Identification of novel adrenomedullin in mammals: a potent cardiovascular and renal regulator

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Abstract **We have identified cDNA encoding a new member of the adrenomedullin (AM) family, AM2, for the first time in mammals (mouse, rat and human). The predicted precursor carried mature AM2 in the C-terminus, which had an intramolecular ring formed by an S–S bond and a possibly amidated C-terminus. Phylogenetic analyses clustered AM2 and AM into two distinct but closely related groups. Similarity of exon–intron structure and synteny of neighboring genes showed that mammalian AM2 is an ortholog of pufferfish AM2 and a paralog of mammalian AM. AM2 mRNA was expressed in submaxillary gland, kidney, stomach, ovary, lymphoid tissues and pancreas of mice, but not in adrenal and testis. Intravenous injection of synthetic mature AM2 decreased arterial pressure more potently than AM, and induced antidiuresis and antinatriuresis in mice. These results show that at least two peptides, AM and AM2, comprise an adrenomedullin family in mammals, and that AM2 may play pivotal roles in cardiovascular and body fluid regulation.**

Key words: Calcitonin gene-related peptide family; Adrenomedullin family; Vasodepressor action; Antidiuretic action; Comparative genomics; Molecular evolution

1. Introduction

Calcitonin gene-related peptide (CGRP), adrenomedullin (AM), amylin (islet amyloid polypeptide), and the recently discovered calcitonin receptor-stimulating peptide are structurally related to each other and form a hormone family named CGRP superfamily [1]. However, their biological functions are much diversified among the peptides. AM is a new regulatory peptide discovered in 1993 [2]. Subsequent studies have shown that AM is expressed in various tissues and exhibits many functions including cardiovascular and fluid regulation [3], regulation of growth and differentiation, and other hormone secretions [4,5]. AM is also implicated in various disease states such as cardiovascular and renal disorders, sepsis, cancer and diabetes [6,7]. This spectrum of functions is thought to be achieved by interaction with multiple receptors and their association with multiple receptor activity-modifying proteins (RAMPs) and receptor component proteins (RCPs) [8–10]. However, there is a possibility that the diverse functions of AM are due to multiple closely related peptides that share their receptors and associated proteins.

Recently, we have isolated cDNAs encoding five AM-like peptides from the pufferfish, *Takifugu rubripes*, and named them AM1–5 [11]. They are apparently derived from a common ancestral gene, and phylogenetic analyses grouped them into a cluster different from that of CGRP genes. Therefore, we suggested the possibility that AM forms an independent family of paralogous peptides that are different from CGRP and amylin in vertebrates including mammals. Among the members of the AM family identified in teleost fishes, AM1 is an ortholog of mammalian AM because linkage analysis showed that the same genes clustered in the vicinity of the AM/AM1 gene of human, mouse and pufferfish. Pufferfish AM1, 4, and 5 genes were expressed in various tissues including the interrenal (adrenal homolog) as mammalian AM. However, AM2 and 3 genes were expressed most abundantly in the brain. Together with the similarity of overall sequences and exon–intron structures, pufferfish AMs were divided roughly into two groups, AM1/4/5 and AM2/3 [11]. However, members of the AM2/3 group have not yet been identified in mammals.

In the present study, we initially attempted to identify mammalian AM2/3 genes in silico using the sequence information of pufferfish peptides. Then the cDNAs of candidate peptides were actually cloned from mouse, rat and human tissues, and the identity to AM2 was determined by their overall similarity, exon–intron structure, synteny of neighboring genes, and pattern of tissue distribution of transcripts. Finally, the putative mature AM2 peptide was chemically synthesized, and its cardiovascular and renal actions were examined in vivo using urethane-anesthetized mice and compared with those of AM.

