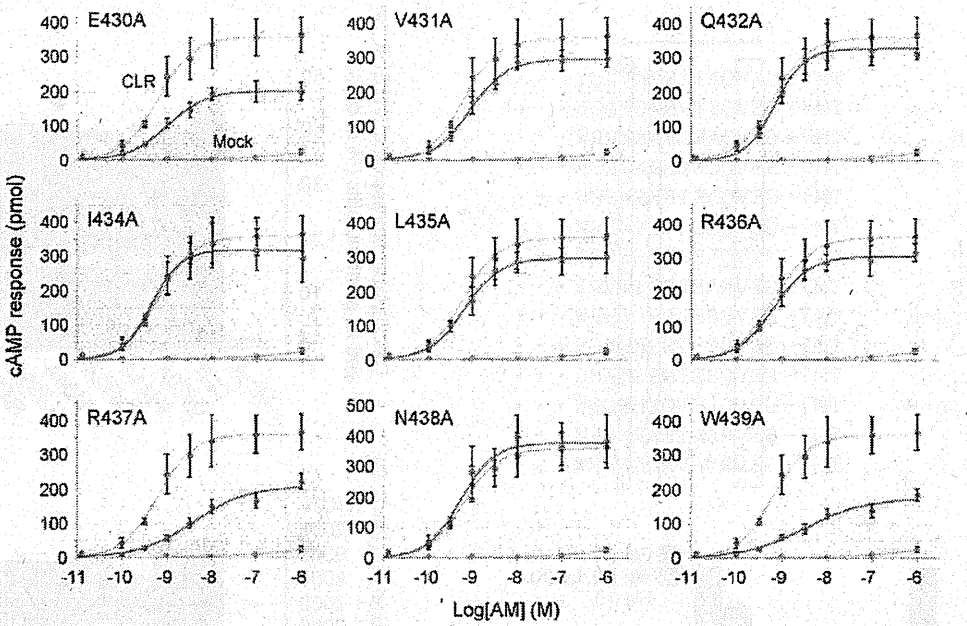

Fig. 3. AM-evoked cAMP production mediated via hCLR helix 8 point mutants in HEK-293 cells stably expressing hCLR. All cells transfected with wild-type or mutant hCLR were simultaneously exposed to the indicated concentrations of hAM for 15 min at 37 °C and then lysed. The resultant lysates were analyzed for cAMP content. Note that the same cAMP responses mediated via wild-type hCLR are shown in every mutant-mediated response. Bars are expressed as means ± SD of five experiments due to limitations of the MasterPlex ReaderFit software used.

surface expression of V431A (35.3 ± 1.0%), R437A (34.1 ± 2.4%) and W439A (27.4 ± 1.0%) was significantly lower than was seen with WT-hCLR.

3.3. Effect of helix 8 point mutation on ^{125}I-AM binding to hCLR

Fig. 2B shows that in cells expressing WT-hCLR (IC_{50} = 22.4 ± 3.0 nM), specific ^{125}I-AM binding was about 20-fold higher than in cells expressing empty vector (*Mock*), and similar levels of ^{125}I-AM binding were seen in cells expressing E430A, Q432A, I434A, L435A or N438A. On the other hand, the IC_{50} values for V431A (13.1 ± 2.0 nM), Q432A (15.0 ± 1.3 nM), R436A (14.4 ± 2.7 nM) and R437A (15.7 ± 1.4 nM) were somewhat lower, and the IC_{50} value for W439A (8.2 ± 1.2 nM) was significantly lower, than was seen with WT-hCLR ($p < 0.05$). The remaining mutants' IC_{50} values ranged from 18.3 to 19.4 nM.

3.4. Functionality of hCLR helix 8 point mutants

The functionality of the mutant receptors was assessed by measuring AM-induced intracellular cAMP production (Fig. 3 and Table 1). AM elicited little or no cAMP production in HEK-293 cells expressing hRAMP2 alone. Following transfection with WT-hCLR, however, AM elicited concentration-dependent increases in cAMP, and comparable responses were seen when cells were transfected with Q432A, I434A, L435A or N438A. The maximum cAMP levels obtained with the remaining five mutants were significantly lower than those obtained with WT-hCLR. In addition, the R437A and W439A mutants exhibited significant increases in their EC_{50} values.

3.5. Internalization of hCLR helix 8 point mutants

Our earlier flow cytometric analysis revealed that AM dose-dependently induced hRAMP2-mediated internalization of hCLR, with the peak of the receptor's internalization occurring after about 60 min [15]. In the same study, we also showed that there was no significant difference in the efficiency of receptor internalization induced by 0.1 μM and 1.0 μM AM [15]. We therefore evaluated the internalization of each point mutant after exposing hRAMP2-expressing cells to 0.1 μM AM for 60 min (Fig. 4). Surprisingly, we found that four mutants, E430A, V431A, Q432A and W439A, significantly enhanced AM-induced internalization of the mutant receptor complexes, despite the fact that E430A and W439A significantly reduced AM signaling (Fig. 3). The remaining mutants had little effect on receptor internalization.

4. Discussion

All family B GPCRs are preferentially coupled to Gs proteins responsible for stimulating cAMP production, and many undergo rapid internalization in response to an agonist. However, the roles of their C-tails in Gs coupling and in internalization have not been fully explored. We recently showed that AM elicits little or no cAMP production or internalization of receptor complexes composed of hRAMP2 and a hCLR truncation mutant totally lacking its

Table 1
AM-induced cAMP production in RAMP2-expressing HEK-293 cells co-transfected with wild-type CLR and its point mutants.

Construct	cAMP production	
	EC_{50} (nM)	Maximum response (pmol)
CLR	0.68 ± 0.10	372.4 ± 21.6
E430A	1.10 ± 0.11*	206.0 ± 9.5*
V431A	0.92 ± 0.05	300.4 ± 10.6*
Q432A	0.82 ± 0.11	328.2 ± 12.6
I434A	0.47 ± 0.04	319.3 ± 24.1
L435A	1.05 ± 0.44	306.8 ± 19.6
R436A	0.68 ± 0.09	305.6 ± 13.6*
R437A	5.15 ± 1.86*	215.7 ± 13.0*
N438A	0.59 ± 0.08	386.8 ± 29.4
W439A	3.99 ± 0.97*	182.8 ± 7.6*

The results represent the mean ± SEM of five independent experiments.
* $p < 0.05$ vs. CLR.

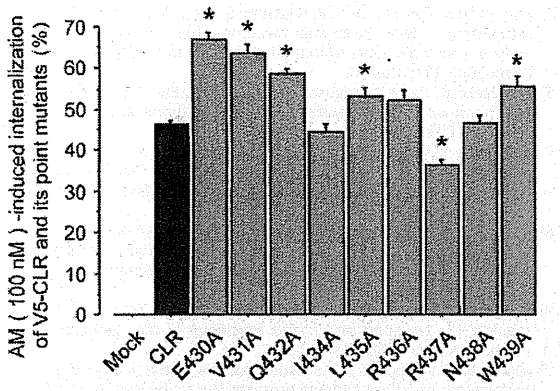

Fig. 4. Flow cytometric analysis of internalization of hCLR helix 8 point mutants in HEK-293 cells stably expressing hRAMP2. Surface expression of each construct was estimated by flow cytometry before and after exposing the cells to 0.1 μM hAM for 60 min. Internalization was estimated as the percentage of receptors lost from the cell surface after AM exposure. Internalization of WT-hCLR and all hCLR helix 8 point mutants was significantly higher than that seen with mock ($p < 0.05$). The results represent the means ± SEM of six independent experiments. *$p < 0.05$ vs. hCLR.

C-tail, despite their full cell surface expression and high-affinity ^{125}I-AM binding [13]. This means the hCLR C-tail is crucial for both AM-induced cAMP production and hAM_1 receptor internalization, which is in contrast to the earlier report that the hCLR C-tail is involved in agonist-mediated receptor internalization, but not cAMP production [2]. That finding was obtained using COS-7 cells transiently co-expressing hRAMP1 and an hCLR mutant in which the C-tail was completely removed. The discrepancies between that earlier study and our present one may reflect differences in the cell backgrounds, transfection methods, cell-surface expression levels of the target receptor complex, cAMP assays and/or the hRAMP isoform.

We observed that substituting Glu430 in putative hCLR helix 8 with Ala (E430A) reduced maximum cAMP levels by ~45% without altering the cell surface expression of the receptor or its affinity for ^{125}I-AM in HEK-293 cells stably expressing hRAMP2. This suggests Glu430 is required for the coupling of hCLR helix 8 to Gs. Notably, this negatively charged residue is strictly conserved among B GPCRs (Fig. 1), and a conserved Glu residue is also reportedly involved in Gs coupling to putative helix 8 in the hVPAC1 receptor in CHO cells [3]. In that case, substituting Glu394 with Ala (E394A) reduced cAMP responses by ~45%, although cell surface expression of the mutant receptor and agonist binding were identical to those in the wild-type receptor [3]. The C-tail of porcine calcitonin receptor is also required for evoked cAMP production [6], but the role of the conserved Glu residue in that receptor remains unknown. On the other hand, evidence suggests that the C-tails of the rat glucagon [33], parathyroid [10] and secretin receptors [9] may not be involved in coupling to Gs proteins. However, all of those studies made use of a truncation approach, which left some or all of helix 8 intact. It is therefore possible that the strictly conserved Glu residue in helix 8 is generally involved in coupling family B GPCRs to Gs.

Putative hCLR helix 8 also possesses two positively charged dibasic residues (Arg436–Arg437), both of which are highly conserved among family B GPCRs; in particular, Arg437 is strictly conserved (Fig. 1). In the present study, R437A mutation of hCLR reduced cell surface expression of the mutant receptor by ~25%, thereby reducing specific ^{125}I-AM binding by ~20%. Moreover, this mutant reduced maximum cAMP levels by ~40%, with an ~8-fold increase in the EC_{50} value. Thus, Arg437 also appears to participate in both the coupling to Gs proteins and the proper surface delivery of hCLR. Although Arg436 is next to Arg437, R436A mutation had little effect on the cell surface expression or the cAMP responses of the mutant receptor complex. By contrast, double mutation of Arg400–Arg401 to Ala400–Ala401 did not affect cell surface expression, radioligand binding or cAMP responses of the mutant hVPAC1 receptor [3]. It remains to be seen whether the highly conserved Arg residues in hCLR helix 8 are also important for other family B GPCR functions.

