and CGRP, and probably of AM2 and amylin also, may have occurred before the divergence of cartilaginous fish lineage (Fig. 1.3). It remains to be determined whether the AM-RG gene is an ortholog of the AM2 gene or the AM5 gene. If the amylin gene is identified in cartilaginous fish, this issue may be addressed by the chromosomal localization with the AM-RG gene. It is also important to determine whether cartilaginous fish possess the AM5 gene or not in order to assess the time of its production (before or after the divergence of cartilaginous fish and bony fish).

We also identified an AM-like sequence in cyclostomes, the hagfish, *Eptatretus burgeri*, and lamprey, *Peteromyzon marinus* (Wong and Takei, 2009). The cyclostome AM-like peptides have apparently higher similarity to AM2 than AM1 at the level of the mature sequence, but overall homology of the precursor sequence (including signal and prohormone sequence) and exon-intron structure rather preferentially classified them into the AM1 group. Consistently, tissue distribution of the transcript showed that this gene is expressed in almost all tissues examined (AM-MT type). Since we could not identify CGRP or amylin in cyclostomes, we cannot tell which of the two combined genes (AM1/CGRP or AM2/amylin) exist on a chromosome of cyclostomes (Fig. 1.3).

As only one AM peptide was identified in the two cyclostome groups as a member of the CGRP family, it needs to be determined whether CGRP or amylin exists in

this most primitive vertebrate group. There is evidence showing that in the hagfish brain, CT-like peptide immunoreactive with antibodies raised against salmon CT exists that has a molecular mass similar to mature CT of other species (ca. 3,500) and the immunoreactive CT exhibits hypocalcemic activity in the rat (Suzuki 2001). Furthermore, there are CT-like peptide and CT receptor-like protein in the chordate (*Ciona intestinalis*), and synthetic CT-like peptide enhances Ca metabolism in the scale of goldfish (T. Sekiguchi and H. Satake, personal communication). The CT-like gene does not transcribe CGRP mRNA by alternative splicing, but it is important to note that the CT-like gene exist in chordates that have not experienced first-round whole genome duplication (1R) that occurred during the transition to vertebrate. Judging from the available molecular data in cyclostomes and a chordate where only an AM peptide and/or a CT-like peptide have been demonstrated, it remains to be determined whether CGRP/amylin or AM1/AM2 is more akin to a prototype of the CGRP family. In the near future, completion of the genome project in the hagfish (*Myxine glutinosa*), sea lamprey (*Peteromyzon marinus*) and amphioxus (*Brachistoma floridae*, Putnam et al. 2008) may give us an answer to the early evolution of the CGRP family.

1.4 Receptors for the CGRP Family Peptides

A detailed account of receptors for the CGRP family peptides will be given in subsequent chapters (e.g. Chapter 3 of this volume). As receptors for CGRP, AM(1), AM2 and AM5, the complex of CT receptor-like receptor (CLR) and receptor activity-modifying protein (RAMP) have been identified, and CT receptor (CTR) and/or the complex of CTR and RAMP are amylin and CRSP receptors (Poyner et al. 2002; Katafuchi et al. 2003). In mammals, three types of RAMPs (RAMP1–3) have been known thus far. CGRP binds to CLR + RAMP1, and AM(1) to CLR + RAMP2/3 with high affinity. AM2 binds to the complex of CLR + RAMP3 with medium affinity (Roh et al. 2004; Takei et al. 2004a). AM5 exhibits weak affinity only to CLR + RAMP1 but the affinity of AM5 is even lower than that of AM(1) (Takei et al. 2008). Amylin binds to CTR + RAMP1/2/3 with high affinity (Poyner et al. 2002; Hay et al. 2005), while CRSP binds CTR alone even with affinity higher than CT (Katafuchi et al. 2003).

