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Effects of adrenomedullin and calcitonin gene-related peptide on contractions of the rat aorta and porcine coronary artery

[1]Ryo Yoshimoto, Minori Mitsui-Saito, Hiroshi Ozaki & Hideaki Karaki

Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

1 Effects of adrenomedullin and α-calcitonin gene-related peptide (CGRP) on the contractions and cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) of the rat aorta and porcine coronary artery were investigated. Characteristics of the receptors mediating the effects of adrenomedullin and α-CGRP were also investigated.

2 Adrenomedullin and α-CGRP caused a concentration-dependent relaxation in the rat aorta contracted with noradrenaline. The IC_{50} values for adrenomedullin and α-CGRP were 2.4 nM and 4.0 nM, respectively. The relaxant effects of these peptides were abolished by removal of the endothelium and significantly attenuated by an inhibitor of nitric oxide synthase, N^G-monomethyl-L-arginine (L-NMMA, 100 μM), but not by a cyclo-oxygenase inhibitor, indomethacin (10 μM).

3 Adrenomedullin and α-CGRP increased the endothelial $[Ca^{2+}]_i$ in the rat aorta with endothelium, whereas they did not change $[Ca^{2+}]_i$ in the smooth muscle.

4 An antagonist of the $CGRP_1$ receptor, CGRP (8–37), antagonized the relaxant effects of α-CGRP and the β-isoform of CGRP (β-CGRP) but not those of adrenomedullin in the rat aorta.

5 In the porcine coronary artery contracted with U46619, adrenomedullin and α-CGRP caused a concentration-dependent relaxation with an IC_{50} of 27.6 and 4.1 nM, respectively. Removal of the endothelium altered neither the IC_{50} values nor the maximal relaxations induced by adrenomedullin or α-CGRP. When the artery was contracted with high K^+ solution (72.7 mM), these peptides caused a small relaxation.

6 Adrenomedullin and α-CGRP increased cyclic AMP content and decreased the smooth muscle $[Ca^{2+}]_i$ in the porcine coronary artery.

7 CGRP (8–37) significantly antagonized the relaxant effects of adrenomedullin and α-CGRP in the porcine coronary artery. However, it had little effect on the relaxations induced by the β-isoform of CGRP (β-CGRP).

8 These results suggest that in the rat aorta, adrenomedullin and α-CGRP increase the endothelial $[Ca^{2+}]_i$, activate nitric oxide synthase and release nitric oxide, without a direct inhibitory action on smooth muscle. In the porcine coronary artery, in contrast, adrenomedullin and α-CGRP directly act on smooth muscle, increase cyclic AMP content, decrease the smooth muscle $[Ca^{2+}]_i$ and inhibit contraction. The rat aortic endothelium seems to express the CGRP receptor which is sensitive to α-CGRP, β-CGRP and CGRP (8–37) and the adrenomedullin specific receptor. The porcine coronary smooth muscle, in contrast, seems to express two types of CGRP receptor; one of which is sensitive to α-CGRP, CGRP (8–37) and adrenomedullin and the other is sensitive only to β-CGRP.

Keywords: Adrenomedullin; α-calcitonin gene-related peptide (α-CGRP): β-CGRP; CGRP (8–37); cyclic AMP; endothelium; smooth muscle; calcium

Introduction

Human adrenomedullin is a peptide with 52 amino acid residues, originally isolated from human phaeochromocytoma cells in the process of probing agents with the ability to increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in human platelets (Kitamura *et al*., 1993a). The mRNA of adrenomedullin is expressed in various tissues (Kitamura *et al*., 1993b; Sakata *et al*., 1993) and the peptide is produced in vascular smooth muscle cells and endothelial cells (Sugo *et al*., 1994a,b), suggesting it has an important role as a local hormone. Previous studies have demonstrated that adrenomedullin has a potent hypotensive and vasorelaxant effect *in vivo* and *in vitro* (Kitamura *et al*., 1993a; Ishiyama *et al*., 1993; Nuki *et al*., 1993; Santiago *et al*., 1995; Nakamura *et al*., 1995; Kureishi *et al*., 1995). The structure of adrenomedullin shows some homology with calcitonin gene-related peptide (CGRP) (Kitamura *et al*., 1993a), which is known to be a potent vasorelaxant (Brain *et al*., 1985; Shoji *et al*., 1987). Both adrenomedullin and CGRP increase cyclic AMP in various tissues, including smooth muscle cells (Kubota *et al*., 1985; Edvinsson *et al*., 1985; Hirata *et al*., 1988; Shoji *et al*., 1987; Kitamura *et al*., 1993a,b; Ishizaka *et al*., 1994; Eguchi *et al*., 1994a,b; Shimekake *et al*., 1995), which is thought to lead to a subsequent vasorelaxation.

CGRP has two isoforms named α- and β-CGRP, which are encoded in different genes and differ in three amino acid sequences (Amara *et al*., 1982; Steenbergh *et al*., 1985). The CGRP receptors have been classified into two subtypes termed $CGRP_1$ and $CGRP_2$, based on their affinity for CGRP (8–37), a truncated peptide of CGRP. CGRP (8–37) shows a high affinity for the $CGRP_1$ receptor but not for the $CGRP_2$ receptor (Chiba *et al*., 1989; Dennis *et al*., 1989; Quirion *et al*., 1992). Previous studies have shown that the elevation of cyclic AMP induced by adrenomedullin and α-

[1] Author for correspondence.

CGRP was antagonized by CGRP (8–37) in rat cultured smooth muscle cells (Eguchi *et al*., 1994a; Ishizaka *et al*., 1994), and human neuroblastoma cell line (SK-N-MC cell) (Zimmermann *et al*., 1995; Entzeroth *et al*., 1995), suggesting that adrenomedullin and CGRP share the $CGRP_1$ receptor. However, in other tissues, like human umbilical vein endothelial cells, CGRP (8–37) failed to antagonize the elevation of cyclic AMP induced by adrenomedullin (Kato *et al*., 1995). These results imply the presence of subtypes of the adrenomedullin receptor.

