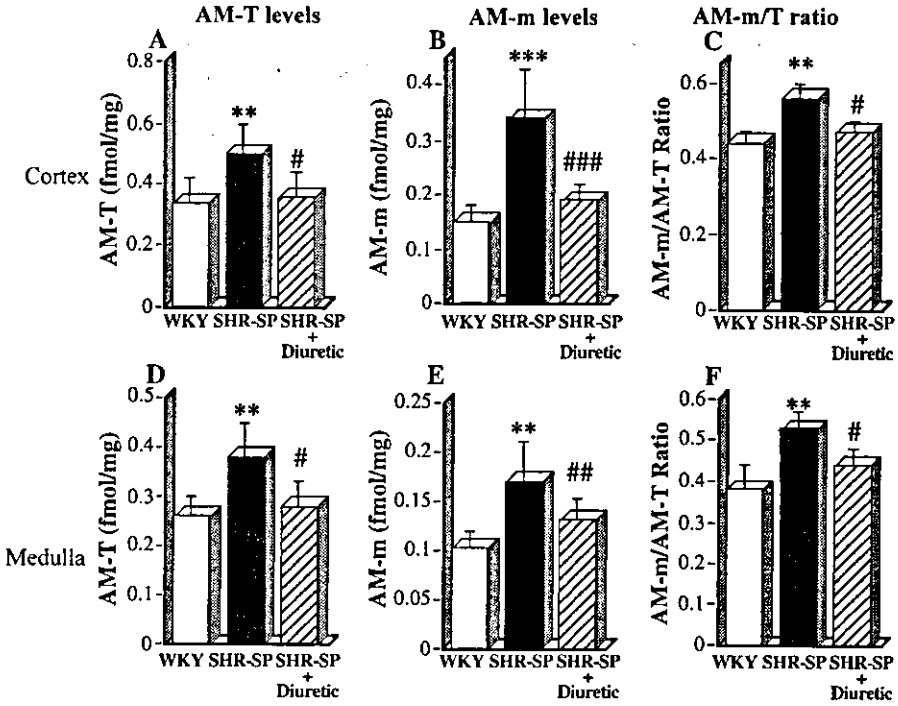

Fig. 2. Tissue AM-T levels (A and D), AM-m levels (B and E), and AM-m/AM-T ratio (C and F) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Data are expressed as mean±S.D. $^{**}P<0.01$ vs. WKY; $^{***}P<0.001$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP; $^{\#\#}P<0.01$ vs. SHR-SP; $^{\#\#\#}P<0.001$ vs. SHR-SP.

PRC still differed significantly between WKY and diuretic-treated SHR-SP.

3.4. Tissue AM-m level, AM-T level, and AM-m/AM-T ratio in renal cortex and medulla in WKY, SHR-SP, and SHR-SP+Diuretic

The tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla in the three groups are presented in Fig. 2. The tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla were significantly higher in SHR-SP than in WKY. Treatment with diuretic significantly decreased the tissue AM-m levels, AM-T levels, and AM-m/AM-T ratios in the renal cortex and medulla of SHR-SP. There were no significant differences in these indices between WKY and diuretic-treated SHR-SP.

3.5. Levels of gene expression of AM in renal cortex and medulla

The expression of rat AM mRNA in the renal cortex and medulla in the three groups was measured by Northern blot analysis. Representative results of Northern blot analysis of AM mRNA from the renal cortex and medulla and the results of quantitative analysis of these blots corrected for the levels of GAPDH mRNA, serving as an internal control, are shown in Fig. 3A and B. The expression of AM mRNA in the renal cortex and medulla was slightly but significantly higher in SHR-SP than in WKY (Fig. 3A and B). Treatment with diuretic decreased the expression of AM mRNA in SHR-SP.

3.6. Levels of gene expression of CRLR, RAMP2, and RAMP3 in renal cortex and medulla

The expression of CRLR, RAMP2, and RAMP3 mRNA in the renal cortex and medulla in the three groups was investigated by RT-PCR analysis with specific CRLR, RAMP2, and RAMP3 primers. Specific bands of the

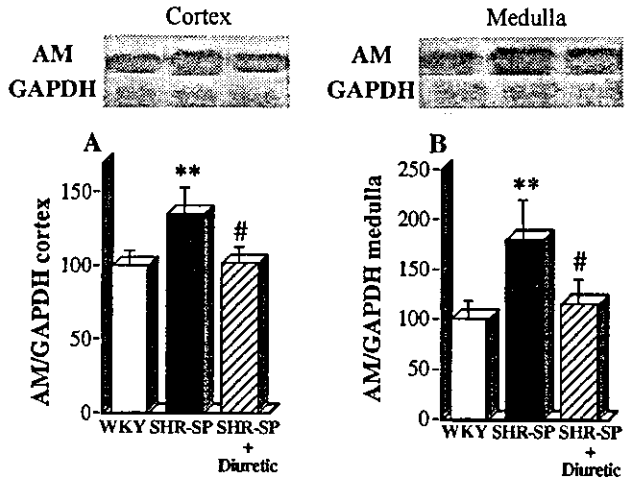

Fig. 3. Levels of AM gene expression in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative autoradiograms of AM and GAPDH mRNA bands in (A) renal cortex and (B) medulla (upper). AM mRNA levels corrected for GAPDH mRNA levels in the renal cortex and medulla (lower). Data are expressed as mean±S.D. $^{**}P<0.01$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP.

predicted length (323, 164, and 416 bp) were obtained with each CRLR-, RAMP2-, and RAMP3-specific primer. Representative electrophoretic profiles of RT-PCR products and quantitative analysis of the levels of these products corrected for the levels of the GAPDH-specific product, serving as an internal control, are shown in Fig. 4A–F. The CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal cortex were higher in SHR-SP than in WKY (Fig. 4A–C); CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal medulla did not differ significantly (Fig. 4D–F). Treatment with the diuretic reduced the CRLR/GAPDH, RAMP2/GAPDH, and RAMP3/GAPDH mRNA levels in the renal cortex of SHR-SP (Fig. 4A–C), but did not alter these levels in the renal medulla.

3.7. Levels of gene expressions of TGF-β, collagen I, and PAM in renal cortex and medulla

The TGF-β/GAPDH mRNA level and collagen I/GAPDH mRNA level in the renal cortex and medulla were higher in SHR-SP than in WKY (Fig. 5A–D). Long-term diuretic treatment significantly reduced the TGF-β/GAPDH and collagen I/GAPDH mRNA levels. In contrast, there were no differences in the mRNA level of PAM in the renal cortex or medulla among the three groups (data not shown).

4. Discussion

To investigate the pathophysiological role of the renal AM system in renal impairment associated with severe hypertension, we used a model of malignant hypertension, SHR-SP. In this model, a high-salt diet causes severe systemic hypertension, resulting in the establishment of renal impairment at the age of 16 weeks. We found that both molecular forms of AM in the plasma and the renal cortex and medulla tissue were higher in SHR-SP than in WKY. In addition, AM-m/AM-T ratio and AM gene expression in the renal cortex and medulla, and gene expression of CRLR, RAMP2, and RAMP3 in the renal cortex were also higher in SHR-SP than in WKY. Long-term diuretic treatment