Unlike helix 8 in rhodopsin-like receptors (family A GPCRs), helix 8 in family B GPCRs, including hCLR, contains no cysteine residues, the palmitoylation of which has been shown to anchor the helix to the plasma membrane. Nonetheless, a recent analysis revealed that a synthetic hCLR helix 8 peptide readily anchors to liposomes in a membrane-parallel orientation via Trp439, which is strictly conserved among family B GPCRs [2]. In the intact receptor, therefore, a tethered Trp439 may partially fulfill the role of the lipid anchor seen at the equivalent position in many family A GPCRs. This function would be unaffected by the hRAMP isoform, as the hCLR C-tail does not interact with any of the three hRAMP isoforms [14]. In the present study, W439A mutation of hCLR reduced cell surface expression of the mutant receptor by ~40%, and there was a corresponding ~25% reduction in specific ^{125}I-AM binding, with a ~3-fold decline in the IC_{50} value, as compared to WT-hCLR. In addition, maximum cAMP levels were reduced by ~50%, with a ~6-fold increase in the EC_{50} value. Thus Trp439 appears to be important for both cell surface expression of the hAM_1 receptor and AM-mediated cAMP responses mediated via the receptor. Analysis of the crystal structure will be necessary to determine whether this hydrophobic residue serves as a lipid anchor in the intact form of hCLR.

It has been shown that CGRP-receptor component protein (RCP), an intracellular peripheral membrane protein, is specifically required for CLR/RAMP signal transduction [5,22]. In mouse NIH3T3 cells endogenously expressing RCP, CLR and RAMP1 or -2, RCP co-immunoprecipitates with CLR and appears to assist CGRP and AM receptor coupling to Gs [5,22]. However, nothing is known about the CLR domains responsible for the interaction with RCP, the mechanism by which RCP couples CLR to the cellular signal transduction pathway, or the role of RCP in vivo. In future experiments, it will be important to clarify whether RCP interacts with the three hCLR point mutants, E430A, R437A and W439A, all of which significantly reduced AM signaling when co-expressed with hRAMP2.

We also found that the E430A and V431A mutations significantly enhanced AM-induced internalization of the mutant receptor complexes, whereas the other mutations had little effect on internalization. As mentioned above, E430A mutation markedly reduced cAMP responses, and V431A also reduced the responses by ~20%. Taken together, these results suggest that internalization of hAM_1 receptors does not depend on Gs coupling, which is consistent with our earlier findings obtained using various C-tail deletion mutants [13]. Our present results are also supported by a recent report showing that introduction of a dileucine into helix 8 of the formyl peptide receptor (a family A GPCR) significantly enhances receptor internalization [27].

We previously showed that four GPCR kinases (GRK-2, -3, -4 and -5) bind to the Ser/Th-rich regions distal to putative hCLR helix 8, and that overexpression of these GRKs significantly enhanced internalization of the hAM_1 receptor in HEK-293 cells expressing endogenous GRKs 2–6 [13]. The helix 8 region of GPCRs moves significantly upon receptor activation [1,26,34], making it a candidate for recognition by GRKs. Perhaps the conformation of the E430A and V431A hCLR mutants favors interaction with intracellular binding partners such as GRKs.

In conclusion, we have shown that within putative hCLR helix 8, the strictly conserved Glu430 residue is crucial for Gs coupling, and that the strictly conserved Arg437 and Trp439 residues are involved in both cell surface expression of the hAM_1 receptor and Gs

coupling. We also suggest that the Glu430–Val431 sequence participates in the negative regulation of hAM_1 receptor internalization, which is not dependent on Gs coupling.

Acknowledgements

This study was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports Science and Technology, Japan and by research grants from the Takeda Science Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research and the Suzuken Memorial Foundation.

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Peptides 32 (2011) 1540–1550

Contents lists available at ScienceDirect

Peptides

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Shared and separate functions of the RAMP-based adrenomedullin receptors

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ARTICLE INFO

Article history:
Received 11 May 2011
Received in revised form 20 May 2011
Accepted 20 May 2011
Available online 27 May 2011

Keywords:
Adrenomedullin
Adrenomedullin 2
Calcitonin-receptor-like receptor
Receptor activity-modifying protein
Genetically engineered mouse
Cardiovascular diseases

ABSTRACT

Adrenomedullin (AM) is a novel hypotensive peptide that exerts a variety of strongly protective effects against multiorgan damage. AM-specific receptors were first identified as heterodimers composed of calcitonin-receptor-like receptor (CLR), a G protein coupled receptor, and one of two receptor activity-modifying proteins (RAMP2 or RAMP3), which are accessory proteins containing a single transmembrane domain. RAMPs are required for the surface delivery of CLR and the determination of its phenotype. CLR/RAMP2 (AM_1 receptor) is more highly AM-specific than CLR/RAMP3 (AM_2 receptor). Although there have been no reports showing differences in intracellular signaling via the two AM receptors, *in vitro* studies have shed light on their distinct trafficking and functionality. In addition, the tissue distributions of RAMP2 and RAMP3 differ, and their gene expression is differentially altered under pathophysiological conditions, which is suggestive of the separate roles played by AM_1 and AM_2 receptors *in vivo*. Both AM and the AM_1 receptor, but not the AM_2 receptor, are crucial for the development of the fetal cardiovascular system and are able to effectively protect against various vascular diseases. However, AM_2 receptors reportedly play an important role in maintaining a normal body weight in old age and may be involved in immune function. In this review article, we focus on the shared and separate functions of the AM receptor subtypes and also discuss the potential for related drug discovery. In addition, we mention their possible function as receptors for AM2 (or intermedin), an AM-related peptide whose biological functions are similar to those of AM.

Contents

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doi:10.1016/j.peptides.2011.05.022

Table 1
Representative biological actions of AM.

Main actions	Reference
Stimulation of intracellular cAMP	[64,80]
Intracellular Ca^{2+} mobilization	[64,80]
Stimulation of nitric oxide (NO) production	[50,64]
Reduction of blood pressure (Vasodilation)	[20,68,132]
Reduction of vascular and cardiac oxidative stress	[90,117]
Protection from vascular hypertrophy and inflammation	[64,82]
Inhibition of vascular apoptosis	[64]
Inhibition of left ventricular hypertrophy	[20,139]
Inhibition of left ventricular remodeling	[90]
Promotion of diuresis and natriuresis	[33,93]
Inhibition of aldosterone secretion	[143,144]
Induction of angiogenesis	[57,88]
Induction of lymphangiogenesis	[37,61]

1. Introduction

Adrenomedullin (AM) and AM2 (also known as intermedin; IMD) belong to the calcitonin (CT)/CT gene-related peptide (CGRP) family [108,114,131,141]. The members of this family share very little sequence homology; only 18 amino acids are conserved between AM and AM2 (Fig. 1). However, each member contains two common structures necessary for their biological activity: a ring structure comprising six amino acids linked by a disulfide bridge and an amidated C-terminus (Fig. 1) [108,141]. AM is a 52 amino acid peptide first identified in human pheochromocytoma tissue extracts as a highly potent vasodilator comparable to CGRP [68]. Its gene expression and binding sites are widely distributed in peripheral tissues and in the central nervous system, which enables AM to exert a variety of biological effects (Table 1) [13,33,59,64,68,81,93,117,141]. Most of these effects are strongly protective against multiorgan damage induced by hypertension, oxidative stress, atherosclerosis, ischemia and sepsis. Notably, the angiogenic properties of AM support blood flow to ischemic tissue, including tumor tissue [58]. Therefore, high-affinity AM antagonists or antibodies could become effective therapeutic tools for the prevention of tumor angiogenesis and progression [28,47,92].

Genomic screens that were conducted by two independent research groups identified a novel AM-related peptide that was named AM2 (or IMD) [114,130]. Although this peptide has not yet been isolated from mammalian tissue extracts and there have been no reports on the effects of an AM2 gene knockout, the biological actions of exogenous AM2 are similar to those of AM [129,130]. Interestingly, several studies suggest that the involvement of AM2 in some pathophysiological processes differs from that of AM [51,129].

The discovery of three receptor activity-modifying proteins (RAMP1, RAMP2 and RAMP3) led to the identification of the human CGRP and AM receptors [84]. These accessory proteins enabled the then-orphan CT receptor-like receptor (CLR), which is a family B G protein coupled receptor (GPCR), to function as the CGRP receptor (CLR/RAMP1) or as the AM_1 or AM_2 receptor (CLR/RAMP2 and CLR/RAMP3, respectively) [84]. The three RAMPs are each composed of about 160 amino acids, and all exhibit a common structure that includes a large extracellular N-terminal domain (ECD), a single transmembrane domain (TM), and a very short cytoplasmic C-terminal tail (C-tail); however, they share less than 30% sequence homology [46,84,115]. Although RAMPs are ubiquitous throughout the body, there are differences in their tissue distributions, and the abundance of each isoform depends on the tissue type [100,107,115,142]. In addition, RAMP gene expression is differentially regulated under various disease conditions in animal models [46,73]. Along with AM, the AM_1 receptor facilitates the development of the fetal cardiovascular system and exerts protective effects against various vascular diseases [37,39,57,82,132]. The AM_2 receptor evidently does not mediate the major effects of AM, although there have been no reports showing the differences in intracellular signaling pathways between the two AM receptors [8].

AM2 can also interact with the CGRP, AM_1 and AM_2 receptors and, as with AM, elicit cAMP production, but the responses appear to be smaller than are seen with AM [19,114,130]. It is therefore still controversial whether the three CLR/RAMP complexes are native receptors for AM2. Interestingly, AM2, but not AM, inhibits growth hormone release from rat anterior pituitary cells [133], which suggests there is a unique AM2 receptor that is unrelated to the CLR/RAMP heterodimers.

2. Structure and function of AM, its related peptides and their peptide antagonists

The members of the CGRP family are peptides composed of 32–52 amino acids, and their receptors are all GPCRs [108] that mediate agonist-induced cAMP production, which is indicative of their G_S coupling [76]. AM is composed of 52 highly conserved amino acids, the sequence of which contains only 1–3 substitutions in humans, pigs, dogs and cows [67]. As compared to the human peptide (hAM), rat (r)AM and mouse (m)AM lack 2 resides at their N-termini, and there are 6 amino acid substitutions in each [67]. However, all of these AM peptides share a ring structure comprising 6 amino acids linked by a disulfide bridge and an amidated C-terminal Tyr residue, both of which are necessary for receptor binding and subsequent signaling [31]. In fact, these 2 structural features are common to all CT/CGRP family peptides, despite their low homology at the level of the primary sequence (Fig. 1) [67,108,141]. The 15 N-terminal residues of hAM are not essential for interaction with its receptor (compared to the hCGRP N-terminus) [31]. In addition, amidation of the C-terminal residue of hAM is more important than the amidation of Tyr52 *per se* [31]. When hAM point mutations in which the 5 residues between Cys16 and Cys21 were separately substituted with Ala were intravenously injected into rats, only Phe18Ala and Thr20Ala significantly impaired the peptide's hypotensive effects, as compared to wild-type hAM. Moreover, both of these residues are completely conserved among the aforementioned 6 species, and

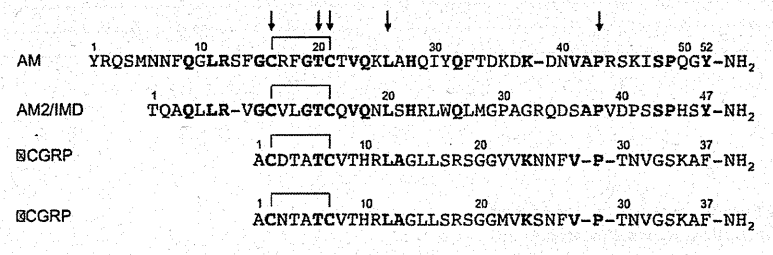

Fig. 1. Amino acid sequence alignment of human adrenomedullins (AM and AM2/IMD) with CGRPs (αCGRP and βCGRP). The human sequences are aligned for maximum homology [113]. *Arrows* indicate conserved amino acids among the AMs and CGRPs. *Bold* residues are conserved in AM.

