The Calcitonin Gene-related Peptide Family

Form, Function and Future Perspectives
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Debbie L. Hay • Ian M. Dickerson
Editors

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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-related 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.

2008

Ian Dickerson

References

Collip JB (1925) The internal secretion of the parathyroid glands. PNAS USA 11:484–485
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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
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 nigroviridis*), 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).

### Adrenomedullin 2

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<tr>
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<td>Chimpanzee</td>
<td>TQAQLLRVGCVLGTCQVQLSHRLWLQMPAGROQDSVPDPSSPHSY–NH2</td>
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<tr>
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<tr>
<td>Rat</td>
<td>PHAQLLRVGCVLGTCQVQLSHRLWLQVRPSARGDSVPDPSSPHSY–NH2</td>
</tr>
<tr>
<td>Mouse</td>
<td>PHAQLLRVGCVLGTCQVQLSHRLWLQVRPGRRDSVPDPSSPHSY–NH2</td>
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<td>Hedgehog*</td>
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### Adrenomedullin 5

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<td>Tupai</td>
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<td>Horse</td>
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<td>Dog</td>
<td>HQVAQRHRRLCSLTGQTHRLPEIYWLSARSTKEPSGKAGREPQDPHSY–NH2</td>
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<tr>
<td>Cat</td>
<td>HQVAQRHRRLCSLTGQTHRLPEIYWLSARSTKEPSGKAGREPQDPHSY–NH2</td>
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**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.