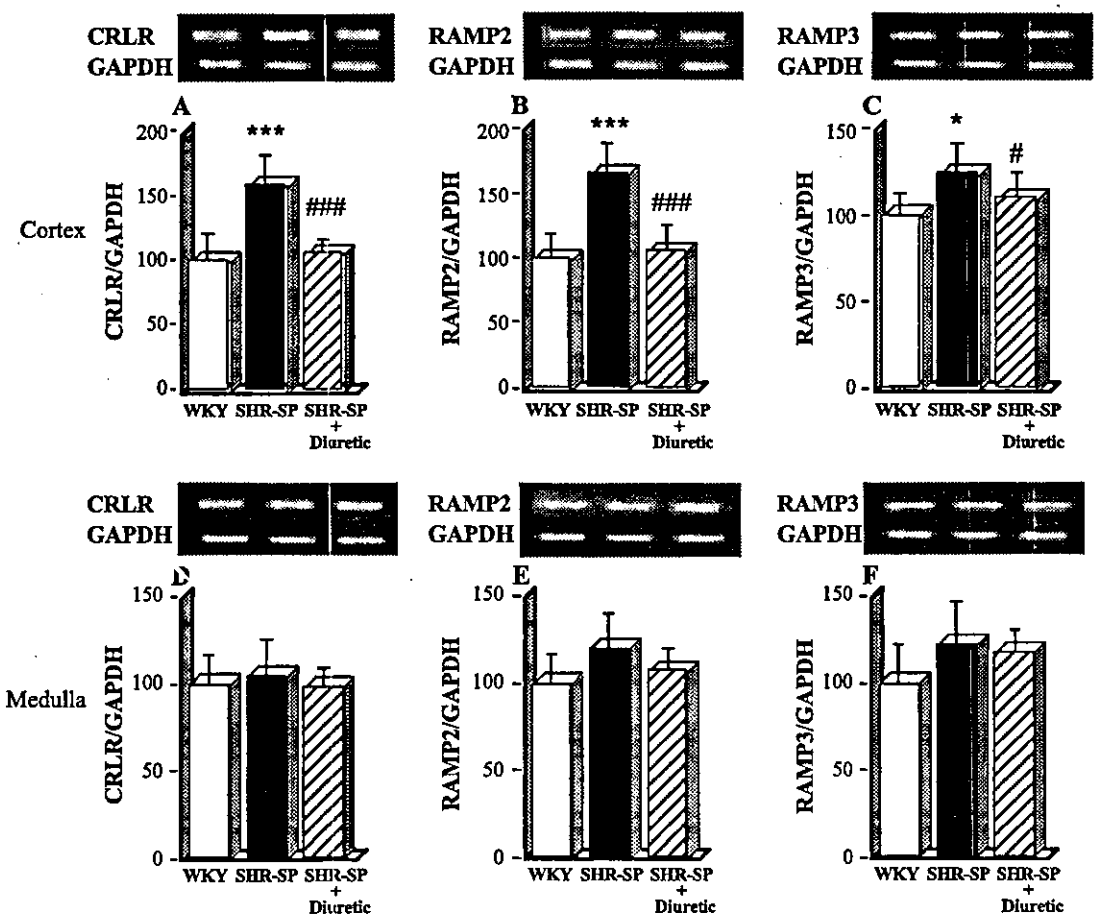

Fig. 4. Gene expression levels of CRLR (A and D), RAMP2 (B and E), and RAMP3 (C and F) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative ethidium bromide-stained agarose gels of RT-PCR products for CRLR, RAMP2, RAMP3, and GAPDH (upper). Quantitative analysis of CRLR, RAMP2, and RAMP3 mRNA levels normalized relative to the GAPDH mRNA level (lower). Data are expressed as mean±S.D. $^{*}P<0.05$ vs. WKY; $^{***}P<0.001$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP; $^{\#\#\#}P<0.001$ vs. SHR-SP.

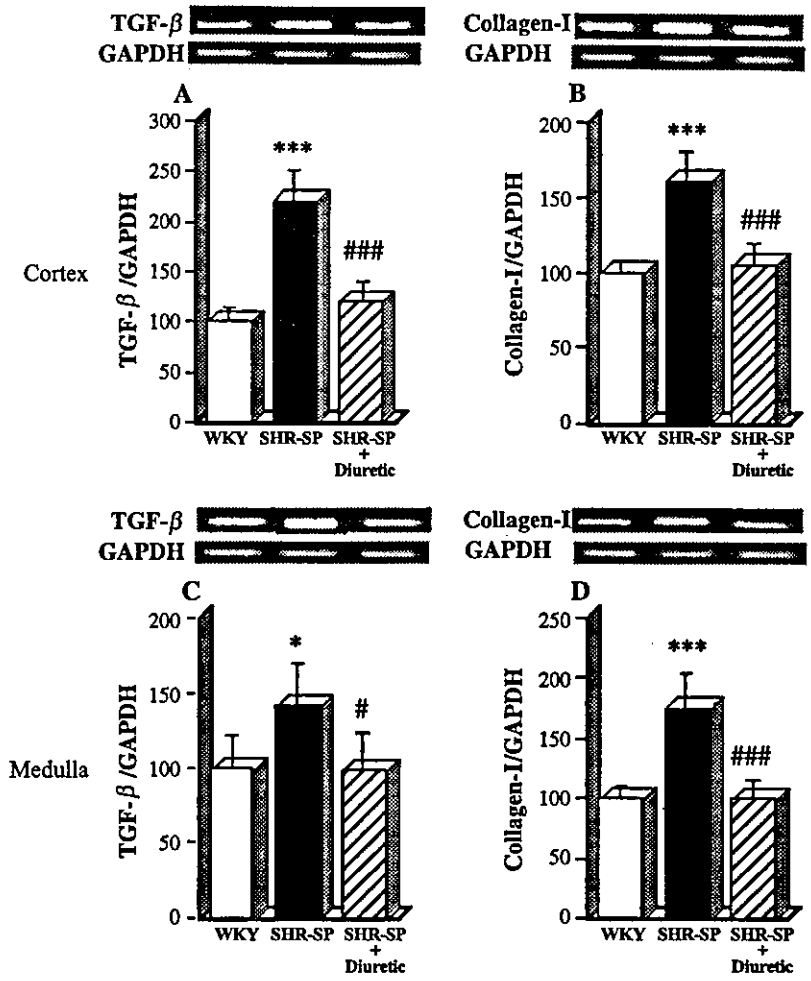

Fig. 5. Gene expression levels of TGF-β (A and C) and collagen I (B and D) in the renal cortex and medulla in WKY, SHR-SP, and diuretic-treated SHR-SP (SHR-SP+Diuretic) are shown. Representative ethidium bromide-stained agarose gels of RT-PCR products for TGF-β, collagen I, and GAPDH (upper). Quantitative analysis of TGF-β and collagen I mRNA levels normalized relative to the GAPDH mRNA level (lower). Data are expressed as mean±S.D. $^{*}P<0.05$ vs. WKY; $^{***}P<0.001$ vs. WKY; $^{\#}P<0.05$ vs. SHR-SP; $^{\#\#\#}P<0.001$ vs. SHR-SP.

reduced blood pressure and improved kidney weight/body weight, serum creatinine level, serum blood urea nitrogen level, urinary excretion of protein, plasma aldosterone level, and PRC in SHR-SP. These improvements were associated with reductions in plasma AM, renal tissue AM, and mRNA of AM and AM receptor component in renal tissues. These results suggest that the AM system, including the ligand, receptor, and amidating activity in the kidneys, is activated in SHR-SP and modulates the pathophysiology of nephrosclerosis in this model.

The active mature form of AM is produced from AM precursor by a two-step enzymatic pathway. First, the AM precursor is converted to C-terminal glycine-extended AM, an inactive intermediate form of AM. Subsequently, inactive AM-Gly is converted by enzymatic amidation to the active form of mature AM, a 52-amino-acid peptide with a C-terminal amide structure [18]. Recent studies have shown that two molecular forms of AM circulate in human plasma and that the major circulating form of AM is AM-Gly [18,19]. We and others have reported that both plasma AM-m and AM-Gly levels increase in parallel in patients with hypertension, chronic renal failure, heart failure, and pulmonary hypertension [18,19,28–30]. In the rat hypertension model, plasma AM level in SHR is not increased compared with WKY [31]. However, it is increased in deoxycorticosterone acetate salt SHR, a severe hypertensive rat model with organ damage [31]. With regard to SHR-SP, plasma level of AM is low at 7 weeks compared with WKY; however, it is increased at 17 weeks [13]. The present study showed that the major circulating form of AM is AM-Gly in WKY and SHR-SP, and that both plasma AM-m and AM-T levels increased in parallel in SHR-SP, as compared with those in WKY. These results are consistent with the previous data [12,13]. Interestingly, long-term diuretic treatment reduced blood pressure levels and circulating AM-m and AM-T levels in salt-loaded SHR-SP. A recent study has reported that chronic salt load increases plasma AM levels in rat [32]. This finding suggests that reduced blood pressure, increased natriuresis, or both effects induced by diuretic treatment may account for the reduction in

circulating AM levels. Thus, plasma AM may be a noninvasive biochemical marker that can be used to monitor blood pressure control during antihypertensive therapy in severe hypertension.

In this study, we also analyzed the molecular forms of AM in renal tissue in WKY and SHR-SP. AM-m/AM-T ratio was obviously higher in renal tissue than in plasma. The reason for this difference is not fully understood. However, if AM-m/AM-T ratio is higher in tissues and AM acts as an autocrine or paracrine factor (or as both), most AM-m produced in tissues may be consumed, with release of primarily an inactive form into the circulation. Therefore, the major molecular form of AM in plasma is inactive. Our study also demonstrated that the AM-m/AM-T ratio in the renal cortex and medulla tissues was higher in SHR-SP than in WKY. This finding suggests that amidating enzyme activity is activated in the impaired kidney. A previous study reported that amidating enzyme mRNA expression is detected in collecting ducts and distal tubules [33]. Our study showed that antihypertensive therapy reduced AM-m and AM-T levels and AM-m/AM-T ratio. The amidating enzyme is an enzyme complex denoted collectively as peptidyl-glycine amidating monooxygenase [34]. In fact, amidation is a two-step process catalyzed by two separate enzyme activities: peptidyl-glycine-hydroxylating monooxygenase and peptidyl-hydroxyglycine-amidating lyase, both encoded by the same gene [35]. In the present study, we measured mRNA levels of PAM and found that there were no differences in mRNA levels of PAM in the renal cortex or medulla between WKY and SHR-SP. Thus, amidating enzyme activity might depend on posttranslational modification, although we did not measure the amidating enzyme activity in this study. Further study is required to elucidate the mechanism of regulation and posttranslational modification and the roles of amidating enzyme in the normal and diseased kidney.