Both adrenomedullin and CGRP have been suggested to be important regulators of vascular contractile mechanism. Therefore, we investigated the direct effects of adrenomedullin and CGRP on isolated vessels. The rat aorta and porcine coronary artery were chosen because CGRP has been shown to induce an endothelium-dependent relaxation (Brain *et al*., 1985) in the rat aorta and an endothelium-independent relaxation (Shoji *et al*., 1987) in the porcine coronary artery. Moreover, we describe here the pharmacological characteristics of the adrenomedullin receptor and the CGRP receptor in these tissues.

Methods

Tissue preparations

Male Wistar rats (6–8 weeks old) were killed by a sharp blow on the neck and exsanguinated. The thoracic aorta was isolated and cut into rings of 2–3 mm wide for the use of tension measurement and into spiral strips of 3–4 mm wide and 10–12 mm long for the fluorometry experiment. In some experiments, the vascular endothelium was removed by gently rubbing the intimal surface with a glass rod moistened with physiological salt solution (PSS). Porcine hearts were obtained at a nearby abattoir and transported to our laboratory in ice-cold PSS. The left descending coronary artery with 2 mm in diameter was isolated and fat and connective tissue were removed. The artery was cut into rings 2 mm wide. In some experiments the endothelium was removed by gently rubbing intimal surface with a glass rod moistened with PSS.

Solutions

PSS contained (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 1.0, $NaHCO_3$ 23.8, $CaCl_2$ 1.5 and glucose 5.5. High K^+ solution was made by increasing KCl concentration to 72.7 mM and decreasing NaCl concentration to 69.6 mM in the PSS. These solutions were saturated with 95% O_2 and 5% CO_2 mixture at 37°C. Ethylenediamine tetra acetic acid (0.01 mM) was added to these solutions to chelate contaminating heavy metals.

Measurements of muscle tension

The force of contraction was recorded isometrically. One end of the preparation was anchored to a stationary support and the other was attached to a force-displacement transducer (Orientec, Tokyo, Japan) connected to an amplifier, under a resting tension of 10 mN. High K^+ solution was repeatedly applied until the peak force was reproducible. In the rat aorta, the functional integrity of endothelium was assessed by checking whether 1 μM carbachol almost completely (>85%) relaxed the contraction induced by 100 nM noradrenaline. In the porcine coronary artery, U46619 (100 nM) and substance P (10 nM) were used to stimulate smooth muscle and endothelium, respectively.

Measurements of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured as previously described by Ozaki *et al*. (1987) and Sato *et al*. (1988, 1990) with a fluorescent Ca^{2+} indicator, fura-PE3 (Vorndran *et al*., 1995). Muscle strips were treated with acetoxymethyl ester of fura-PE3 (fura-PE3/AM, 5 μM) containing 0.02% cremophor EL at room temperature for about 6 h.

Measurements of cyclic AMP

Each muscle strip was stabilized in PSS for more than 60 min before the experiment. The muscle strips were then treated with adrenomedullin, α-CGRP, or forskolin in the presence of phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 1 mM). Ten minutes after the drug treatments, the muscle strips were quickly frozen in liquid nitrogen, crushed and homogenized in PSS containing 10% trichloroacetic acid. After centrifugation at 1,500 *g* for 15 min, trichloroacetic acid in the supernatant was removed by washing with water-saturated ether and cyclic AMP was assayed with an enzyme-immunoassay kit (Cayman Chemical Company, MI, USA). Protein content of each sample was determined by bovine serum albumin as the standard (Bradford, 1976).

Chemicals

Human adrenomedullin, human α-CGRP and human α-CGRP (8–37) were obtained from Peptide Institute (Osaka, Japan), β-CGRP was purchased from Sigma Chemicals (MO, U.S.A.) and dissolved in water. U46619 (1,5,5-hydroxy-11α,9α-(epoxymethano) prosta-5z, 13E-dienoic acid; Cayman Chemical Company, MI, U.S.A.) was dissolved in ethanol. Other drugs used were noradrenaline N^G-monomethyl-L-arginine (L-NMMA), (Wako Pure Chemicals, Osaka, Japan), carbachol (CCh), isobutyl methylxanthine (IBMX), substance P (Sigma Chemicals), fura-PE3/AM (Teflabs, TX, U.S.A.) and cremophor EL (Nacalai Tesque, Kyto, Japan).

Statistics

The results are given as the mean and the s.e.mean. Student's *t* test was used to evaluate the significance of the results and a *P* value less than 0.05 was considered significant.

Results

Effect of adrenomedullin and α-CGRP on the rat aorta

When the rat aorta was contracted with noradrenaline (100 nM), a tonic contraction was observed. Cumulative addition of adrenomedullin or α-CGRP (10 pM–100 nM) during the steady state of contraction caused a concentration-dependent relaxation (Figure 1). Removal of the endothelium abolished the relaxation (Figure 1a,b). The IC_{50} values for adrenomedullin and α-CGRP were 2.4 nM and 4.0 nM, respectively. The maximal relaxations induced by adrenomedullin and α-CGRP were 45.5% and 48.0%, respectively (Table 1). When the rat aorta was treated for 20 min with a cyclo-oxygenase inhibitor, indomethacin (10 μM), the relaxant effects of the peptides were not significantly altered; the maximal relaxations induced by adrenomedullin (100 nM) and α-CGRP (100 nM) were 31.9% and 41.7% and the IC_{50}s were 4.1 and 1.9 nM, respectively (Figure 2 and Table 1). On the other hand, pretreatment for