2. Materials and methods

2.1. Molecular biological studies

2.1.1. RNA extraction. Two male mice (40.5 and 45.0 g) and two female mice (35.8 and 32.6 g) of the ICR strain, and a male rat of the Wistar strain (245 g) were purchased from a commercial source. After anesthesia with diethylether, animals were pithed, and the brain, pituitary, submaxillary gland, heart, thymus, lung, liver, spleen, pancreas, stomach, small intestine, large intestine, mesentery, adrenal

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

Name	Sequence
AM2-a	GGACGCCCGTGCCCAGCTTGCCA
AM2-b	CCATGGCCCGGATCCCGACG
AM2-e	TCAGCCATAGCTGTGGGGGCT
AM2-f	GCAGCTCTGGACGGGGGCACAGCA
GAPDH-A3	ATCGAAGGTGGAAGAGTGGGAGT
GAPDH-S3	TACATGGTCTACATGTTCCAGTATGA
mAM1-F	GTATCAGACCATCGCCACAG
mAM1-F2	GGCCAGATACTCCTTCGCAGTTC
mAM1-R	GCCCGACTGTTCAATGCTGT
mAM2-F1	CAAACTGGTTTTCCGCTGG
mAM2-R1	TCATAGGTTTAGGGTATCCAAAGCC
rAM2-F1	CAAACTAGTTTTCCGCTGAACC

glands, vertebra, and gonads were dissected out and immediately frozen in liquid nitrogen. The uterus and mammary gland were also collected from female mice. Total RNA was extracted from each tissue using Isogen (Nippon Gene).

2.1.2. cDNA cloning. Initially, we screened the EST and genome sequence files released by the National Center for Biotechnology Information (NCBI), ftp://ftp.ncbi.nlm.nih.gov/blast/db/, to obtain homologs of the pufferfish AM family based on the amino acid sequences of mature peptides. For this purpose, we have developed a program, named BiogrepX (http://biocaml.org:8080/~bannai/bg/), which allows searching for Perl-style regular expressions of protein sequences, from various protein and nucleotide sequence databases. In the case of nucleotide sequence databases, BiogrepX searches for regular expression on all six translation frames. The use of regular expressions enables searching for more specifically conserved sequences, which may be highly degenerate overall and would be overlooked by more general methods such as BLAST [12]. The screening detected AM-like sequences in the mouse, rat and human databases, which were distinct from AM of each species but apparently homologs of pufferfish AM2. Therefore, we named them mouse, rat and human AM2 (see below). The primers for cDNA cloning were designed based on these sequences (Table 1).

Double-strand cDNA pool was prepared by the SMART system (Clontech) using 2 μg total RNA of the mouse or rat kidney. The coding regions of mouse and rat AM2 cDNAs were cloned by the 3′-rapid amplification of cDNA ends method using the CDSIII/3′-PCR primer of the kit and specific primers mAM2-F1 or rAM2-F1 for mouse and rat, respectively (Table 1). The polymerase chain reaction (PCR) was done for 35 cycles at the annealing temperature of 60°C under the conditions reported previously [11]. Human kidney first strand cDNA was purchased from BD Biosciences. Human AM2 was amplified by PCR with primers in the 3′ and 5′ non-coding regions (Table 1). After an initial PCR reaction with primers AM2-a and AM2-f for 25 cycles at the annealing temperature of 55°C, the signal was further amplified by nested PCR with primers AM2-b and AM2-e for 30 cycles at 70°C. After electrophoresis, the products were purified, subcloned into pT7 Blue (Novagen), and sequenced.

2.1.3. Phylogenetic analysis. Precursor sequences of mouse, rat, human and/or pufferfish AM2, AM/AM1, CGRP and amylin were aligned using the CLUSTAL W alignment program at http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html/. A phylogenetic tree was constructed using the neighbor-joining method.

2.1.4. Tissue distribution of transcripts. The tissue distribution of AM2 and AM gene transcripts was analyzed in two male and two female mice by reverse transcription (RT)-PCR. One microgram of total RNA from each tissue was reverse-transcribed as described previously [11]. The primers used for PCR were mAM2-F1 and mAM2-R1 for AM2 and mAM1-F2 and mAM1-R for AM (Table 1). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were amplified using the primers GAPDH-S3 and GAPDH-A3. The PCR reaction was performed for 35 cycles for AM2 and AM and 28 cycles for GAPDH under the condition reported previously [11]. Amplified products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining.

2.1.5. Exon–intron structure. The exon–intron structure of mouse, human and pufferfish AM2 genes was determined by comparing the cDNAs with genomic DNA sequences, which were obtained from the website at http://www.ncbi.nih.gov/Genomes/index.html.

