

ADRENOMEDULLIN RECEPTOR AND SIGNAL TRANSDUCTION

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INTRODUCTION

Adrenomedullin (AM) was originally isolated from acid extract of human pheochromocytoma as a bioactive peptide characterized by its ability to raise intracellular cAMP levels in rat platelet (Kitamura et al., 1993). AM shows a partial structural homology with calcitonin gene-related peptide (CGRP) and amylin in terms of the six amino acids ring structure by disulfide bridge and C-terminal amidation, hence AM is classified as a member of calcitonin supergene family (reviewed in Hinson et al., 2000; Poyner et al., 2002). AM was initially characterized as a potent vasodilatory peptide: AM relaxes precontracted vascular bed *in vitro* and intravenous bolus injection of AM caused a potent and long-lasting hypotensive effect *in vivo* (Ishiyama et al., 1993; Kitamura et al., 1993; Nuki et al., 1993; Parkes and May, 1997). However, a growing body of evidence has been accumulated that AM exerts pleiotropic actions, including cell proliferation (Iwasaki et al., 1998a; Shichiri et al., 2003), migration (Fukai et al., 2003; Horio et al., 1995), apoptosis (Kato et al., 1997; Shichiri et al., 1999), inflammation (Hirata et al., 1996; Sugo et al., 1995), angiogenesis (Kim et al., 2003), and hormone secretion (Nussdorfer et al., 1997).

Biological action of AM was initially considered to be mediated solely via cAMP/PKA pathway via its specific receptor, but subsequent studies have revealed that pleiotropic effects of AM are mediated through diverse intracellular signal transduction pathways other than cAMP/PKA pathway, such as ERK/MAPK, PI3K/AKT, and NO/cGMP/PKG pathway. Recently, three novel isoforms of receptor activity-modifying proteins (RAMP) were

isolated (McLatchie et al., 1998), and it has been proposed that calcitonin receptor-like receptor (CRLR), a seven-transmembrane receptor, can function as either CGRP receptor or AM receptor, depending on which RAMP isoforms are co-expressed: co-expression of RAMP1 and CRLR confers CGRP1 receptor, whereas RAMP2 or RAMP3 co-expressed with CRLR generates AM receptor (Born et al., 2002; McLatchie et al., 1998; Poyner et al., 2002).

This chapter specifically focuses on the biochemical and molecular characterization of AM receptor, the intracellular signal transduction mechanism of AM in relation to its pleiotropic effects, and current concept of RAMP/CRLR receptor system.

1. CHARACTERIZATION OF AM RECEPTOR

Since biological function of AM was initially characterized as its vasodilator effect *in vitro* and *in vivo*, the receptor of AM was initially studied by its binding to rat VSMCs. By competitive binding assay using [¹²⁵I] rat (r)AM, we first demonstrated the presence of a single class of binding sites for AM in cultured rat aortic VSMC with the apparent dissociation constant (K_d) of 1.3×10^{-8} M and the maximal binding capacity (B_{max}) of 19,000 sites/cell (Eguchi et al., 1994b). The apparent K_d value is almost comparable concentration to that for AM to induce vasodilation in the perfused rat mesenteric vascular bed (Nuki et al., 1993). Affinity-labeling of VSMC membrane fraction with [¹²⁵I] rAM revealed two distinct major labeled bands with apparent molecular weight of 120 and 70kDa; both bands disappeared in the presence of excess unlabeled rAM or rat CGRP, suggesting that AM receptor is identical and/or very similar to CGRP receptor (Eguchi et al., 1994b).

Since AM has been shown to increase cAMP generation in rat platelet (Kitamura et al., 1993) and cAMP is a well-known major second messenger for vasodilation, cAMP is originally considered to be a major intracellular signaling molecule for AM. In consistent with this notion, we clearly demonstrated that human (h)AM and rAM dose-dependently (10^{-9} - 10^{-6} M) stimulates cAMP formation in rat VSMC with the approximate EC_{50} value (6×10^{-9} M) lower than its K_d value (Eguchi et al., 1994b). CGRP1 receptor

antagonist, CGRP(8-37) dose-dependently inhibited cAMP response by rAM and rCGRP (Eguchi et al., 1994b). In agreement with this, AM-induced vasodilator response in rat mesenteric vascular bed was markedly inhibited by CGRP(8-37) (Nuki et al., 1993). Similar inhibitory effect of CGRP(8-37) on AM-induced vasodilation was reported in the isolated rat heart and microvasculature (Entzeroth et al., 1995 ; Hall et al., 1995). AM has been shown to bind with high affinity to, and stimulate cAMP production in neuroblast cell line (SK-N-MC), a cell model exclusively expressing CGRP1 receptor (Zimmermann et al., 1995). These data suggest that at least some biological effect by AM is mediated via CGRP1 receptor. In fact, AM has been shown to compete with [125 I]CGRP binding in a variety of tissues, whose affinities are one- or two-orders of magnitude lower than CGRP (Table 1-A and 1-B), suggesting the preferential binding of AM to CGRP1 receptor.