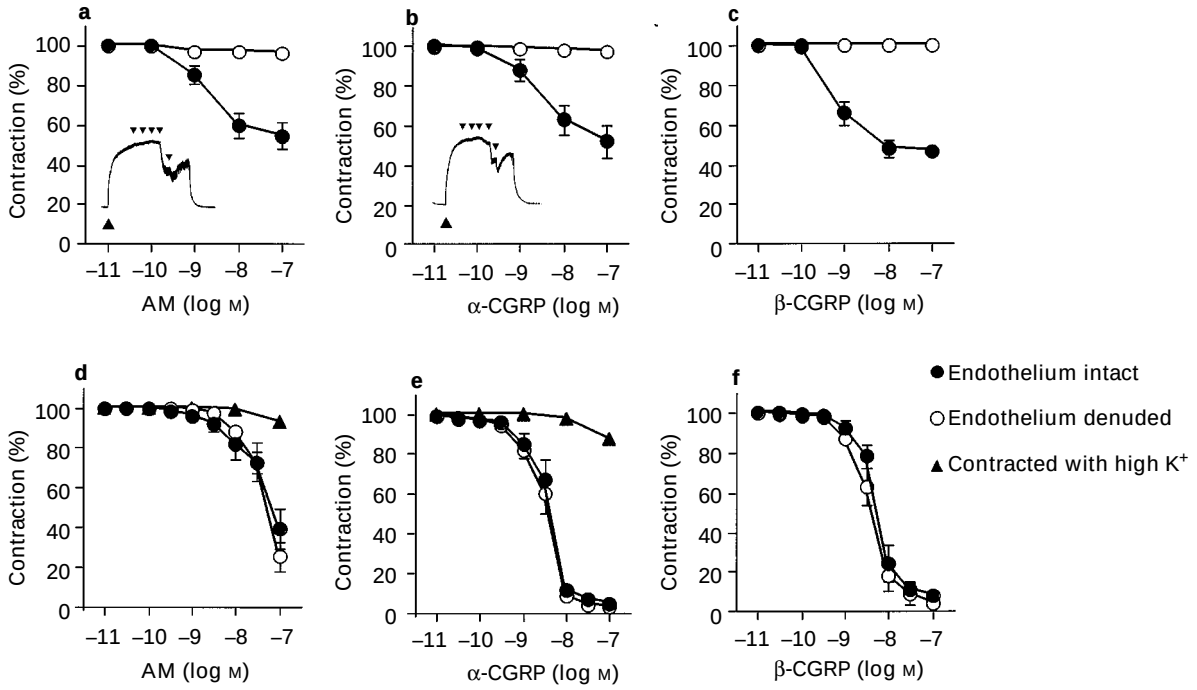

Figure 1 Concentration-response curves for adrenomedullin (AM), α- and β-CGRP in the rat aorta (a, b, c) and the porcine coronary artery (d, e, f) with or without endothelium. Rings of the rat aorta and porcine coronary artery were contracted with noradrenaline (NA, 100 nM) and U46619 (100 nM) or high K^+, respectively. The insets represent the recordings of the relaxant effects of AM or α-CGRP in the rat aorta; 100% represents the contractile response to each stimulant before the addition of AM or CGRP. Each point represents the mean of 4 to 9 experiments and the s.e.mean is shown by vertical line when larger than each symbol.

Table 1 Effects of L-NMMA (100 μM) and indomethacin (10 μM) on the IC_{50} and E_{max} values for adrenomedullin and α-CGRP in the rat aorta

Conditions	IC_{50}	E_{max}
Adrenomedullin	2.4±0.8	45.5±6.5
+L-NMMA	NE	8.3±9.6**
+indomethacin	4.1±0.2^{NS}	31.9±9.6^{NS}
α-CGRP	4.0±0.9	48.0±7.3
+L-NMMA	NE	11.3±4.5**
+indomethacin	1.9±0.3^{NS}	41.7±6.3^{NS}

NE, not estimated. NS, not statistically different. **Significantly different from the value in the absence of L-NMMA or indomethacin with $P<0.01$.

10 min with an inhibitor of nitric oxide synthase, L-NMMA (100 μM), significantly attenuated the relaxant effects of the peptides. The maximal relaxations induced by adrenomedullin (100 nM) and α-CGRP (100 nM) were 8.3% and 11.3%, respectively (Figure 2 and Table 1).

We next investigated the effects of adrenomedullin and α-CGRP on $[Ca^{2+}]_i$ in the rat aorta. When adrenomedullin (100 nM), α-CGRP (100 nM) and carbachol (1 μM) were added to a resting muscle, adrenomedullin and α-CGRP caused a transient elevation of $[Ca^{2+}]_i$. In contrast, carbachol caused a rather sustained increase in $[Ca^{2+}]_i$ (Figure 3a) as has been shown by Sato *et al*. (1990). The peak $[Ca^{2+}]_i$ values caused by adrenomedullin and α-CGRP were 28.6±10.0% and 79.5±16.6%, respectively, of the increment induced by carbachol (Figure 3b). Adrenomedullin, α-CGRP and CCh were inactive in the resting muscle without endothelium (data not shown). We also simultaneously measured $[Ca^{2+}]_i$ and muscle tension in the aorta contracted with noradrenaline (100 nM). Addition of noradrenaline caused a tonic increase in both muscle tension and $[Ca^{2+}]_i$. Addition of adrenomedullin (100 nM) or α-CGRP (100 nM) during the steady state of the contraction caused a transient elevation of $[Ca^{2+}]_i$ followed by an inhibition of the contraction (Figure 3c,d, left panel). Removal of the endothelium abolished the changes in muscle tension and $[Ca^{2+}]_i$ induced by adrenomedullin or α-CGRP (Figure 3c,d, right panel).

In order to identify the receptors activated by adrenomedullin and α-CGRP, a $CGRP_1$ receptor antagonist, CGRP (8–37), was used. CGRP (8–37) (1 μM) was applied after the contraction induced by noradrenaline (100 nM) reached a steady state, and then adrenomedullin or α-CGRP was cumulatively added. CGRP (8–37) alone had no effect on the contraction induced by noradrenaline. As shown in Figure 4a, CGRP (8–37) had no effect on the relaxation induced by adrenomedullin. The IC_{50} value was 2.3 nM, which was not different from that in the absence of CGRP (8–37) (Table 2). On the other hand, CGRP (8–37) produced a rightward shift of the concentration-response curve for α-CGRP and the relaxant effect of 10 nM α-CGRP was significantly inhibited (Figure 4b and Table 2). Another isoform of CGRP, β-CGRP, was also used to investigate further the receptors mediating the relaxant effect of CGRP. β-CGRP induced a concentration-dependent relaxation in the absence of CGRP (8–37) (1 μM) with an IC_{50} of 0.9±0.1 nM and in the presence of CGRP (8–37) with an IC_{50} of 9.5±1.2 nM. β-CGRP failed to induce a relaxation when the endothelium was absent (Figures 1c and 4c and Table 2).

