Central and peripheral cardiovascular actions of adrenomedullin 5, a novel member of the calcitonin gene-related peptide family, in mammals

Y Takei, H Hashimoto1, K Inoue, T Osaki2, K Yoshizawa-Kumagaye3, M Tsunemi3, T X Watanabe3, M Ogoshi, N Minamino2 and Y Ueta1

Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164-8639, Japan
1Department of Physiology, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Fukuoka 807-8555, Japan
2Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan
3Peptide Institute Inc., Protein Research Foundation, Minoh, Osaka 562-8686, Japan
(Correspondence should be addressed to Y Takei; Email: takei@ori.u-tokyo.ac.jp)

Abstract

Adrenomedullin 5 (AM5) is a new member of the calcitonin gene-related peptide (CGRP) family identified in teleost fish. Although its presence was suggested in the genome database of mammals, molecular identity and biological function of AM5 have not been examined yet. In this study, we cloned a cDNA encoding AM5 in the pig and examined its cardiovascular and renal effects. Putative mature AM5 was localized in the middle of the prohormone and had potential signals for intermolecular ring formation and C-terminal amidation. The AM5 gene was expressed most abundantly in the spleen and thymus. Several AM5 genes were newly identified in the database of mammals, which revealed that the AM5 gene exists in primates, carnivores, and undulates but could not be identified in rodents. In primates, nucleotide deletion occurred in the mature AM5 sequence in anthropoids (human and chimp) during transition from the rhesus monkey. Synthetic mature AM5 injected intravenously into rats induced dose-dependent decreases in arterial pressure at 0.1–1 nmol/kg without apparent changes in heart rate. The decrease was maximal in 1 min and AM5 was approximately half as potent as AM. AM5 did not cause significant changes in urine flow and urine Na⁺ concentration at any dose. In contrast to the peripheral vasodepressor action, AM5 injected into the cerebral ventricle dose-dependently increased arterial pressure and heart rate at 0.1–1 nmol. The increase reached maximum more quickly after AM5 (5 min) than AM (15–20 min). AM5 added to the culture cells expressing calcitonin receptor-like receptor (CLR) or calcitonin receptor (CTR) together with one of the receptor activity-modifying proteins (RAMPs), the combination of which forms major receptors for the CGRP family, did not induce appreciable increases in cAMP production in any combination, although AM increased it at $10^{-10}$–$10^{-9}$ M when added to the CLR and RAMP2/3 combination. These data indicate that AM5 seems to act on as yet unknown receptor(s) for AM5, other than CLR/CTR+RAMP, to exert central and peripheral cardiovascular actions in mammals.

Journal of Endocrinology (2008) 197, 391–400

Introduction

Adrenomedullin (AM) has been known as a member of the calcitonin gene-related peptide (CGRP) family that is composed of CGRP, AM, and amylin (López & Martínez 2001, Brain & Grant 2004, Muff et al. 2004). However, AM was found to be diversified in teleost fish and forms an independent subfamily consisting of five paralogs, AM1–AM5 (Ogoshi et al. 2003). Synteny and phylogenetic analyses indicated that members of the AM subfamily can be divided into three groups, AM1/4, AM2/3, and AM5, and that teleost AM1 is an ortholog of mammalian AM. Comparative genomic analyses further showed that two paralogs of each group, AM1 and AM4, and AM2 and AM3, are generated at the third-round whole-genome duplication (3R) that occurred in the teleost lineage (Vandepoele et al. 2004), but the counterpart of AM5 may have disappeared during teleost evolution (Ogoshi et al. 2006). Since AM2/3 and AM5 should have existed when tetrapods (lobe-finned fishes) diverged from ray-finned fishes, we searched them in the genome and expressed sequence tag (EST) databases and identified AM2 and AM5 genes in mammals (Takei et al. 2004b, Ogoshi et al. 2006). The AM2 gene was also identified in mammals by a similar comparative approach and named intermedin (Roh et al. 2004, Takei 2006). In addition, another member of the CGRP family, calcitonin receptor-stimulating peptide (CRSP), was identified in the pig (Katafuchi et al. 2003), which was generated by tandem
duplication of the CGRP gene (Ogoshi et al. 2006). Thus, the CGRP family appears to be more diversified than it was previously thought.