In contrast, using [125 I] AM binding studies, the presence of specific AM binding sites with much higher affinity to AM than CGRP, has been reported (Table 1). For example, [125 I] rAM binding to rat lung and heart membranes was competed by rAM with high affinity (K_i : 5.8 and 0.2nM, respectively), whereas rCGRP could not or scarcely compete with [125 I] rAM binding to these tissues (Owji et al., 1995). It is of note, however, that rAM competed with [125 I]CGRP binding to the same tissue preparations with relatively high affinity (Owji et al., 1995). These data clearly demonstrated the presence of specific AM receptors in lung and heart, in addition to the specific CGRP receptors with relative high-affinity to AM. Similar results were also noted in L6 cells and spinal cord (Coppock et al., 1996; Owji et al., 1996). [125 I]AM binding studies obviously revealed that CGRP was about three orders of magnitude less than AM, suggesting highly specific receptors for AM. Since a wide variety of cells and tissues, including lung, heart, and vascular tissue, have both specific CGRP and AM binding sites, it is difficult to distinguish between two distinct, but very similar receptors in these tissues. Collectively, AM appears to exert its biological effect through its specific AM receptor and/or CGRP1 receptor.

Table 1-A and 1-B Characterization of AM and CGRP binding site

1-A. [125 I]CGRP binding site

Tissue or cell	CGRP affinity (nM)	AM affinity (nM)	Ref
Rat lung	0.3 (IC ₅₀)	5.0 (IC ₅₀)	Owji et al., 1995
Rat heart	0.05 (IC ₅₀)	0.8 (IC ₅₀)	Owji et al., 1995
SK-N-MC cells	0.04 (Ki) 3.5 (Ki for CGRP(8-37))	0.37(Ki)	Entzeroth et al., 1995
L6 cells	0.13 (IC ₅₀)	8.7 (IC ₅₀)	Coppock et al., 1996
Rat spinal cord	0.18 (Kd)	34.6 (Ki)	Owji et al., 1996
Rat uterus	0.14 (Kd)	1.67 (Ki)	Upton et al., 1997
Rat hypothalamus	0.1 (Kd) 4.0 (Ki for CGRP(8-37))	4.6 (Ki)	Taylor et al., 1996

1-B. [¹²⁵I]AM binding site

Tissue or cell	AM affinity(nM)	CGRP affinity(nM)	Ref
Bovine aortic EC	10*	>1000	Shimekake et al., 1995
Rat lung	1.3 (IC ₅₀)	>1000	Owji et al., 1995
Rat heart	0.5 (IC ₅₀)	1050	Owji et al., 1995
Swiss 3T3 cell	3.5 (IC ₅₀)	>1000	Withers et al., 1996
Rat aorta	1.38 (Kd)	>1000	Nandha et al., 1996
Rabbit kidney	0.45 (IC ₅₀)	>1000	Hjelmqvist et al., 1997
Rat-2 fibroblast	0.43 (Kd)	>1000	Coppock et al., 1999
Human brain	0.17 (Kd)	>1000	Sone et al., 1997
L6 cells	0.22 (IC ₅₀)	>1000	Coppeck et al., 1996
Rat uterus	0.08 (Kd)	>1000	Upton et al., 1997

IC₅₀: concentration inhibiting binding by 50%; Ki: absolute inhibition constant; Kd: dissociation constant; *exact IC₅₀ could not be calculated.

2. STRUCTURE-ACTIVITY RELATIONSHIP OF AM

Although the structural homology between AM and CGRP is low (~30%), calcitonin supergene family share the ring structure formed by an intramolecular disulfide bond and amidated C-terminal Tyr⁵² residue in common (reviewed in Hinson et al., 2000; Poyner et al., 2002). Hence, we studied structure/activity relationship of AM molecule using various synthetic human AM analogs in rat VSMCs (Table 2) (Eguchi et al., 1994a). Comparison between binding affinities and potencies of adenylate cyclase activity by synthetic AM analogs are summarized in Table 2. N-terminal-truncated derivatives, hAM-(13-52)-NH₂ and hAM-(16-52)-NH₂, both

retaining the cyclic structure and the amidated C-terminus showed comparable Ki and cAMP-generating activities to those of mature form hAM-(1-52)-NH₂, whereas N-terminal fragment hAM-(1-10)-OH had no effect. These data indicate that N-terminal residues (1-15) of AM molecule are not essential for interaction with its receptor. Both removal of the C-terminal Tyr⁵² residue [hAM-(1-51)-OH] and [hAM-(1-52)-OH] resulted in a remarkable decrease in receptor-binding activity and cAMP response. The amidation of the C-terminal Gly⁵¹ residue [hAM-(1-51)-NH₂] retained some, although less potent than hAM-(1-52)-NH₂, receptor-binding activity and cAMP response, indicating the importance of C-terminal amidation rather than the amidated Tyr⁵² residue *per se*. Cleavage of the disulfide bond between the Cys¹⁶ and Cys²¹ residue by carbamoylmethylation [[Cys(CAM)^{16,21}]hAM-NH₂] retained some, although less potent than hAM-(1-52)-NH₂, receptor-binding activity, but completely lost cAMP response. Collectively, our data indicates that amidation of C-terminal residue and the cyclic structure of AM molecule are critical for receptor binding and cAMP response.