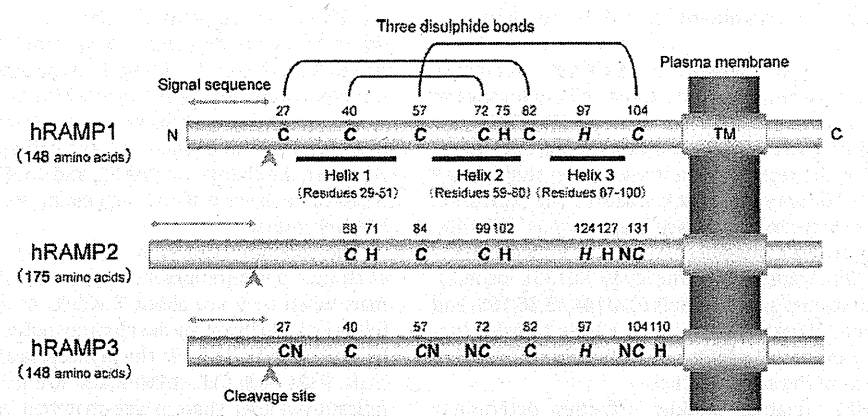

Fig. 2. Schematic representation of the structure of the three human RAMPs. Each human (h)RAMP possesses a large extracellular N-terminal domain (ECD) with a signaling sequence and cleavage site, a single transmembrane (TM) domain and a very short cytoplasmic C-terminal tail. The three hRAMPs share ~30% sequence homology, and RAMP2 is 27 amino acids longer than RAMP1 and RAMP3. Recent crystal structural analysis of the hRAMP1 ECD revealed the presence of three α-helices (helix 1, 2 and 3) and three disulfide bonds (Cys27–Cys82, Cys40–Cys72, and Cys57–Cys104) [71]. The four Cys(C) residues and one His (H) residue are conserved among all three RAMPs (shown in *bold italic*); the N-terminal glycosylation sites (N; Asn) are also shown. Note that the number of His residues is the smallest among the amino acids making up each RAMP.

Thr20 in hAM is also strictly conserved among the CT/CGRP family peptides (Fig. 1) [67,108,130]. Interestingly, Phe18 in hAM is less well conserved; it is substituted by Leu in hAM2 and hCT and by Thr in human αCGRP, βCGRP and amylin, suggesting that these residues may contribute to the determination of agonist specificity.

A C-terminal hAM fragment (residues 22–52; hAM_{22-52}) lacking the disulfide bond has been shown to act as a selective AM receptor antagonist [31]. This first report showed that the binding affinity of hAM_{22-52} was ~70-fold lower than that of hAM in cultured rat vascular smooth muscle cells (VSMCs), which express AM-specific receptors [31,32]. Similarly, the N-terminally truncated peptide $\alpha CGRP_{8-37}$ has been used as a selective CGRP receptor antagonist since 1989 [23,25]. Among CGRP peptides, αCGRP was first cloned from the CT gene [4], while βCGRP was discovered to be a second CGRP analog derived from its own unique gene [123]. Nevertheless, hβCGRP differs from hαCGRP by only three amino acids (Fig. 1), and the two display similar biological activities. Both hAM_{22-52} and $h\alpha CGRP_{8-37}$ have also been used to investigate the function of AM2. So far, there have been no reports of an AM2 peptide antagonist (e.g., $AM2/IMD_{16-47}$).

Circular dichroismspectroscopic analysis showed that the secondary structures of AM, AM2 and αCGRP differ considerably [113]. The α-helical contents of AM, AM2 and αCGRP are 31%, 51% and 80%, respectively. AM_{22-52} contains 31% α-helix and 12% β-sheet, which is identical to AM. By contrast, $\alpha CGRP_{8-37}$contains 61% less α-helical content than αCGRP, which leads to a compensatory increase in β-sheets (from 0% to 32%). Thus, the absence of the N-terminus, including the ring structure, produces a large change in the secondary structure of αCGRP but not AM.

3. Discovery of the three RAMPs and their structural characterization

Since its discovery, AM has been shown to share a number of pharmacological features with α- and βCGRP. Indeed, many effects of AM are blocked by $\alpha CGRP_{8-37}$ as well as by AM_{22-52} [41,73,89,94,137]. For these reasons, AM is believed to bind to its specific receptors, as well to receptors that bind both CGRP and AM.

The three hRAMPs were initially identified as chaperones promoting the forward trafficking of CLR from the endoplasmic reticulum to the cell surface [84]. Once at the cell surface, each RAMP governs the expression of the CLR phenotype: CLR/RAMP1, CLR/RAMP2 and CLR/RAMP3 form functional CGRP, AM_1 and AM_2 receptors, respectively [44,73,84,86,108]. The differences in the pharmacology of these three receptors are described in the next section.

Schematic diagrams showing the basic structures of the three hRAMPs are presented in Fig. 2. As mentioned earlier, all three possess a large ECD (91 amino acids in hRAMP1 and hRAMP3, 102 amino acids in hRAMP2), a single TM domain (22 amino acids), and a very short C-terminal tail (9 amino acids) [46,84,110]. There is also a predicted signal peptide (26 amino acids in hRAMP1, 44 amino acids in hRAMP2, and 27 amino acids in hRAMP3) [46,110]. Overall, the three hRAMPs share only ~30% sequence identity overall and~32% identity in their TM regions.

In 2008, Kusano et al. [71] reported the first crystal structure of the hRAMP1 ECD (residues 22–112) in the absence of hCLR. The hRAMP1 ECD is composed of three α-helices (α-helix 1, 2 and 3) that are stabilized by 3 disulfide bonds (Cys27–Cys82, Cys40–Cys72 and Cys57–Cys104). As shown in Fig. 2, the four Cys residues at positions 40, 57, 72 and 104 are strictly conserved among hRAMPs [46]. In hRAMP1 and hRAMP2, these conserved residues are required for the efficient transport of hCLR to the cell surface [79,119,120,124]. The remaining two Cys residues within the hRAMP1 ECD (Cys27 and Cys82), which are conserved in hRAMP3 but not hRAMP2, are not involved in the cell surface expression of hCLR or in CGRP potency [120]. There have been no studies examining the effects of substituting any or all of these 6 Cys residues in the hRAMP3 ECD. Interestingly, the hRAMP1 ECD possesses no putative N-terminal glycosylation sites (Asn residues), while the hRAMP2 ECD contains 1 Asn residue, and the RAMP3 ECD contains four Asn residues. Note that all of these Asn residues are located next to highly conserved Cys residues [46], although the function in the AM_1 and AM_2 receptors remains unclear.

Recently, ter Haar et al. [134] reported the crystal structure of an unbound heterodimer composed of the CLR ECD and the RAMP1 ECD. Within this structure, α-helices 2 and 3 of RAMP1 interact with the N-terminal α-helix of CLR. Surprisingly, this heterodimerization induces no significant conformational rearrangement of the RAMP1ECD structure. But this report contains no information on the crystal structures of other regions of RAMP1 and CLR or the effect of ligand binding. Consequently, the full extent of RAMP1-CLR interactions remains unknown, as does whether the formation of the CLR/RAMP1 dimer causes a conformational change in the subunits.

4. The pharmacology of recombinant CLR/RAMP complexes

The pharmacological characteristics of CLR/RAMP complexes have been extensively studied after their transfection into several cultured cell lines, including human embryonic (HEK)-293 cells, African monkey kidney (COS-7) cells and Chinese hamster ovary (CHO) cells. Although the results of the transfection studies may have been affected by factors such as the cell source, the expression levels of endogenous receptors and intracellular proteins [135,140], passage number (e.g., continuous cell passage may reduce levels of the target protein) [24], transfection efficacy [5,74,135], inconsistencies in the animal species and materials used [44,45,86,108] and the position of ligand iodination [74], there is little transfection-dependent variation in the selectivity of recombinant CLR/RAMP complexes derived from the same species.

For hCLR/hRAMP1, relative binding affinities determined based on the competitive inhibition of ^{125}I-CGRP binding are as follows: βCGRP $\geq$ αCGRP > αCGRP$_{8-37}$ > AM $\gg$ AM$_{22-52}$ [2,3,84]. Similar results are obtained with cells expressing rCLR/rRAMP1 [100]. However, with a combination of rCLR and mRAMP1, the affinity rαCGRP$_{8-37}$ binding is ~3-fold greater than rαCGRP binding [56]. In cells expressing hCLR/hRAMP2, the rank order of inhibition of ^{125}I-AM binding is as follows: AM > AM$_{22-52}$ > hαCGRP$_{8-37}$ $\geq$ hβCGRP > hαCGRP [2,36,84]. This relation is also seen in cells expressing the exogenous rodent AM$_1$ receptor [56,100]. The relative binding affinity for recombinant hCLR/hRAMP3 is AM > AM$_{22-52}$ = hαCGRP$_{8-37}$ = hβCGRP > hαCGRP [2,36]. Notably, in cells transfected with rCLR/mRAMP3, the binding affinity of rαCGRP$_{8-37}$ is more than or equal to rAM [56].

Only CLR/RAMP2 (AM$_1$ receptor) acts as a highly AM-specific receptor and is particularly sensitive to hAM$_{22-52}$ or rAM$_{20-50}$, irrespective of animal species. In contrast, CLR/RAMP3 (AM$_2$ receptor) cross-reacts with CGRP peptides at lower concentrations and is more sensitive to αCGRP$_{8-37}$ than the AM$_1$ receptor. With the rodent AM$_2$ receptor, in particular, AM binding is more effectively blocked by αCGRP$_{8-37}$ than by hAM$_{22-52}$ or rAM$_{20-50}$, which is not the case with the hAM$_2$ receptor. At higher concentrations, AM, but not hAM$_{22-52}$orrAM$_{20-50}$, can also interact with CLR/RAMP1 (CGRP receptor); indeed, there have been several reports on the effect of αCGRP$_{8-37}$ on AM-mediated responses via the recombinant CLR/RAMP1 complex [1,24,55,85,89]. However, there is no evidence that the CGRP receptor functions as a αCGRP$_{8-37}$-sensitive AM receptor *in vivo*.