All CGRP family peptides exhibit cardiovascular actions, although relative potency differs among the members, CGRP > AM ≥ AM2 > amylin (Ando et al. 1990, Charles et al. 1997, Hall & Brain 1999, Fujisawa et al. 2004). CGRP is a neuropeptide in the brain and periphery, which is released from axon terminals innervating the vascular smooth muscles (see Brain & Grant 2004 for review). AM is synthesized in both vascular endothelial cells and smooth muscle cells and exerts potent hypotensive actions by relaxation of microvessels in various peripheral tissues (see López & Martínez (2001) for review). Expression of the AM gene is enhanced in various forms of cardiac failure and renal dysfunction, causing protective actions on the heart and kidney (Tsuruda & Grant 2004, Takei et al. 2005). AM2 forms of cardiac failure and renal dysfunction, causing protective actions on cardiovascular and body fluid regulation (Roh & Grant 2004).

AM2 is a neuropeptide in the brain and periphery, which is released from axon terminals innervating the vascular smooth muscles (see Brain & Grant 2004 for review). AM is synthesized in both vascular endothelial cells and smooth muscle cells and exerts potent hypotensive actions by relaxation of microvessels in various peripheral tissues (see López & Martínez (2001) for review). Expression of the AM gene is enhanced in various forms of cardiac failure and renal dysfunction, causing protective actions on the heart and kidney (Tsuruda & Burnett 2002). The AM2 gene is expressed in the brain and kidney of mammals and exerts various central and peripheral actions on cardiovascular and body fluid regulation (Roh et al. 2004, Takei et al. 2004a, Taylor et al. 2005, Yang et al. 2005, Takahashi et al. 2006). Amylin, a pancreatic hormone that is secreted with insulin from β cells (Cluck et al. 2005), exhibits a weak vasorelaxant effect (1/100 of CGRP).

Biological actions of the CGRP family peptides are principally mediated by the complex of calcitonin receptor (CTR) or CTR-like receptor (CLR) associated with one of the three receptor activity-modifying proteins (RAMPs); CLR–RAMP1 is a receptor for CGRP, CLR–RAMP2/3 for AM, CLR–RAMP3 for AM2, and CTR–RAMP2 for amylin (Brain & Grant 2004, Conner et al. 2004). After ligand binding, the receptor complex increases intracellular cAMP to mediate biological actions. However, AM2 has lower affinity than AM to CLR–RAMP3 complex, while central action of AM2 was not blocked by CTR or CLR-like receptor (CLR) associated with one of the three receptor activity-modifying proteins (RAMPs); CLR–RAMP1 is a receptor for CGRP, CLR–RAMP2/3 for AM, CLR–RAMP3 for AM2, and CTR–RAMP2 for amylin (Brain & Grant 2004, Conner et al. 2004). After ligand binding, the receptor complex increases intracellular cAMP to mediate biological actions. However, AM2 has lower affinity than AM to CLR–RAMP3 complex, while central action of AM2 was more potent than AM in the rat (Hashimoto et al. 2007). Furthermore, the central AM2 effect was not blocked by AM22–52 and CGRP 8–37, antagonists for CGRP family receptors. Therefore, a specific receptor for AM2 other than CLR–RAMP3 may exist in the rat (Taylor et al. 2006).

Judging from the potent cardiovascular actions of AM and AM2 in mammals, a new member of the AM subfamily, AM5, may also have similar actions through the CLR/CTR–RAMP complex. However, only the presence of the AM5 gene is predicted in the database and it is not known whether it is actually expressed as a functional protein in mammalian tissues. In this study, we cloned a cDNA encoding AM5 and examined the tissue distribution of transcripts in the pig. Then, inferred mature AM5 was chemically synthesized and its cardiovascular effects examined after peripheral and central injections in the rats. In parallel with the cardiovascular actions, we examined the renal effect of AM5 as AM and AM2 have diuretic and natriuretic actions in the rats (Fujisawa et al. 2004). We used rats as an experimental species, because we have an established technique to examine cardiovascular and renal effects in rats (Watanabe et al. 1988) and because most cardiovascular effects of AM and AM2 after peripheral and central administrations have been investigated in this species (Allen et al. 1997, Taylor et al. 2005, Hashimoto et al. 2007). Finally, specific AM5 receptors were sought in culture cells expressing homologous porcine CTR/CLR and RAMP using cAMP production as a marker.