2.1.6. Synteny of neighboring genes. To determine the relationship among mammalian and pufferfish AM2s, we surveyed neighboring genes on mouse chromosome 15, human chromosome 22 and pufferfish genome scaffold M000040 using the websites at http://www.ncbi.nlm.nih.gov/mapview/ and http://fugu.hgmp.mrc.ac.uk/fugu-bin/clonesearch/. In addition, loci around the AM gene on human chromosome 11 were also surveyed to determine the relationship between human AM and AM2 genes.

2.2. Physiological studies

All physiological experiments were conducted in line with the Guidelines for the Care and Use of Laboratory Animals approved by the Committee for Animal Experiments of the University of Tokyo.

2.2.1. Surgery. Eight male mice of the ICR strain at 6–8 weeks of age (35.1 ± 0.52 g) were anesthetized by an intramuscular injection of urethane at 2.25 g/kg and tracheotomy was performed. The carotid artery and external jugular vein were cannulated with PE 10 tubes (Clay Adams), the tips of which were tapered after heating and pulling. The urinary bladder was cannulated with a funnel-like catheter made from a PE 10 tube.

2.2.2. Experimental protocol. The arterial catheter was used for monitoring blood pressure and heart rate in a polygraph (NEC San-ei), and the venous catheter for continuous infusion of Ringer solution (NaCl 130, KCl 5, $CaCl_2$ 5.3, $NaHCO_3$ 2 in mM) at 10 μl/min. Urine flow rate was monitored by a drop counter, and urine was collected every 20 min by a fraction collector. Urine Na^+ concentration was measured by an atomic absorption spectrophotometer (Z5300, Hitachi).

Predicted mature human AM2 with amidated C-terminus (see below) was synthesized by the Peptide Institute. Human AM was purchased from the Peptide Institute. Each peptide was dissolved in 0.9% NaCl containing 0.01% Triton X-100 and injected through a venous catheter at the dose of 0.1, 1 or 10 nmol/kg.

2.2.3. Statistical analyses. The vasodepressor effects of AM2 and AM were compared by analysis of variance (ANOVA) followed by Tukey's test. Changes in urine volume and urinary Na^+ excretion (volume × concentration) were compared every 20 min after AM or AM2 injection with the pre-injection values by repeated measures ANOVA. Significance was set at $P < 0.05$. All data are expressed as means ± S.E.M.

3. Results and discussion

3.1. Molecular biological studies

We have cloned AM2-like cDNAs from three mammalian species and named them mouse, rat and human AM2 (Fig. 1a). The overall identity of predicted precursor sequences including the signal peptide was 90% between mouse and rat, which is much higher than that of AM (79%) [5]. The inferred mature sequence was even more conserved and only one of 48 amino acids including C-terminal glycine differed between mouse and rat (Fig. 1b). The mammalian AM2 possessed structures common to the AM family including the presence of an intramolecular ring of six amino acid residues flanked by an S–S bond and a potential amidation signal at the C-terminus. The highly conserved sequence of AM2 was particularly obvious across different classes of vertebrates; sequence identity was 71% between mouse and pufferfish, which was much higher than that of AM/AM1 (50%). The sequence identity between AM2 and AM was rather low; the identity was 33% in the case of mice (Fig. 1b).

The phylogenetic tree depicted based on the precursor sequence showed that AM2 and AM/AM1 of mammals and pufferfish were clustered into the respective groups (Fig. 2). A close relationship between mammalian and pufferfish AM2 was further supported by the similar termination pattern of