In parallel with the diversification of the CGRP family peptides, CLR and RAMP are also diversified in teleost fish; three CLRs (CLR1–3) and five RAMPs (RAMP1–5) have been identified in the pufferfish, *Takifugu obscurus* (Nag et al. 2006) compared with a single CLR and three RAMPs in mammals. Examination of the ligand selectivity using transiently expressed CLR and RAMP combinations revealed that CGRP increased cAMP accumulation when applied to the complex of CLR1 + RAMP1/4, AM1 to CLR1 + RAMP2/3/5 and CLR2 + RAMP2, and AM2 and AM5 only to CLR1 + RAMP3. By contrast, only one CTR has been identified in teleost fish although two distinct CTs are produced as functional hormones (Nag et al. 2007). The teleost CTR has characteristics of having three to four hormone

binding domains in its long N-terminal extracellular sequence, although only the most proximal domain is essential for the CT binding. The cAMP experiment using transient expression system showed that CT binds to CTR alone, and amylin to the combination of CTR + RAMP1/3/4 with high affinity. Interestingly, CGRP also binds preferentially to the combination of CTR + RAMP1/4 with high affinity just like amylin, as observed in the human system where α-CGRP binds a CTR and RAMP1 complex that is designated as AMY_1 receptor (Hay et al. 2005).

1.5 Biological Actions of the AM Family

The biological actions of AM(1) have been intensively investigated in mammals, which showed that AM(1) is a multifunctional hormone that is involved in various aspects of homeostatic regulation. In particular, special emphasis is laid on the cardiovascular and renal effects as this peptide seems to be beneficial in management of cardiac and renal failure (Nagaya et al. 1999). As 15 years have passed since its discovery, a number of extensive reviews have been published to delineate its biological actions (e.g. López and Martínez, 2002; Brain and Grant 2003; Burton et al. 2004; Muff et al. 2004).

1.5.1 Biological Actions in Mammals

Because of the presence of previous reviews, the biological actions of AM(1) will be described only briefly here. AM(1) is synthesized in various peripheral tissues and exerts local actions on these tissues in a paracrine/autocrine fashion as expected from the ubiquitous distribution. However, significant amounts of AM(1) circulate in the blood of rat and human in both mature form and immature form without C-terminal amidation (Kitamura et al. 1994; Kato et al. 1999). The circulating AM(1) is probably secreted from the endothelial cells of various vasculatures. AM(1) actions are modulated also by binding to a plasma binding protein, later identified as complement factor H (Pio et al. 2001). The bound AM(1) cannot be measured by radioimmunoassay but exhibits enhanced biological actions in some circumstances. Collecting all functional data obtained thus far, AM(1) seems to be a defensive peptide that protects the tissues and cells from various types of damages including sepsis (Jiang et al. 2004), ischemia/reperfusion injury (Gonzalo et al. 2007), cardiac and renal failure (Tsuruda and Burnett 2002), etc. Its cytoprotective properties are also confirmed in vivo by works on genetically modified animals (Shindo et al. 2000; Nishimatsu et al. 2002). Another notable action is on body fluid regulation, acting directly on the brain to suppress thirst (Murphy and Samson 1995) and on the kidney to induce diuresis and natriuresis (Jougasaki et al., 1995), and indirectly through inhibition of vasopressin secretion (Yokoi et al. 1996), stimulation of renin release and plasma

angiotensin II (Rademaker et al. 2003), and inhibition of angiotensin II-stimulated aldosterone production (Yamaguchi et al. 1996).

AM2 is synthesized in the brain and pituitary (Takahashi et al. 2006; Morimoto et al. 2007; Hashimoto et al. 2008) and exerts potent central effects similar to that of AM(1), such as regulation of drinking (Taylor et al. 2005), pituitary hormone secretion (Taylor et al. 2005, 2006; Hashimoto et al. 2005), sympathetic activity (Taylor et al. 2005; Ren et al. 2006; Hashimoto et al. 2007), and blood–brain barrier function (Chen et al. 2006). AM2 also exhibits potent peripheral cardiovascular (Takei et al. 2004b; Roh et al. 2004; Pan et al. 2005; Abdelrahman and Pang 2006; Dong et al. 2006; Kandilci et al. 2006; Fujisawa et al. 2006; Chauhan et al. 2007) and renal actions (Fujisawa et al. 2004), but its potency is somewhat lower than AM(1) when compared between homologous peptides, probably reflecting its lower affinity to their known receptor, CLR and RAMP2/3 complex. Although AM2 was identified only 4 years ago, an extensive review has been published recently on the structure and function of this new member of the CGRP family (Bell and McDermott 2008). AM5 has just been identified in mammals, and thus little is known about its biological actions except for central and peripheral cardiovascular effects and a renal effect (Takei et al. 2008). As expected from the low affinity to known AM receptor complexes, AM5 is less effective than AM(1) except for central vasopressor actions.