Materials and Methods

Molecular studies

All animal experiments reported in this paper have been approved by the Committee for Animal Experiments of the University of Tokyo and of the University of Occupational and Environmental Health. Immature female pig of 7.8 kg was purchased from a local dealer. After decapitation, the brain, pituitary, heart, lung, thymus, spleen, kidney, adrenal, stomach, and liver were excised, cut into small pieces, and frozen in liquid nitrogen. Total RNA was extracted from the frozen tissues using Isogen (Nippon Gene, Toyama, Japan). A double-stranded cDNA pool was prepared from 1 μg total RNA from the spleen using SMART cDNA library construction kit (Clontech). The whole coding region of pig AM5 cDNA was obtained by PCR, under the condition described previously (Ogoshi et al. 2003), using primers that correspond to putative 5′- and 3′-untranslated region (Table 1). Amplified fragments were subcloned and sequenced more than ten clones.

The tissue distribution of AM5 transcripts was examined by RT-PCR. Total RNA from each tissue (2 μg) was reverse transcribed as above, and PCR was performed using gene-specific primers for porcine AM5 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that was used for internal control (Table 1). Annealing temperature and number of amplification cycles were 65 °C and 40 cycles for AM5 and 58 °C and 35 cycles for GAPDH.

AM5 genes were sought in the genome and EST databases of various vertebrate species using BioGrepX program established by Dr Hideo Bannai of Kyushu University (see Takei et al. 2004b). Phylogenetic analyses of newly identified AMs were performed using a Bayesian method in MrBayes program (version 3.1.2; Ronquist & Huelsenbeck 2003) to confirm their identity in the AM subfamily.

Table 1 List of primers used for cloning and RT-PCR analyses

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH-A4</td>
<td>5′-CACGATCAAAGGTAGAAGTGAGTGT-3′</td>
</tr>
<tr>
<td>GAPDH-S3</td>
<td>5′-TACATGGTCTACATGTCAGATGA-3′</td>
</tr>
<tr>
<td>pigAM5-A1</td>
<td>5′-GCATTCTCTATTTTATCAGCTTGA-3′</td>
</tr>
<tr>
<td>pigAM5-A2</td>
<td>5′-CGAGTTAGGGCCAAAGAAACTTGGAGT-3′</td>
</tr>
<tr>
<td>pigAM5-S1</td>
<td>5′-ACACCCGTAGGGGTCCCGTGA-3′</td>
</tr>
<tr>
<td>pigAM5-S2</td>
<td>5′-GTGGGCTTTCGGCCTGAGT-3′</td>
</tr>
</tbody>
</table>
Physiological studies
Predicted mature peptide of porcine AM5, which consists of 50 amino acid residues with an intramolecular ring formed by a disulfide bond and an amidated C terminus, was synthesized by a peptide synthesizer (Applied Biosystems, 430A) with p-methyl-benzhydrylamine resin as a solid support. The correct sequence was confirmed by mass analysis, amino acid analysis, and reverse-phase HPLC. Human AM was purchased from the Peptide Institute Inc. (Osaka, Japan). We used human AM to compare the effect with porcine AM5 because porcine AM was not commercially available and human and porcine AM differs by only one of 52 amino acid residues.

For i.v. injection experiments, adult male Sprague–Dawley rats weighing 285.3 ± 3.9 g (n = 7) were purchased from a commercial source. After anesthesia, cannulae were inserted in the femoral artery, femoral vein, and urinary bladder (Watanabe et al. 1988). The arterial cannula was connected to a pressure transducer and tachometer for continuous measurement of arterial pressure and heart rate. The venous cannula was connected to a syringe for continuous infusion of a Ringer solution (NaCl, 130; KCl, 5; CaCl2, 5·3; NaHCO3, 2 in mM) and for injection of peptides. Urine was collected every 10 min, and its volume and Na+ and Cl− concentrations were determined. AM5 and AM were injected as a bolus at 0·1, 0·3, and 1 nmol/kg in 0·5 ml NaCl solution containing 0·01% Triton X-305 (n = 7). Vehicle served as control. Injection interval was 20 min at 0·1 and 0·3 nmol/kg, and 40 min at 1 nmol/kg. Arterial pressure returned to a pre-injection level in 10 min after injection of the highest dose.