AM2 also reportedly activates the three CLR/RAMP complexes, but its affinity and selectivity remain unclear [42,109,114,130]. In contrast to AM, the pharmacology of AM2 has not yet been well explored. We therefore examined the effect of hAM2 on cAMP responses using recombinant human AM$_1$ and AM$_2$ receptors expressed in HEK-293 cells. As shown in Fig. 3, this peptide exerted potent effects via both AM receptors: AM > AM2/IMD > αCGRP for AM$_1$ receptors and AM = AM2/IMD > αCGRP for AM$_2$ receptors. Hay et al. obtained similar results with COS-7 cells and also showed that AM2 can activate the CGRP receptor (CLR/RAMP1) with the same potency as AM [47]. These findings support the idea that many effects of AM2 are similar to those of AM, although there is evidence of a unique, unidentified AM2 receptor in rat anterior pituitary cells [133].

5. Pathophysiological functions of endogenous AM receptors in experimental rat models

There is now extensive evidence that AM and AM2 are both potent cardiorenoprotective mediators [8,13,19,51,59,64,93]. Upon their release, these peptides are well known to act locally as autocrine/paracrine regulators. Changes in the tissue expression of mRNAs encoding AMs and their receptors have been examined under various pathophysiological conditions using experimental rat models. As shown in Table 2, tissue expression of AMs and their receptor components (CLR and RAMP2 and RAMP3) is upregulated in various cardiorenal disease states, suggesting that AMs act via their receptors to compensate for pathophysiological conditions. However, the changes in RAMP2 and RAMP3 mRNA expression differ in some disease states, suggesting separate roles for AM$_1$ and AM$_2$ receptors.

The spontaneously hypertensive rat (SHR) is a useful model of chronic pressure loading. Hypertension develops in these animals when they are about 7 weeks of age (young adult) and is followed initially by the development of compensated left ventricular hypertrophy (LVH). In the myocardia and aortas of 11-week-old male SHRs, AM, CLR, RAMP2 and RAMP3 gene expression is significantly higher than in age-matched Wistar-Kyoto (WKY) rats (control) [104]. Moreover, AM gene delivery significantly reduces blood pressure and the extent of LVH in 11-week-old SHRs [20], and AM attenuates angiotens in II-induced cardiomyocyte hypertrophy *in vitro* [139]. By 20 weeks, however, left ventricular levels of AM and RAMP3 mRNAs are no longer elevated, despite the presence of myocardial concentric hypertrophy and oxidative stress. By contrast, robust induction of AM2 and RAMP3 gene expression is observed in the hypertrophied left ventricle [9]. Expression of neutral endopeptidase is known to be diminished in left ventricular tissue from 10-week-old SHRs. Downregulation of this enzyme may therefore have resulted in the augmented expression of AM2.

Chronic inhibition of nitric oxide (NO) synthesis through treatment with N^G-nitro-L-arginine-methyl-ester (L-NAME) also leads to hypertension and myocardial hypertrophy, ischemia and oxidative stress [14]. In the hypertrophied left ventricle of L-NAME-treated rats, expression of mRNAs for the two AMs and their two receptors is significantly augmented, and notably, the levels of AM2 mRNA are much higher than those of AM mRNA [10–12,145]. This suggests AM2 may play a more prominent role than AM as a local endogenous negative feedback regulator of cardiac hypertrophy. Interestingly, two antioxidants (Vitamin C and Tempol) normalized the augmented expression of myocardial AM2 and CLR (along with RAMP1) without affecting blood pressure in this model, but the expression of AM, RAMP2 and RAMP3 were unaffected. This suggests that induction of AM2 and CGRP receptor expression is influenced to a much greater extent by cardiac oxidative stress than by pressure loading [10]. Hypertension induced by L-NAME also elevates levels of mRNAs for AM and its receptor components in the aorta [105].

In the Dahl salt-sensitive (DS) rat model, systemic hypertension causes LVH by the age of 11 weeks and then heart failure by17 weeks [96]. The levels of mRNAs for AM and its two receptors in the left ventricle of DS rats were significantly increased at both the LVH stage and heart failure stage, as compared to age-matched Dahl salt-resistant (DR) rats [93]. Similar increases in the mRNA expression of AM and its receptors were also observed in the hypertrophic left ventricle in the deoxycorticosterone acetate (DOCA)-salt model, another rat model of malignant hypertension [127]. Thus, AM and both AM$_1$ and AM$_2$ receptors are upregulated in the hypertrophied left ventricle in various hypertensive rat models, but it remains unclear whether the behavior of the receptors differ depending the pathophysiological state.

In rats with congestive heart failure induced by myocardial infarction (MI; resulting from left coronary artery ligation), mRNA expression of the two AMs (AM2 > AM) and the two AM receptors was increased in both the non-infarcted and infarcted regions of the left ventricle, although the induction was significantly greater in the infarcted areas [52]. Induction of AM signaling may be beneficial because early infusion of AM (for a week) markedly inhibits left ventricular remodeling after MI in rats [90]. In this MI model, atrial

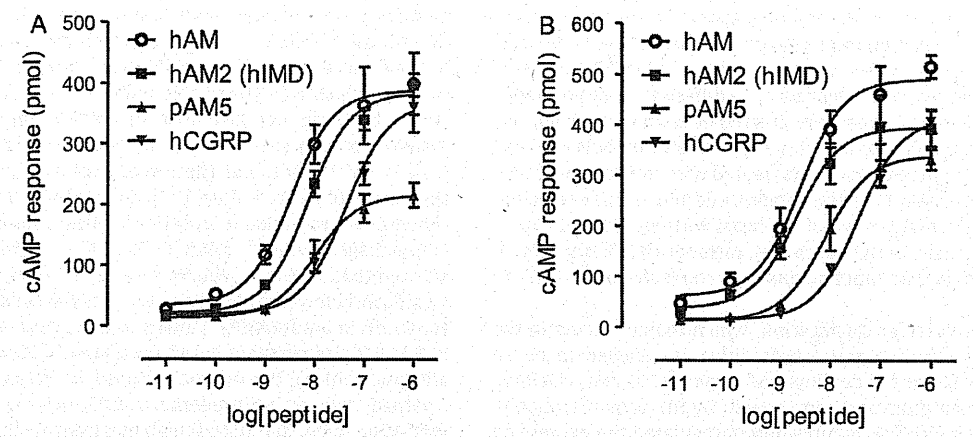

Fig. 3. AM-evoked cAMP production in HEK-293 cells transiently overexpressing hCLR/hRAMP2 (A) or hCLR/hRAMP3 (B). Following transient transfection, the cells were incubated for 15 min at 37 °C in Hanks' buffer with 20 mM HEPES, 0.2% BSA, 0.5 mM 3-isobutyl-1-methylxanthine and the indicated concentrations of hAM, hAM2, haCGRP or porcine (p)AM5 and were then lysed. The cell lysates were then analyzed for intracellular cAMP content using a commercial enzyme immunoassay kit. None of the agonists elicited cAMP production in HEK-293 cells transfected with empty vector up to a concentration of 10^{7} M (data not shown). Symbols represent the means ±S.E. from three separate identical experiments. Data were analyzed using Prism 5.02 (GrapghPad Software Inc., San Diego, CA, USA).

RAMP3 mRNA levels were unaffected, despite significantly elevated levels of AM and AM2 mRNA [52]. In addition, no increases in the expression of AM receptor mRNA were seen in the right ventricles of the MI rats, although AM and AM2 mRNAs were both significantly increased (AM2 > AM) [52]. Renal expression of mRNA for AMs and their receptors was not upregulated during heart failure in MI rats [52], despite the fact that intra-renal infusion of AM or AM2 causes diuresis and natriuresis without a significant reduction in systolic blood pressure in rats [33,38,93]. Interestingly, severe MI-induced heart failure diminished the expression of mRNA encoding the AM_1 receptor but not the AM_2 receptor in the rat lung. As a result, the numbers of pulmonary AM receptors were greatly reduced, whereas pulmonary AM mRNA and peptide expression were greatly increased [98]. In fact, the release of AM from the lungs into circulation has been observed in patients with severe systolic dysfunction [98]. Alveolar macrophages are likely the main source of increased AM expression in the lungs, as their expression of AM mRNA is substantially elevated in rats and humans with severe heart failure [98]. The elevated AM levels may limit pulmonary infiltration of neutrophils because AM inhibits the cytokine-induced secretion of neutrophil chemoattractant from rat alveolar macrophages *in vitro* [62].

Hemodynamic stress and angiotensin II are both key mediators of cardiac hypertrophy induced by pressure overload (POL; by aortic banding) or volume overload (VOL; by aortocaval shunt). Angiotensin II, rather than hemodynamic stress, is reportedly a critical stimulator of the left ventricular mRNA expression of AM and its receptors in rat POL and VOL models [101]. In this report, acute POL increasedAM_1 and AM_2 receptor mRNA expression, whereas acute VOL increased only AM_1 receptor mRNA; moreover, the induction

Table 2
Changes in gene expression of AM and its receptor components under various disease conditions in rat models.