1.5.2 Biological Actions in Teleost Fish

As AM peptides are diversified in teleost fish and most members are still retained, it seems that they have important functions in this advanced fish group. As we have an established system to examine cardiovascular and osmoregulatory actions using conscious eels, we cloned cDNAs coding for eel AM1, AM2 and AM5, and synthesized the inferred mature peptides based on the catalytic signal of the processing enzyme. Unexpectedly, eel AM1 had little effect on the arterial blood pressure of the eel, while eel AM2 and AM5 profoundly decreased it in a dose-dependent manner and the decrease reached 50% at 1 nmol/kg (Fig. 1.5), which is much greater than the effect of other vasodepressor hormones thus far known in eel including ANP (Nobata et al. 2008). In mammals, AM(1) is generally more potent and efficacious than AM2 and AM5 for the cardiovascular effects when administered peripherally in the rat (Fujisawa et al. 2006; Takei et al. 2008). The degree of depression is greater in the dorsal aorta than in the ventral aorta of eel, indicating the relaxant effect on the systemic resistance vessels but not on the branchial vessels. In addition to the cardiovascular effects, eel AM2 and AM5, but not AM1, exhibited potent osmoregulatory effects in the eel; they induced vigorous drinking and inhibited urine flow and sodium concentration in a dose-dependent manner (Ogoshi et al. 2008). The dipsogenic effect of AM2 and AM5 was as potent as that of angiotensin II, the most potent dipsogenic hormone thus far known in vertebrates (Fitzsimons 1998). Slow infusion of AM2 and AM5 at

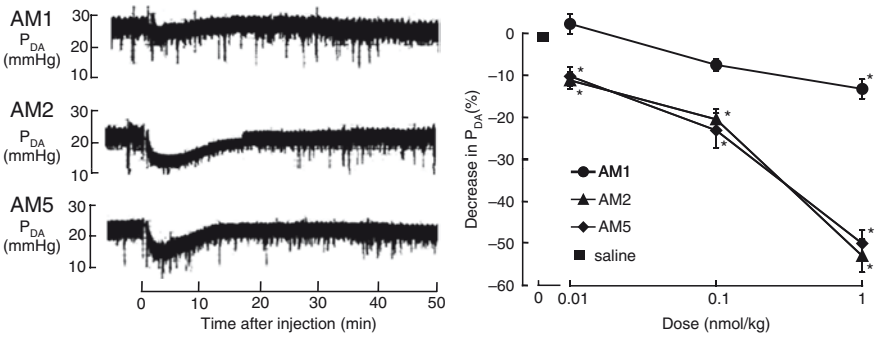

Fig. 1.5 Changes in arterial blood pressure after injection of eel adrenomedullin 1, 2 and 5 in conscious eels. *Left*, time-course of arterial pressure changes; *right*, dose–response relationship. The decrease is significant (*$p < 0.05$) compared with the change after saline injection (0 dose) (modified from Nobata et al. 2008)

non-depressor doses also induced drinking but not antidiuresis, showing that the renal effect is due to depression-induced decreases in GFR. Injection of AM2 into the third cerebral ventricle increased arterial pressure in the eel as in mammals but AM5 decreased it (Ogoshi et al. 2008). Thus different receptors may be present for AM2 and AM5 in the eel brain. Collectively, AM2 and AM5 are major cardiovascular and osmoregulatory hormones in the eel, which contrasts to more profound effects of AM(1) in mammals.