For i.c.v. injection experiments, adult male Wistar rats (273·8 ± 9·8 g, n = 34) were anesthetized, and stainless steel cannula implanted stereotaxically (o.d. = 0·55 mm) aimed at the left lateral ventricle at the following coordinates: 0·8 mm posterior to the bregma, 1·4 mm lateral to the midline, and 2·0 mm below the surface of left cortex such that a tip of the cannula was 1·0 mm above the left cerebral ventricle (Hashimoto et al. 2005). Two anchoring screws were fixed to the skull and the cannula was secured in place by acrylic dental cement. Seven days postoperation, the animals were anesthetized with urethane (1·4 g/kg) and catheter inserted into the femoral artery for continuous recording of arterial pressure. The arterial pressure and heart rate were recorded before and after i.c.v. injection of AM5 or AM at doses of 0, 0·1, 0·3, or 1 nmol/ rat in 10 µl. After experiment, i.c.v. injection was confirmed by a dye injection.

Cyclic AMP accumulation in culture cells expressing CGRP receptors
Synthetic porcine calcitonin was purchased from Bachem AG (Bubendorf, Switzerland), and human AM from Peptide Institute Inc. Porcine CGRP was custom synthesized by American Peptide Company Inc. (Sunnyvale, CA, USA). The sequence identity of porcine and human AM is 94%, while those of CGRP and calcitonin are 84 and 44% respectively.

Porcine CTR, CLR, and three isoforms of RAMP cDNAs encoding complete open reading frames were isolated from the porcine lung and hypothalamus cDNA libraries and ligated into pcDNA 3·1 (+) expression vector as described previously (Katafuchi et al. 2003). The CLR or CTR either alone or in combination of one of the three RAMPs (cDNA ratio 1:4) were co-transfected into COS-7 cells with Lipofectamine Plus reagent (Invitrogen Corp.) according to the manufacturer’s protocol. The transfected cells were then used for cAMP assay.

COS-7 cells were plated at 40 000 cells/well in 48-well plate and cultured for 24 h. Subsequently, the transfected cells were washed twice with Dulbecco’s modified Eagle’s medium dissolved in 20 mM HEPES, pH 7·4, containing 0·5 mM 3-isobutyl-1-methylxanthine and 0·05% BSA, followed by incubation in this medium for 30 min at 37 °C. The incubation medium was then replaced by 200 µl medium containing 10−11–10−6 M of hormones, and incubated at 37 °C for another 30 min. Aliquots (100 µl) of the medium were used for cAMP measurement by RIA as reported previously (Katafuchi et al. 2003).

Statistical analyses
All data were expressed as means ± S.E.M. Time-course changes in cardiovascular and renal parameters after injection of AM5 were statistically compared with controls by the ANOVA, followed by the Tukey’s test at each time point. The dose–response relationship was examined by the Dunnett’s test. In case normal distribution was not demonstrable, Steel–Dwass test was used for comparison (Takagi et al. 2003). Significance was set at P < 0·05.