Table 2 Comparison between binding affinities and potencies of adenylate cyclase activity by hAM analogs

hAM analogs	Ki(M) ^a	EC ₅₀ (M) ^b
hAM-(1-52)-NH ₂	0.023	0.03
hAM-(13-52)-NH ₂	0.039	0.03
hAM-(16-52)-v	0.062	0.03
hAM-(1-51)-NH ₂	0.27	0.24
hAM-(1-52)-OH	1.0	>1.0
hAM-(1-51)-OH	1.1	>1.0
Cys(CAM) ^{16,21}]hAM-NH ₂	1.3	>1.0
hAM-(22-52)-NH ₂	1.6	>1.0
hAM-(33-52)-NH ₂	>1.0	>1.0
hAM-(1-10)-OH	>1.0	>1.0
hAM-(1-10)-OH	>1.0	>1.0

^aThe apparent inhibition constant (Ki) obtained from [¹²⁵I] hAM binding studies.

^bThe appropriate half-maximal effective concentration (EC50) obtained from adenylate cyclase stimulation. Reproduced with permission from ref. (Eguchi et al., 1994a).

CGRP(8-37), which lacks N-terminal ring structure but retains C-terminal portion of CGRP, has been widely recognized and used as a CGRP1 receptor antagonist. In the same analogy, we designed hAM-(22-52)-NH₂, which lacks N-terminal ring structure but retains C-terminal portion. We found that hAM-(22-52)-NH₂ retain receptor binding activity, but devoid of cAMP response (Eguchi et al., 1994a). In addition, hAM-(22-52)-NH₂ inhibited hAM-stimulated cAMP formation with apparent IC₅₀ (4x10⁻⁶M). Hence, we proposed that hAM-(22-52)-NH₂ serves as a selective antagonist for AM receptor.

3. DISCOVERY OF CRLR/RAMP SYSTEM

In 1991, calcitonin (CT) receptor was identified as the first CT supergene family receptor by the expression cloning system from the cDNA library of a porcine kidney epithelial cell line (Lin et al., 1991). CT receptor belongs to type B (Class II) family of G protein coupled receptor (GPCR), including PTH, glucagon, and CRF receptors. These receptors are characterized by the long extracellular N-terminal domain containing several cysteine, the intracellular carboxy-terminal domain lacking the palmitoylation site, and capability of cAMP generation through activation of Gs (Gether, 2000). Shortly after the molecular cloning of CT receptor, calcitonin receptor-like receptor (CRLR) was identified by PCR cloning from rat hypothalamus by utilizing the homology between type B family GPCRs (Njuki et al., 1993). Subsequently, human and other mammalian CRLR homologues were also cloned (Aiyar et al., 1996; Elshourbagy et al., 1998; Fluhmann et al., 1995), which exhibit 50~60% sequence homology with the CT receptor of the same species. In initial transfection studies using several mammalian cells, CRLR showed no affinity to any member of known CT supergene family (Fluhmann et al., 1995; Njuki et al., 1993), thus initially considered as an orphan GPCR. However, Aiyar et al. have shown that human CRLR exhibits the pharmacology of CGRP1 receptor when stably transfected into the cell line derived from human embryonic kidney cell line, HEK293 (Aiyar et al., 1996). This finding was further confirmed using rat and porcine CRLR in transfected HEK293 (Elshourbagy et al., 1998; Han et al., 1997), suggesting that HEK293 cells could express certain endogenous factor(s) essential for the functional expression of CRLR.

This assumption was then confirmed by the discovery of a novel transmembrane protein, termed receptor activity modifying protein (RAMP) (McLatchie et al., 1998). The introduction of cRNA from SK-N-MC cells, where CGRP1 receptor is abundantly present, into *Xenopus* oocyte induced the novel cAMP response to CGRP. The identified cDNA encoded the 148 amino-acid residues with a single transmembrane domain, named RAMP1. Subsequent study showed that co-transfection of CRLR and RAMP1 into COS-7 cells exhibits functional CGRP1 receptor, whereas the transfection of either gene alone shows no effect (McLatchie et al., 1998). In addition, two additional members of RAMP family were also identified through database search, named RAMP2 and RAMP3 (McLatchie et al., 1998). Although sequence similarity between three RAMP isoforms are less than 30% within the same species, all three isoforms share the conserved cysteine residues at their extracellular domain and DPPXX and LVVWXXSK sequences flanking the transmembrane domain, whose functional role remains unknown (Born et al., 2002).

