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Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes

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Abstract

The purpose of the present study was to determine if cardiac myocytes and nonmyocytes secrete adrenomedullin, to investigate the effects of adrenomedullin on cAMP and cGMP levels in cardiac myocytes and nonmyocytes, to study the effect of calcitonin gene-related peptide (CGRP) receptor antagonist CGRP-(8–37) and adrenomedullin-specific receptor antagonist, adrenomedullin-(22–52) on response to adrenomedullin and CGRP. Neonatal (days 1–2) cardiac myocytes and nonmyocytes were prepared from the ventricle of Wistar rats. Not only cardiac myocytes, but also nonmyocytes secrete almost equal amounts of adrenomedullin into the media. Both adrenomedullin and CGRP increased the cAMP levels, not the cGMP levels, both in the myocytes and nonmyocytes. In myocytes, CGRP-(8–37), almost completely inhibited the adrenomedullin- and CGRP-induced cAMP formation. In nonmyocytes, CGRP-(8–37) completely inhibited the cAMP levels induced by adrenomedullin and CGRP. More profound antagonistic effect of CGRP-(8–37) on cAMP levels induced by adrenomedullin was observed in nonmyocytes than in myocytes. In contrast, antagonistic effect of adrenomedullin-(22–52) for adrenomedullin-stimulated cAMP formation was considerably less potent than CGRP-(8–37) both in myocytes and nonmyocytes. Adrenomedullin-(22–52) did not affect the cAMP formation induced by CGRP either in myocytes or nonmyocytes. These results suggest that myocytes and nonmyocytes secrete adrenomedullin and that adrenomedullin increases cAMP levels possibly via different receptors in myocytes and nonmyocytes.

Keywords: Adrenomedullin; Myocyte; Nonmyocyte; CGRP (calcitonin gene-related peptide); cAMP; cGMP

1. Introduction

Adrenomedullin is a 52-amino-acid peptide that was originally discovered in acid extracts from a human pheochromocytoma (Kitamura et al., 1993a). Subsequent studies have demonstrated that adrenomedullin peptide is widely distributed in various tissues and organs, including the heart (Ichiki et al., 1994; Sakata et al., 1994). Cloning of the cDNA encoding the adrenomedullin precursor has shown that this gene is highly expressed in the heart, adrenals, kidneys, and lungs of both rats and humans (Kitamura et al., 1993b; Sakata et al., 1993). Binding studies have demonstrated that abundant and specific receptors for this peptide are highly present in the heart, lungs, spleen and kidneys (Owji et al., 1995). Thus, the considerable overlap between the expression of the adrenomedullin peptide and mRNA with the expression of adrenomedullin receptors suggests that this peptide may directly influence cardiac function. Indeed, a recent study showed that adrenomedullin decreased both contractility and calcium ion concentrations in isolated rabbit adult ventricular myocytes in a dose-dependent manner (Ikenouchi et al., 1997). Another recent study revealed that the heart is a target organ of adrenomedullin and that adrenomedullin augments nitric oxide synthesis in the heart (Ikeda et al., 1996). In addition, we recently found that adrenomedullin peptide and mRNA levels are increased in the ventricles in rats with heart failure (Nishikimi et al., 1997). Increased plasma adrenomedullin levels also have been reported in patients with heart failure (Jougasaki et al., 1995; Nishikimi et al., 1995) and in hypertensive patients with cardiac hypertrophy (Sumimoto et al., 1997). These findings suggest that adrenomedullin in the heart

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may be involved in the regulation of cardiac function or the pathophysiology of heart failure and cardiac hypertrophy.

However, information on the receptors and the mechanism involved in the action of adrenomedullin on heart is limited. Ikenouchi et al. (1997) recently reported that adrenomedullin increases cGMP levels in isolated rabbit adult ventricular myocytes without changing cAMP levels. Other studies reported that adrenomedullin increases cAMP levels in neonatal rat cardiac myocytes (Ikeda et al., 1996; Sato et al., 1997). Thus, the second messenger of adrenomedullin in the heart is controversial. Furthermore, whether cardiac myocytes or nonmyocytes secrete adrenomedullin, the effect of adrenomedullin on cAMP and cGMP levels in cardiac nonmyocytes remains unknown. In some systems, adrenomedullin and CGRP (calcitonin gene-related peptide) share a common receptor and signalling mechanism (Zimmermann et al., 1996), whereas in other tissues, the effects of adrenomedullin and CGRP can be clearly separated (Osajima et al., 1995; Kato et al., 1995). The purposes of the present study were to determine whether cardiac myocytes and/or nonmyocytes secrete adrenomedullin, to investigate the effects of adrenomedullin on the cAMP and cGMP levels in cardiac myocytes and nonmyocytes, to study whether the effect of adrenomedullin on a second messenger is mediated via a receptor with high affinity for CGRP-(8–37) or adrenomedullin-(22–52).

2. Materials and Methods

2.1. Cell cultures

Enriched cultures of neonatal (days 1–2) cardiac myocytes and nonmyocytes were prepared from the hearts of Wistar rats by a method previously reported (Iwaki et al., 1990) with minor modifications. Apical halves of cardiac ventricles were recovered and minced in a chilled balanced salt solution (116 mM NaCl, 20 mM HEPES, 12.5 mM NaH_2PO_4 5.6 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$, pH 7.35). Ventricular cardiac myocytes were dispersed in the balanced salt solution containing 0.06% collagenase II (Worthington Biochemical, Freehold, NJ) with agitation for 6 min at 37°C and then pipetted approximately 20 times. This procedure was repeated for five to six times. Each collected cell suspension was minced with 1/5 volume of chilled fetal calf serum (Gibco Laboratories, Grand Island, NY) and then pelleted by centrifugation at 1000 rpm for 5 min. The pellets were combined in chilled fetal calf serum and kept at 4°C.