A new family of single-transmembrane-domain proteins, which are called RAMP1, RAMP2, and RAMP3, has been cloned [11]. RAMPs are required to transport CRLR to the plasma membrane [11]. Previous studies demonstrated that the RAMP1/CRLR complex serves as a CGRP receptor, while RAMP2/CRLR and RAMP3/CRLR serve as AM receptors [11,36,37]. Several groups of investigators have reported that mRNA, binding sites, and immunoreactivity of AM exist in the kidney, and that AM has many physiological effects on the kidney [2,3]. However, whether the level of renal AM receptor is modulated by transcriptional regulation in hypertensive nephrosclerosis remains unknown. Furthermore, few studies have examined the expression of the mRNAs of CRLR and RAMPs in cardiovascular disease. One such study showed that RAMP1, RAMP2, and CRLR gene expression is markedly upregulated in obstructive nephropathy, whereas RAMP3 expression is unchanged [16]. We previously reported that mRNA levels of RAMP2 and RAMP3 in renal medulla were upregulated, whereas mRNA levels of CRLR in renal medulla or CRLR, RAMP2, and RAMP3 in renal cortex were not upregulated in deoxycorticosterone acetate salt SHR compared with WKY [31]. The present study showed that the mRNA levels of CRLR, RAMP2, and RAMP3 in the renal cortex were higher in SHR-SP than in WKY. Thus, the changes of mRNA levels of AM receptor component appear to be model-dependent. Further study is required to elucidate the exact mechanism of the regulation and role of AM receptor component in hypertensive nephrosclerosis. A recent study demonstrates that CRLR immunoreactivity is found in the distal tubules and glomerulus in renal cortex [38]. Previous studies have also shown that AM immunoreactivity is present in the distal tubules and glomerulus in renal cortex [2,3]. Taken together, the combined upregulation of receptor and ligand seems to enhance the effect of AM in the impaired kidney. Thus, not only upregulation of the ligand and amidating activity of AM, but also upregulation of the receptor system of AM may be involved in the pathophysiology of nephrosclerosis in severe hypertension.

To date, the pathophysiological role of increased AM system activity in the impaired kidney in hypertension is not fully understood. Previous studies demonstrated that AM is not only a natriuretic and diuretic peptide [2,3], but also an antigrowth peptide able to inhibit angiotensin II-induced proliferation of cultured mesangial cells, fibroblasts, and vascular smooth muscle cells [2,3,39]. Stimulation by IL-1β or TNF-α induces the expression of AM in these cells. These findings suggest a possible role of AM as an autocrine or paracrine cytoprotective factor (or as both) for nephrosclerosis [40]. In addition, we recently have shown that chronic AM infusion significantly improves renal function, histological findings, and biochemical and molecular markers in malignant hypertensive rats and salt-induced hypertensive rats without changing blood pressure [24,25]. Moreover, recent studies have indicated that AM gene delivery markedly increases plasma and renal tissue AM levels and attenuates the renal fibrosis induced by hypertension in rats [41,42] Furthermore, a very recent study shows that angiotensin II infusion causes more pronounced renal damage in AM+/− mice than AM+/+ mice, suggesting that endogenous AM exerts a protective effect against stress-induced renal injury [43] Taken together, these results suggest that increased activity of the AM system, including peptide, mRNA, and receptor, may have a compensatory effect on renal impairment in hypertensive nephrosclerosis.

In conclusion, our study shows that plasma AM levels, renal AM levels, renal gene expression of AM, and renal expression of the AM receptor system are all upregulated in impaired kidneys in severe hypertension. Diuretic therapy may attenuate increased activity of the AM system. These results suggest that induction of the AM system, including ligand, receptor, and amidating activity, as observed here, may modulate the pathophysiology of nephrosclerosis in the severely hypertensive rat.

Acknowledgments

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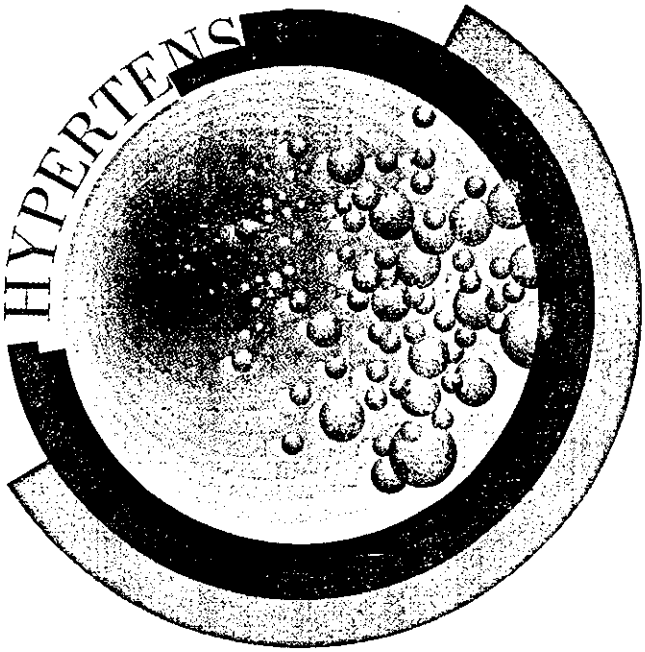

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アドレノメデュリン　Adrenomedullin

*北村和雄（きたむらかずお）

アドレノメデュリン（AM）は，ヒト褐色細胞腫組織から分離された強力な血管拡張性ペプチドであり，特徴として，分子内に6個のアミノ酸よりなるリング構造とC末端のアミド構造をもっている(図)。生理活性に必須であるこのリング構造とアミド構造は，calcitonin gene-related peptide（CGRP）やアミリン，およびごく最近発見されたAM2などと共通しており，一つのスーパーファミリーを形成している。さらに，AM前駆体からはproadrenomedullin N-terminal 20 peptide（PAMP）が降圧作用を有した別の生理活性ペプチドとして生合成されている(図)。AMは副腎髄質だけでなく，全身の臓器で発現・分泌されており，肺，腎臓，心臓，血管など循環調節に重要な臓器での発現量も多い。特に，血管内皮細胞や血管平滑筋細胞で遺伝子発現がきわめて高く，受容体もこれらの細胞に豊富に存在している。

AMの主作用は，強力で持続時間の長い血管拡張作用であるが，それ以外にアルドステロンなどの分泌抑制作用や利尿作用ももち，全体として血圧を低下させる方向に作用する。現在までに明らかにされたAMの作用は多彩であり，それらを昇圧因子であるアンジオテンシンⅡと比較してみると，AMのほとんどの作用はアンジオテンシンⅡに拮抗することが

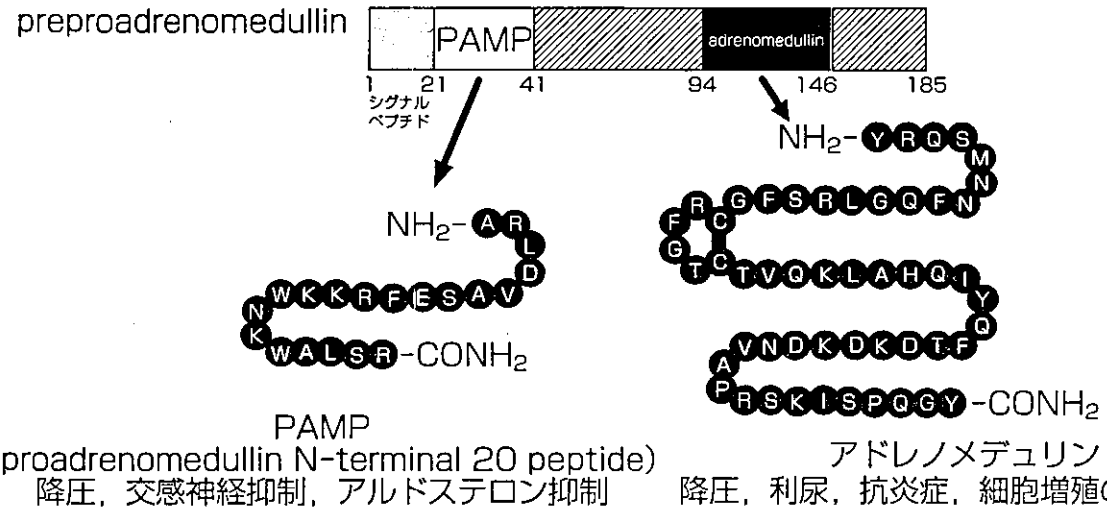

図　ヒトアドレノメデュリン前駆体の構造とアドレノメデュリンとPAMPの生合成

ヒトAM前駆体は，185個のアミノ酸よりなる。AM前駆体からはAM以外に，proadrenomedullin N-terminal 20 peptide（PAMP）が別の生理活性ペプチドとして生合成される。両ペプチドとも降圧作用を示すペプチドであるが，それらの作用機序は異なっている。