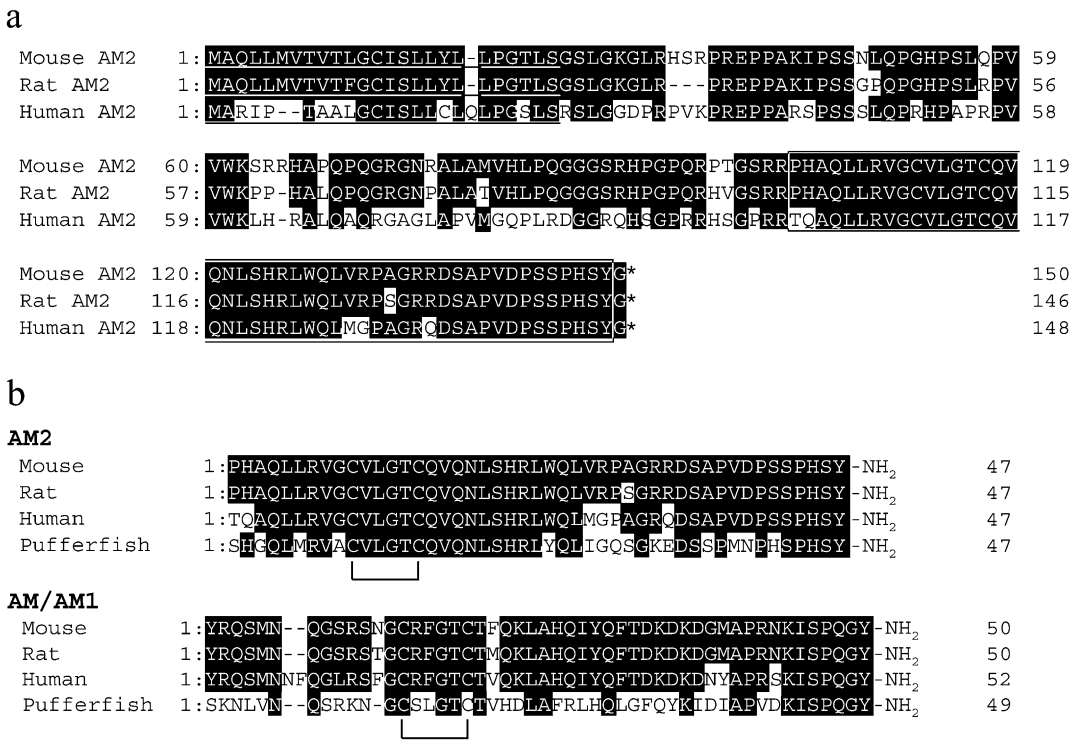

Fig. 1. Amino acid sequences of AMs. a: Precursor sequences of mouse, rat and human AM2 deduced from the cloned cDNA. Sequences identical to mouse AM2 are reversed. Putative signal sequence is underlined [33]. Putative mature peptide is boxed. Accession numbers: mouse AM2, AB121035; rat AM2, AB101236; human AM2, AB121034. Asterisks represent stop codons. b: Comparison of mature AM2 and AM/AM1 sequences from mammals and pufferfish. Amino acid residues identical to the mouse peptide are reversed in each group. Brackets show disulfide bonds.

the precursor C-terminus and exon–intron architecture of their genes. The C-terminal glycine of AM2 was followed by a stop codon (Fig. 1a) and AM2/3 genes were interrupted by only one intron in the coding region (Fig. 3a). AM/AM1 precursors are followed by four consecutive arginine residues that are removed after processing [13], and AM/AM1 genes are interrupted by two introns in the coding region [11]. Comparative genomic analyses concluded that mammalian AM2 is the ortholog of pufferfish AM2; synteny of AM genes and at least three neighboring genes was conserved among mouse, human and pufferfish (Fig. 3b).

Tissue distribution of mRNA showed that AM2 is expressed in various tissues of mice; high expression was detected in the submaxillary gland, kidney, stomach and mesentery, followed by the pituitary, lung, pancreas, intestines, spleen and thymus (Fig. 4). This expression pattern is quite different from that of pufferfish AM2, which is expressed almost exclusively in the brain [11]. It is possible that mammalian AM2 is expressed only in a restricted region of the brain, which may have masked its expression in the whole brain. The brisk expression in the pituitary suggests its paracrine/autocrine role in pituitary hormone secretion as suggested for AM in mammals [14–16]. In addition, AM2 was expressed in the ovary but not in the testis, indicating some specific role for the female reproductive system as suggested for AM [17]. AM2 appears to be expressed more than AM in the immune system [4].