Since previous workers have demonstrated that adrenomedullin and α-CGRP increased cyclic AMP in cultured smooth muscle cells from the rat thoracic aorta (Kubota *et al*., 1985; Hirata *et al*., 1988; Eguchi *et al*., 1994a,b; Ishizaka *et al*., 1994; Shimekake *et al*., 1995), we investigated the effects of

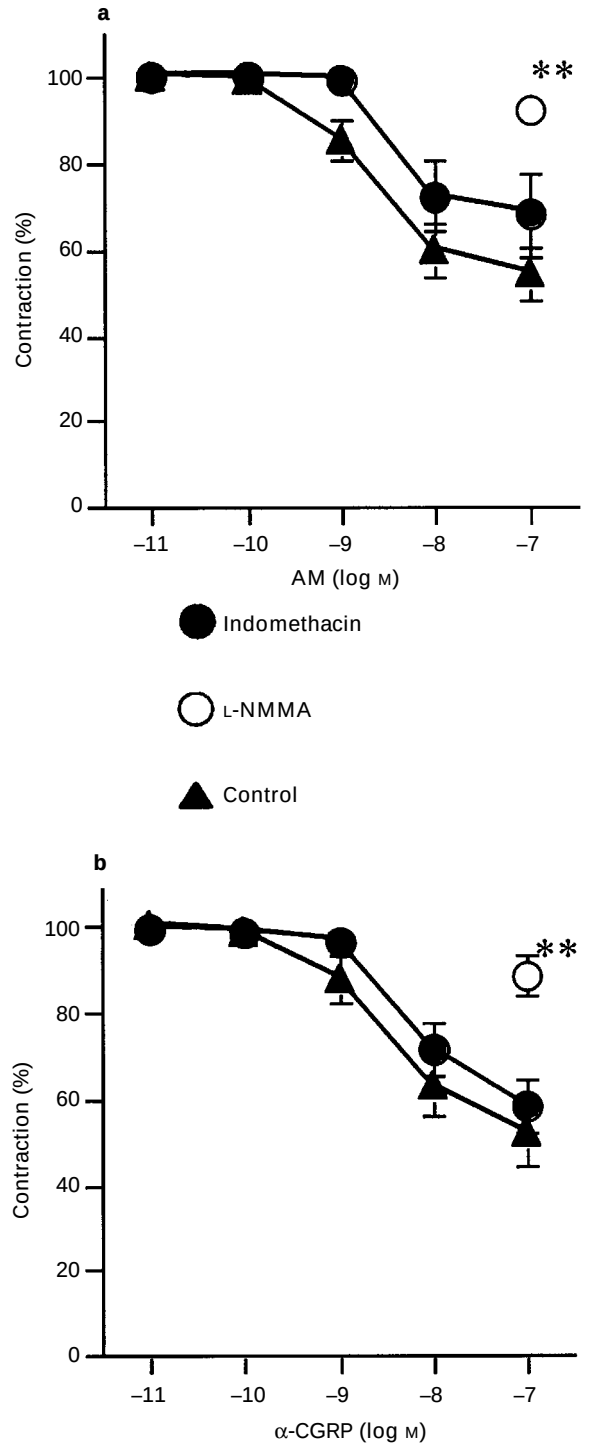

Figure 2 Vasorelaxant effects of adrenomedullin (AM) and α-CGRP in the endothelium-intact rat aorta contracted with noradrenaline (100 nM). AM and α-CGRP were added in the absence (control, data replotted from Figure 1), or the presence of 10 μM indomethacin, or 100 nM of the peptides were added in the presence of L-NMMA (100 μM). 100% represents the contractile response to noradrenaline. Each point represents the mean of 4 to 6 experiments and the s.e.means are shown by vertical lines. **Significantly different from control with $P<0.01$.

adrenomedullin (100 nM), α-CGRP (100 nM) and forskolin (1 μM) on cyclic AMP content in the rat aorta without endothelium. The rat aorta was incubated with the relaxants in the presence of IBMX (1 mM) for 10 min. The results indicated that neither adrenomedullin nor α-CGRP increased cyclic AMP content, whereas forskolin increased it (Figure 5).

Effect of adrenomedullin and α-CGRP on the porcine coronary artery

Cumulative addition of adrenomedullin or α-CGRP in the porcine coronary artery without endothelium contracted with high K^+ solution caused a small relaxation (7.3 ± 0.7% at 100 nM of adrenomedullin and 12.6 ± 2.1% at 100 nM of α-CGRP, Figure 1d,e). In the next series of experiments, the porcine coronary artery was contracted with a thromboxane A_2 analogue, U46619 (100 nM), and then adrenomedullin or α-CGRP was cumulatively added. In the endothelium-intact coronary artery, adrenomedullin or α-CGRP caused a concentration-dependent relaxation with an IC_{50} of 27.6 and 4.1 nM, respectively. The relaxations induced by adrenomedullin (100 nM) and α-CGRP (100 nM) were 60.5% and 95.4%, respectively (Figure 1d,e and Table 2). When the endothelium was denuded, adrenomedullin and α-CGRP also caused a concentration-dependent relaxation with an IC_{50} of 32.3 and 3.4 nM, respectively. The maximal relaxations induced by adrenomedullin (100 nM) and α-CGRP (100 nM) were 75.1% and 97.4%, respectively. Therefore, the removal of the endothelium did not significantly alter the IC_{50} values and the maximal relaxations induced by adrenomedullin and α-CGRP (Table 2).

We also simultaneously measured $[Ca^{2+}]_i$ and muscle tension in the porcine coronary artery without endothelium. Addition of U46619 (100 nM) caused a sustained increase in muscle tension and $[Ca^{2+}]_i$. When adrenomedullin (100 nM) was added during the steady state of the contraction, it decreased the contraction and $[Ca^{2+}]_i$ below the resting level ($[Ca^{2+}]_i$; -20.0 ± 11.0%), which soon recovered above the resting level ($[Ca^{2+}]_i$; 18.0 ± 11.7%). When α-CGRP (10 nM) was added, the contraction and $[Ca^{2+}]_i$ decreased below the resting level ($[Ca^{2+}]_i$; -41.5 ± 11.0%), which soon recovered towards the resting level ($[Ca^{2+}]_i$; 0%) (Figure 6).