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●key words：アドレノメデュリン，PAMP，降圧ペプチド，アンジオテンシンⅡ，アドレノメデュリン受容体

表　アドレノメデュリンとアンジオテンシンⅡの作用の比較

	アドレノメデュリン	アンジオテンシンⅡ
血管	平滑筋弛緩	平滑筋収縮
	増殖抑制	増殖促進・肥大・過形成
心臓	肥大抑制	肥大促進
腎	ナトリウム利尿	ナトリウム再吸収
アルドステロン	分泌抑制	分泌促進
バゾプレシン	分泌抑制	分泌促進
飲水行動	抑制	促進
カテコラミン	分泌抑制	分泌促進
血圧	低下	上昇
体液量	減少	増加

わかる(表)。さらにAMは，慢性投与したときには *in vivo* で血漿レニン活性を抑制することも明らかにされており，AMがアンジオテンシンⅡに拮抗する内在性の降圧因子として，きわめて重要な役割を果たしていることが予想される。さらに，血管平滑筋や心筋増殖抑制作用，血管内皮細胞のアポトーシス抑制作用を有し，各種の臓器障害に対し臓器保護的に作用していると考えられる。なお，AMのトランスジェニックマウスが開発されており，血圧の低下が確認されている。

一方，AM遺伝子欠損マウスは3つのグループより報告されており，いずれもホモ接合体では，胎生致死であることが示されている。AMのヘテロ接合体では，外見上，行動上の明らかな差異は認められていないが，AMの発現レベルが心臓，腎臓，血中で低下しており，ヘテロ接合体で約10 mmHgの高血圧が認められ，AMの降圧因子としての重要性が再認識されている。さらに，アンジオテンシンⅡと食塩負荷を行うと，ヘテロ接合体では心肥大と冠動脈の線維化と内腔の狭窄が認められ，内在性AMの心保護作用が注目されている。

AM受容体に関しては，AMとCGRPがレセプターとしてCRLR（calcitonin-receptor-like receptor）という7回膜貫通G蛋白共役型レセプターを共有することが明らかにされた。AMとCGRPに対する特異性は，1回膜貫通型のRAMP（receptor-activity-modifying protein）とよばれる膜蛋白により規定され，RAMPがなければCRLR自体も膜表面に移行せず活性も示さない。RAMPにはRAMP1-3の相互に相同性を有する3種類の蛋白の存在が明らかにされており，CRLR＋RAMP1でCGRPの，CRLR＋RAMP2またはCRLR＋RAMP3でAMの受容体を形成する。今後，CRLR-RAMP系レセプターが，AMの多彩な作用にどのように関与しているのか解明が待たれる。

PAMPもAMと同じく副腎髄質や心房に高濃度に存在しているが，肺や腎臓でのPAMP濃度は低値である。PAMPを麻酔下ラットに投与すると，速やかで強力な降圧活性が認められるが，持続時間はAMと比較して短時間である。PAMPの降圧機序として，交感神経末梢からのカテコールアミン放出抑制作用が報告されている。これらのことから，AMとPAMPという共通の前駆体から生成されたペプチドが異なる機序で降圧活性を示し，協調して循環調節に関与していると考えられる(図)。

本態性高血圧症患者の血中 AM 濃度は，正常血圧者と比較して高値であることが示されており，さらに，臓器障害の重症度が増すに従って，血中 AM 濃度が増加していることが明らかになっており，高血圧における血中 AM 濃度の上昇と臓器障害との関係が示唆される。また，二次性高血圧のなかでは，腎血管性高血圧や原発性アルドステロン症において，血中 AM 濃度が高値であることが示されている。

Dahl 食塩感受性高血圧ラットモデルにおいて，左室の AM 濃度と血中 AM 濃度との間によい相関のあることが報告されている。また，2 腎 1 クリップによる腎血管性高血圧ラットでも，左室の AM 濃度が収縮期血圧ならびに肥大の程度と相関することが示されている。臨床的にも，腎機能障害のない本態性高血圧症では，左室肥大例で心肥大のない例よりも血中 AM 濃度が高く，左室重量係数と血中 AM 濃度との間に有意な相関を報告している。高血圧による左室肥大例では心室の AM mRNA の発現が亢進し，降圧とともに心肥大に拮抗することで，臓器保護的に働くことが推定される。

悪性高血圧では，本態性高血圧症と比較して血中 AM 濃度は著増し，降圧とともにその濃度は低下し，AM の高血圧における寄与を示す証拠の一つと考えられる。また，悪性高血圧のモデル動物に AM の慢性投与を行うことで，心臓の線維化を抑制し，腎機能を保持するなどの臓器保護効果が観察されている[10]。

以上より，AM は高血圧症で重症度とともに血中および組織中濃度が増加し，増加した AM は高血圧に対する降圧因子および臓器保護因子として重要な役割を果たしていると考えられる。

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Review

Adrenomedullin receptors: pharmacological features and possible pathophysiological roles☆

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Abstract

Three receptor activity modifying proteins (RAMPs) chaperone calcitonin-like receptor (CLR) to the cell surface. RAMP2 enables CLR to form an adrenomedullin (AM)-specific receptor that is sensitive to AM-(22–52) (AM_1 receptor). RAMP3 enables CLR to form an AM receptor sensitive to both calcitonin gene-related peptide (CGRP)-(8–37) and AM-(22–52) (AM_2 receptor), though rat and mouse AM_2 receptors show a clear preference for CGRPα-(8–37) over AM-(22–52). RAMP1 enables CRL to form the CGRP-(8–37)-sensitive $CGRP_1$ receptor, which can also be activated by higher concentrations of AM. Here we review the available information on the pharmacological features and possible pathophysiological roles of the aforementioned AM receptors.

Keywords: Adrenomedullin; Antagonist; Calcitonin-like receptor; Heterodimer; Receptor activity modifying protein; Transfection

1. Introduction

Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) belong to the calcitonin (CT) superfamily of regulatory peptides and are comprised of 52 and 37 amino acids, respectively [5,38]. CGRP is expressed in α- and β-forms (CGRPα and CGRPβ, respectively), which differ by one amino acid in rats and by three in humans [6,54,76]. CGRPα is generated by alternate tissue-specific splicing of the calcitonin gene [38]; CGRPβ is not derived from the calcitonin gene, despite its high sequence homology with CGRPα [6]. Although the sequence identity between the AM and CGRP peptides is only 30%, the two structures required for biological activity are totally conserved: a C-terminal amid and a ring structure comprised of six amino acids linked by a disulfide bridge between cysteine residues at positions 16 and 21 of AM and 2 and 7 of CGRP [92]. Both peptides and their specific and common binding sites are widely distributed among peripheral tissues and in the central nervous system, enabling them to exert a wide variety of biological effects, including potent vasorelaxation [21,92]. To evaluate the pharmacological characteristics of AM and CGRP, many laboratories have used the $CGRP_1$ receptor antagonist CGRP-(8–37) [13] and the AM receptor antagonist AM-(22–52) [19], both of which contain no disulfide bond.

In 1998, McLatchie et al. [50] identified and cloned human receptor activity modifying protein 1 (RAMP1; 148 amino acids), which enhances the activity of endogenous CGRP receptors in *Xenopus* oocytes. cDNAs encoding human RAMP2 and -3 (175 and 148 amino acids, respectively) were then cloned by expressed sequence tag analysis [50], and eventually all three isoforms were also cloned in rat and mouse [58,71]. RAMP1 was found to serve as an accessory protein involved in the transport of an orphan receptor, calcitonin-like receptor (CLR), to the cell surface, and when co-transfected into mammalian cells with RAMP2 or -3, CLR was found to function as an AM receptor. All three CLR/RAMP complexes exhibit a 1:1 stoichiometry [33,50]. And although they share less than 30% sequence identity, they all exhibit a common structure that includes a large extracellular N-terminal domain, a single membrane-spanning domain, and a very short cytoplasmic domain [79], and all three CLR/RAMP heterodimeric receptors mediate intracellular cAMP production and Ca^{2+} mobilization [41]. We recently identified the individual RAMP domains responsible for agonist binding to CLR/RAMP heterodimers, and their

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deletion yielded a group of dominant-negative (DN) RAMP mutants [42–44].