Phylogenetic analyses showed that AM/AM1 and AM2 are more closely related to each other than to CGRP and amylin (Fig. 2). Therefore, AM/AM1 and AM2 appear to belong to the same hormone family independent of CGRP and amylin

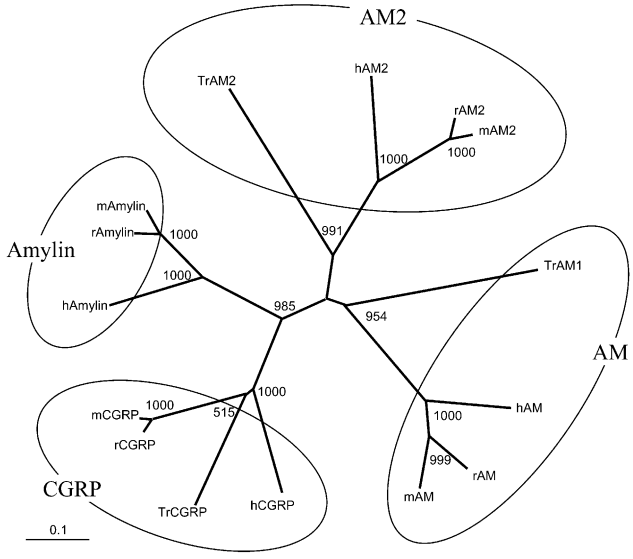

Fig. 2. Phylogenetic tree of mouse (m), rat (r), human (h) and pufferfish (Tr) AM2, AM, CGRP, and/or amylin depicted by the neighbor-joining method. Precursor sequences were used for the analysis. Bootstrap values after 1000 trials are indicated on the tree. Accession numbers: mouse AM, NM_009627; CGRP, AF330212; amylin, NM_010491; rat AM, NM_012715; CGRP, M11597; amylin, NM_012586; human AM, NM_001124; CGRP, X02330; amylin, NM_000415; pufferfish AM1, AB120295; AM2, AB120296; CGRP, AJ309015.

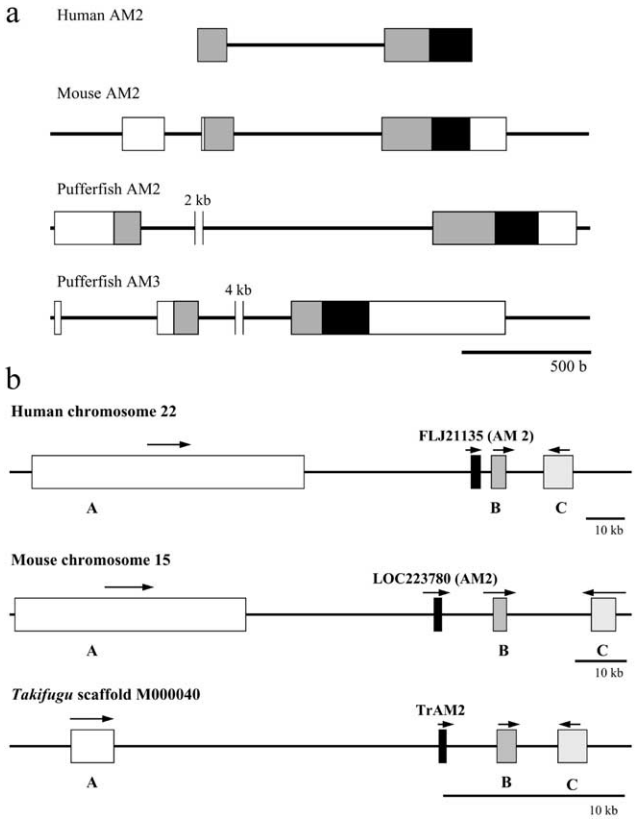

Fig. 3. Structure of AM2 genes. a: Exon–intron structures of mouse, human and pufferfish AM2 and 3 genes. Exons are shown as boxes. Mature AM sequences are in black, and coding regions are shadowed. Blank boxes indicate untranslated sequences. b: Conserved synteny of genes around the AM2 gene among mouse, human and pufferfish. Arrows indicate the direction of each gene. A, BC024879 for mouse and KIAA0685 for human; B, ALDRL6; C, AI451006 for mouse and BC002942 for human. A and C have different names in mouse and human but are orthologs.

as suggested in the pufferfish [11]. A closer relationship of AM2 and AM/AM1 genes was further supported by the presence of paralogous genes on the same chromosome; MAPK8IP2, ALDRL6, PARVB and SOX10 co-localized with the AM2 gene on human chromosome 22, while MAPK8IP1,