The effect of CGRP (8–37) on the relaxation induced by adrenomedullin or α-CGRP was investigated in the porcine coronary artery without endothelium. CGRP (8–37) (1 μM) was applied after the contraction induced by U46619 (100 nM) reached a steady state and then adrenomedullin or α-CGRP was cumulatively added. CGRP (8–37) alone had no effect on the contraction. As shown in Figure 4d and e, CGRP (8–37) antagonized the relaxant effects of adrenomedullin and α-CGRP. β-CGRP also induced a concentration-dependent relaxation both in the presence and the absence of the endothelium (Figure 1f and Table 2). The maximal relaxations induced by β-CGRP were comparable in the absence and presence of the endothelium. In the endothelium-denuded artery, the IC_{50} (7.1 ± 1.1 nM) for β-CGRP in the presence of CGRP (8–37) (1 μM) was similar to that of in the absence of CGRP (8–37), although the relaxation induced by 300 nM β-CGRP was slightly inhibited by CGRP (8–37) (1 μM) (Table 2 and Figure 4f).

Figure 5 shows the effect of adrenomedullin (100 nM), α-CGRP (100 nM) and forskolin (1 μM) on the content of cyclic AMP in the porcine coronary artery without endothelium. The coronary artery was incubated with the relaxants in the presence of IBMX (1 mM) for 10 min. Results indicated that adrenomedullin, α-CGRP and forskolin increased cyclic AMP content.

Discussion

In the present experiments, we found that adrenomedullin and α-CGRP caused a relaxation only in the presence of the

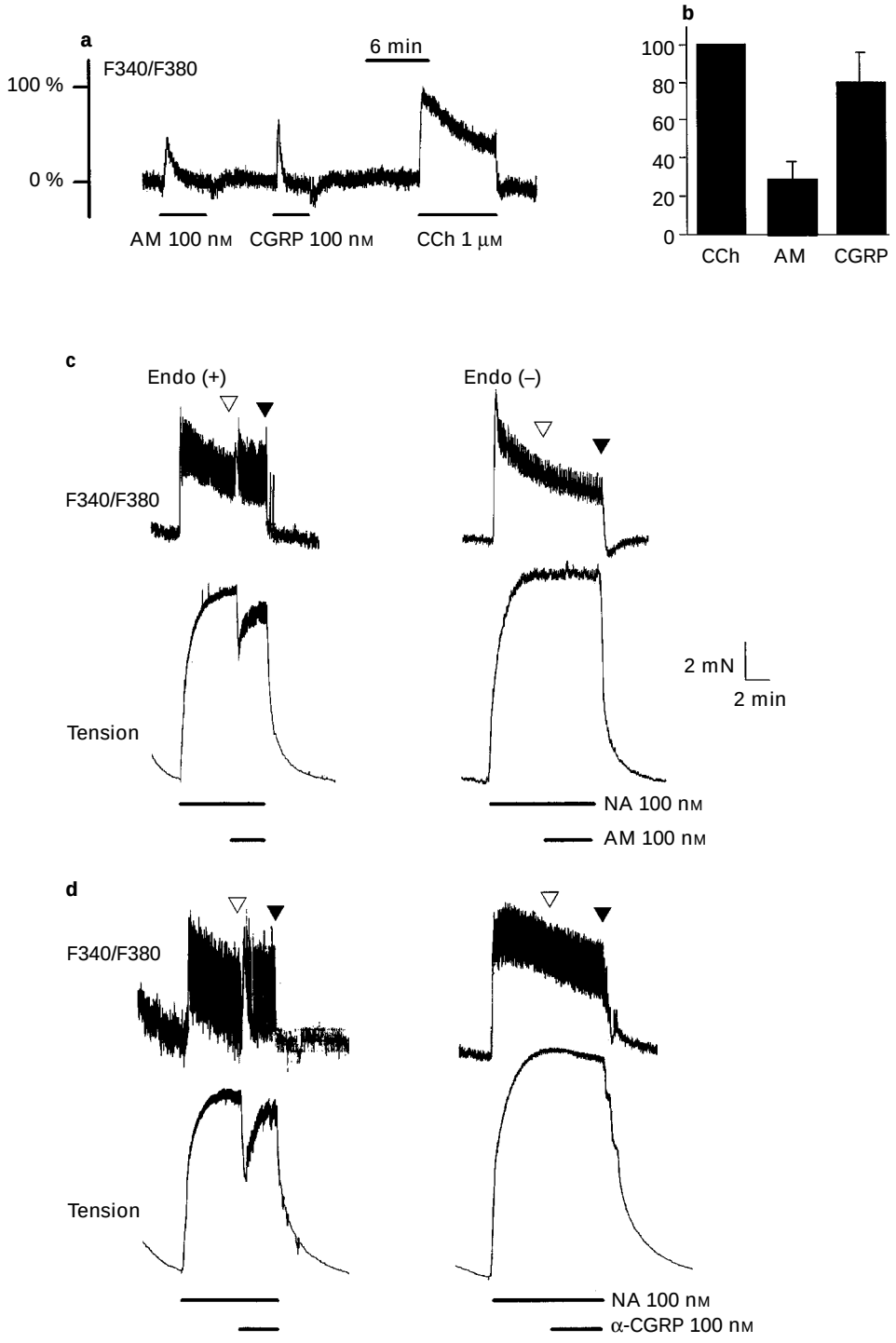

Figure 3 (a) Effect of adrenomedullin (AM, 100 nM), α-CGRP (100 nM) and carbachol (CCh, 1 μM) on $[Ca^{2+}]_i$ in the endothelium-intact rat aorta. (b) Averaged increases in $[Ca^{2+}]_i$ induced by AM, α-CGRP and carbachol; 100% represents the maximal increase in $[Ca^{2+}]_i$ induced by 1 μM carbachol. Each column represents the mean of 7 to 8 experiments and the s.e.mean is shown by vertical line. (c) and (d) Typical traces of the simultaneous recordings of $[Ca^{2+}]_i$ and muscle tension of the effect of AM (100 nM) and α-CGRP (100 nM). Muscle strips were contracted with 100 nM noradrenaline (NA). Application of peptides and washout are indicated by open and closed triangles, respectively. Endothelium-intact (left panel,) and endothelium-denuded (right panel,) strips.