Like CLR, calcitonin (CT) receptor belongs to the Class II (or Class B) family of G protein-coupled receptors, members of which share a number of structural features and are all activated by peptide ligands [74]. CT receptor shares ~55% overall amino acid sequence identity with CLR, though the transmembrane domains are almost 80% identical [74]. The best characterized splice variants of human CT receptor differ depending on the presence ($CT_{(b)}$ receptor) or absence ($CT_{(a)}$ receptor) of 16 amino acids in the first intracellular loop [74]. In that regard, co-transfection of COS-7 cells with a RAMP plus the most common variant, the $CT_{(a)}$ receptor, leads to formation of a 1:1 dimer at the cell surface [16] and augmentation of responses evoked by amylin or CGRP [16,47,56,85]. AM can also elicit cAMP production via $CT_{(a)}$ receptor/RAMP1, -2 or -3 overexpressed in human embryonic kidney (HEK)-293 cells, though the responses are at least 10 to 100-fold weaker than those elicited by amylin or CGRPα [45,46]. In this review, we will summarize the pharmacological characteristics of the AM receptors associated with RAMP accessory proteins and their possible pathophysiological roles.

2. Factors influencing CLR/RAMP transfection

Before transfecting CLR or a RAMP accessory protein into mammalian cells, the cells' endogenous gene expression and function should be adequately characterized because native receptor components can modify the binding to and function of overexpressed CLR/RAMP heterodimers. In addition, we have found that the outcomes of transfection studies may vary due to such factors as the source, background and passage of the cells used [15], the growth hormones present in the medium, transfection efficacy, inconsistencies in the animal species and materials used [31], and the position of ligand iodination [46].

Because of their high transfection efficacy, HEK-293 and monkey COS-7 cells have been frequently used in a variety of transfection studies. In some HEK-293 cell lines that endogenously express RAMP1, transfection of hCLR led to significant increases in CGRP-evoked cAMP production [20,59]. Another HEK-293 cell line was shown to endogenously express low levels of RAMP2, but in that case transfection of hCLR or hRAMP1 did not augment cAMP production stimulated by CGRPα or AM, indicating that the RAMP2 was totally inactive [42,44,59]. Thus, the functionality of endogenous RAMPs likely affects the efficacy of transfected CLR [1,20,44].

Like HEK-293 cells, monkey COS-7 cells were derived from kidney, and the finding that transfection of either CLR or RAMP1 alone increased CGRP binding by ~30% suggested that these cells endogenously express CLR and one or more RAMPs ([31], unpublished observation). Indeed, we were able to detect both CLR and RAMP2 mRNAs in these cells using RT-PCR with primers for human cDNAs ([59], unpublished observation). Moreover, an RT-PCR analysis carried out by Tilakaratne et al. [85] showed that the yield of RAMP2 was lower than that of RAMP1 or RAMP3; apparently, however, none of the RAMP proteins was functional. Another consideration is that Choksi et al. [15] showed that in SK-N-MC neuroblastoma cells, which endogenously express CLR, RAMP1 and -2 (RAMP1 > RAMP2), basal expression of receptor proteins, CGRP binding and CGRP-evoked cAMP production were all markedly diminished with continuous cell passage. This raises the possibility that some data obtained from general-purpose cultured cells, including HEK-293 and COS-7 cells, may not reflect the native cellular background due to their high passage.

Finally, Hay et al. [31] showed the importance of carrying out pharmacological characterizations using CLR and RAMP cDNAs from the same animal species. In addition, it is desirable to use cell lines, agonists, antagonists and radioligands that are derived from the same species whenever possible. Ideally, moreover, the cell lines used for these transfection studies should not endogenously express RAMPs, CLR or CT receptors.

3. Recombinant AM_1 and AM_2 receptors

The pharmacological characteristics of AM_1 and AM_2 receptor subtypes (CLR/RAMP2 and CLR/RAMP3, respectively) were well summarized by Muff et al. ([35], for review see Ref. [57]). So we will summarize them only briefly here. In HEK-293 and COS-7 cells co-transfected with RAMP2 and human (h)-, rat (r)-, mouse (m)- or bovine (b)CLR, the rank order of inhibition of ^{125}I-AM binding was as follows: AM > AM receptor antagonist > CGRP-(8–37) > CGRPα. Amylin and CT were without effect. The CGRPα/AM EC_{50} ratios for cAMP production in HEK-293 and COS-7 cells transfected with these receptors are ~40- to 150 and >180, respectively. The lower selectivity for AM seen in some HEK-293 cells could be due in part to the presence of endogenous functional hRAMP1 or hCT receptor. In HEK-293 cells co-expressing hRAMP2 and h- or bCLR, hAM-(22–52) antagonized hAM-stimulated cAMP production with IC_{50}'s of 125–400 nM; hCGRPα-(8–37) was >20-fold weaker [3,59]. rAM-(20–50) inhibited rAM- and rCGRPα-evoked cAMP production with similar K_i's, whereas rCGRPα-(8–37) failed to antagonize either response [35]. In COS-7 cells co-transfected with mCLR and mRAMP2, 1 μM rAM-(20–50) raised the EC_{50} for rAM and rCGRPα 11- and >14-fold, respectively, and the corresponding K_i was 131 nM for rAM-evoked cAMP production [35], while 1 μM rCGRPα-(8–37) raised the K_i for rAM-evoked responses to 380 nM [35]. Although the $CGRP_1$ receptor antagonist CGRPα-(8–37) also weakly antagonized AM-evoked cAMP production in mammalian cultured cells transfected with CLR/RAMP2, the antagonist

Table 1
Pharmacological features of recombinant CLR/RAMP heterodimers

Receptor subtype	Agonist specificity		Antagonist potency for AM-evoked responses	
	AM	CGRP α	hAM-(22–52)or rAM-(20–50)	CGRP-(8–37)
CLR/RAMP1 ($CGRP_1$ receptor)		<	≪	
CLR/RAMP2 (AM_1 receptor)		≫	≫	
CLR/RAMP3 (AM_2 receptor)				
Humans, pigs, cows		≫	>	
Rats, mice		>	≪	

had no effect on responses evoked by 200 nM rAM in yeast cells transfected with hCLR/hRAMP2, which endogenously express no CLR/RAMP system [51].

Thus, the CLR/RAMP2 heterodimer defines the AM_1 receptor, which recognizes AM over CGRP with a selectively of two to three orders of magnitude, and which is more effectively antagonized by either hAM-(22–52) or rAM-(20–50) than by CGRP-(8–37) (Table 1).

The available data on AM binding to the AM_2 receptor and the resultant signaling are somewhat limited. In HEK-293 or COS-7 cells expressing h-, m-, b- or pAM_2 receptor, inhibition of ^{125}I-AM binding (IC_{50} = 1.4–4.6 nM) by AM was comparable to that seen with AM_1 receptors [35,57]. Again, the IC_{50} values observed with AM_1 and AM_2 receptors were comparable to those for AM-evoked cAMP production via either receptor. hAM-(22–52) and hCGRPα-(8–37) inhibited ^{125}I-AM binding to the hAM_2 receptor with equal potency (IC_{50} = 16–38 nM and 12–35 nM, respectively), and hCGRPα was only ~4-fold less potent. This differs from the profile obtained with rodent AM_2 receptor expressed in COS-7 cells, where rAM = rCGRPα-(8–37) > rAM-(20–50) = rCGRPα [35]. When co-transfected with hRAMP3 into HEK-293 cells, p- and bCLR were even more selective for AM over CGRPα, and hCGRPα-(8–37) was insensitive. In HEK-293 cells co-transfected with hRAMP3 and h-, p- or bCLR, hCGRPα was 30- to 100-fold less potent than hAM with respect to evoked cAMP production, which is similar to results obtained with AM_1 receptors. By contrast, in COS-7 cells co-expressing mRAMP3 and r- or mCLR, CGRPα was only 12- to 16-fold less potent than AM, and amylin and CT were inactive. In HEK-293 cells expressing bCLR/hRAMP3, hAM-(22–52) antagonized hAM-stimulated cAMP production with an IC_{50} of 85 nM; that hCGRPα-(8–37) was 20-fold less potent [3] is consistent with the inhibitory effects of both antagonists on bAM_1 receptor. In COS-7 cells expressing mCLR/mRAMP3, 1 μM rCGRPα-(8–37) increased the EC_{50}'s of rAM and rCGRPα 10- and 25-fold, respectively, giving K_i's of 127 nM and 47 nM [35]. On the other hand, 1 μM rAM-(20–50) raised the EC_{50} of rAM and rCGRPα 4- and 12-fold, respectively, giving K_i's of 480 nM and 120 nM [35]. In yeast cells expressing hCLR/hRAMP3, AM-(22–52) but not CGRP-(8–37) antagonized rAM-mediated responses [51].