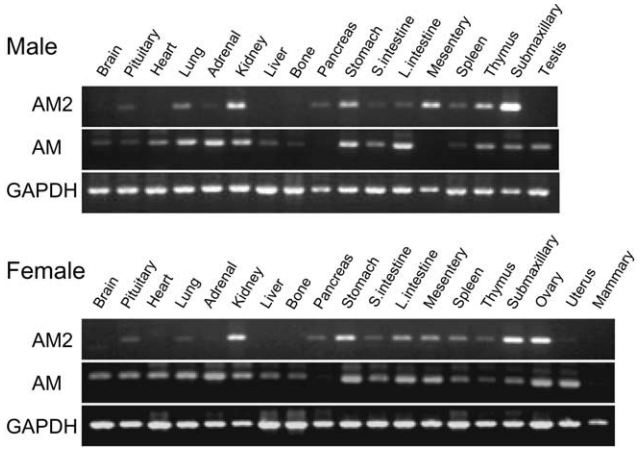

Fig. 4. Expression of AM2 and AM genes in various tissues of male and female mice examined by RT-PCR. GAPDH is used as an internal control. Similar results were obtained in two males or two females.

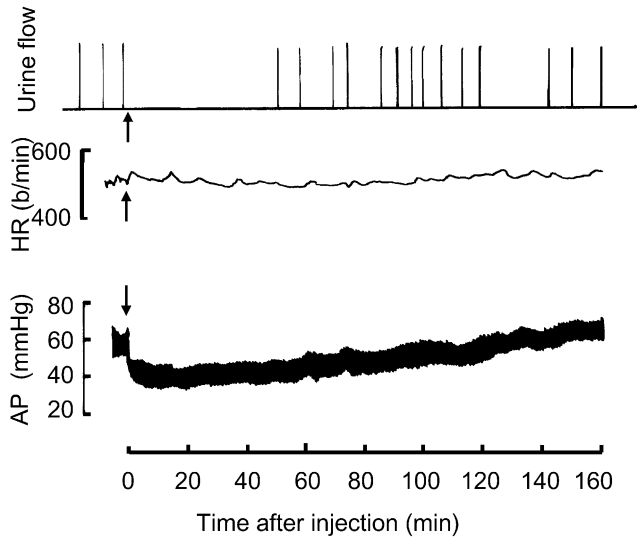

Fig. 5. An example of changes in arterial pressure (AP), heart rate (HR) and urine flow rate after injection of AM2 at 10 nmol/kg in urethane-anesthetized mouse. Each spike represents 15 μl of urine dropped from a bladder catheter. Arrows show the point of injection.

ALDRL3, PARVA and SOX6 co-localized with the AM gene on human chromosome 11.

3.2. Physiological studies

The average arterial pressure and heart rate of urethane-anesthetized mice were 66.0 ± 2.8 mm Hg and 579 ± 31 beats/min ($n = 8$), respectively. Synthetic human AM2 exhibited potent and long-lasting hypotensive actions in the mouse (Fig. 5) as reported for AM in several species of mammals [2,18–20]. The AM2 effect was dose-dependent and was more potent than human AM (Fig. 6a). Since the amino acid sequences of AM2 and AM are highly conserved between mouse and human (Fig. 1b), human peptides may have full activities in the mouse. The hypotensive effect continued for 166.0 ± 16.9 min ($n = 4$) at 10 nmol/kg, which is much longer than that of atrial natriuretic peptide (ANP) [21]. This may be due to the longer half-life of AM (22.0 min) than ANP (1.5 min) [14]. AM2 also exhibited a chronotropic effect in the mouse (Fig. 5) as AM does in the rabbit [18].

Since the mice originate from a dry area in Central Asia [22], they urinated scarcely and it took more than 4 h to obtain constant and significant urine flow after Ringer infusion at 10 μl/min. The average urine flow rate and Na^+ excretion rate after equilibrium was 135 ± 18 μl/h and 8.13 ± 0.91 μmol/h ($n = 8$), respectively. A bolus injection of AM2 transiently inhibited urine flow in the mouse (Fig. 5). Since urine Na^+ concentration decreased after AM2 injection, the antinatriuretic effect continued longer than the antidiuretic effect (Fig. 6b). The transient decrease in urine flow rate also occurred after AM injection, but to a much lesser extent compared with AM2 (Fig. 6b). Since diuretic and natriuretic effects of AM have been reported in other mammalian species [23–26], the inhibitory effect may be specific to the mice. However, it is to be noted that AM2 and AM induced diuresis and delayed natriuresis in over-hydrated mice to which Ringer was infused at 30 μl/min (data not shown). In no case did diuresis and natriuresis occur in mice infused at 10 μl/min.