Results
Molecular studies
A cDNA coding for porcine AM5 was cloned from the spleen (Fig. 1A). The cDNA had a single nucleotide polymorphism (C and T) in the coding region, which changes amino acid from Ser to Phe in the mature sequence. As both types of cDNAs occurred in equal numbers, they may originate from two haploid genomes of parents. Prohormone excluding a putative signal peptide consists of only 91 amino acid residues, and mature AM5 may be cleaved off by furin at a typical processing signal (Arg-X-X-Arg) in the N-terminal region and a processing signal peptide consists of only 91 amino acid residues, and mature AM5 may be cleaved off by furin at a typical processing signal (Arg-X-X-Arg) in the N-terminal region and at consecutive Arg residues that connect the C-terminal peptide (Fig. 1A). Accordingly, mature AM5 of 50 amino acid residues may be formed after disulfide bond formation by two Cys residues in the N-terminal region and C-terminal amidation using a Gly residue after removal of C-terminal Arg residues. The AM5 gene was expressed abundantly in the spleen and thymus, and slightly in the adrenal and pituitary (Fig. 1B). The signal was undetectable in the brain, heart, kidney, liver, and stomach.
We identified AM5 sequences in the genome database of ungulates (artiodactyls; pig, ox, and sheep and perissodactyles; horse), carnivores (dog and cat), primates (prosimians; Tupaia belangeri and simians; Macaca malatta) (Fig. 2), but we could not identify such sequences in rodents (mice and rats). In primates, AM5-like sequence exists in human (AC011495) and in the chimp (Pan troglodytes, NW_001228245), but the sequence changes in the middle of mature peptide because of the deletion of two nucleotides. The AM5 sequences were highly conserved in mammals. In amphibians, AM5 was identified in two Xenopus species, X. tropicalis and X. laevis (Fig. 2). The putative mature sequences differed from each other by four amino acid residues. In teleost fish, AM5 sequences were identified in nine species (Fig. 2). The AM5 sequence is fairly variable (83% identity) in two species of pufferfish (Takifugu rubripes and Tetraodon nigroviridis), but it is identical in two salmonid fishes (Oncorhyncus mykiss and Salmo salar). In general, AM5 sequences are more conserved in mammals than in teleost fish (Fig. 2). Xenopus AM5 is more similar to teleost AM5 than to mammalian AM5.

Physiological studies
Cardiovascular and/or renal parameters in rats before AM injections are shown in Table 2. While i.v. injections of AM5 and AM decreased arterial pressure immediately, i.c.v. injections increased it slowly and for a longer period (Fig. 3A and B). Control vehicle injection did not alter arterial pressure in both i.v. and i.c.v. injections. The peak increase occurred 5 min after i.c.v. injection of AM5, and the increase was slower and lasted longer after AM injection. Even with such a profound hypotension after i.v. injection of AM5, heart rate did not increase simultaneously at the highest dose (Fig. 3C). After i.c.v. injection, on the other hand, heart rate increased profoundly,
and the peak occurred more quickly with AM5 than with AM as is the case for the increase in arterial pressure (Fig. 3D).

The dose–response analyses showed that AM5 was approximately half as potent as AM in terms of peripheral vasodepressor effect (Fig. 4A). The increase in heart rate was significant at 1 nmol/kg AM, probably because of the reflex tachycardia in response to hypotension (Fig. 4B). The urine volume tends to decrease at 1 nmol/kg, which may be due also to the profound hypotension, while urinary Na\(^+\) excretion did not change after i.v. injections of AM5 and AM (Fig. 4C and D). Urinary Cl\(^-\) excretion did not change either (data not shown). The dose–response analyses for the i.c.v. vasopressor effect showed that AM5 is more potent than AM 5 min after injection (Fig. 5A), but the relationship was reversed at 20 min because the pressure returned to the pre-injection level 20 min after AM5 injection (Fig. 5B). When the maximal increase was compared, the vasopressor potency was comparable between AM5 and AM. The tendency was similar with chronotropic effect; AM5 was more effective at 5 min and AM was more effective at 20 min (Fig. 5C and D).

Neither AM5, AM, nor CGRP increased cAMP concentration appreciably in the medium when added to the culture cells that express porcine CLR alone (Fig. 6A), but CGRP increased it profoundly in cells co-expressing CLR and RAMP1 at 10\(^{-11}\) M (Fig. 6B). AM and AM5 also increased cAMP production with CLR–RAMP1, but the increase was significant only at 10\(^{-8}\) M for AM and 10\(^{-7}\) M for AM5. AM increased cAMP production with CLR–RAMP2 at 10\(^{-10}\) M and CLR–RAMP3 at 10\(^{-9}\) M, but AM5 was much less potent and efficacious than AM (Fig. 6C and D). AM5 and AM increased cAMP accumulation slightly in cells co-expressing CTR and RAMP, with AM more potent and efficacious than AM5 (Fig. 7). Calcitonin increased cAMP accumulation in cells co-expressing CTR and/or RAMP at 10\(^{-10}\) M and the increase was more than tenfold at 10\(^{-7}\) M (Fig. 7).