Thus, unlike the pharmacological features of the AM_1 receptor, those of the AM_2 receptor, differ among species (Table 1). In h-, p- and bAM_2 receptors, AM antagonists are more potent than CGRP-(8–37), which is not so different from that seen with AM_1 receptors. By contrast, r- and mAM_2 receptors recognize AM over CGRP with a selectivity of one order of magnitude, and are more effectively antagonized by CGRP-(8–37) than by AM antagonists.

4. A recombinant $CGRP_1$ receptor able to respond to AM

In HEK-293 cells co-transfected with hRAMP1 and h-, p- or bCLR, ^{125}I-CGRP binding was inhibited by CGRPα with an IC_{50} of 0.1–2.2 nM [2,3,24,50]. Likewise, CGRPβ showed a high-affinity (IC_{50} = 0.01–0.3 nM) for these receptors. Although the $CGRP_1$ receptor antagonist CGRP-(8–37) lacks the ring structure required for CGRP signaling, it was capable of binding to CLR/RAMP1 heterodimers with an affinity comparable to that of CGRPα. By contrast, AM was 25- to 500-fold less potent than CGRPα, and the AM receptor antagonist AM-(22–52) was largely inactive. When COS-7 or Drosophia Schneider 2 cells were transfected into r- or mCLR plus m- or hRAMP1, CGRPα, CGRPβ and CGRP-(8–37) all strongly inhibited ^{125}I-CGRP binding with very similar potencies (IC_{50} = 1.2–7 nM) [4,9,34,35]. In those cases, AM was ~20- to 40-fold less potent than CGRPα, and AM-(22–52) was totally inactive. Taken together, these results indicate that, for the CLR/RAMP1 dimer, binding affinities determined as a function of competitive inhibition of ^{125}I-CGRP binding is as follows: CGRPα = CGRPβ = CGRP-(8–37) > AM ≫ hAM-(22–52) or rAM-(20–50).

When hRAMP1 and h-, p- or bCLR were co-transfected into HEK-293 cells, CGRPα and CGRPβ augmented cAMP production with similar potencies (EC_{50} = 0.3–7.8 nM) [2,3,24,41,42], and AM was only ~2- to 8-fold less potent than CGRPα [2,3,24,42]. In COS-7 cells transfected with r- or mCLR plus m- or hRAMP1, AM was ~100- to 300-fold less potent than CGRPα, though the EC_{50}'s of CGRPα and CGRPβ (0.1–0.9 nM) were about the same as that seen in HEK-293 CLR/RAMP1 transfectants [9,34,35]. There has been only a single study in which functional expression of hCLR/hRAMP heterodimeric receptors was evaluated using a null system, the yeast *Saccharomyces cerevisiae* [51]. In control cells, neither CGRP nor AM (1 μM) elicited β-galactosidase activity, but after co-transfection of hCLR and hRAMP1, both hCGRPα and hAM elicited β-galactosidase activity, and hAM was ~20–30-fold less potent than hCGRPα. Taken together, these results indicate that the rank order of agonists eliciting functional responses in CLR/RAMP1 transfectants is as follows: CGRPα

= CGRPβ > AM, which is consistent with the profile of inhibition of ^{125}I-CGRP binding.

There have been several reports on the effect of CGRP-(8–37) on AM-mediated responses [15,35,59]. In HEK-293 cells co-transfected with hCLR and hRAMP1, hCGRPα-(8–37), but not hAM-(22–52), dose-dependently inhibited cAMP production evoked by 10 nM hAM or 1 nM hCGRPα with estimated IC_{50}'s of ~30 nM and ~200 nM, respectively [59]. Comparable results were obtained in HEK-293 cells transfected with hCLR/hRAMP1; IC_{50}'s for hCGRPα-(8–37)-mediated inhibition of hAM and hCGRPα responses were 35 nM and 110 nM, respectively [3]. In hCLR/hRAMP1-transfected yeast cells, hCGRPα-(8–37) blocked the response to 2 μM rAM and 100 nM hCGRPβ with IC_{50}'s of ~1 and 4 μM, respectively, whereas hAM-(22–52) had no effect [51]. In COS-7 cells co-transfected with mCLR and mRAMP1, EC_{50}'s for rAM- and rCGRPα-evoked cAMP production were increased 209- and 100-fold in the presence of 1 μM rCGRPα-(8–37), but 1 μM rAM-(20–50) was without effect; the calculated K_i's for rCGRPα-(8–37) were 12 nM and 5 nM for rCGRPα and rAM-evoked cAMP production, respectively [35]. Thus, a $CGRP_1$ receptor antagonist can inhibit AM responses ~3- to 7-fold more effectively than CGRP responses in CLR/RAMP1 transfectants.

The findings summarized above indicate that, irrespective of its animal species, AM can interact with $CGRP_1$ receptors and that AM responses mediated via this receptor are antagonized by CGRPα-(8–37) but not by a selective AM receptor antagonist (Table 1). Still, although higher concentrations of AM can activate this receptor, there is no evidence that the $CGRP_1$ receptor functions as a CGRP-(8–37)-sensitive AM receptor in vivo.

5. Possible CT-(8–32)-sensitive AM receptors

Recent studies have shown that CT receptors can also interact with RAMPs, thereby forming heterodimeric receptors that are highly sensitive to amylin and CGRP [16,45–47,56,85]. Of the human CT receptor isoforms, $CT_{(a)}$ receptor is the more strongly expressed and the more widely distributed.

Co-expression of human $CT_{(a)}$ receptor with any hRAMP in HEK-293 cells led to significant increases in hAM-evoked cAMP production (EC_{50} > 1–5 nM) [45], though hAM remained at least 10 to 100-fold less potent than hCGRPα [45,46]. Salmon (s)CT-(8–32), but not hCGRPα-(8–37) or hAM-(22–52), antagonized hAM-evoked cAMP production via human $CT_{(a)}$ receptor/RAMP1, -2 or -3 with IC_{50}'s of 40–100 nM, but had little effect on AM responses mediated via the CT receptor alone. By contrast, 1 μM sCT-(8–32) had no effect on hCGRPα- or hAM-evoked cAMP production in cells expressing CLR/RAMP1, -2 or -3 [45]. Thus, AM can also activate sCT-(8–37)-sensitive receptors comprised of human $CT_{(a)}$ receptor with any RAMP in HEK-293 cells. Whether this receptor behaves as a functional AM receptor in vivo remains unclear, however.

6. Endogenous AM receptors in tissues and cell lines

CGRP and AM have been reported to share a number of pharmacological features; for example, many effects of AM are apparently blocked by CGRPα-(8–37). In basilar arteries isolated from Wistar-Kyoto and stroke-prone spontaneously hypertensive rats, relaxations elicited by AM and CGRP were markedly inhibited by 1 μM hCGRP-(8–37) [65]; likewise, treatment with 100 nM CGRP-(8–37) inhibited relaxations induced by either AM or CGRP in isolated canine retinal arteries [69], and 1 μM CGRP-(8–37) inhibited AM- and CGRPα-evoked relaxations in porcine coronary arteries [97]. The effects of AM, which was 4- to 100-fold less potent than CGRP, were all endothelium-independent.

There have been several reports that CGRPα-(8–37), but not AM-(22–52), blocks AM-evoked cellular responses. For instance, in neonatal cardiac myocytes and non-myocytes, AM elicited concentration-dependent increases in cAMP, and CGRP was about 20–60 times more potent than AM [64]. CGRP-(8–37) dose-dependently blocked cAMP production induced by AM or CGRP in both cell types, whereas AM-(22–52) at concentrations up to 1 μM did not [64]. In non-myocytes, the estimated IC_{50}'s for CGRP-(8–37)-mediated inhibition of cAMP production elicited by 10 nM AM and 1 nM CGRP were ~1 and ~10 nM, respectively [64]. Tomoda et al. [86] demonstrated that in rat non-myocytes, which abundantly express RAMP1 but express only low levels of RAMP2 and CLR, AM was ~100-fold less potent than CGRP with respect to stimulation of cAMP production, and that a CGRP receptor antagonist dose-dependently reduced IL-6 secretion stimulated by AM plus IL-1β, but an AM receptor antagonist did not. These findings suggest that endogenous co-expression of RAMP1 with CLR may yield a receptor common to both CGRP and AM.

On the other hand, AM was ~100-fold more potent than CGRPα with respect to evoked cAMP production in human and rabbit aortic endothelial cells [37,55], human aortic smooth muscle cells [37] or rat cerebral microvessels [40], all of which endogenously express AM_1 receptors comprised almost exclusively of CLR and RAMP2. In rabbit aortic endothelial cells, hAM-(22–52) inhibited AM-evoked cAMP production with a K_i of 3 nM [55]. Moreover, Kobayashi et al. [40] demonstrated that in rat cerebral microvessels AM-(22–52) (1 μM), but not CGRP-(8–37) (1 μM), blocked AM (10 nM)-evoked cAMP production with an IC_{50} of 1.8 μM. In this case, ^{125}I-AM binding was inhibited by AM and AM-(22–52) with IC_{50}'s of 0.3 and 7.6 nM, respectively; CGRP and CGRP-(8–37) were much less potent (IC_{50} > 100 nM). These results are compatible with those obtained with NG108-15 neuroblastoma × glioma hybrid cells, which are believed to endogenously express AM_1 re-

ceptor [50] and various CLR/RAMP2-transfected cells [57]. Such AM-specific effects were also observed in mesangial cells [72,73], mesenteric arteries [26], cerebral parenchymal microvessels [83], iris sphincter [98], oculus [17] and testicular peritubular myoid cells [78]. Among them, rat mesangial cells were recently found to express CLR as well as all three RAMPs (RAMP2 > RAMP1 = RAMP3) [67].