endothelium in the rat aorta. We also found that the endothelium-dependent relaxations induced by adrenomedullin and α-CGRP were significantly attenuated by L-NMMA but not by indomethacin. It has been shown that an inhibitor of nitric oxide synthase, N^{ω}-nitro-L-arginine methyl ester, reduced the adrenomedullin-induced hypotension in systemic arterial pressure and hindquarters perfusion pressure of the rat (Feng *et al*., 1994; Nossaman *et al*., 1996). Meanwhile, the relaxant effect of α-CGRP in the rat thoracic aorta is known to be completely dependent on the presence of the endothelium (Brain *et al*., 1985) and blocked by haemoglobin, methylene blue (Fiscus *et al*., 1991) and L-arginine analogues (Gray & Marshall, 1992a; Hao *et al*., 1994), but not by indomethacin (Fiscus *et al*., 1991). These results, together with ours, suggest that adrenomedullin and α-CGRP possess similar inhibitory effects on the rat aorta and that these effects are mediated by

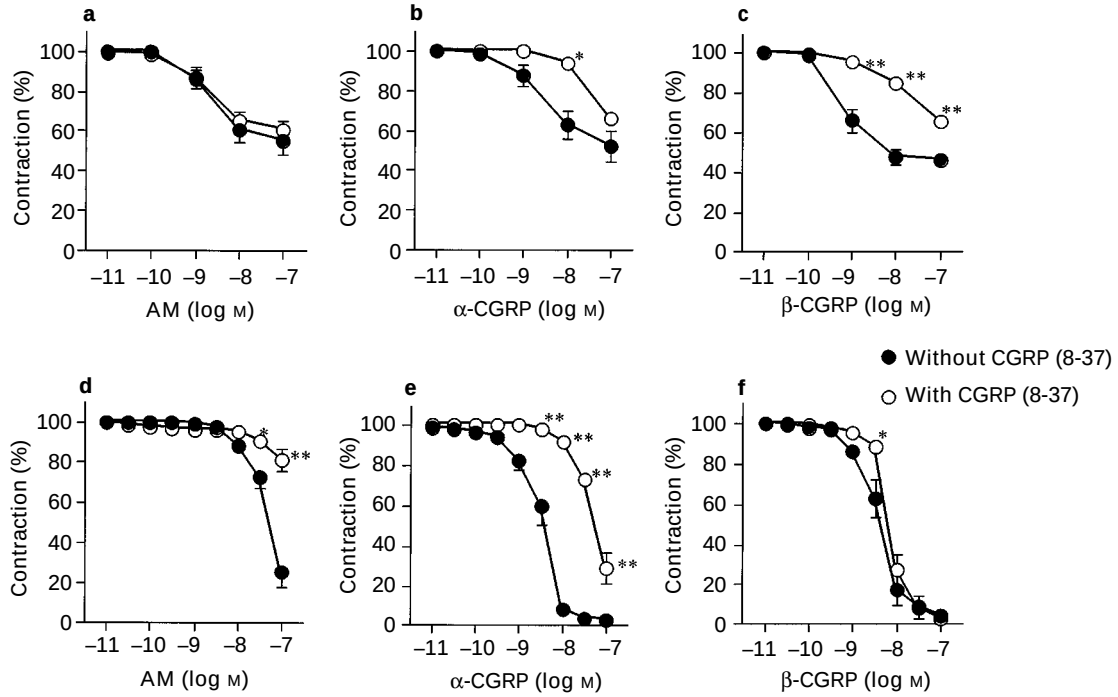

Figure 4 Effect of CGRP (8–37) (1 μM) on the concentration-response curves for adrenomedullin (AM), α- and β-CGRP in the rat aorta with endothelium (a, b, c) and in the porcine coronary artery without endothelium (d, e, f). AM, α- or β-CGRP was cumulatively added in the absence or presence of CGRP (8–37). Rings of the rat aorta and porcine coronary artery were contracted with noradrenaline (100 nM) and U46619 (100 nM), respectively. 100% represents the contractile response to noradrenaline or U46619 before the addition of the peptides. Each point represents the mean of 4 to 9 experiments and the s.e.mean is shown by vertical lines. Significance of difference from control, $*P<0.05$ and $**P<0.01$.

Table 2 The IC_{50}, apparent pA_2 and E_{max} values for the relaxant effects of AM and CGRPs

Vessels	*Relaxants*	*Endo*	*CGRP (8–37)*	*IC_{50}* (nM)		*Apparent pA_2*	*E_{max}*		n	*Possible receptor*
Rat aorta	AM	+	–	2.4±0.8	NS	–	45.5±6.5	NS	9	Adrenomedullin
		+	+	2.3±0.7		ND	39.9±4.8		6	
	α-CGRP	+	–	4.0±0.9	**	–	48.0±7.3	NS	6	$CGRP_1$
		+	+	45.7±7.3		7.0	33.7±2.4		4	
	β-CGRP	+	–	0.9±0.1	**	–	53.7±3.2	**	5	$CGRP_1$
		+	+	9.5±1.2		7.0	34.4±1.9		5	
Porcine coronary artery	AM	+	–	27.6±4.8	NS	–	60.5±10.0	NS	6	$CGRP_1$
		–	–	32.3±2.3	**	–	75.1±7.5	**	7	
		–	+	>>100		ND	44.1±6.2		4	
	α-CGRP	+	–	4.1±0.7	NS	–	95.4±1.4	NS	9	$CGRP_1$
		–	–	3.4±0.6	**	–	97.4±1.5	**	7	
		–	+	54.1±9.7		7.2	74.8±9.2		4	
	β-CGRP	+	–	6.0±1.1	NS	–	96.2±2.7	NS	5	$CGRP_2$
		–	–	4.8±1.2	NS	–	92.7±2.4	NS	6	
		–	+	7.1±1.1		5.7	97.6±1.2		6	

n, number of experiments. ND, not determined. NS, not significantly different. E_{max}, maximal relaxation. AM, adrenomedullin. Endo, endothelium. **Significantly different from the value in the absence of CGRP (8–37) with $P<0.01$.

nitric oxide but not by prostaglandins or other cyclo-oxygenase products. However, Plane & Garland (1996) have shown that the choice of vasoconstrictor determined the mechanism of the subsequent endothelium-dependent relaxation, such as nitric oxide-dependent and -independent relaxation. Therefore, adrenomedullin and α-CGRP may induce L-NMMA-resistant relaxations in the rat aorta when stimulated with other agonists.

