

## Specific receptors for adrenomedullin in cultured rat vascular smooth muscle cells

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### Abstract

The effects of synthetic rat adrenomedullin (rAM), a novel vasorelaxant peptide originally isolated from human pheochromocytoma, on receptor binding and cAMP generation were studied in cultured rat vascular smooth muscle cells (VSMC). A binding study using [<sup>125</sup>I]rAM revealed the presence of a single class of high-affinity ( $K_d$   $1.3 \times 10^{-8}$  M) binding sites for rAM in VSMC. The apparent  $K_i$  of rat calcitonin gene-related peptide (rCGRP) was  $3 \times 10^{-7}$  M. Affinity labeling of VSMC membranes with [<sup>125</sup>I]rAM revealed two distinct labeled bands with apparent molecular weights of 120 and 70 kDa, both of which were abolished by excess unlabeled rAM or rCGRP. rAM stimulated cAMP formation with an approximate  $EC_{50}$  of  $10^{-8}$  M, the effect of which was additive with isoproterenol, but not with rCGRP. The rAM-induced cAMP response was unaffected by propranolol, indomethacin, or quinacrine, but inhibited by a CGRP receptor antagonist, human CGRP[8–37]. These data suggest that VSMC possesses specific AM receptors functionally coupled to adenylate cyclase with which CGRP interacts.

**Key words:** Adrenomedullin; Calcitonin gene-related peptide; Receptor; Cyclic AMP; Affinity labelling; Rat vascular smooth muscle cell

### 1. Introduction

A novel vasorelaxant peptide, termed adrenomedullin (AM), has recently been isolated from the acid extract of human pheochromocytoma [1]. Human AM with 52 amino-acid residues shows structural homology with calcitonin gene-related peptide (CGRP) and amylin in terms of the ring structure by disulfide bridge and C-terminal amide. Human AM stimulates cAMP generation in rat platelets in vitro [1]. Intravenous bolus injection of AM, like CGRP, caused a potent and long-lasting hypotensive effect in anesthetized rats in vivo [1]. It has been shown that AM-like immunoreactivity (LI) [1] and AM mRNA [2] are present not only in normal human adrenal medulla, but also in lung and kidney. Furthermore, AM-LI circulates in human plasma [1], suggesting its role as a vasodilatory hormone.

However, no information is yet available about the mechanism of vasodilatory action by AM. To elucidate whether there exists vascular AM receptor and its transmembrane signal, the present study was designed to examine the effects of synthetic AM on receptor binding

and cAMP generation in cultured rat vascular smooth muscle cells (VSMC).

### 2. Materials and methods

#### 2.1. Materials

Synthetic rat (r) and human (h) AM were synthesized by the solid phase method and purified by reverse-phase HPLC. rCGRP, rat amylin and hCGRP[8–37] were purchased from Peptide Institute Inc. (Osaka, Japan), isoproterenol, propranolol, quinacrine, 3-isobutyl-1-methylxanthine (IBMX), phenylmethylsulphonyl fluoride (PMSF) and disuccinimidyl suberate (DSS) from Sigma Chemical (St. Louis, MO), and indomethacin from Wako Chemical (Osaka, Japan). Iodination of synthetic rAM was performed by chloramine-T method and purified by reverse-phase HPLC: monoiodinated [<sup>125</sup>I]rAM (specific activity: ~150 Ci/mmol) was used in the experiments.

#### 2.2. Cell culture

VSMCs from the thoracic aorta of 15-week-old male Wistar rats prepared by the explant method were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air–5%CO<sub>2</sub> as described [3]. Subcultured VSMCs (10–15th passages) were used in the experiments.

#### 2.3. Binding study

Confluent cells (~10<sup>6</sup> cells/well) were usually incubated with 160 pM [<sup>125</sup>I]rAM at 4°C in Hanks' balanced salt solution (HBSS) containing 0.1% bovine serum albumin in the same manner as reported [4]. After completion, cells were extensively washed, solubilized in 0.5 N NaOH, and the cell-bound radioactivity was determined. Specific binding was obtained by subtracting nonspecific binding in the presence of an excess ( $5 \times 10^{-6}$  M) unlabeled rAM from total binding.