Because RAMPs are so ubiquitously expressed [50,58, 71,79], their combined presence make interpreting some AM-mediated responses difficult. For instance, in porcine coronary arteries, where both RAMP1 and RAMP2 are expressed (RAMP1 > RAMP2), 1 μM CGRPα-(8–37) inhibited AM-induced vasodilation, whereas 1 μM AM-(22–52) had little effect [29]. And although human coronary arteries express equal levels of RAMP2 and RAMP1, the vasorelaxant effect of AM was ~100-fold less potent than that of CGRPα and was antagonized by CGRPα-(8–37) but not by AM-(22–52) [30]. This may be explained by the finding that the interaction between CLR and RAMP1 predominates over that between CLR and RAMP2 [9,34]. On the other hand, based on data obtained in rabbit aortic endothelial cells, it has been suggested that RAMP3 has great affinity for CLR [55]. In isolated rat uterus, where all three RAMPs are highly expressed at equal levels, 1 μM CGRPα-(8–37) or AM-(22–52) totally blocked the inhibitory effects of AM on bradykinin-induced periodic contractions [95]. In that case, CGRPα-(8–37)- and AM-(22–52)-sensitive AM_2 receptors may predominate among the three AM receptors present. It remains unclear, however, whether these phenomena reflect competition among RAMPs for interaction with CLR, interaction among RAMPs themselves (RAMP homo- or heterodimerization) or both.

To our knowledge, there has been only one previous observation of CT-(8–32)-sensitive AM responses that were insensitive to hCGRP-(8–37) and hAM-(22–52) [18]. In that case, CT receptor was endogenously expressed in human T47D breast cancer cells, and the observed EC_{50} values for AM and CT were 132 and 0.5 nM, respectively. Whether T47D cells also express RAMP is unknown, however.

Champion et al. [12] demonstrated that in the hindlimb vascular bed of the cat, neither CGRP-(8–37) nor AM-(22–52) antagonized the vasodilatory effect of AM, but they suppressed CGRP-mediated responses equally. In another study, moreover, AM-induced relaxation of mouse aortic rings was only slightly (3-fold) shifted by AM-(22–52), and not at all by CGRP-(8–37), and both antagonists failed to inhibit CGRP-mediated vasorelaxation [7]. However, those studies provided no information as to whether CT induces vasorelaxation and CT-(8–32) inhibits AM-mediated responses. In general, the vasodilation elicited by CT is substantially weaker than that elicited by CGRP or AM [8]. It is thus unlikely that the vasorelaxant effects of AM are mediated via three AM receptors comprised of RAMP1, -2 or -3 plus CLR or CT receptors. Instead, another AM receptor may be present there.

7. Assessment of endogenous CLR expression

An earlier Northern blot analysis revealed that, in humans, the mRNA encoding CLR was predominantly expressed in the lung, less so in heart, and not at all in brain and other peripheral tissues [1]. Later, RT-PCR analysis showed CLR mRNA to be present in a variety of human tissues and cells, including cerebral arteries [77], adrenal cortex [84], uterus [63], hairy skin [27], SK-N-MC neuroblastoma cells [15,50], KG1C oligodendroglial cells [90] and coronary artery endothelial and smooth muscle cells [60], and that at least one RAMP isoform was also expressed. L6 myoblastic cells also showed no CLR signals in Northern blot analyses, despite the presence of sufficient CLR protein to measure high-affinity binding of AM and CGRP [50]. Apparently, the fact that CLR mRNA may be undetectable by Northern analysis does not exclude its presence. Furthermore, in situ hybridization did not reveal rCLR signals in the cerebellum or spleen, despite the presence of densely distributed CGRP binding sites [23]; the authors suggested the existence of another CGRP receptor. In yet another study, however, CLR mRNA was readily detectable in rat cerebellum and spleen by Northern analysis [11]. In that case, the CLR mRNA correlated significantly with RAMP2 mRNA ($R = 0.94$, $P = 0.016$) in eight rat tissues, including cerebellum and spleen, and ^{125}I-AM binding tended to correlate with CLR mRNA ($R = 0.93$, $P = 0.11$) and RAMP2 mRNA ($R = 0.95$, $P = 0.14$), but not RAMP3 mRNA ($R = 0.32$, $P = 0.40$) [11].

There also have been several recent reports showing widespread distribution of CLR-like immunoreactivity (CLR-LI) in human organs, including lung, heart ventricle and kidney [27,28,63]. CLR-LI was also detected in the endothelium of all blood vessels and tended to be more strongly detected in venous than arterial vessels, with the exception of the pulmonary artery and vein [28]. In human middle cerebral arteries, levels of CLR mRNA were independent of artery diameter, but levels of RAMP1 and RAMP2 mRNAs tended to increase with increases in artery size and decreases in responsiveness to CGRP and AM [77]. In that case, CLR protein levels should also be evaluated in arteries of varying size. Nonetheless, it appears that CLR is widely expressed along with RAMP, leading to formation of functional AM receptors in vivo.

8. Expression of mRNAs encoding CLR, RAMPs and AM under various pathologic conditions

As shown in Table 2, changes in the gene expression of the three RAMP isoforms do indeed vary under different pathological conditions. In patients with pregnancy-induced hypertension, for example, the level of RAMP2 mRNA was increased in the fetal membrane, but was reduced, along with CRLR mRNA, in umbilical artery and uterus—i.e., the expression of RAMP2 mRNA was negatively correlated with

Table 2
Alterations of mRNAs encoding CLR, RAMPs and AM under various pathologic conditions

Pathology	Materials		CLR	RAMP1	RAMP2	RAMP3	AM	References
Hypertension	Women (+pregnancy)	Umbilical artery	↓	ND	↓	ND	↑	[48]
		Uterine muscle	↓	ND	↓	ND	ND	[48]
	Rat (+phenylephrine)	PVN/NTS	ND	ND	↓	ND	ND	[82]
	SHR-SP	Left ventricle	↑	ND	↑	ND	↑	[91]
Heart failure	Rat (+myocarial infarction)	Left ventricle	ND	ND	↑	ND	↑	[68]
		Atrium	↑	ND	↑	ND	↑	[87]
		Ventricle	↑	ND	↑	ND	↑	[87]
		Kidney	→	ND	→	ND	→	[87]
	Rat (+aortic banding)	Left ventricle	↑	ND	↑	↑	↑	[70]
	Rat (+aortocaval shunt)	Left ventricle	↑	ND	↑	→	↑	[70]
		Kidney	→	ND	→	→	↑	[96]
Cardiomyopathy	Rat (+isoproterenol)	Heart	ND	ND	↑	ND	↑	[75]
Renal failure	Rat (+5/6 nephrectomy)	Kidney	↓	ND	→	↓	→	[88]
Nephropathy	Rat(+ureteral obstruction)	Obstructed kidney	↑	↑	↑	→	→	[58]
Diabetes	Rat (+streptozotocin)	Kidney	ND	ND	↑	ND	↑	[32]
Salt loading	Rat (+8% NaCl)	Adrenal gland	↑	↑	↑	→	↑	[10]
		Kidney	↑	→	→	↑	↑	[10]
Sepsis	Rat (+lipopolysaccharide)	Lung/Spleen/Thymus	↓	↓	↓	↑	ND	[71]
Others (in vitro)	Human neuroblastoma cell (+hypoxia)		ND	→	↓	(-)	↑	[39]
	Human CAEC (+C-reactive protein)		→	→	→	(-)	↓	[60]
	Human CASMC (+C-reactive protein)		→	→	→	(-)	↓	[60]
	Human CASMC (+tumor necrosis factor-α		↓	↓	↓	(-)	↑	[61]
	HumanCASMC(+dexamethasone)		↑	↑	→	ND	↑	[25]
	Rat cardiomyocyte (+endothelin I)		↑	→	↓	↑	↑	[52]
	Rat cardiomyocyte (+angiotensin II)		→	↑	→	↑	↑	[53]
	Rat mesangial cell (+PDGF)		ND	ND	→	↑	↑	[67]

ND: not determined; CLR: calcitonin-like receptor; RAMP: receptor activity modifying protein, adrenomedullin; SHR-SP: spontaneously hypertensive stroke-prone rat; PVN: paraventricular nucleus; NTS: nucleus tractus solitarius; CAEC: coronary artery endothelial cell; CASMC: coronary artery smooth muscle cell; PDGF: platelet-derived growth factor.

the systolic and diastolic pressures in both tissues [48]. The reduced expression of AM_1 receptor mRNA in umbilical artery may have contributed to the increased resistance in the umbilical circulation seen in these cases, though it remains unclear whether the increased AM downregulates its own receptors.