Pathology	Experimental model	Tissue	AM	AM2	CLR	RAMP2	RAMP3	Reference
Hypertension with cardiac hypertrophy	+ Chronic pressure loading (SHRs, 11-week-old)	Myocardium	↑	ND	↑	↑	↑	[104]
		Aorta	↑	ND	↑	↑	↑	[104]
	+ Chronic pressure loading (SHRs, 20-week-old)	Left ventricle	→	↑	→	→	↑	[9]
		Right ventricle	→	→	→	→	→	[9]
	+ L-NAME (SD rats)	Left ventricle	↑	↑	↑	↑	↑	[11,12,145]
		Right ventricle	→	↑	→	→	→	[145]
		Aorta	↑	ND	↑	↑	↑	[145]
	+ 8% NaCl (DS rats, 11-week-old)	Left ventricle	↑	ND	↑	↑	↑	[93]
Malignant hypertension	+ DOCA-salt (SHRs)	Left ventricle	↑	ND	↑	↑	↑	[127]
Heart failure	+ 8% NaCl (DS rats, 18-week-old)	Left ventricle	↑	ND	↑	↑	↑	[93]
	+ Myocardial infarction	Left ventricle	↑	↑	↑	↑	↑	[52]
		Right ventricle	↑	↑	→	→	→	[52]
		Atrium	↑	↑	↑	↑	→	[52]
		Lung	↑	ND	↓	↓	→	[98]
	+ Aortic banding	Left ventricle	↑	ND	↑	↑	↑	[62]
	+ Aortocaval shunt	Left ventricle	↑	ND	↑	↑	↑	[62]
Cardiomyopathy	+ Isoproterenol	Myocardium	↑	ND	↑	↑	↑	[60,112]
Cardiovascular calcification	+ Vitamin D_3 plus nicotine	Myocardium	↑	ND	↑	↑	↓	[106]
		Aorta	↑	ND	↑	↑	↑	[106]
Renal failure	+ 5/6 nephrectomy	Kidney	→	ND	↓	→	↓	[138]
Nephropathy	+ Ureteral obstruction	Obstructed kidney	→	ND	↑	↑	→	[87]
Diabetes	+ Streptozotocin (STZ)	Kidney	↑	ND	ND	↑	→	[50]
Salt loading	+ 8% NaCl (Wistar rats)	Adrenal gland	↑	ND	↑	↑	→	[17
		Kidney	↑	ND	↑	→	↑	[17]
Sepsis	+ Lipopolysaccharide	Lung	↑	ND	↓	↓	↑	[102]

Abbreviations: AM, adrenomedullin; CLR, calcitonin-receptor-like receptor; RAMP, receptor activity-modifying protein; SHR, spontaneously hypertensive rat; L-NAME, N^{G}-nitro-L-arginine methyl ester; SD, Sprague–Dawley; DS, Dahl salt-sensitive; DOCA, Deoxycorticosterone acetate; ND, not determined.

of AM receptor mRNAs in POL models appears to be greater than in VOL models. These findings suggest that the effect of VOL on RAMP3 transcription differs from its effect on RAMP2 transcription and that the two AM receptors have different functions under the pathophysiological conditions created by cardiac overload. Significant induction of both AM_1 and AM_2 receptor mRNAs was also observed in the myocardium of rats treated with isoproterenol, a β-adrenergic agonist [60,112]. Isoproterenol administration is known to cause marked enlargement of the heart with myocardial hypertrophy and necrosis. In this rat model, intraperitoneal injection of AM2 improved cardiac function and prevented myocardial injury [60].

Cardiac and vascular calcification, which mainly occurs in the aorta, coronary arteries and myocardium, is believed to be an important risk factor for cardiovascular events. In rats, calcification of the myocardium and aorta caused by the administration of Vitamin D_3 plus nicotine significantly upregulated the expression of AM and AM_1 receptor mRNAs [106]. Notably, myocardial and aortic calcification had the opposite effect on RAMP3 mRNA [106].

Local levels of RAMP2 and -3 mRNAs are also affected by renal failure. Expression of CLR and RAMP3 mRNAs is downregulated in the remnant kidney after 5/6 nephrectomy, which causes acute renal failure, although expression of RAMP2 mRNA is unaltered [138]. CLR and RAMP2 mRNAs, but not RAMP3 mRNA, are upregulated in kidneys with ureteral obstruction, despite demonstrating no change in the expression of AM mRNA [87]. The upregulation of AM_1 receptors might provide counter-regulatory actions against the proliferative and/or fibrotic changes in the obstructed kidney. Streptozocin (STZ)-induced diabetic rats show upregulated expression of AM and RAMP2 mRNAs, but not RAMP3 mRNA, in hypertrophied glomeruli and in afferent arterioles and enhanced urinary excretion of nitric oxide (NO_2^- and NO_3^-), probably due to AM stimulation [50]. In this model, adenovirus-mediated AM gene transfer improved cardiac function and prevented renal damage [30]. In addition, chronic salt loading led to the increased expression of AM and CLR mRNAs in rat adrenal glands and kidneys, without a significant rise in blood pressure [17]. In that experiment, RAMP1 and -2 mRNAs were upregulated in the adrenal glands, while RAMP3 mRNA was increased in the kidneys. In addition to its diuretic and natriuretic effects, AM can also inhibit angiotensin II- or potassium-induced aldosterone secretion from the adrenal glands [143,144]. Following salt loading, some secondary mediators may differentially regulate AM receptor expression to restore water and electrolyte balance.

During sepsis, which induces the most conspicuous increases in both circulating and local AM levels in both humans and rats [83,97], mRNA expression of CLR and the three RAMPs is markedly downregulated in a number of tissues [102]. This most likely reflects substantial increases in the levels of various agonists, cytokines or both. Most notably, RAMP3 mRNA in the lung, spleen and thymus show accelerated upregulation during the late stage of sepsis [102], suggesting that RAMP3 may be involved in some aspect of immune function *in vivo*.

Taken together, the findings summarized in this section suggest that the shared and separate functions of the AM_1 and AM_2 receptors in disease are dependent on their location *in vivo* and the pathophysiological conditions.

6. Knockout mouse models for RAMP2 or RAMP3

Two research groups have now characterized homozygous and heterozygous RAMP2 knockout (KO) mice (*RAMP2*$^{-/-}$ and *RAMP2*$^{-/+}$, respectively) [26,37,57]. *RAMP2*$^{-/-}$ embryos die *in utero* by mid-gestation after developing severe interstitial edema due to abnormalities in their vascular development [37,57]. Similar findings were obtained with homozygous AM [18,118] and CLR [27] KO mice. *RAMP2*$^{-/-}$ mice generated by Ichikawa-Shindo et al. [57] exhibited vascular fragility that resulted in severe hemorrhaging, which was observable under the skin and within organs. Severe bleeding was also seen in *AM*$^{-/-}$ embryos [118], but the systemic edema seen in *RAMP2*$^{-/-}$ mice was much more severe than in *AM*$^{-/-}$ mice, and there was excessive pericardial effusion suggestive of heart failure [57]. By contrast, Fritz-Six et al. [37] showed the remarkable absence of heart failure and embryonic hemorrhage in *AM*$^{-/-}$, *RAMP2*$^{-/-}$ and *CLR*$^{-/-}$ mice [37]. They also showed that, in *AM*$^{-/-}$, *RAMP2*$^{-/-}$ and *CLR*$^{-/-}$ embryos, there were significantly fewer proliferative endothelial cells in the lymph sacs. However, in adult *RAMP2*$^{-/+}$ mice, which show reduced expression of RAMP2 and elevated blood pressures (~10 mmHg higher than wild-type mice), the edema induced in various disease models (footpad, skin or brain edema model) was more severe than in wild-type mice, due to vascular hyperpermeability and impaired neovascularization [57]. Human dermal lymphatic microvascular endothelial cells (HLMVECs) strongly express AM, CLR and RAMP2 mRNAs, such that their expression is ~4-fold higher than in human umbilical vein endothelial cells (HUVECs) [37]. AM promoted cell proliferation and migration as well as network formation in cultured HLMVECs. The increased cell proliferation was cAMP- and MEK/ERK-dependent, and *in vivo*, AM treatment increased the numbers of lymphatic vessels and blood vessels in injured mouse tails, thereby reducing lymphedema [61]. This suggests that the AM-AM_1 receptor system could be a novel therapeutic target for patients with secondary lymphedema. Although AM2 has also emerged as a powerful angiogenic growth factor [88,122], it remains unknown whether AM2 is required for lymphangiogenesis.

Interestingly, AM2 did not rescue the deficiency of AM in *AM*$^{-/-}$ mice. It has been proposed that *AM*$^{-/-}$ embryos die *in utero* at mid-gestation due to vascular fragility, which leads to severe edema and particularly hydrocephalus resulting from the immature blood–brain barrier (BBB) [18,118]. Indeed, AM is actively secreted from cerebral endothelial cells (CECs) [65,66], the major cellular component of the BBB, and improves the BBB function through the expression of Claudin-5 [54]. It is well known cAMP is an important second messenger in the regulation of BBB functions. AM2 has been shown to induce cAMP production in the CECs, which is comparable to AM [22]. This suggests that exogenous AM2 can interact with the same receptor as AM. In general, AM2-staining in the peripheral vascular endothelial cells is weaker than that of AM2; however, little is known about AM2 production in the CECs. There is a possibility that the CECs produce little or no AM2, despite the complete loss of the AM gene *in vivo*. Another possibility is that endogenous AM2 in the CECs may bind to another unique receptor that is not involved in the development of the BBB during fetal life. Studies of AM2 KO mice will be necessary to investigate the embryonic functions of AM2.

Surprisingly, a complete absence of RAMP3 has no affect the survival of *RAMP3*$^{-/-}$ mice, at least up to ~6 months of age [26]. Older *RAMP3*$^{-/-}$ mice (9–10 months old) weigh ~25% less than age-matched wild-type mice, but they survive to at least 18 months of age with no obvious decline in health [26]. In addition, aged *RAMP2*$^{-/+}$ and *CLR*$^{-/+}$ mice do not differ significantly in body weight from their respective wild-type littermates [26]. Although RAMP3, as with AM and AM2 [128], is strongly expressed in the proximal renal tubule, there are no obvious differences in urine volume or protein/creatinine between *RAMP3*$^{-/-}$ and wild-type mice [26].

Thus, studies of RAMP KO mice have shown that RAMP2 and RAMP3 have distinct physiological functions throughout embryogenesis, adulthood and old age, despite mediating similar AM and AM2 signaling in complexes with CLR.

7. Transgenic mouse models for RAMP2

RAMP function has also been studied using transgenic (TG) mice. So far, there have been two reports of myc epitope-tagged mouse RAMP2 (myc-mRAMP2) overexpression in smooth muscle cells induced by the mouse α-actin gene [82,132]. AM is reported to be actively secreted from cultured vascular endothelial cells and VSMCs [136], while CLR is mainly expressed in the vascular endothelium [40]. Expression of myc-mRAMP2 was identified in the smooth muscle cell layers of the aorta, stomach and urinary bladder [132], and the morphology of the aorta and mesenteric microvessels in wild-type and RAMP2 TG mice were histologically indistinguishable. Basal blood pressure and cardiac hemodynamics were also similar in wild-type and RAMP2 TG mice. However, the hypotensive effects of intravenous bolus injection of AM were significantly enhanced in conscious RAMP2 TG mice compared to wild-type mice, whereas the hypotensive effects of αCGRP did not differ between TG and wild-type mice [132]. In addition, exogenous AM more potently relaxed noradrenaline-precontracted aortic rings from RAMP2 TG mice than from wild-type mice. These responses were inhibited by AM_{22-52}, but not by $\alpha CGRP_{8-37}$, in both wild-type and TG mice [132], reflecting the higher numbers of functionally active AM_1 receptors in the aortas of RAMP2 TG mice. This is consistent with the findings that the adult rat aorta expresses RAMP2 mRNA but not RAMP3 mRNA [143].