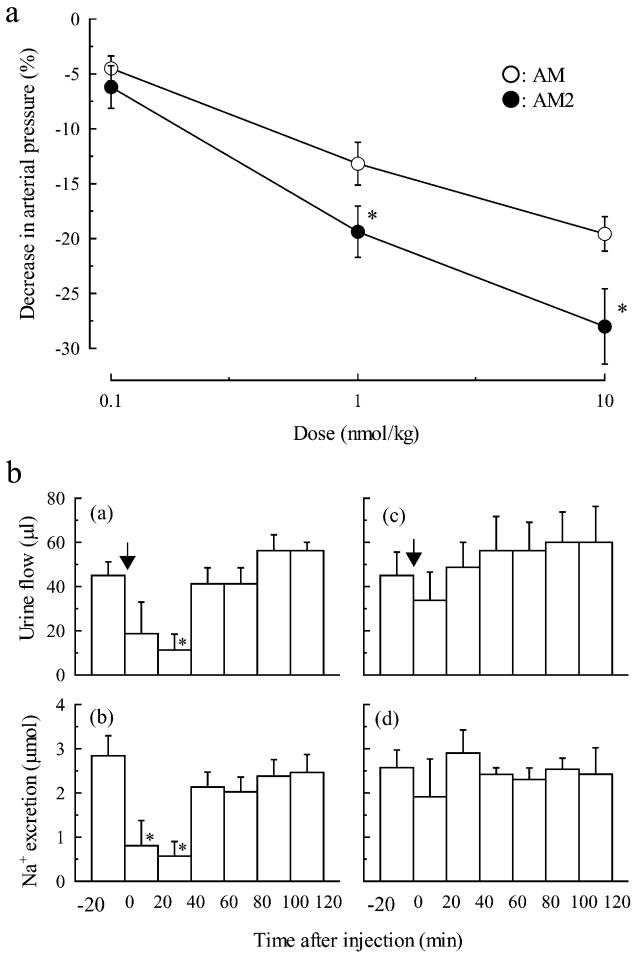

Fig. 6. Comparison of cardiovascular and renal actions of AM2 and AM in mice ($n=4$). a: Dose–response relationship for vasodepressor effects of AM2 and AM. The difference between the two peptides is significant as determined by ANOVA. $^*P<0.05$ by Tukey's test. b: Effects of AM2 (a,b) and AM (c,d) on urine flow rate and urinary Na^+ excretion at 10 nmol/kg. The decrease in Na^+ excretion was more prominent because of a decrease in urine Na^+ concentration. $^*P<0.05$ compared with the pre-injection value.

After the initial discovery of AM from human pheochromocytoma [2], extensive studies have shown that AM is a multifunctional peptide that plays pivotal roles not only in cardiovascular and fluid homeostasis but also in growth/development, carcinogenesis and others [3–5]. The present identification of AM2 in mammals indicates that some of the multiple AM functions may be shared by the paralogous AM2 through the same receptors. To elucidate this issue, it needs to be determined whether AM2 actions are mediated by its own specific receptors or shared by calcitonin receptor-like receptor and related proteins such as RAMPs and RCPs [27–29].

The current study identified a novel hormone in mammals based on its identification in a teleost fish. Similar discoveries of fish hormones in mammals have already been reported for several hormones such as melanin-concentrating hormone, stanniocalcin, urotensin and others, and these new hormones have been shown to play important roles in mammals [30–32]. With the availability of new tools and techniques for molecular biological discovery, such contributions of comparative studies to general (mammalian) endocrinology will further increase in the future.