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### 2.4. Determination of intracellular cAMP

Confluent cells were incubated at 37°C for 10 min in HBSS containing 0.5 mM IBMX. Incubation was terminated by the addition of HCl and intracellular cAMP was measured by a radioimmunoassay kit (New England Nuclear, Boston, MA) as described [4].

### 2.5. Affinity labeling of [<sup>125</sup>I]rAM with VSMC membranes

The membrane suspension from VSMCs was prepared and affinity labeling of [<sup>125</sup>I]rAM was performed essentially in the same manner as described previously [5]. Briefly, cells were homogenized in 20 mM phosphate buffer (130 mM NaCl, 10 mM EDTA, 0.2 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin), pH 7.5, and the homogenate was washed, centrifuged, and resuspended in the same buffer. The membrane suspension (10 µg protein) was incubated with 2 nM [<sup>125</sup>I]rAM at 4°C for 1 h in the absence and presence of excess ( $5 \times 10^{-6}$  M) unlabeled rAM or rCGRP; DSS was added to a final concentration of 0.5 mM and incubated at 25°C for 20 min. After completion, the reaction mixtures were subjected to SDS-PAGE in the presence of 5% 2-mercaptoethanol. After electrophoresis, the gels were dried and exposed to film at -70°C for 5 days.

## 3. Results

Binding of [<sup>125</sup>I]rAM at 4°C to cultured rat VSMC was a time-dependent process (Fig. 1). An apparent equilibrium binding was reached after 1–2 h; the specific binding was about 60% of the total binding. Binding at 37°C was about one-tenth that at 4°C after 1 h, and the specific binding was only 10% of the total binding (data not shown). Therefore, the subsequent binding study was performed at 4°C for 2 h.

Competitive binding of [<sup>125</sup>I]rAM to rat VSMC by unlabeled rAM and rCGRP is shown in Fig. 2A. Unlabeled rAM competitively inhibited the binding of [<sup>125</sup>I]rAM; Scatchard analysis of the binding data (Fig.

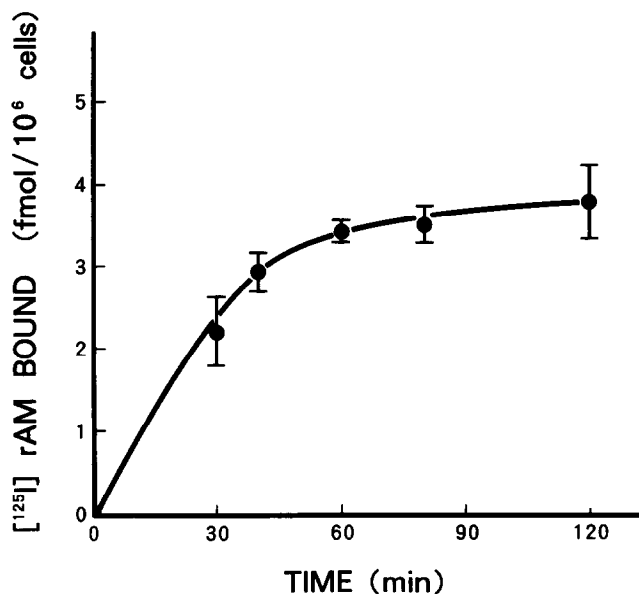


Fig. 1. Specific binding of [<sup>125</sup>I]rAM to cultured rat VSMC as a function of time. Confluent cells were incubated with [<sup>125</sup>I]rAM at 4°C for the time as indicated. Specific binding was obtained by subtracting nonspecific binding in the presence of  $5 \times 10^{-6}$  M unlabeled rAM from total binding. Each point is the mean of triplicate dishes; bars show S.E.M.