This RAMP2 TG mouse model remains susceptible to angiotensin II-induced increases in blood pressure and cardiac hypertrophy [82]. Nevertheless, the RAMP2 TG mice were almost completely protected from aortic vascular hypertrophy and inflammation caused by chronic angiotensin II infusion [82]. Moreover, cultured VSMCs from aortic explants from RAMP2 TG mice grew more slowly than those from wild-type mice, even in the presence of angiotensin II [82], and AM_{22-52} was able to enhance angiotensin II-stimulated proliferation of RAMP2 TG VSMCs to a greater degree than wild-type cells [82]. This suggests that, in the TG cells, endogenous AM is acting more effectively to inhibit aortic VSMC proliferation, thanks to the greater numbers of functional AM_1 receptors. Thus, the overexpression of RAMP2 in VSMCs exerts a powerful protective effect against vascular hypertrophy and inflammation by enhancing the vascular response to AM. This suggests that the vascular AM-AM_1 receptor system could be an important target for novel therapeutic approaches. In addition, AM2 was recently shown to be expressed in VSMCs from human renal arterioles [128], suggesting that AM2 may also exert protective effects via the AM_1 receptors.

8. Molecular basis of RAMP ECD function

Since their discovery in 1998, the structure–function relationships of hRAMPs have been extensively investigated using various point and deletion mutants and chimeras. The hRAMP ECD is known to be the major determinant of hCLR surface delivery and agonist binding specificity [43,75,77,78,109,119], the details of which are well summarized by Qi and Hay [110]. They reported the crystal structure of the RAMP1 ECD in complex with the CLR ECD and revealed the hydrophobic and electrostatic interactions between the N-terminal α-helix of CLR and α-helices 2 and 3 of RAMP1 [134]. In addition, studies of the small molecule CGRP receptor antagonistsolcegepant (BIBN409BS6) [99] and telcagepant (MK0974) [53], which are both used in the treatment of acute migraine, form hydrophobic interactions with Trp74 in α-helix 2 of the RAMP1 ECD and with Trp72 in the CLR ECD [134]. Interestingly, however, Trp74 in hRAMP1 does not affect the affinity of αCGRP for the CLR/RAMP1 complex, suggesting that Trp74 does not participate in CGRP binding, although it may be in close proximity to the CGRP binding site. Reciprocal replacement of the equivalent residue (Glu74) in hRAMP3 with Trp (CLR/RAMP3-Glu74Trp) elicited a ~10-fold reduction in AM potency without affecting αCGRP potency in humans [43,109]. The opposite effect was seen with the reciprocal hRAMP1-Trp74Glu mutant in complex with CLR; that is, AM potency increased ~10-fold, while αCGRP potency was unaffected [109]. These findings are indicative of a direct interaction between residue 74 and AM. Subsequent analysis showed that the most important requirement for this interaction is a residue with a full negative charge at position 74 in RAMP1 or RAMP3 [111]. The CLR/RAMP1-Trp74Glu mutant also enhanced the potency of AM2 to the same degree as AM, while CLR/RAMP3-Glu74Trp slightly reduced AM2 potency. This suggests that AM2 may interact with CGRP and AM_2 receptors differently than AM. Human RAMP2 also possesses a Glu residue at the position equivalent to Glu74 (Glu101) in hRAMP3 [46]. It remains to be determined whether CLR/RAMP2-Glu101Trp behaves pharmacologically similar to CLR/RAMP3-Glu74Trp. There are another 7 residues that are conserved in hRAMP2 and hRAMP3 but not in hRAMP1 [109]. However, these residues (Glu35, Asp46, Pro87, Leu88, Ala89, Ile93 and Asn103) in hRAMP3 are not involved in AM binding to the AM_2 receptor [109,110].

So far, Glu74 is the only residue in hRAMP3 known to be involved in AM binding. However, recent data [111] suggest that this residue does not play a key role in determining the selectivity of agonist binding (AM vs. CGRP) to AM_2 and CGRP receptors. In many family B GPCRs, agonist specificity is primarily associated with the ECD, with secondary recognition by a TM domain; the N-terminal and C-terminal portions of the agonist are recognized by the TM and ECD, respectively [121]. This "two domain model" is thought to apply to CLR/RAMP complexes because the N-terminus of CLR is known to be important for agonist binding [6,7,21,69,70]. It is noteworthy that the CLR N-terminus contains a sequence that contributes to the selective interaction of AM with the AM_1 receptor but not CGRP with its receptor [69,70]. How then do the three RAMP ECDs critically govern the CGRP vs. AM specificity of CLR? An earlier cross-linking study showed that ^{125}I-αCGRP is incorporated into RAMP1, while ^{125}I-AM is incorporated into RAMP2 or RAMP3 [49]. This suggests that the three RAMPs lie close to the agonist binding pocket within the CLR/RAMP complex. From the analysis of the crystal structure of hRAMP1 ECD, it was predicted that Arg67, Asp71, Trp74, Glu78 and Trp84, which, except for Trp84, are all situated in α-helix 2, comprise part of the agonist binding pocket [71]. Subsequent analysis revealed that the formation of a heterodimer with the RAMP1 ECD does not cause a significant conformational rearrangement of the structural features of the CLR ECD [134]. Additional information on the crystal structure of the entire CLR protein in complex with RAMP1 will be needed to clarify whether RAMP1 confers CGRP vs. AM selectivity through the allosteric modulation of the conformation of CLR or by directly contributing to the structure of the agonist binding pocket.

The affinity of CGRP for the AM_2 receptor is at least 10-fold greater than for the AM_1 receptor. It has been suggested that the CGRP vs. AM selectivity of the RAMPs may be regulated in part by the disulfide bond linking Cys27 and Cys82, which is strictly conserved in RAMP1 and RAMP3 but not RAMP2. That is, this bond may create a putative CGRP binding domain. It should be noted that several amino acid residues close to these Cys residues are also highly conserved in RAMP1 and RAMP3, but not RAMP2, and that the N-terminus of the mature RAMP2 sequence is ~20 residues longer than those of the other 2 RAMPs. This sequence variation likely contributes to the different pharmacological features of the AM_1 and AM_2 receptors.

Among the amino acid residues making up RAMPs, His residues exhibit the lowest content percentage; hRAMP1, -2 and -3 possess 2, 4 and 2 His residues, respectively, all of which are located in their ECDs (Fig. 2) [46]. A RAMP1-His71Ala mutant mediated nor-

mal CLR surface delivery, but the resultant heterodimers showed significantly diminished AM binding and potency [77]. It is therefore likely that His71 of RAMP1 is directly or indirectly involved in AM binding, although its contribution appears minor. Expression of RAMP2-H is 124Ala and -His127Ala leads to poor surface expression of CLR, thereby abolishing AM binding and signaling [109]. His124 in hRAMP2 is also conserved at position 97 in hRAMP1 and RAMP3 (Fig. 2). Notably, His97 in RAMP1 was identified as one of the three residues (Phe93, His97 and Phe101), all located in α-helix 3, that constitute the CLR binding interface [71]. Co-expression of CLR with RAMP1-His97Ala or RAMP3-His97Ala also reduced the cell surface expression and function of the receptors, making them less effective than the CLR/RAMP2-His124Ala receptor complex [77,78]. By contrast, RAMP2-His102Ala and RAMP3-His110Ala mutations had little effect on receptor expression or function [77].

9. Receptor trafficking regulated by the RAMP C-tail

Upon binding to their respective agonists, human CLR/RAMP complexes stably expressed in HEK-293 cells are rapidly internalized, without dissociation, via clathrin-coated vesicles [48,80]. CLR/RAMP1 receptor internalization is also reported lydynamin- and β-arrestin 2-dependent [48]. In that regard, it is well known that G protein coupled receptor kinases (GRKs) phosphorylate Ser/Thr sites located in many GPCR C-tails, enabling β-arrestins to bind at these sites [34]. Interestingly, AM_1 receptors display greater internalization than AM_2 receptors when expressed in HEK-293 cells [72], although the underlying mechanism remains unclear. Once internalized, GPCRs are often recycled back to the plasma membrane resulting in resensitization. However, unlike many GPCRs, after internalization endogenous and recombinant CLR/RAMP complexes are targeted to lysosomes where they are degraded [48,80,91]. It has been suggested that although they are short, RAMP C-tails are important for CLR surface expression, internalization and recycling [15,16,35,72,125]. Deletion of the C-tail from hRAMP2 disrupts transport of hCLR to the cell surface, leading to the significant loss of receptor function, while deleting the C-tail from hRAMP3 enhances AM-stimulated receptor internalization with no change in AM affinity or potency [72]. By contrast, deletion of the C-tail from hRAMP1 has little effect on receptor expression, function or intracellular trafficking [35,72].

RAMP C-tails may contain sites of potential interaction with other proteins [46,115]. For instance, the hRAMP3 C-tail, but not the hRAMP1 or RAMP2C-tails, possesses a classical type I PDZ binding motif (Thr-Leu-Leu), and the binding of *N*-ethylmaleimide-sensitive factor (NSF) to the PDZ motif of hRAMP3 was shown to promote slow recycling of internalized AM_2 receptors in HEK-293 cells [15]. Likewise, Na^+/H^+ exchanger regulatory factor-1 can also interact with the PDZ motif of hRAMP3, resulting in the complete inhibition of the internalization of the AM_2 receptor but not the CGRP or AM_1 receptor [16]. The C-tails of RAMPs, similar to that of CLR, also contain potential phosphorylation and ubiquitination sites [46,115]. In HEK-293 cells overexpressing the hCLR/hRAMP1 complex, agonists promote rapid phosphorylation of CLR but not RAMP1 [48]. In addition, deleting the respective C-tails from hRAMPs had no effect on lysosomal sorting of CLR [72]. It is therefore unlikely that the hRAMP C-tails participate in either phosphorylation or ubiquitination.

Taken together, these findings indicate that the hRAMP2 C-tail is critically involved in AM_1 receptor expression and function, while the hRAMP3 C-tail affects AM_2 receptor internalization and recycling. Thus, the C-tails of hRAMP2 and RAMP3 differentially govern AM receptor trafficking.

10. Perspectives

The hypotensive peptides AM and AM2 exert potent protective effects against multiorgan damage. Most notably, both peptides appear to inhibit cardiovascular oxidative stress, remodeling and apoptosis and to promote angiogenesis, although the data for AM2 are limited. Given the efficacy of these peptides, much effort has been devoted to developing practical clinical uses for AM in the treatment of acute MI [63], heart failure [95,103], arteriosclerosis obliterans [126] and pulmonary hypertension [29]. Furthermore, recent studies have clearly shown that the endogenous AM-AM_1 receptor system is required for both lymphangiogenesis and angiogenesis and that AM treatment can significantly improve rat-tail edema caused by injury. Consequently, this receptor system has also been regarded as a potential therapeutic target for patients with secondary lymphedema. On the contrary, selective local blockade of lymphangiogenesis mediated via AM_1 receptors could be an important therapeutic strategy for inhibiting tumor metastasis. Unfortunately, no selective non-peptide or peptide AM receptor agonists or antagonists have been identified. The two non-peptide CGRP receptor antagonists, olcegepant (BIBN409BS6) [99] and telcagepant (MK0974) [53], both of which are currently available for the treatment of migraines, have little or no affinity for AM_1 and AM_2 receptors [116].