Table 2 Cardiovascular and renal parameters in Nembutal-anesthetized rats infused with Ringer and used for i.v. injection (n=7) and cardiovascular parameters in urethane-anesthetized rats used for i.v. injection (n=34)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>i.v. injection</th>
<th>i.c.v. injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial pressure (mmHg)</td>
<td>112 ± 4</td>
<td>74 ± 9</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>428 ± 3</td>
<td>350 ± 8</td>
</tr>
<tr>
<td>Urine volume (μl/h)</td>
<td>400 ± 1</td>
<td>48 ± 9</td>
</tr>
<tr>
<td>Urine Na(^+) concentration (mM)</td>
<td>46 ± 1</td>
<td>8 ± 6</td>
</tr>
<tr>
<td>Urine Cl(^-) concentration (mM)</td>
<td>70 ± 1</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>Urinary Na(^+) excretion (μmol/h)</td>
<td>15 ± 3</td>
<td>4 ± 6</td>
</tr>
<tr>
<td>Urinary Cl(^-) excretion (μmol/h)</td>
<td>24 ± 6</td>
<td>1 ± 6</td>
</tr>
</tbody>
</table>

Figure 2 Amino acid sequences of adrenomedullin 5 thus far identified in vertebrates. Bracket shows a disulfide bond. Amino acid residues identical in more than half the species are shaded. Accession numbers: pig, AB287333; ox, AV664186; sheep, NW_001800322; dog, DN365983; cat, AANG01678840; tupai (Tupaia belangeri), scaffold_50755; monkey (Macaca mulatta), NW_001106523; Xenopus laevis, CD301659; Xenopus tropicalis, DN011652; Takifugu rubripes, AB120299; Tetraodon nigroviridis, SCAF14992; flounder (Paralichthys olivaceus), AU091250; medaka, AB257078; zebratilis, NW_634155; rainbow trout, BX878389; Atlantic salmon, DY716166; eel (Anguilla japonica), AB363988.

Table 3 Parameters of cardiovascular action of adrenomedullin 5. *Y TAKEI and others 395 www.endocrinology-journals.org Journal of Endocrinology (2008) 197, 391–400 Downloaded from Bioscientifica.com at 02/09/2019 06:55:35PM via free access
Discussion

The CGRP family peptides have been shown to exert wide spectra of biological actions in various tissues and have attracted attention of both basic and clinical endocrinologists because of their important roles in various forms of diseases. For example, CGRP seems to be a major cause of migraine through local vasodilatation of cerebral vessels by release from trigeminal sensory nerve terminals (Arulumani et al. 2004). The AM gene is up-regulated in response to cardiac and renal failure or septic shock in proportion to the severity of the disease state (Eto et al. 2003, Rademaker et al. 2003). Amylin, co-secreted with insulin from pancreatic β cells, is now used for the treatment of type 2 diabetes (Cluck et al. 2005). The second AM, named AM2/intermedin, was recently identified in the selected species of mammals based on the discovery of AM subfamily in teleost fish (Roh et al. 2004, Takei et al. 2004b). AM2 seems to be a multifunctional peptide as is AM but has more potent central actions than AM (Taylor et al. 2005, Hashimoto et al. 2007). CRSP is a brain peptide that stimulates CTR alone, and its function is now under investigation (Katafuchi et al. 2003). In addition, the present study showed that the AM5 gene exists in primates, carnivores, and ungulates and is expressed as mRNA in the pig tissues. Furthermore, AM5 exhibited brisk cardiovascular actions when administered in the brain or periphery as did AM and AM2. Therefore, the CGRP family is now consisted of CGRP, AM, amylin, AM2, CRSP, and AM5 in mammals.

Calcitonin, hypocalcemic hormone produced by alternative splicing of the CGRP gene, is also occasionally included in the CGRP family (Muff et al. 2004).