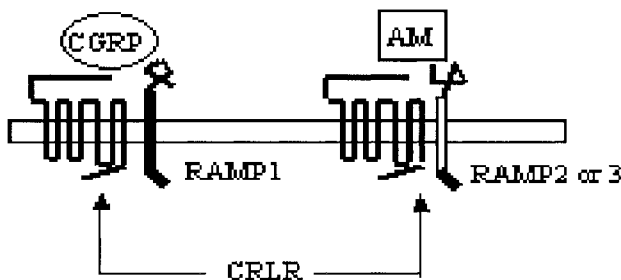


Fig. 1 CRLR and RAMP isoform determine the ligand selectivity for CGRP and AM. Extracellular domain of RAMPs plays an important role in ligand selectivity. Glycosylation of CRLR is important for its cell surface localization, but not for its ligand selectivity.

Co-transfection of CRLR and RAMP2 or RAMP3 exhibits functional AM receptor with high affinity to AM, but not to CGRP. RAMP2 and RAMP3 appears to be indistinguishable from each other in terms of AM binding and cAMP response (McLatchie et al., 1998). Collectively, co-expression of

CRLR with RAMP1 and RAMP2/3 exhibits the CGRP1 receptor and AM receptor pharmacology, respectively (Fig. 1).

4. CRLR/RAMP INTERACTION: RECEPTOR MODIFICATION AND THE MECHANISM OF LIGAND SPECIFICITY

McLatchie et al. initially showed that RAMPs are required to transport CRLR to the plasma membrane (McLatchie et al., 1998). Co-expression of CRLR and RAMP1 exhibited mature glycosylated receptor with CGRP1 receptor property, whereas co-expression of CRLR and RAMP2 or 3 resulted in core-glycosylated receptor with AM receptor property. Hence, the ligand specificity of the receptor formed by CRLR/RAMPs has been initially thought to be determined by the glycosylation pattern of the receptor (McLatchie et al., 1998). However, in *Drosophila* Schneider (S2) cells stably co-transfected with CRLR and RAMP1 or 2 revealed the pharmacology of CGRP1 receptor and AM receptor, respectively, although both receptors were uniformly glycosylated independent of RAMP1 and 2 expression (Aldecoa et al., 2000). The subsequent study confirmed that RAMP2 and 3 also facilitate full maturation and terminal glycosylation of CRLR, and only the fully-processed receptor is capable of interacting with AM and CGRP. In other words, although co-expression of CRLR and RAMP2 resulted in increase in core-glycosylated CRLR at whole cell levels, only the fully-processed CRLR could express on cell surface (Hilairet et al., 2001). Thus, it is currently recognized that the glycosylation pattern itself does not appear to determine the ligand selectivity of CRLR/RAMP system as initially speculated, but the glycosylation of CRLR is essential for the cell surface localization of CRLR (Flahaut et al., 2002). By contrast, the CRLR-RAMP heterodimer assembly is indispensable for the cell surface localization of RAMP1 (Christopoulos et al., 1999; Flahaut et al., 2002).

The co-transfection of CT receptor and RAMP1 or 3 was shown to exhibit the pharmacology of amylin receptor, suggesting that RAMP is involved in the determination of the functional property of Class II GPCR (Christopoulos et al., 1999; Muff et al., 1999). The ligand-receptor crosslinking study revealed RAMP-ligand complex as well as CRLR-ligand complex, suggesting that not only CRLR but also RAMP comprises the ligand recognition site of the receptor with the formation of ligand-CRLR-RAMP complex. The study using confocal microscopy also revealed the

ligand-CRLR-RAMP co-localization on the cell surface (Leuthauser et al., 2000). Recent work using RAMP1 extracellular domain as a chimera with PDGF receptor transmembrane domain clearly reveals that extracellular domain of RAMP is sufficient for assembling functional CGRP receptor (Fitzsimmons TJ et al., 2003). It has been very recently revealed that transfection of deletion mutants (residues 91-94, 96-100, or 101-103) of hRAMP1 blocked CGRP binding and cAMP response, whereas substituting alanine for the residues 91-103 one at a time had little effects on CGRP response (Kuwasako et al., 2003). It is therefore suggested that structural conformation of RAMP or its allosteric effect on CRLR rather than individual amino acid sequence play a pivotal role in the determination of ligand specificity.

It has been also shown that CRLR interacts with another protein, receptor component protein (RCP), identified as the protein which confers the CGRP-mediated cAMP response in *Xenopus laevis* oocyte, in a similar manner as does RAMP1 (Luebke et al., 1996). There is no sequence similarity between RCP and RAMPs. It is thus intriguing that RCP could interact with CRLR and anti-sense oligonucleotides for RCP decreased the CGRP response in NIH3T3 cell line (Prado et al., 2001; Evans et al., 2000). Furthermore, RAMP/CRLR/RCP complex formation was confirmed by immunoprecipitation experiment (Prado et al., 2001). These data suggest that RCP may play a pivotal role in AM/CGRP receptor system, however, further study is required to establish its biological significance.