So far, there is no evidence of a difference in the affinity of recombinant AM_1 and AM_2 receptors for AM or in their abilities to transduce the AM signal [8,80]. These AM receptors exist together by their location *in vivo* [100,107,115,142], although tissue expression of RAMP2 and RAMP3 mRNAs appears to vary under different pathophysiological conditions. In addition, the effects of AM2 can also be mediated by the 2 AM receptors. To complicate matters further, hRAMP2 and hRAMP3 can also strongly interact with the CT receptor and the vasoactive intestinal peptide (VIP)/pituitary adenylatecyclase-activating polypeptide type 1 receptor (VPAC1), both of which are family B GPCRs [46,116]. Consequently, the development of non-peptide AM receptor-specific agonists and antagonists would be highly desirable, not only to clarify the shared and separate functions of AM_1 and AM_2 receptors *in vivo* but also to realize the clinical application of AM. To that end, further work to elucidate the entire crystal structure of RAMP2 and RAMP3 in complex with CLR would be valuable because it is anticipated that each CLR/RAMP complex contains numerous crevices that could contribute to drug binding and discovery. It would also be interesting to know the structural basis for the preference of the three RAMPs for interaction with CLR, as opposed to other GPCRs (e.g., CT receptor, VPAC1) in the same tissues and cells. If the molecular mechanism(s) underlying the preferential association between RAMPs and GPCRs is solved, it may enable us to provide selective treatment for a targeted GPCR/RAMP.

After internalization in vascular endothelial cells, endogenous hAM_1 receptors are targeted for degradation in lysosomes [91], which is consistent with the intracellular trafficking of hAM_1 receptors stably overexpressed in HEK-293 cells [48,80]. At present, the mechanism of lysosomal sorting of AM_1 receptors remains unknown, but the establishment of strategies for promoting receptor recycling could be an important means of sustaining AM signaling.

11. Conclusion

In summary, AM and AM2 are potent hypotensive, antioxidative and anti-atherosclerotic factors in the cardiovascular system. In particular, much attention has been paid to the clinical application of AM, many of the effects of which are mediated via the RAMP2-based AM_1 receptor. However, the evidence now

suggests that the AM_2 receptor also has distinct functions *in vivo*. Further studies are needed to clarify the shared and differing roles of the 2 AM receptors to realize their potential clinical applications.

Conflict of interest

None.

Acknowledgements

This study was supported in part by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and by research grants from the Takeda Science Foundation.

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Current Hypertension Reviews, 2011, 7, 268-272

Adrenomedullin: Roles for Structure and Function in Cardiac or Vascular Tissues

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Abstract: Adrenomedullin (AM) is a unique bioactive molecule, originally isolated from human pheochromocytoma by monitoring cyclic adenosine monophosphate (cAMP) elevation in platelets. PreproAM mRNA and its translated peptide have been recognized to be widely distributed in the organs of rodents and humans, including heart and vasculature. AM exhibits vasorelaxant activity working on vascular endothelial cells and smooth muscle cells. In addition, AM modulates left ventricular contractility and remodeling in the hypertrophied/failing heart, and alters the structural integrity of the vascular wall. Furthermore, immunocompetent cells, such as macrophage-, and mast cell-derived AM might contribute to the pathogenesis of cardiovascular disorders. Most biological actions mediate cAMP-protein kinase A signaling, whereas cAMP-independent pathways, such as the nitric oxide/soluble guanylate cyclase/cGMP pathway, modulated in molecules/signaling associated with anti-oxidative stress and anti-apoptotic pathways, are also reported. Overall, the actions of AM are assumed to be beneficial against vasoconstrictive factors activated in the diseased heart and vascular wall, whereas some reports imply that the biological activity of AM might be dependent on circumstances. Specifically, inotrophic action, activation of adhesion molecules and smooth muscle proliferation by AM has been debated. In this review, we will present the recent advances in AM research, and discuss the controversy of AM actions in cardiac and vascular tissues.

Keywords: Atherosclerosis, contractility, fibrosis, heart failure, myocyte hypertrophy, remodeling.

INTRODUCTION

Adrenomedullin (AM) is a potent vasodilatory peptide that was originally isolated from human pheochromocytoma [1]; however, we now know that preproAM mRNA and its translated peptide are widely distributed in tissues and organs, including heart and vasculature [2]. Specifically, AM synthesis and peptide concentration are increased in the hypertrophied and failing heart, and injured vasculature [3-5]. In this review article, we introduce the recent advances in AM research and discuss controversies in cardiac or vascular tissues.

Is the AM Effect on LV Remodeling Beneficial or Detrimental?

It is fundamental for the heart to adapt the cellular response to mechanical load by hypertrophy of cardiomyocytes and hyperplasia of cardiac fibroblasts in order to maintain structural integrity and function; however, it becomes maladaptative to long-term, inadequate stimuli. It is of great interest that AM production is augmented by hypertrophic/hyperplastic stimuli, such as angiotensin II [6-8], endothelin-1 [9] and mechanical stretching [10]. AM is capable of inhibiting cardiomyocyte hypertrophy and fibroblast proliferation/collagen synthesis *in vitro* [6, 7, 11]. A number of animal studies have support the *in vitro* observation that AM exerts beneficial effects on cardiac hypertrophy without affecting systemic blood pressure in models of chronic nitric oxide deficiency combined with pressure overload [12], pressure overload or angiotensin II stimulation in genetically modified AM heterozygotes [13], and of ischemia/reperfusion [14]. However, the anti-remodeling property of AM might be dependent on the situation; overexpression of AM by adenovirus-mediated gene transfer resulted in beneficial effects on angiotensin II-induced cardiac hypertrophy in rats; in contrast, the authors found profound dilatation in post-infarcted LV following AM overexpression [15]. Potential mechanisms include a delayed healing process, probably due to anti-inflammatory action [16], decreased proliferation [7, 11] and activation of fibroblasts [17], and the stimulation of extracellular matrix enzymes [18]; therefore, the question remains to be resolved of "when" AM should be administered to the diseased heart. The "amount" of AM necessary to administer seems to be another concern for future clinical application.

Does AM have a Stronger or Weaker Effect on Myocardial Contractility?

Activation of the stimulatory G protein (Gαs)-adenylate cyclase-cyclic adenosine monophosphate (cAMP) system is one of the major pathways for the stimulation of cardiac

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1573-4021/11 $58.00+.00

contractility in the mammalian heart. The level of Gαs is inactivated and internalized in the failing heart, whereas the level of inhibitory G protein (Gαi) is increased [19]. Because AM exerts biological action by cAMP elevation, it is supposed to directly increase the contractility by Gαs-mediated adenylate cyclase-cAMP; however, the inotrophic effects of AM are inconclusive; a positive inotrophic effect of AM was reported in papillary muscles (trabecullae) of rats [20] and human atrium and ventricles [21], and in perfused whole rat heart [22]. On the other hand, negative inotrophic effects have been reported in the rabbit papillary muscle [23], isolated ventricular myocyte of adult rats [24], rabbits [25] and humans [26]. Lainchbury *et al.* [27] reported that AM did not influence contractility in dogs. Although the preparation of myocardial tissues from different species, experimental conditions, and concentration of AM used might have affected the inconsistent results, Mittra and Bourreau [24] reported that long-time exposure to AM by adult rat cardiomyocytes switched the sensitivity of receptor activity-modifying protein (RAMP) coupled with Gαs to Gαi, resulting in changing positive inotrophic effects to negative effects [24].

Are Non-myocytes (Fibroblasts) a Dominant Target for AM?

The effect of AM on inhibiting proliferation/collagen in non-myocytes (cardiac fibroblasts) was consistent in Horio *et al.*'s [11] and our results [7], whereas the biological activity of AM on cardiac myocyte hypertrophy was inconsistent between the two groups [6, 11]. It could be argued that AM preferentially acts on fibroblasts, but not on cardiomyocytes, and contaminated fibroblasts (~10%) by the isolation technique of cardiomyocytes would lead to the decreased incorporation of ^{14}C-labeled phenylalanine; however, we confirmed that cardiomyocyte size was decreased by AM under microscopic observation [28]. Harada *et al.* [29] reported a critical role for fibroblasts in cardiomyocyte hypertrophy, and we speculate that AM reduced cardiomyocyte hypertrophy in part though the modulation of fibroblast activation, and that undetermined substances secreted from fibroblasts might have influenced myocyte hypertrophy. Subsequently, we reported that a sub-depressor dose of AM administration to angiotensin II-induced hypertensive rats for two weeks preferentially inhibited collagen synthesis and deposition without affecting cardiocyte hypertrophy [17]. Taken together, AM exhibited the biological activity in cultured fibroblasts with a lower concentration ($\geq 10^{-8}$ mol/L) than in myocytes ($\geq 10^{-7}$ mol/L), and we support the hypothesis that the main cellular target for AM is fibroblasts in the heart [30].

Is AM Good, Bad or Ugly for Vascular Structural Integrity?