1.6 Conclusions and Future Perspectives

An interesting difference in biological actions of the AM family between mammals and teleost fish is that AM(1) appears to be a major hormone for cardiovascular and body fluid regulation in mammals, but AM2 and AM5 are dominant in the eel, although their affinity to the known AM receptors (CLR and RAMP complexes) is much less than AM1 in a teleost (Nag et al. 2006). Therefore, it is highly probable that yet unknown receptor(s) specific for AM2 and/or AM5 exist in teleost fish, although a possibility remains that new molecular chaperones other than RAMP, or a new receptor component protein (RCP) as suggested for AM receptors (Prado et al. 2001), exist in teleost fish. Since AM2 was vasopressor in action but AM5 was vasodepressor when administered into the eel brain ventricle (Ogoshi et al. 2008), AM2 and AM5 may have distinct receptors specific for respective peptides in teleost fish.

The presence of a new AM2 receptor has also been suggested in mammals. Taylor et al. (2005) showed that only AM2, and not CGRP, AM(1) and amylin, inhibited GH release from the rat dispersed anterior pituitary cells. It is also shown that the effects on sympathetic activation and neurohypophysial hormone secretion

after intracerebroventricular injection of AM2 are obviously greater than those of AM(1), and these central effects are only partially inhibited by pretreatments of both CGRP receptor antagonist ($CGRP_{8–37}$) and AM receptor antagonist ($AM_{22–52}$) (Taylor et al. 2005; Hashimoto et al. 2005, 2007). The data suggesting cGMP as an intracellular messenger for the AM2 effect in the rat mesenteric artery indirectly support the presence of receptors other than CLR and RAMP complex, which most likely exploit cAMP (Chauhan et al. 2007). Radioligand binding assay demonstrates a single class receptor for AM2 in the sarcolemmal membrane of the rat heart (Jia et al. 2006). If a specific receptor for AM2 does exist in vertebrates, the eel appears to be an excellent model for identification of this novel receptor as AM2 exhibits much greater potency and efficacy in cardiovascular and osmoregulatory actions than AM1 in this teleost species. We expect that identification of new receptors for the CGRP family peptides should enhance the value of a 'reverse' phylogenetic approach, which we previously demonstrated to be useful for identification of new hormones such as AM2 and AM5 (Takei et al. 2007).

Another promising approach to elucidate AM2 and AM5 function is modification of their genes. Disruption or transfer of the CGRP family genes has provided important information about their functions (Muff et al. 2004). Disruption of the CGRP gene in mice suggests its physiological role in blood pressure regulation, pain perception and inflammatory processes, but double (α-CGRP and β-CGRP) knockout has not been performed yet. Disruption of the amylin gene confirmed its protective function of pancreatic β-cells. Unlike other CGRP peptides, homozygotes of AM(1) knockout mice are lethal in utero because of impaired vascular development, resulting in hydrops fetalis (Caron and Smithies 2001). The studies using heterozygotes showed that AM(1) is important for maintenance of peripheral vascular resistance and protection against oxidative stress. Judging from the close relationship with AM(1), AM2 may also be important for embryonic development of vascular and other tissues and disruption of its gene is lethal as is the case with AM(1). However, knockout of the AM2 gene is absolutely necessary to assess its physiological role in mammals. With respect to AM5, mutation of the AM5 gene occurred after divergence of rhesus monkey in the primate lineage, although it is not yet determined whether anthropoids other than human and chimp possess normal AM5 gene or not. As human and chimp are natural knockout models of the AM5 gene compared with rhesus monkey, comparison of the functional differences between these primates may provide an important insight into the physiological function of AM5. Further, as AM5 receptor may still be retained in human and chimp, the examination of the effect of AM5 in these animals may help elucidate AM5's basic function in mammals.

Acknowledgement The authors thank Dr. D. L. Hay and I. M. Dickerson for the opportunity to introduce our comparative studies on the CGRP family. We also thank Dr. N. Minamino of National Cardiovascular Center Research Institute and Dr. Y. Ueta of University of Occupational and Environmental Health for collaboration, and Dr. Christopher A. Loretz of State University of New York at Buffalo for polishing the manuscript.