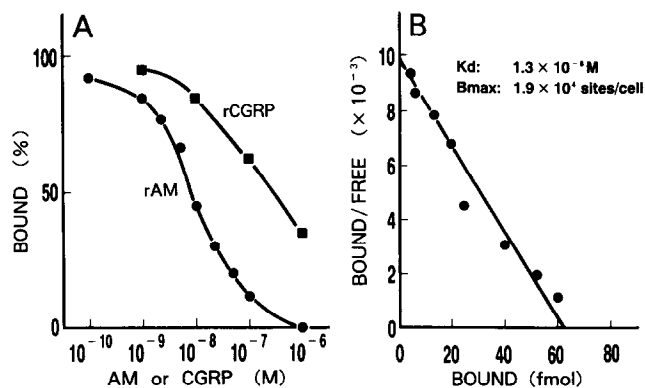


Fig. 2. Competitive binding of [<sup>125</sup>I]rAM to cultured rat VSMC by unlabeled rAM and rCGRP. (A) Confluent cells were incubated with [<sup>125</sup>I]rAM at 4°C for 2 h in the absence and presence of unlabeled rAM (●) and rCGRP (■) in indicated concentrations. Each point is the mean of triplicate dishes. (B) Scatchard plot of rAM binding. The ratio of bound to free rAM is plotted against the concentrations of bound rAM.

2B) revealed the presence of a single class of binding sites for rAM with the apparent dissociation constant ( $K_d$ ) of  $1.3 \times 10^{-8}$  M and the maximal binding capacity ( $B_{max}$ ) of 19,000 sites/cell. rCGRP showed a less steep inhibition curve than rAM with the apparent inhibition constant ( $K_i$ ) of  $3 \times 10^{-7}$  M. Affinity labeling of VSMC membranes with [<sup>125</sup>I]rAM revealed two major labeled bands with apparent molecular weights ( $M_r$ ) of 120 and 70 kDa; both bands disappeared in the presence of excess unlabeled rAM or rCGRP (Fig. 3).

The effects of rAM, hAM, rCGRP and rat amylin on cAMP formation in cultured rat VSMC are shown in Fig. 4. rAM and hAM dose-dependently ( $10^{-9}$ – $10^{-6}$  M) stimulated cAMP formation, the approximate concentrations of which for half-maximal stimulation ( $EC_{50}$ ) were  $6 \times 10^{-9}$  M and  $10^{-8}$  M, respectively, whereas rCGRP was less potent than AM with the apparent  $EC_{50}$  of  $3 \times 10^{-8}$  M; rat amylin had no effect. The effect of CGRP receptor antagonist (hCGRP[8–37]) on cAMP generation stimulated by rAM and rCGRP is shown in Fig. 5. hCGRP[8–37] dose-dependently ( $10^{-8}$ – $10^{-5}$  M) inhibited cAMP generation stimulated by rCGRP ( $10^{-6}$  M) and by rAM ( $10^{-7}$  M), the approximate concentrations of which for half-maximal inhibition ( $IC_{50}$ ) were  $3 \times 10^{-7}$  M and  $2 \times 10^{-6}$  M, respectively.

The effects of various compounds on rAM-induced cAMP formation in cultured rat VSMC were tested (Table 1). The stimulatory effect by rAM ( $10^{-6}$  M) was additive with isoproterenol ( $10^{-6}$  M), but not with rCGRP ( $10^{-6}$  M). The rAM-induced cAMP response was not affected by propranolol ( $10^{-6}$  M), indomethacin ( $10^{-5}$  M), or quinacrine ( $10^{-5}$  M).