There is also a recent study showing a similar correlation between systemic blood pressure and levels of RAMP2 mRNA [82]. Phenylephrine-induced blood pressure elevation decreased expression of RAMP2 mRNA in the paraventricular nucleus (PVN) and the nucleus tractus solitarius (NTS) [82], whereas nitroprusside-induced hypotension increased RAMP2 mRNA expression in the NTS and decreased AM expression in the PVN [82]. Alterations in the levels of RAMP2 in these autonomic nuclei may affect the ability of central AM to regulate sympathetic activity. On the other hand, in the spontaneously hypertensive stroke-prone rat (SHR-SP), CLR, RAMP2 and AM mRNAs were all upregulated in the hypertrophied left ventricle (LV), and decreasing blood pressure using captopril or trichlormethiazide resulted in reduced expression of all the upregulated genes [91]. In addition, the fact that AM inhibits angiotensin II-induced hypertrophy of cultured rat cardiomyocytes suggests the increased expression of AM_1 receptor may ameliorate the LV hypertrophy [89].

During heart failure induced by myocardial infarction in rat, expression of AM and RAMP2 or RAMP3 mRNA was increased in both the nonischemic and ischemic regions of the LV [68,87], and treatment with the endothelin receptor antagonist bosentan prevented the increase in RAMP2 mRNA [68]. The induction of the AM signaling system may be beneficial, as early infusion of AM (for a week) powerfully inhibited ventricular remodeling after myocardial infarction in rats [62]. In rat cardiac overload models, both pressure overload (by aortic banding) and volume overload (by aortocaval shunt) led to increased expression of AM and AM_1 receptor mRNAs, but unlike pressure overload, volume overload did not enhance RAMP3 mRNA expression [70]. Thus, LV AM_1 and AM_2 receptors might play different roles during cardiac overload. In addition, AM protected rat myocardium from isoproterenol-induced hypertrophy and necrosis [36], and RAMP2 mRNA was upregulated in the myocardium of isoproterenol-treated rats [75].

Although AM exerts both diuretic and natriuretic effects [21], renal expression of AM_1 or AM_2 receptor mRNA was unchanged during heart failure [87,96]. Expression of CLR and RAMP3 mRNAs was downregulated in the remnant kidney after 5/6 nephrectomy, which is a rat model of acute renal failure, while RAMP2 mRNA was unchanged [88]. Despite no change in AM mRNA expression, CLR,

RAMP1 and RAMP2 mRNAs, but not RAMP3 mRNA, were upregulated in kidneys with ureteral obstruction [58]. The increase in AM_1 receptor may provide protection against proliferative or fibrotic changes in the obstructed kidney. Streptozocin-induced diabetic rats showed upregulated expression of AM and RAMP2 in hypertrophied glomeruli and in afferent arterioles and increased urinary excretion of nitric oxide (NO_2^- and NO_3^-) due partly to AM stimulation [32]. In this model, adenovirus-mediated AM gene transfer improved cardiac function and prevented renal damage [32]. Chronic salt loading led to increased expression of AM and mRNA CLR in the adrenal glands and kidneys of the rat, without significant elevation of blood pressure [10]. In that case, RAMP1 and RAMP2 mRNAs were increased in adrenal glands, while RAMP3 mRNA was increased in kidneys [10]. In addition to its diuretic and natriuretic effects, AM can also inhibit angiotensin II- or potassium-induced aldosterone secretion from adrenal glands [93,94]. After salt loading, therefore, some secondary mediators may differentially regulate the expression of AM receptor components to restore water and electrolyte balance.

During sepsis, levels of both circulating and local AM were markedly increased in humans and rats [49,66]. By contrast, expression of CLR and the three RAMP mRNAs were markedly downregulated in many tissues [71]. This is most likely due to the marked increases in the level of various agonists, cytokines or both. It is noteworthy that in lung, spleen and thymus the upregulation of RAMP3 mRNA was accelerated during the late stage of sepsis, suggesting that RAMP3 may be involved in immune function.

There have been several in vitro studies showing the effects of various agents on AM receptor components. In cultured human neuroblastoma cells, hypoxia induced downregulation of RAMP2 mRNA with no effect on RAMP1 mRNA [39], though the mechanisms remain unclear. C-reactive protein (CRP) dose-dependently decreased AM release from human coronary artery endothelial and smooth muscle cells (HCAECs and HCASMCs), but did not affect expression of CLR, RAMP1 and RAMP2 mRNA or AM-evoked cAMP production in either cell type [60]. In HCASMCs, low concentrations of TNF-α downregulated CLR, RAMP1 and RAMP2 mRNAs, thereby decreasing AM-evoked cAMP production, while higher concentrations of TNF-α elicited secretion of small amounts of AM from the cells [61]. It is, therefore, likely that the increased AM contributes little to the downregulation of AM receptors.

Aside from vasorelaxation, the most powerful effect of AM is the inhibition of the vascular oxidative stress known to contribute significantly to the development of atherosclerosis [80,81]. This suggests that downregulation of AM by CRP, together with downregulation of AM receptors by TNF-α, contributes to the progression of coronary atherosclerosis. By contrast, dexamethasone (≥10 nM) increased CLR and RAMP1 mRNAs, but not RAMP2 mRNA, in HCASMCs [25], though no effect on AM_1 receptor function was reported. Endothelin I and angiotensin II differentially regulated the expression of CLR and all three RAMP mRNAs in rat cardiomyocytes, resulting in increases in AM-evoked cAMP production [52,53]. In addition, AM has been shown to decrease platelet-derived growth factor (PDGF)-induced mesangial cell proliferation [14], and Nowak et al. clearly showed that in mesangial cells PDGF stimulated expression of RAMP3 mRNA and protein, as well as AM-stimulated adenylate cyclase activity [67]. The increase in RAMP3 mRNA was dependent on mRNA stability, but not on transcription, and was mitogen-activated protein kinase (MAPK) kinase (MEK)- and p38 MAPK-dependent [67]. They also demonstrated that transfection of RAMP2 and RAMP3 enhances AM-mediated inhibition of mesangial cell hypertrophy. By contrast, AM promoted migration and invasion of human umbilical vein endothelial cells, which express endogenous AM_1 and AM_2 receptors [22]. Interestingly, both phenomena were strongly inhibited by pretreatment with anti-CLR/anti-RAMP2 or anti-CLR/anti-RAMP3 antibodies, all of which were raised against amino acids in the respective extracellular N-terminal domains [22]. Such blocking antibodies should be useful for clarification of the pathophysiological roles of endogenous AM receptor subtypes.

9. Concluding remarks and future perspectives

Co-expression of CLR and RAMP2 or RAMP3 can produce functional AM receptors (Table 1), but only CLR/RAMP2 comprises an AM-specific receptor that is particularly sensitive to AM-(22–52)—i.e., this heterodimer defines the AM_1 receptor subtype. CLR/RAMP3 defines the AM_2 receptor, which cross-reacts with CGRP at lower concentrations and is more sensitive to CGRP-(8–37) than is the AM_1 receptor. With the r- or m AM_2 receptor, in particular, AM-evoked responses are more effectively blocked by CGRP-(8–37) than by AM-(22–52), though this is not observed with the h-, p- or bAM_2 receptor. Overexpressed $CGRP_1$ receptor (CLR/RAMP1) can also respond to AM at higher concentrations, and the responses are blocked by CGRP-(8–37) but not by AM-(22–52) (Table 1), though there has been no evidence that $CGRP_1$ receptor can also act as a CGRP-(8–37)-sensitive AM receptor in vivo. Thus, in tissues where only $CGRP_1$ and AM_1 receptors are present, combined use of the both antagonists will be useful for defining these receptor subtypes. But in cases where the AM_2 receptor is also present, the antagonist selectivity will be impaired.

Actually, RAMPs commonly coexist in native tissues and cells [74,79]. We recently identified three dominant-negative RAMP mutants able to inhibit endogenous CLR/RAMP function [42–44]. However, the coexistence of multiple RAMP isoforms means that these mutants cannot selectively inhibit the corresponding RAMPs, most likely due to competitive interactions among RAMPs. The identification of specific, selective negative-regulators for CLR/RAMPs will be needed to clarify the role of individual AM receptors

under various pathophysiological conditions and to examine whether other AM receptors may be present in some vascular beds [7,12].

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