5. cAMP/PROTEIN KINASE A (PKA) PATHWAY

(1) VASODILATION

Since the discovery of AM (Kitamura et al., 1993), cAMP was initially considered to be a major intracellular signaling molecule for AM. We have first demonstrated that AM activates adenylate cyclase in rat VSMC (Eguchi et al., 1994a; Eguchi et al., 1994b); cAMP response by AM was enhanced by exogenous addition of GTP, and AM receptor binding was inhibited by a non-hydrolyzable GTP analog, GTP- γ S (Eguchi et al., 1994a). Furthermore, the cAMP response by AM was shown to be abrogated by pretreatment with cholera toxin, but not by pertussis toxin, suggesting that

AM receptors are functionally coupled to adenylate cyclase via the stimulatory G-protein (Gs) (Eguchi et al., 1994a). Our data from the pharmacological experiments have been confirmed by the molecular identification of AM receptor, CRLR-RAMP system (Born et al., 2002; Poyner et al., 2002), where CRLR belongs to family B of GPCR as characterized by functional coupling to Gs (Gether, 2000). Co-expression of CRLR and RAMP1 exhibits the cAMP response by CGRP, whereas RAMP2 or 3 co-expressed with CRLR exhibits cAMP response specifically by AM (McLatchie et al., 1998).

(2) MITOGENIC ACTION

AM exerts its mitogenic action via cAMP/PKA pathway in Swiss 3T3 cells; AM increase DNA synthesis and cell proliferation, whose effect was abolished by a PKA inhibitor (H-89) (Withers et al., 1996). Furthermore, transfection of constitutive active mutant of Gs_i mimicked the mitogenic response by AM (Withers et al., 1996). Similar mitogenic response by AM through cAMP/PKA pathway has been shown in human oral keratinocyte (Kapas et al., 1997). On the other hand, cAMP/PKA pathway is widely recognized as a negative regulator of cell growth. In accordance with this, it has been shown that AM inhibits DNA synthesis via cAMP-dependent pathway in serum stimulated growing VSMCs (Kano et al., 1996). A similar anti-mitogenic action by AM has been reported in cultured mesangial cells (Chini et al., 1995; Segawa et al., ; Togawa et al., 1997), whose effect was mediated via cAMP/PKA pathway. We also confirmed that AM caused a partial inhibition of cell growth when AM is added to asynchronously growing VSMC supplemented with serum containing several growth factors (Iwasaki et al., 1998a; Shichiri et al., 2003). Collectively, it is suggested that bi-functional role of AM for cell growth control; in quiescent cells AM exerts its mitogenic action via PTK/ERK pathway (as discussed in the following section), whereas AM exerts its anti-mitogenic action via cAMP/PKA pathway in asynchronously growing cells (Fig. 2). Thus, AM appears to play dual roles in cell growth, depending on the cell type and cell cycle stage.

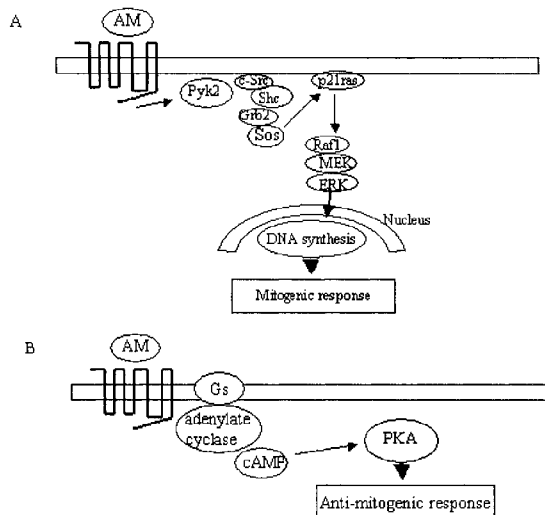


Fig. 2 Dual effect of AM on cell proliferation in VSMC. A: AM activate Pyk2/c-src non-receptor tyrosine kinase pathway, leading to activation of Ras/MEK/ERK pathway and cell proliferation. B: AM stimulate cAMP-PKA pathway, leading to growth inhibition in cells, in which ERK activation pathway is not linked to AM response.

(3) ANTI-OXIDANT ACTION

It has recently been shown that administration of salt and angiotensin II (Ang II) to AM deficient mice generated by gene targeting resulted in perivascular inflammation in coronary artery and increases in systemic and local oxidative stress and reversal of increased urinary isoprostane excretion, an oxidative stress marker, by exogenous AM supplementation (Shimosawa et al., 2002). These data postulate the notion that AM may play a protective role against oxidative stress as an endogenous anti-oxidant *in vivo*. However, the underlying cellular mechanism and the mode of action by AM have not been clarified yet. Therefore, we have explored the underlying molecular mechanism of the putative anti-oxidant action of AM against Ang II-induced reactive oxygen species (ROS) generation in rat VSMCs

(Yoshimoto et al., 2004). Using DCF fluorescence that detects ROS, AM inhibited intracellular ROS generation by Ang II, whose effect was mimicked by dibutyl cAMP and inhibited by PKA inhibitor (H-89) and a receptor antagonist, CGRP(8-37). Thus, our data demonstrate that AM directly inhibits intracellular ROS generation via AM receptor-mediated and cAMP/PKA pathway in VSMC, suggesting its protective role as an endogenous anti-oxidant in Ang II-induced vascular injury (Fig. 3).