## 4. Discussion

AM, a novel 52-amino-acid residue peptide originally

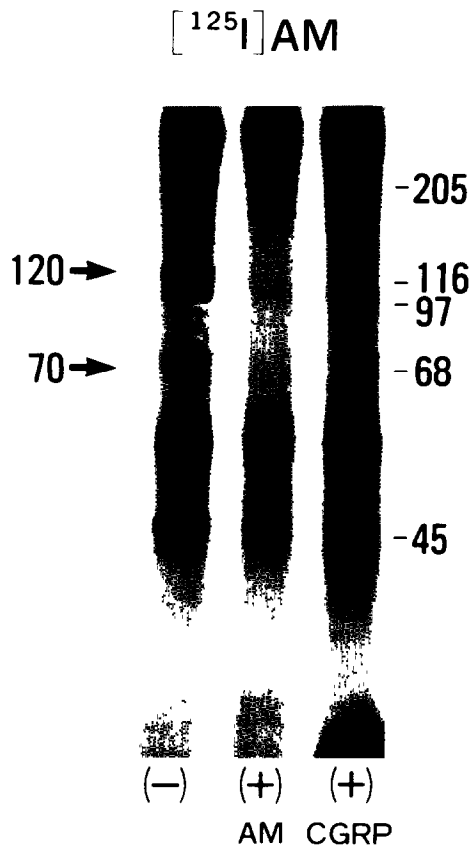


Fig. 3. Autoradiograph after SDS-PAGE of affinity labeling of [ $^{125}$ I]rAM to rat VSMC membranes. VSMC membrane suspensions were incubated with [ $^{125}$ I]rAM at 4°C for 1 h in the absence and presence of  $5 \times 10^{-6}$  M rAM or rCGRP, cross-linked by DSS, and subjected to SDS-PAGE. Molecular size markers are denoted on the left; arrows indicate positions of the 120- and 70-kDa labeled bands.

isolated from human pheochromocytoma, is one of the most potent vasodilator in vivo [1]. hAM relaxes precontracted rat mesenteric vascular bed in vitro, whose effect is not mediated by adrenergic or cholinergic receptors [6], suggesting the presence of distinct AM receptors in rat VSMC. The present study demonstrates for the first time the presence of specific binding sites for rAM in cultured rat VSMC.

The equilibrium binding of [ $^{125}$ I]rAM to VSMC was obtained at 4°C, whereas specific binding at 37°C was smaller than that at 4°C. The greater nonspecific binding of [ $^{125}$ I]rAM at 37°C is most likely due to degradation and/or internalization of [ $^{125}$ I]rAM during incubation as is the case of [ $^{125}$ I]rCGRP binding to rat VSMC [4]. The present competitive binding of [ $^{125}$ I]rAM to VSMC under the equilibrium condition revealed the presence of a single class of high-affinity binding sites for rAM. The apparent  $K_d$  value ( $1.3 \times 10^{-8}$  M) estimated from Scatchard analysis is comparable to that of PD2 (8.32) to induce vasodilation in the perfused rat mesenteric vascular bed [6].

hAM [1] as well as rAM [7] stimulates cAMP formation in rat platelets. The present study clearly shows that

rAM dose-dependently increased intracellular cAMP concentrations in cultured rat VSMC. The approximate  $EC_{50}$  by rAM ( $6 \times 10^{-9}$  M) to induce a cAMP response appears to be almost comparable to the apparent  $K_d$  value from the binding study. Furthermore, the stimulatory effect on cAMP formation by rAM was additive with isoproterenol, whereas  $\beta$ -adrenergic antagonist (propranolol), cyclooxygenase inhibitor (indomethacin) and phospholipase A2 inhibitor (quinacrine) failed to inhibit the stimulatory effect by rAM. Therefore, it is suggested that rAM-induced cAMP formation in VSMC is not due to a prostanoid-dependent mechanism, but directly mediated by specific AM receptors functionally coupled to adenylate cyclase, distinct from  $\beta$ -adrenergic receptors. Since cAMP is an intracellular mediator of vasorelaxation, it seems most likely that the vasorelaxant effect by AM is exerted by cAMP in VSMC. Since there exists apparent structural homology between AM, CGRP and amylin which share the ring structure formed by an intramolecular disulfide linkage and the C-terminal amide [1], it is of great interest to compare the cAMP-generating activities by these three peptides. The rank order of potency to induce cAMP formation in rat