The evolutionary history of the CGRP family has been unveiled in fishes and tetrapods by comparative genomic analyses (Ogoshi et al. 2006). It has been suggested that CGRP–AM(1), amylin–AM2, and AM5 existed on three different proto-chromosomes before divergence of ray-finned fishes and lobe-finned fishes that lead to tetrapods. In teleosts, all genes were duplicated at the 3R, and thus AM(1) and AM4, AM2 and AM3, and CRSP1 and CRSP2 genes were produced in the teleost lineage. One of the duplicated amylin and AM5 genes appear to have been silenced after the 3R. Before the 3R, two chromosomes on which CGRP–AM(1) and amylin–AM2 exist may be duplicated between CGRP and amylin and between AM(1) and AM2 at the second-round whole-genome duplication that occurred during transition from agnathans to gnathostomes. The origin of the AM5 gene is not known, but it seems to be produced from the AM(1) or AM2 gene as judged by the high sequence similarity between AM5 and AM(1)/AM2.

AM5 sequences were highly conserved within mammalian species. However, the homology is low between mammals and teleost fishes, although CGRP, AM(1), AM2, and amylin sequences are well conserved even across different classes of vertebrate. Xenopus AM5 sequences were more similar to teleost AM5 than to mammalian AM5, although amphibians are phylogenetically closer to mammals than to teleost and have orthologous hormones similar to mammals.

Figure 3 Time-course changes in (A and B) arterial pressure and (C and D) heart rate after i.v. injection of 0.3 nmol/kg and i.c.v. injection of 1 nmol/rat of adrenomedullin 5 (open circles), adrenomedullin (open squares), or vehicle (closed circles) in the rat. *P<0.03 compared with the value of controls.
Figure 4 Dose–response relationship for the effects of adrenomedullin 5 (open circles) and adrenomedullin (closed circles) on (A) arterial pressure, (B) heart rate, (C) urine volume, and (D) urinary Na⁺ excretion after i.v. injection in the rat. *P<0.05 compared with the value of vehicle injection.

Figure 5 Dose–response relationship for the effects of adrenomedullin 5 (open circles) and adrenomedullin (closed circles) on (A and B) arterial pressure and (C and D) heart rate 5 min (A and C) and 20 min (B and D) after i.c.v. injection in the rat. *P<0.05 compared with the value of vehicle injection.
It seems that *Xenopus* AM5 has specific sequences that are important for the aquatic life. In primates, nucleotide deletion occurred in the AM5 gene in the region that codes for mature sequence during the transition from rhesus monkey (*Macaca*) to anthropoids (chimp and human). In anthropoids, the AM5 gene with deletion seems to be expressed and registered as carnitine acyltransferase-like protein 1 in the EST database (AF331918). The AM5 gene was not detectable in mice and rats, but the potent cardiovascular actions shown in this study indicate that the gene is present with sequences altered at conserved amino acid residues in rodents, or it was silenced recently in rodents as in anthropoids.

It is suggested that the CGRP family peptides act principally in a paracrine fashion to exhibit biological activity (Brain & Grant 2004). Judging from the high affinity of CGRP, AM, and amylin to CLR/CLR coupled with one of RAMPs (10⁻¹¹–10⁻⁹ M), which is in a range of endogenous variation of plasma concentrations, these peptides may act also as an endocrine hormone from plasma (Eto et al. 2003). However, the potency of AM2 for cAMP accumulation in CLR–RAMP was much lower than AM (Roh et al. 2004, Takei et al. 2004a), while its central actions are even greater than AM (Hashimoto et al. 2007). In addition, the AM2 effect was blocked only partially by CGRP₈–₃₇ and AM₂₂–₅₂, although the AM effect was totally blocked by combination of the two blockers. Furthermore, the inhibitory effect of AM2 on GH secretion from pituitary cells in vitro was not demonstrated by AM (Taylor et al. 2006). Therefore, the presence of AM2-specific receptor(s) that differs from CLR–RAMP3 has been suggested. In this study, we showed that AM5 is much less potent and efficacious than AM in any CTR/CLR and RAMP combinations, although cardiovascular effect of AM5 after i.v. and i.c.v. injections were comparable with that of AM. Thus, AM5 seems to have yet unidentified specific receptor(s) that differ from CTR and CLR.