As for the regulation of vascular AM receptor, we have demonstrated distinct desensitization of adenylate cyclase coupled to AM receptor (Iwasaki et al., 1998b). The cAMP response by AM was markedly decreased by pretreatment with AM in a dose-dependent manner. Receptor desensitization occurs in a variety of GPCRs (Wang et al., 1990). For example, phosphorylation of beta-adrenoceptor by PKA and by a GPCR kinase mediates heterologous and homologous desensitization of the adenylate cyclase in response to beta-adrenergic agonist, respectively (Benovic et al., 1985; Hausdorff et al., 1989). However, the homologous desensitization of vascular AM receptor was independent of PKA, protein kinase C (PKC), protein tyrosine kinase (PTK) or receptor sequestration, since pretreatment with each of these inhibitors failed to affect the AM-stimulated cAMP response (Iwasaki et al., 1998b). Thus, the mechanism(s) responsible for the homologous desensitization of vascular AM receptor remains to be determined.

Intracellular cAMP generation by AM and other CT supergene family has been recognized as a major signal transduction for their biological effects. However, a growing body of evidence has been accumulated and showing that cAMP/PKA pathway does not necessarily account for all of the biological actions by AM. Although cardiovascular effects by AM, such as vasodilation, and positive inotropic action, are usually accompanied by increased cAMP response, there have been only a few studies showing that such AM's actions could be blocked by either cAMP antagonists or PKA inhibitors. Furthermore, we have shown that AM has pleiotropic effects other than cardiovascular effects, such as cell growth (Iwasaki et al., 1998a; Shichiri et al., 2003), migration (Fukai et al., 2003), and apoptosis (Kato et al., 1997; Shichiri et al., 1999), whose effects are independent of cAMP/PKA pathway. Thus cAMP/PKA pathway accounts for many, but not all, biological actions by AM, however, the importance of signal transduction pathways other than cAMP/PKA pathway needs to be taken

into much consideration.

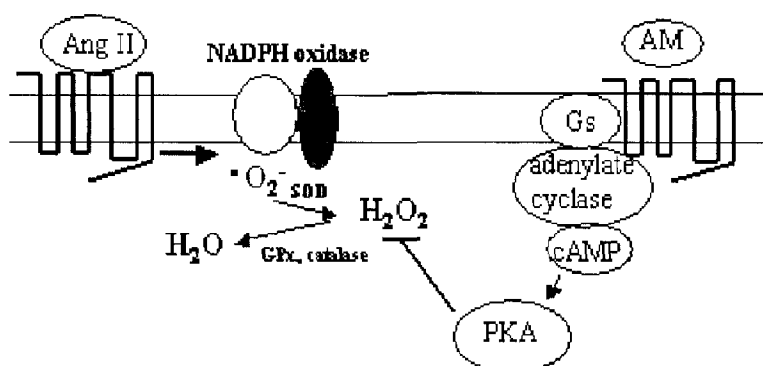


Fig. 3 Anti-oxidant effect of AM. In VSMC AM directly inhibits intracellular ROS generation by Ang II via its receptor-mediated and cAMP/PKA dependent mechanism.

6. PROTEIN TYROSINE KINASE (PTK)/ EXTRACELLULAR REGULATED KINASE (ERK) PATHWAY

The mitogenic effect of AM has been demonstrated in a variety of cells, including certain tumor cell lines, VSMC, fibroblasts, keratinocytes, osteoblasts, adrenal zona glomerulosa cells and so forth. The possible role of AM as an autocrine/paracrine growth factor has been suggested for various tumor cell lines (Martinez et al., 1997; Miller et al., 1996; Takahashi et al., 1997a; Takahashi et al., 1997b). We demonstrated for the first time that AM stimulated DNA synthesis and cell proliferation in quiescent rat VSMC, whose effect was inhibited by CGRP(8-37) (Iwasaki et al., 1998a; Shichiri et al., 2003). Furthermore, AM rapidly and transiently stimulated ERK activities; the AM-induced mitogenic effect and ERK activation was completely blocked by a MEK inhibitor (PD98059) and a protein tyrosine kinase (PTK) inhibitor (genestein). Our data strongly suggest that the mitogenic effect of AM on VSMCs is mediated via PTK/ERK pathway. It is of note that a cAMP antagonist (Rp-cAMP-S) and a highly selective PKA inhibitor (KT5720) failed to inhibit the mitogenic effect and ERK activation by AM, despite of the AM-mediated cAMP response. In our study, AM had no effect on either intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) or inositol 1,4,5,-

triphosphate formation, and a selective PKC inhibitor (GF109203X) and a dihydropyridine-sensitive Ca^{2+} channel blocker (nicardipine) failed to block AM-induced ERK activation. Collectively, our data suggest that AM exerts its mitogenic activity via PTK-mediated ERK activation in quiescent VSMC (Iwasaki et al., 1998a).