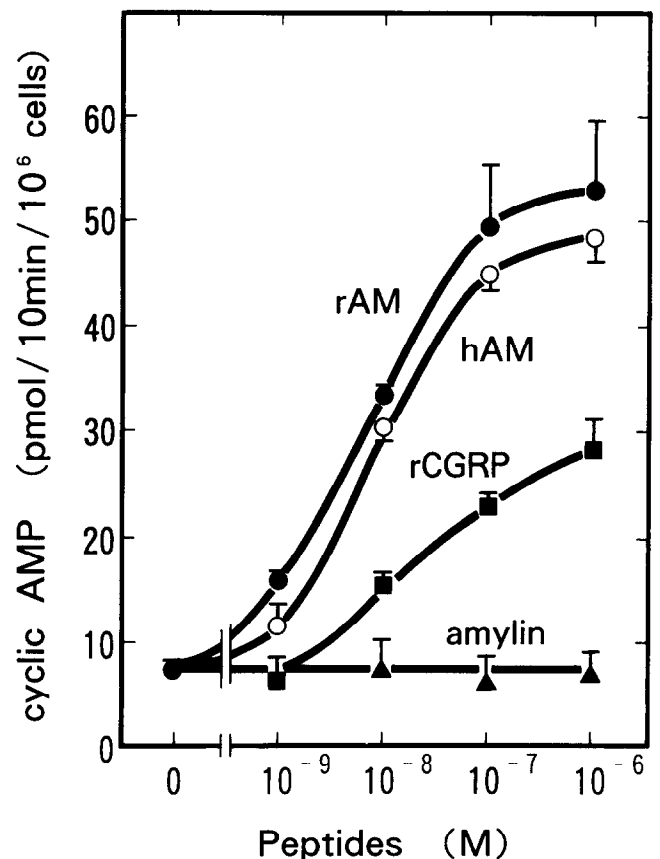


Fig. 4. Effects of AM-related peptides on intracellular cAMP formation in cultured rat VSMC. Confluent cells were incubated at 37°C for 10 min with or without various concentrations of rAM (●), hAM (○), rCGRP (■) and rat amylin (▲). Each point is the mean of four samples; bars show S.E.M.

VSMC in the present study is  $rAM \cong hAM > rCGRP \gg$  rat amylin, whereas that in the rat platelet is  $hCGRP \cong rAM \cong hAM \gg$  human amylin [1,7]. Such discrepancy may be due to the different tissue expression of receptor subtype and/or species difference of ligands.

The present study further demonstrates that rCGRP has lower binding affinity than rAM, and that rCGRP-induced cAMP formation was non-additive with rAM. The present study also showed that a CGRP receptor antagonist (hCGRP[8–37]) dose-dependently inhibited rCGRP- and rAM-induced cAMP formation in VSMC. These data are in agreement with a recent study in which hAM-induced vasodilator response in the rat mesenteric vascular bed was markedly inhibited by hCGRP[8–37] [6]. Taken together with the presence of specific CGRP receptors functionally coupled to adenylate cyclase in VSMC [4], it is possible to speculate that AM and CGRP may interact with the same receptors coupled to adenylate cyclase in VSMC.

The affinity labeling experiment to visualize receptor molecules on SDS-PAGE after covalent binding of rAM to VSMC membranes revealed two major labeled proteins with apparent  $M_r$  of 120 and 70 kDa, both of which