A comparative study also suggests the presence of specific receptors for AM2 and AM5. In the eel, homologous eel AM2 and AM5 are 100-fold more potent and efficacious than AM(1) for the vasodepressor effect when injected into the circulation (Nobata et al. 2008). In the pufferfish (*Takifugu obscurus*), three CLRs and five RAMPs have been identified (Nag et al. 2006). However, homologous AM5 and AM2 bind only to CLR1–RAMP3 with low affinity, while AM(1) bind to CLR1–RAMP2/3/5 and CLR2–RAMP2 combinations with much higher affinity as determined by the COS cells expressing these proteins. Therefore, specific receptors for AM5 and AM2 should exist in the vascular smooth muscles of teleosts. It seems that teleost fish serve as excellent materials for identification of new AM5 and/or AM2 receptor other than CLR–RAMP in vertebrates.

AM5 caused immediate hypotension when injected into the periphery and caused long-lasting hypertension when injected centrally in this study as observed with AM. The hypertension and tachycardia after i.c.v. injection of AM5...
may be due to the sympathetic activation as shown by AM and AM2 (Taylor et al. 2005, Hashimoto et al. 2007). The peripheral vasodepressor effect of AM5 is half as potent as AM, but the central vasopressor effect was comparable between the two peptides. It is possible that the peripheral vasodepressor effect was ameliorated by the central vasopressor action mediated via the circumventricular organ that has incomplete blood–brain barrier. It has been shown that AM acts on the area postrema, one of such organs, to elevate arterial pressure (Allen et al. 1997), and the area postrema possesses AM receptors and responds to AM by modulating excitability of the neurons (Yang & Ferguson 2003). The difference in the time course of central vasopressor effect also indicates that AM5 and AM may act on different receptors or use different signal transduction systems. The lack of the renal effect may be due to the injection of hormones into the general circulation as AM was diuretic and natriuretic only when infused directly into the renal artery in rats (Fujisawa et al. 2004). The concomitant hypotension after i.v. injection of AM5 may have decreased GFR and thus ameliorated the possible diuretic effect. The renal effect of AM5 may be minor compared with ANP because ANP is diuretic and natriuretic after a bolus systemic injection or infusion even with concomitant hypotension (Watanabe et al. 1988).

The present study showed that the AM5 gene is expressed abundantly in the spleen and thymus of pig. In the pufferfish, AM5 transcripts were identified in the spleen, head kidney (hematopoietic tissue equivalent to bone marrow), gill, and skin, all of which are implicated in defensive functions against infection (Ogoshi et al. 2003). Therefore, AM5 is expressed abundantly in the defense-related organs or hematopoietic organs in both mammals and fishes. The AM family of peptides have diverse functions that include regulation of cardiovascular, body fluid, and immune systems (López & Martínez 2000, Brogden et al. 2005), of which AM(1) is potent in peripheral actions and AM2 in central actions. It is intriguing to examine what is the major function of AM5 in the AM family. The AM5 gene was disrupted very recently in human and may be silenced in rodents. Therefore, it is of interest to examine how the loss of the AM5 gene has influenced their biological systems, particularly in the immune and hematopoietic system.

Acknowledgements

We thank Dr Junyou Li of Animal Resource Science Center, Graduate School of Agricultural and Life Sciences, the University of Tokyo and Dr Tadaomi Naka of Ocean Research Institute for their help in sampling pig tissues. We also thank Ms. Sanae Hasegawa for measurements of urinary ion concentrations. This work was supported in part by a...
grant from the Japan Society for the Promotion of Science (16207004) to Y T. There is no conflict of interest that would prejudice its impartiality.

References


Brain SD & Grant AD 2004 Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiological Reviews 84 903–934.


Hashimoto H, Hyodo S, Kawasaki M, Shabata M, Saito T, Suzuki H, Otsubo H, Yokoyama T, Fujihara H, Higuchi T et al. 2007 Adrenomedullin 2 is a more potent activator of hypothalamic oxytocin–secreting neurons than adrenomedullin in rats, and its effects are only partially blocked by antagonists for adrenomedullin and calcitonin gene-related peptide receptors. Peptides 28 1104–1112.