Adrenomedullin and α-CGRP elevated $[Ca^{2+}]_i$ in the rat aorta with endothelium at the resting condition. Although we simultaneously measured $[Ca^{2+}]_i$ in the endothelium and smooth muscle, the elevation of $[Ca^{2+}]_i$ in response to adrenomedullin and α-CGRP seems to be derived from endothelium, because neither of the peptides changed $[Ca^{2+}]_i$ in the endothelium-denuded aorta. These results are consistent with a previous study demonstrating that adrenomedullin increased $[Ca^{2+}]_i$ in bovine cultured endothelial cells (Nakamura *et al*., 1995). In noradrenaline-stimulated aorta, an oscillatory change in $[Ca^{2+}]_i$ was observed together with the sustained increase in $[Ca^{2+}]_i$. Both adrenomedullin and α-CGRP induced a transient increase in $[Ca^{2+}]_i$ followed by an inhibition of muscle tension. The transient increase in $[Ca^{2+}]_i$ may reflect endothelial $[Ca^{2+}]_i$ signal, because neither adrenomedullin nor α-CGRP was effective in the absence of

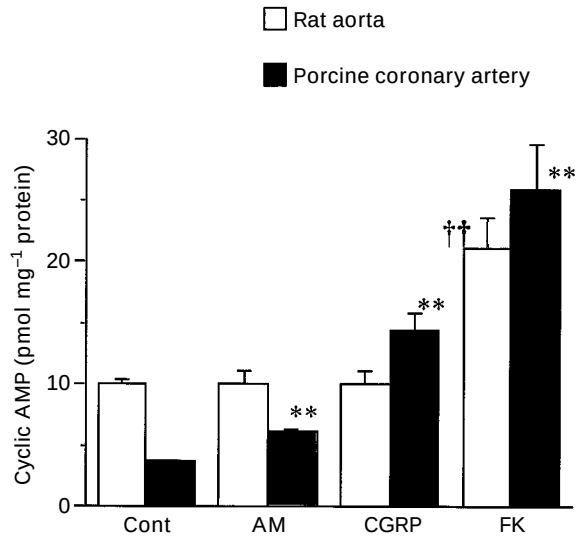

Figure 5 Effect of adrenomedullin (AM, 100 nM), α-CGRP (100 nM) and forskolin (FK, 1 μM) on cyclic AMP content in the rat aorta without endothelium and the porcine coronary artery without endothelium. Relaxants were added in the presence of IBMX (1 mM) for 10 min. Each column represents the mean of 4 experiments and the s.e.mean is shown by vertical lines. **Significantly different from control with $P<0.01$.

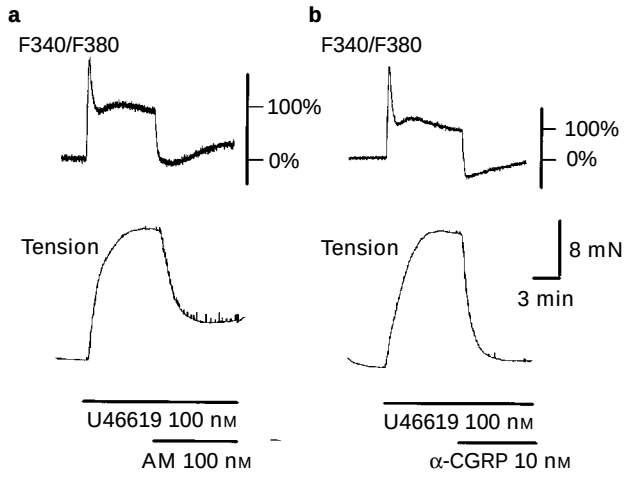

Figure 6 Representative recordings of the effects of adrenomedullin (AM, a, 100 nM) and α-CGRP (b, 100 nM) on the contraction and $[Ca^{2+}]_i$ in the porcine coronary artery without endothelium contracted with U46619 (100 nM).

the endothelium. Adrenomedullin and α-CGRP, therefore, may increase $[Ca^{2+}]_i$ in the endothelium, activate nitric oxide synthase and release nitric oxide.

Although the relaxant potencies of adrenomedullin and α-CGRP were comparable, α-CGRP increased endothelial $[Ca^{2+}]_i$ almost twice as much as adrenomedullin. In general, agonist-induced activation of nitric oxide synthase requires an elevation of endothelial $[Ca^{2+}]_i$ (Moncada *et al*., 1991). In addition, Gray & Marshall (1992b,c) have suggested that the elevation of endothelial cyclic AMP induced by α-CGRP and other cyclic AMP-elevating agents, such as β-adrenoceptor agonists and forskolin, results in activation of nitric oxide synthase. It has also been demonstrated that adrenomedullin increases cyclic AMP more potently than α-CGRP in human vascular endothelial cells (Kato *et al*., 1995). It is possible, therefore, that adrenomedullin and α-CGRP release nitric oxide from the endothelium by increasing both endothelial cyclic AMP and $[Ca^{2+}]_i$.

Previous studies have shown that adrenomedullin elevated cyclic AMP in cultured smooth muscle cells from the rat aorta (Eguchi *et al*., 1994a,b; Ishizaka *et al*., 1994; Shimekake *et al*., 1995). However, in the present study, adrenomedullin did not change cyclic AMP content in the rat aorta without endothelium. In cultured vascular smooth muscle cells of the rat aorta, α-CGRP has also been shown to increase cyclic AMP (Kubota *et al*., 1985; Hirata *et al*., 1988), although it was inactive in the rat freshly isolated aorta without endothelium (Wang *et al*., 1991; Gray & Marshall, 1992c; present study). These conflicting results may be due to different preparations and different experimental conditions.