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References

[1] Katafuchi, T., Kikumoto, K., Hamano, K., Kangawa, K., Matsuo, H. and Minamino, N. (2003) J. Biol. Chem. 278, 12046–12054.
[2] Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 192, 553–560.
[3] Samson, W.K. (1999) Annu. Rev. Physiol. 61, 363–389.
[4] Hinson, J.P., Kapas, S. and Smith, D.M. (2000) Endocr. Rev. 21, 138–167.
[5] López, J. and Martínez, A. (2002) Int. Rev. Cytol. 221, 1–92.
[6] Wang, C., Dobrzynski, E., Chao, J. and Chao, L. (2001) Am. J. Physiol. 280, F964–F971.
[7] Dixon, I.M.C. (2002) Cardiovasc. Res. 56, 347–349.
[8] Kapas, S., Catt, K.J. and Clark, A.J.L. (1995) J. Biol. Chem. 270, 25344–25347.
[9] Yallampalli, C., Chauhan, M., Thota, C.S., Kondapaka, S. and Wimalawansa, S.J. (2002) Trends Endocrinol. Metab. 13, 263–269.
[10] Hay, D.L., Poyner, D. and Dickerson, I. (2003) Trends Endocrinol. Metab. 14, 3–4.
[11] Ogoshi, M., Inoue, K. and Takei, Y. (2003) Biochem. Biophys. Res. Commun. 311, 1072–1077.
[12] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[13] Kitamura, K., Sakata, J., Kangawa, K., Kojima, M., Matsuo, H. and Eto, T. (1993) Biochem. Biophys. Res. Commun. 194, 720–725.
[14] Meeran, K., O'Shea, D., Upton, P.D., Small, C.J., Ghatei, M.A., Byfield, P.H. and Bloom, S.R. (1997) J. Clin. Endocrinol. Metab. 82, 95–100.
[15] Nakamura, Y., Shimatsu, A., Murabe, H., Mizuta, H., Ihara, C. and Nakao, K. (1998) Brain Res. 807, 203–207.
[16] Nussdorfer, G.G. (2001) Int. Rev. Cytol. 206, 249–284.
[17] Abe, K., Minegishi, T., Tano, M., Hirakawa, T., Tsuchiya, M., Kangawa, K., Kojima, M. and Ibuki, Y. (1998) Endocrinology 139, 5263–5266.
[18] Fukuhara, M., Tsuchihashi, T., Abe, I. and Fujishima, M. (1995) Am. J. Physiol. 269, R1289–R1293.
[19] Parkes, D.G. and May, C.N. (1997) Br. J. Pharmacol. 120, 1179–1185.
[20] Lainchbury, J.G., Cooper, G.J.S., Coy, D.H., Jiang, N.-Y., Lewis, L.K., Yandle, T.G., Richards, A.M. and Nicholls, M.G. (1997) Clin. Sci. 92, 467–472.
[21] Takei, Y. (2000) Int. Rev. Cytol. 194, 1–66.
[22] Schwarz, E. and Schwarz, H.K. (1943) J. Mammal. 24, 59–72.
[23] Ebara, T., Miura, K., Okumura, M., Matsuura, T., Kim, S., Yukimura, T. and Iwao, H. (1994) Eur. J. Pharmacol. 263, 69–73.
[24] Rademaker, M.T., Charles, C.J., Lewis, L.K., Yandle, T.G., Cooper, G.J.S., Coy, D.H., Richards, A.M. and Nicholls, M.G. (1997) Circulation 96, 1983–1990.
[25] Nagaya, N., Nishikimi, T., Horio, T., Yoshihara, F., Kanazawa, A., Matsuo, H. and Kangawa, K. (1999) Am. J. Physiol. 276, R213–R218.
[26] Lainchbury, J.G., Troughton, R.W., Lewis, L.K., Yandle, T.G., Richards, A.M. and Nicholls, M.G. (2000) J. Clin. Endocrinol. Metab. 85, 1016–1020.
[27] McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J.,

Thompson, N., Solari, R., Lee, M.G. and Foord, S.M. (1998) Nature 393, 333–339.
[28] Kamitani, S., Asakawa, M., Shimekake, Y., Kuwasako, K., Nakahara, K. and Sakata, T. (1999) FEBS Lett. 448, 111–114.
[29] Aiyar, N., Disa, J., Pullen, M. and Nambi, P. (2001) Mol. Cell. Biochem. 224, 123–133.
[30] Knigge, K.M., Baxter-Grillo, D., Speciale, J. and Wagner, J. (1996) Peptides 17, 1063–1073.
[31] Ishibashi, K. and Imai, M. (2002) Am. J. Physiol. 282, F367–F375.
[32] Douglas, S.A. (2003) Curr. Opin. Pharmacol. 3, 159–167.
[33] Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Protein Eng. 10, 1–6.