Our results with PTK-dependent ERK activation by AM independent of Ca^{2+} and PKC, suggests that AM activates receptor and/or non-receptor PTK cascade, thereby leading to Ras-dependent MEKK-MEK-ERK pathway. In consistent with this notion, AM rapidly induced tyrosine phosphorylation of several proteins (120kDa, 90kDa, 50kDa) (Iwasaki et al., 1998a). Subsequently, we identified the 120kDa tyrosine-phosphorylated protein as proline-rich protein kinase 2 (Pyk 2), a non-receptor PTK (Iwasaki et al., 2001). AM rapidly (within 1 min) phosphorylates Pyk 2, followed by a gradual decline to the basal level by 10 min; the time course of Pyk 2 phosphorylation was identical with its kinase activity as confirmed by immuno-complex kinase assay. Furthermore, immunoprecipitation studies revealed that Pyk 2 activation induced interaction with and activation of c-Src, a non-receptor PTK, subsequently leading to complex formation with adaptor proteins (Shc and Grb2). These adaptor protein complex recruits SOS, a GTP exchange protein for Ras, finally leading to sequential activation of Ras-MEKK-MEK-ERK cascade (Iwasaki et al., 2001). Our data demonstrated for the first time the involvement of non-receptor PTK/ERK pathway in VSMCs in the mitogenic action by AM (Fig. 2). A growing body of evidence thus far accumulated has revealed that AM is a potent mitogen for activity on various tumor and non-tumor cells (Hinson et al., 2000; Shichiri and Hirata, 2003).

7. NO/cGMP/PROTEIN KINASE G (PKG) PATHWAY

Several lines of evidence have been accumulated showing that vasodilator action of AM is mainly mediated via endothelial nitric oxide (NO) production, because vasodilatory effects by AM in several vasculatures are blocked by NO synthase (NOS) inhibitors (Hinson et al., 2000; Kitamura et al., 2002). In bovine endothelial cells, AM was shown to activate phospholipase C via Gq protein to increase $[\text{Ca}^{2+}]_i$, possibly leading to activation of Ca^{2+} -dependent endothelial NOS (Shimekake et al., 1995). Since NO activates soluble guanylate cyclase to increase intracellular cGMP

levels in VSMC, cGMP-dependent protein kinase (PKG) could function as a major signaling molecule for AM-induced vasorelaxation.

8. PI3 KINASE/AKT PATHWAY

A recent study has shown that AM exerts NO-dependent vasodilation through PI3 kinase/AKT pathway (Nishimatsu et al., 2001). Furthermore, several studies have shown that AM plays a protective role in ischemia-reperfusion myocardial injury via PI3 kinase/AKT pathway (Okumura et al., 2004; Yin et al., 2004). We also observed that mitogenic response of AM in VSMCs is blocked by PI3 kinase inhibitors (wortmannin, LY294002) in the same manner as MEK inhibitor (Shichiri et al., 2003). These data are consistent with the notion that PI3 kinase/AKT pathway plays a pivotal role in certain biological actions by AM, although further studies are required to elucidate how the PI3 kinase is activated following AM stimulation.

9. UNKNOWN SIGNAL TRANSDUCTION PATHWAY(S) FOR APOPTOSIS AND MIGRATION

(1) ANTI-APOPTOTIC EFFECT

After serum-deprivation, endothelial cells undergo apoptosis. We demonstrated for the first time that AM acts as an anti-apoptotic factor for rat aortic endothelial cells (RAECs) in a dose-dependent manner (Kato et al., 1997), although it has no mitogenic effect. Neutralization experiment using anti-AM antibody revealed a marked enhancement of endothelial apoptosis after serum starvation. Since AM is synthesized by and secreted from RAEC in an autocrine/paracrine manner, our data are consistent with the notion that autocrine-secreted endogenous AM functions as a survival factor for endothelial cells (Kato et al., 1997). In spite of cAMP response by AM in RAECs, cAMP antagonist (Rp-cAMPS) failed to inhibit, and cAMP-elevating agonists (forskolin, PGI₂) could not mimic, the anti-apoptotic effect by AM (Kato et al., 1997), suggesting that AM exerts its anti-apoptotic effect through the mechanism independent of cAMP/PKA pathway.

Then, we clarified the molecular mechanism of anti-apoptotic action by AM

(Shichiri et al., 1999). Myc is recognized as one of the key proto-oncogenes regulating cell cycle progression and cell proliferation, while it can promote apoptosis in the absence of survival factor (Pelengaris et al., 2002). Myc, when heterodimerized with its binding partner, Max, exerts transcriptional activity on its target genes through binding to its cognate DNA binding site (E-box), whereas both Max-Max homodimer and heterodimer with alternative partner (Mad, Mxi) can also bind to E-box, but do not show any transcriptional activity on the target gene, thus Myc functioning as a transcriptional repressor (Pelengaris et al., 2002; Yin et al., 1998). In serum-starved quiescent RAECs, AM upregulates Max gene expression without affecting Myc gene expression, thereby leading to the increased Max/Myc ratio. In addition, overexpression of Max gene mimicked the anti-apoptotic effect of AM in serum-starved RAEC, whose effect was abrogated by antisense oligonucleotides against Max (Shichiri et al., 1999). Collectively, these data clearly demonstrates that AM exerts its anti-apoptotic effect through the upregulation of Max gene to block the c-Myc-mediated apoptosis independently from cAMP/PKA pathway (Fig. 4).