The arterial wall is composed of 3 layers (intima, media and adventitia). The intima consists of a monolayer of endothelial cells. The media is the main structural component supported by smooth muscle cells and extra-cellular matrix elements, such as elastin, collagen and fibronectin [31]. The adventitia is populated by terminal nerve fibers, vasa vasorum and surrounding connective tissue, which contains a few resident fibroblasts and inflammatory cells. Vascular protection of AM was previously reviewed [32], and we discuss the action of AM categorized into 3 layers in this review. **Intima:** Leukocyte adhesion to vascular endothelium is an essential event in the development of atherosclerosis, in which E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 play important roles in the process. The effect of AM on the activation of adhesion molecules is under debate. AM has been reported to be capable of inhibiting the expressions of E-selectin, VCAM-1 and ICAM-1 induced by vascular endothelial growth factor in human umbilical vein endothelial cells (HUVEC) [33]. On the other hand, Hagi-Pavli *et al.* [34] reported that AM increased these expressions in a dose-dependent manner in the cells. The stimulation period (4 hour) and concentration of AM (10^{-12} ~10^{-8} mol/L), and the cell source (HUVEC) were consistent in the two studies. The only difference was in the experimental protocol; whether HUVEC treated by AM was stimulated with a pharmacological agent such as vascular endothelial growth factor. **Media:** The effect of AM on smooth muscle proliferation also seems controversial. Kohno *et al.* [35, 36] showed that AM inhibited rat aortic smooth muscle cell proliferation in serum-containing medium or attenuated migration in angiotensin II-stimulated human coronary artery smooth muscle cells. On the other hand, Shichiri *et al.* [37] reported that AM is mitogenic in rat aortic vascular smooth muscle cells. They added synthetic AM directly to the culture medium in the serum-free medium without any growth-promoting agents. In an animal study, AM administration/gene delivery has been reported to inhibit neointimal hyperplasia by attenuating smooth muscle migration/proliferation and by inducing the apoptosis of myofibroblasts in a rat injured carotid artery [38, 39]. In addition, neointimal hyperplasia induced by a cuff on the femoral artery was attenuated in AM transgenic mice [40]. Moreover, overexpression of RAMP2 exhibited reduced aortic medial thickness without affecting systemic blood pressure induced by angiotensin II in mice [41]. The authors also demonstrated that cultured vascular smooth muscle cells obtained from RAMP2 transgenic mice showed a slower growth compared with wild types, and blocking the action of endogenous AM with an AM receptor antagonist, AM_{22-52}, promoted cell proliferation in the presence of angiotensin II in these cells. However, Shichiri's group extended their *in vitro* study, showing that the blockade of AM action with another type of AM receptor antagonist, calcitonin gene-related peptide $(CGRP)_{8-37}$, led to the inhibition of neointimal hyperplasia following a balloon injury in the rat carotid artery [42]. Although the majority of reports addressed the issue supporting the anti-proliferative action of AM in smooth muscle cells, a concern might be raised whether AM has a divergent action that is dependent on the circumstances. **Adventitia:** A few data are available for AM action on the adventitial layer. We and others have consistently suggested that AM might have anti-remodeling effects by inhibiting the proliferation and activation of

adventitial fibroblasts [17, 39, 43], and by stimulating matrix metalloproteinase-2 activity [18]. Considering the importance of extracellular matrix formation in the adventitial layer in determining the stiffness of the vascular wall [44], AM may exert a beneficial action alleviating vascular stiffness; however, extracellular matrix degraded by excessive activity of AM might cause the aortic structure to weaken, exaggerating outward remodeling.

Decreased uptake of AM to the arteries along with reduced RAMP2 expression was reported in a model of pulmonary hypertension induced by monocrotaline [45]. In addition, vasodilatory action of AM diminishes in the setting of heart failure [27], probably due to digestion of the amino-acid sequence of AM by vasopeptidase [46] and/or by matrix metalloproteinase-2 [47]. This suggests that significant roles of AM and its receptor system contribute to the pathogenesis of cardiovascular disorders, and that AM infusion would complement the relatively insufficient activity of this peptide under these situations.

Is Immunoregulatory Cell-Derived AM an Additional Player?

Atherosclerotic plaque contains a number of macrophages (foam cells) that contribute to inflammatory processes that promote thrombosis by stimulating the production of collagen-degrading proteinases [48]. Mast cell number is increased to distribute and activate in the interstitium of ischemic/dilated cardiomyopathy [49] and in the adventitia of the atherosclerotic aorta and aneurysm [50]. AM was reported to be produced in macrophages [51-53] and mast cells [54, 55]. There are few reports with regards to the biological activity of AM on macrophages (ex. M1, M2 phenotypes, matrix metalloproteinase). Mast cells release a number of substances such as histamine, trypsin and chymase as well as proinflammatory cytokines (interleukin-6 and interferon-γ) to possibly modulate the atherosclerotic process [56]. More interestingly, mast cells contain vasoactive peptides, such as atrial natriuretic peptide, B-type natriuretic peptide, AM, endothelin-1 and relaxin [54]. Degranulated mast cells are assumed to evoke myocardial and vascular injury by stimulating vasoconstriction, leukocyte recruitment and extracellular matrix alternation, whereas mast cell-derived natriuretic peptides, AM or relaxin might be protective against vasoconstrictive factors [57]. We have demonstrated that synthetic AM inhibited collagen synthesis in the co-culture of a mast cell line with adventitial fibroblasts, whereas neutralization of AM secreted from the cells stimulated collagen synthesis [55]. It remains to be elucidated to what extent AM derived from these immuno-competant cells contributes to the pathogenesis, because the concentration appears to be lower than in other cell types of the myocardium or vascular wall.

Does AM have Multiple Pathways for Cardiovascular Protection?

The underlying mechanisms by which AM protects against cellular damage remain unknown. AM was initially isolated from human pheochromocytoma by monitoring cAMP elevation in platelets [1]; however, there are several reports showing that the action of AM is cAMP-independent [22, 58], in which the nitric oxide/soluble guanylate cyclase/cGMP pathway is dominant [59]. The mechanisms also involve the decrease in oxidative stress [59], apoptosis determined by TUNEL-positive nuclei in myocytes as well as non-myocytes [14], accompanied by reduced levels of BAX, cleaved caspase-3 and phosphorylation of p38, and by increased phosphorylation of Akt and Bad [59], and by the opening of large conductance Ca^{2+}-activated K^{+} channels in the mitochondrial inner membrane [60] and the induction of heat-shock protein 72 [61].

What is the New Era for AM2/Intermedin in Heart and Vasculature Research?

AM2/intermedin is a newly identified member of the CGRP superfamily [62, 63]. The putative mature bioactive AM2 peptide consists of 47 amino acids, which share 34% sequence homology with AM and <20% similarity with CGRP. Similarly to AM, AM2/intermedin expression was increased in the hypertensive left ventricle and aorta in rats

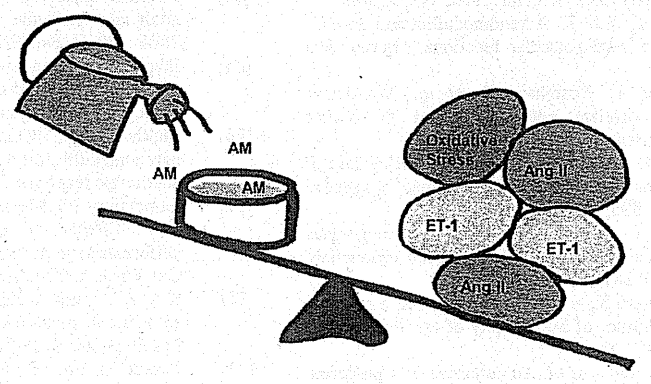

Fig. (1). AM supplementation for potential clinical application in cardiovascular diseases. AM, adrenomedullin; Ang II, angiotensin II; ET-1, endothelin-1

[64, 65], and was reported to stimulate its production under angiotensin II stimulation. It exerts an anti-hypertrophic response in cultured neonatal cardiomyocytes [66], and protects against myocardial ischemia-reperfusion injury [67]. Intravenous infusion of AM2/intermedin showed beneficial effects on hemodynamics (increased cardiac output, decreased vascular resistance) and endocrine profiles (decreased plasma renin activity and brain natriuretic peptide concentration) [68]. So far, AM2/intermedin seems to have similar biological properties to AM.

CONCLUSION

Considering with the published articles, we agree that AM works as a protective factor in the damaged heart and vasculature. AM appears to relieve the "scene of fire from a raging storm". On the other hand, we understand that the action of AM might be dependent on the circumstance. Fig. (1) illustrates the relatively insufficient activity of AM in cardiovascular diseases. The adequate amount and appropriate timing of AM supplementation could overcome maladaptative stimuli, such as oxidative stress, angiotensin II and endothelin-1, resulting in attenuating of the progression of the disorders.

CONFLICTS OF INTEREST

Declared none.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Mariko Tokashiki for the illustration. This study was supported by grants-in-aid for Scientific Research (C) (23591052) from the Ministry of Education, Culture, Sport, Science and Technology, Japan, Mitsubishi Pharma Research Foundation and Takeda Science Foundation.

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Received: July 06, 2011 Revised: August 18, 2011 Accepted: August 30, 2011

8. アドレノメデュリンの内分泌・代謝系へのユニークな作用

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アドレノメデュリンは血管拡張性のペプチドで，血圧低下，体液量減少，細胞増殖抑制，抗酸化，炎症調節作用など多彩な作用を有している．内分泌関係では，アルドステロンの過剰分泌を抑制するとともに，アルドステロンの腎外作用を抑制し，内因性の抗アルドステロン因子となっている．代謝系ではインスリン分泌を抑制し，抗酸化作用によりインスリン抵抗性を改善する作用があるが，一方，インスリン抵抗性が亢進すると，アドレノメデュリンの作用は減弱してしまう．これらのユニークな作用は，今後，新しい治療ターゲットとなるかもしれない．

はじめに

アドレノメデュリン（AM）は血管拡張性の降圧ペプチドであり，全身の血管系に広く発現しており，加えて心臓，肺，腎臓，脳，副腎などの重要臓器にも分布している．血管拡張による血圧調整以外に，免疫・炎症調整作用，抗動脈硬化作用（抗酸化作用，細胞増殖抑制作用），内分泌調整作用など多彩な作用を有している[1]．本稿では，内分泌・代謝に関連したAMの作用，特にAMとアルドステロンの相互作用，AMとインスリン抵抗性について解説したい．

[キーワード&略語]
アドレノメデュリン，アルドステロン，インスリン抵抗性，アンジオテンシンⅡ

A-Ⅱ：angiotensin-Ⅱ（アンジオテンシンⅡ）
AM：adrenomedullin（アドレノメデュリン）
MS：metabolic syndrome（メタボリックシンドローム）
PA：primary aldosteronism（原発性アルドステロン症）
ZG：zona glomerulosa（副腎皮質球状層）

1 アドレノメデュリンの構造と主要な作用

AMはカルシトニン関連ペプチド（CGRP）やアミリンと同じスーパーファミリーに属するペプチドで，C末端のアミド構造と分子内リング構造が共通しており，これらは生理活性に必須である．AMはcAMPをセカンドメッセンジャーとして作用を発現するが，AMとCGRPには構造上の共通性があり，CRLR（calcitonin-receptor-like receptor）という7回膜貫通型受容体を共有している．加えてRAMP（receptor-activity-modifying protein）という1回膜貫通型のタンパクが，受容体の親和性を規定し，さらには細胞膜での発現を調整している．RAMPには相互に相同性を示す3種類のタンパクが存在し，CRLR＋RAMP1でCGRPの，CRLR＋RAMP2またはCRLR＋RAMP3でAMの受容体を形成する．さらに，RAMP1とcalcitonin receptorの組み合わせはアミリンの受容体を形成し，これらの相互関係は複雑である[2]．

Unique effects of adrenomedullin for endocrine and metabolic system
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