Accumulation of cyclic AMP in smooth muscle leads to an inhibition of contraction by decreasing $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contraction elements of smooth muscle (Karaki, 1989; Karaki *et al*., 1997). Abe & Karaki (1989) showed that forskolin decreased $[Ca^{2+}]_i$, relaxed the resting or contracted rat aorta and inhibited noradrenaline-induced contraction more potently than high K^+-induced contraction. In the present study, adrenomedullin and α-CGRP increased cyclic AMP and decreased both $[Ca^{2+}]_i$ and muscle tension at the resting or contracted muscle. Furthermore, adrenomedullin and α-CGRP inhibited U46619-induced contraction more potently than high K^+-induced contraction. These results suggest that adrenomedullin and α-CGRP inhibit muscle contraction through the accumulation of cyclic AMP. Previous studies have suggested that adrenomedullin and α-CGRP decrease $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contractile elements in intact and α-toxin-permeabilized smooth muscle strips (Kureishi *et al*., 1995; Fukuzumi *et al*., 1996). It is likely, therefore, that adrenomedullin and α-CGRP relax the porcine coronary artery by increasing cyclic AMP, resulting in a decrease in $[Ca^{2+}]_i$ and Ca^{2+} sensitivity. However, both adrenomedullin (100 nM) and α-CGRP (100 nM) were far less effective than forskolin (1 μM) in increasing cyclic AMP content, although 1 μM concentration of forskolin is equipotent to adrenomedullin (100 nM) in decreasing muscle contraction (data not shown). Mechanisms other than cyclic AMP might be involved in the vasorelaxations induced by adrenomedullin and α-CGRP and/or cyclic AMP might be localized in the smooth muscle cell.

Previous pharmacological studies have proposed that the CGRP receptor is classified into two subtypes, the CGRP (8–37)-sensitive $CGRP_1$ receptor and -insensitive $CGRP_2$ receptor (Chiba *et al*., 1989; Dennis *et al*., 1989; 1990; Mimeault *et al*., 1991; Quirion *et al*., 1992; Poyner, 1992; 1995 see for review). In the present study, α-CGRP induced an endothelium-dependent relaxation in the rat aorta with an IC_{50} of 4.0 nM and this effect was strongly antagonized by CGRP (8–37) with a pA_2 of 7.0. Furthermore, β-CGRP relaxed the rat aorta with the similar IC_{50} and E_{max} value to those for α-CGRP and this effect was also antagonized by CGRP (8–37) with a pA_2 of 7.0. It is suggested, therefore, that the rat aortic endothelium expresses the $CGRP_1$ receptor, via which both α-CGRP and β-CGRP mediate vasorelaxations (Table 2). Previous studies have also suggested that α-CGRP and β-CGRP may act on the $CGRP_1$ receptor in the guinea pig atrium (Giuliani *et al*., 1992) and rat kidney (Quirion *et al*., 1992; Castelluci *et al*., 1993).

In the porcine coronary artery, α-CGRP caused a relaxation and this effect was antagonized by CGRP (8–37). Although β-CGRP also induced a relaxation, this effect was not antagonized by CGRP (8–37). Previous studies have also shown that α-CGRP and β-CGRP act on the CGRP (8–37)-

sensitive receptor and the CGRP (8–37)-insensitive receptor, respectively, in guinea-pig basilar artery and ileum and human cerebral artery (Jansen, 1992; Jansen *et al*., 1996; Tomlinson & Poyner, 1996). Therefore these tissues seem to express not only the $CGRP_1$ receptor but also the $CGRP_2$ receptor which is not sensitive to CGRP (8–37). Sano *et al*. (1989) have demonstrated that the biding of ^{125}I-labelled α-CGRP to solubilized binding sites is inhibited by unlabelled α- and β-CGRP in the porcine coronary artery and suggested that both α-CGRP and β-CGRP may act on the same receptor. Therefore, it is likely that in the porcine coronary artery, α-CGRP acts on both the $CGRP_1$ and $CGRP_2$ receptor whereas β-CGRP acts only on the $CGRP_2$ receptor (Table 2).

In the rat aorta, adrenomedullin-induced relaxations were not antagonized by CGRP (8–37). This result is consistent with previous *in vivo* and *in vitro* studies (Kato *et al*., 1995; Heaton *et al*., 1995; Edward *et al*., 1996; Nandha *et al*., 1996; Pinto *et al*., 1996; Champion *et al*., 1997). Therefore, the rat aortic endothelium seems to express the adrenomedullin-specific receptor. In the porcine coronary artery, in contrast, CGRP (8–37) antagonized the relaxant effect of not only α-CGRP but also adrenomedullin, suggesting that adrenomedullin and α-CGRP activate the $CGRP_1$ receptor as has been shown previously (Nuki *et al*., 1993; Eguchi *et al*., 1994a; Ishizaka *et al*., 1994; Entzeroth *et al*., 1995; Zimmermann *et al*., 1995; Okumura *et al*., 1997; Mori *et al*., 1997).

Recent studies have identified the cDNA clones for the adrenomedullin receptor and the $CGRP_1$ receptor (Kapas *et al*., 1995; Kapas & Clark 1995; Aiyar *et al*., 1996). In these studies, it was shown that the adrenomedullin receptor does not have affinity for α-CGRP and CGRP (8–37) whereas the $CGRP_1$ receptor has affinity for α-CGRP, CGRP (8–37) and adrenomedullin. These results are consistent with our study suggesting the presence of an adrenomedullin-specific receptor in the rat aortic endothelium. However, our results indicated that the $CGRP_1$ receptor in the rat aortic endothelium is activated by both α-CGRP and β-CGRP but not by adrenomedullin whereas the $CGRP_1$ receptor in the porcine coronary artery is activated by both α-CGRP and adrenomedullin but not by β-CGRP. Furthermore, the CGRP (8–37)-insensitive $CGRP_2$ receptor in the porcine coronary artery was activated only by β-CGRP (Table 2). These results imply the presence of subtypes of the $CGRP_1$ receptor, although further experiments are needed to examine this possibility.

In conclusion, adrenomedullin and α-CGRP induced endothelium-dependent relaxations in the rat aorta whereas these peptides induced endothelium-independent relaxations in the porcine coronary artery. The relaxations in the rat aorta are likely to be mediated by the release of nitric oxide from endothelium whereas those in the porcine coronary artery are likely to be due to the elevation of cytosolic cyclic AMP leading to a decrease in $[Ca^{2+}]_i$. The porcine coronary artery seems to express the $CGRP_1$ receptor which is activated by adrenomedullin and α-CGRP, and another type of CGRP receptor which is activated by β-CGRP, possibly the $CGRP_2$ receptor. The rat aortic endothelium seems to express an adrenomedullin-specific receptor and the $CGRP_1$ receptor. However, the $CGRP_1$ receptor in the rat aorta is not activated by adrenomedullin.

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