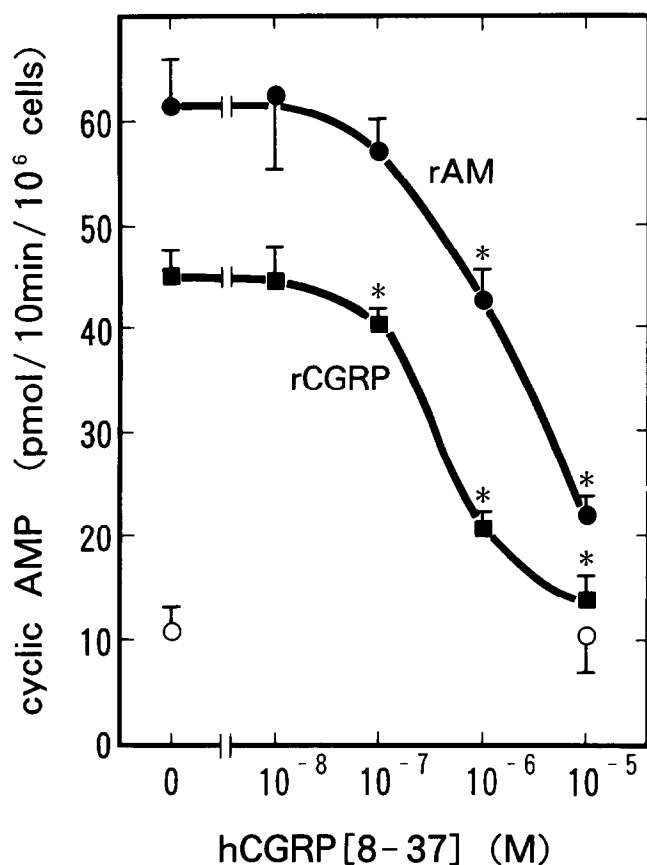


Fig. 5. Effects of CGRP receptor antagonist on cAMP formation stimulated by rAM and rCGRP in cultured rat VSMC. Confluent cells were incubated without (○) or with  $10^{-7}$  M rAM (●) and  $10^{-6}$  M rCGRP (■) in the absence and presence of hCGRP[8–37] in concentrations as indicated. Each point is the mean of four samples; bars show S.E.M. \* $P < 0.05$  vs. control.

Table 1

Effects of various compounds on rAM-induced cAMP formation in cultured rat VSMC

Drugs	(concentration)	cAMP Formed (pmol/10 min/10 <sup>6</sup> cells)
None		5.1 ± 1.0
rAM	(10 <sup>-6</sup> M)	64.6 ± 1.6*
rCGRP	(10 <sup>-6</sup> M)	36.4 ± 1.7*
rCGRP	(10 <sup>-6</sup> M) + rAM (10 <sup>-6</sup> M)	67.0 ± 4.1***
Isoproterenol	(10 <sup>-6</sup> M)	126.0 ± 6.0*
Isoproterenol	(10 <sup>-6</sup> M) + rAM (10 <sup>-6</sup> M)	156.0 ± 3.1***
Propranolol	(10 <sup>-6</sup> M)	5.3 ± 1.4
Propranolol	(10 <sup>-6</sup> M) + rAM (10 <sup>-6</sup> M)	66.6 ± 2.1***
Indomethacin	(10 <sup>-5</sup> M)	5.2 ± 0.9
Indomethacin	(10 <sup>-5</sup> M) + rAM (10 <sup>-6</sup> M)	62.4 ± 3.2***
Quinacrine	(10 <sup>-5</sup> M)	4.9 ± 0.8
Quinacrine	(10 <sup>-5</sup> M) + rAM (10 <sup>-6</sup> M)	59.5 ± 5.3***

Confluent cells were incubated at 37°C for 10 min with various compounds in the absence and presence of rAM. Each value is the mean ± S.E.M. ( $n = 4$ ). \* $P < 0.05$  vs. none. \*\*\* $P < 0.05$  vs. rAM-non-treated group.

were equally abolished by unlabeled rAM and rCGRP. These data are consistent with those of cardiac CGRP receptors in which [<sup>125</sup>I]rCGRP specifically labeled 120 and 70 kDa proteins in porcine cardiac muscles [8]. It has been suggested that there may exist multiple CGRP receptor subtypes, as based on pharmacological and binding studies [9,10]. It should be noted that the 70 kDa CGRP receptor is consistently present in various tissues as shown by affinity labeling studies [10]. In fact, we have already reported the existence of the 60–70 kDa CGRP receptor in cultured rat VSMC [4]. The 120 kDa protein observed in the present study and other study [8] may represent a precursor or intermediate form of the 70 kDa receptor and/or a 70 kDa receptor cross-linked to other proteins.

From the present study, it is evident that AM exerts its vasorelaxant effect through its specific receptors in VSMC with which CGRP interacts, and that cAMP plays their intracellular mediator role. The physiological and pathophysiological role of AM in the regulation of the cardiovascular system remains to be determined.

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