Subsequently, the anti-apoptotic effect by AM has been reported in various cell types, including endometrial cancer cells (Oehler et al., 2001), human adrenal zona glomerulosa cells (Rebuffat et al., 2002), human skin keratinocyte and fibroblast (Albertin et al., 2003). However, the biological significance of the anti-apoptotic effect by AM has not been fully understood. Recent gene targeting experiments of AM gene reveals that the AM null mice are embryonic lethal due to the abnormal development of cardiovascular system (Shindo et al., 2001). Thus endothelial anti-apoptotic effect by AM may play an integral role in embryogenesis, especially cardiovascular development.

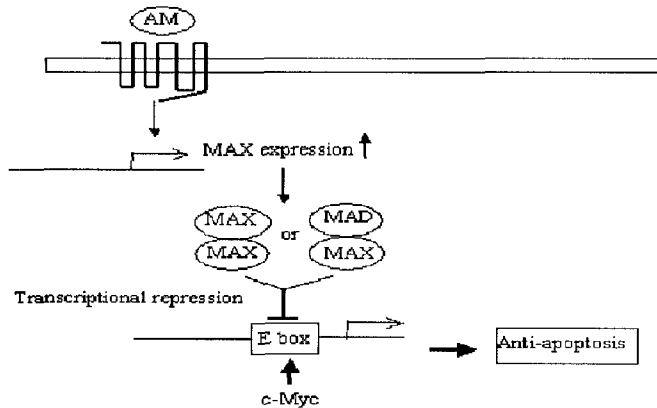


Fig. 4 The mechanism of anti-apoptotic action by AM

AM upregulates MAX expression, but not that of c-MYC, which results in increase in MAX/MYC ratio. This relative excess of MAX leads to predominant formation of MAX-MAX and MAD-MAX dimers rather than MYC-MAX, which function as E-box transcriptional repressor, thereby leading to anti-apoptotic effect.

(2) ANTI-MIGRATION EFFECT

The effect of AM on cell migration has been studied in VSMCs using Boyden chamber (Horio T et al., 1995; Kohno M et al., 1997; Kohno M et al., 1999). In these studies, AM has been shown to inhibit serum-, Ang II-, and PDGF-stimulated VSMC migration. The mechanism of anti-migratory effect by AM appears to be mediated in cAMP/PKA pathway, since its anti-migratory effect as accompanied by cAMP response, and cAMP-elevating agonists (8-bromo cAMP, forskolin, PGI_2) mimicked the effect.

We investigated the differential role of CRLR/RAMP system in the anti-migratory effect using monolayer-wounding of rat VSMC and fibroblasts (Fukai et al., 2003). AM potently exhibited the anti-migratory effect in VSMC where CRLR and RAMP isoforms (1, 2, 3) were co-expressed,

whereas it did not show any effect in fibroblasts where CRLR and RAMP1, but not RAMP2/3, were co-expressed. Failure of CGRP to induce anti-migratory response in VSMC and fibroblasts strongly suggests that the anti-migratory effect is mediated via a specific AM receptors comprising CRLR/RAMP2 or 3. Furthermore, co-transfection of RAMP2 or RAMP3 with CRLR revealed slower cell migration in VSMC and fibroblasts, whose effect was further enhanced by AM. Thus specific AM receptor consisting of both CRLR and RAMP (2, 3) mediates AM-induced anti-migratory response. However, cAMP-elevating agonists failed to mimic, and cAMP antagonist could not block the anti-migratory effect by AM. Furthermore, any of several inhibitors for well-known signaling molecules, such as cGMP, ERK, p38 MAPK, PTK, and PI3 kinase, all failed to block the anti-migratory response by AM. Thus molecular mechanisms of anti-migratory response by AM other than cAMP/PKA pathway remains to be determined (Fukai et al., 2003). The reasons for the apparent discrepancy between our results and those of previous studies could be accounted for by the different experimental methods (wound healing vs. Boyden chamber).

CONCLUDING REMARKS

Expression of AM and its receptor has been shown to be not confined to cardiovascular system, but widely and ubiquitously distributed in variety of tissues. Since its discovery as a potent vasodilator, there have been extensive investigations revealing pleiotropic effects of AM. Recent discovery of CRLR/RAMP system that confers ligand selectivity for AM and CGRP facilitates the understanding of new-facet of AM/CGRP receptors and their signaling pathways. However, further characterization of CRLR/RAMP system is required to connect the missing link between molecular mechanism and experimental data of pleiotropic functions by AM. Development of receptor antagonists highly selective for AM with higher-affinity and their application in experimental and clinical settings could help the understanding of complex pharmacology of AM *in vivo*.

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