The differentiation of myocytes from nonmyocytes was performed by the discontinuous Percoll gradient method (Iwaki et al., 1990). The discontinuous gradient of Percoll (Sigma, St. Louis, MO) consisting of 40.5 and 58.5% of Percoll in the balanced salt solution described above was prepared, and ventricular cells were suspended in the layer of 58.5% Percoll. After centrifugation at 3000 rpm for 30 min at 15°C, the cardiac myocytes selectively migrated to the interface of the discontinuous layers, and the nonmyocytes selectively migrated to the upper layer. Both the myocytes and nonmyocytes were resuspended in Dulbecco's modified Eagle medium (DMEM) (Gibco) and washed twice by centrifugation and resuspended to remove all traces of Percoll. The nonmyocytes were resuspended in DMEM with 10% fetal calf serum and plated to uncoated 10-cm dishes for 30 min. After this plating period, nonadherent cells and debris were washed away and fresh medium was added. The cells were allowed to grow to confluence and were then trypsinized and passaged 1:3. This procedure yielded cultures of cells that were almost exclusively fibroblasts by the first passage, as determined by an immunocytochemical technique previously reported (Fujisaki et al., 1995). Subconfluent nonmyocytes from the third passage were used for the experiments. The myocytes were resuspended in DMEM with 10% fetal calf serum and plated to uncoated 10-cm dishes for 30 min to allow the differential attachment of nonmyocytes. The myocytes-enriched suspensions were again plated to uncoated 10-cm dishes to remove remaining nonmyocytes. This procedure yielded cultures of cells that were almost exclusively cardiac myocytes. After the number of myo-

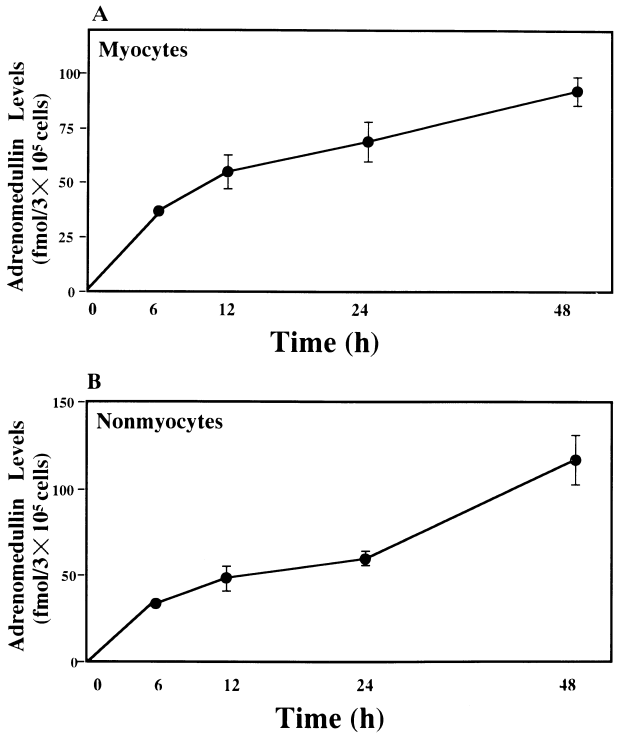

Fig. 1. (A), (B) Line graph shows the time-course of adrenomedullin secretion in the medium in cardiac myocytes and nonmyocytes. The cells were incubated for the indicated time-periods in serum-free medium. Means ± S.D.; $n = 3–4$.

cytes was counted in a hemocytometer, the purified myocytes were plated at a density of 3.2×10^4 cells/cm^2 in a gelatin-coated six-well plate (Iwaki, Chiba, Japan) in DMEM supplemented with 10% fetal calf serum and antibiotics (50 U/ml penicillin G and 50 μg/ml streptomycin). The culture medium was changed to DMEM with 10% fetal calf serum 24 h later. Following a 24-h incubation, the cells were maintained in serum-free DMEM for 12 h. After the preconditioned periods, the cultures were incubated in serum-free DMEM containing 1 mg/ml bovine serum albumin (Seikagaku, Tokyo, Japan) with the following agents or vehicles: synthetic adrenomedullin, CGRP, adrenomedullin-(22–52), and CGRP-(8–37) (Peptide Institute, Osaka, Japan) for the indicated times. The use of laboratory animals for scientific purposes was approved by the ethical committee of our institution.

2.2. Measurements of adrenomedullin

A toltal of 1 ml of the conditioned medium of the cardiac myocytes or nonmyocytes was collected at 6 h, 12 h, 24 h, and 48 h. Each medium sample for radioimmunoassay (RIA) was boiled in 10% volumes of 1 mol/l acetic acid for 10 min to inactivate intrinsic proteases. After cooling, the boiled medium was evaporated in a vacuum until dry. The RIA for rat adrenomedullin was performed as previously reported (Sakata et al., 1994). Anti-adrenomedullin antiserum (No. 172CI-7), which recognizes the C-terminal region of rat adrenomedullin, was used in this RIA. The incubation buffer for the RIA contained 50 mM sodium phosphate buffer (pH 7.4), 0.5% bovine serum albumin, 0.5% Triton X-100, 80 mM NaCl, 25 mM disodium EDTA, and 0.05% NaN_3. The RIA incubation mixture consisted of 100 μl adrenomedullin or unknown sample solution, 50 μl of antiserum at a dilution of 1:140 000, and 50 μl of ^{125}I-labelled peptide (18 000 cpm). After incubation for 40 h, free and bound tracers were separated by the polyethyleneglycol method. The radioactivity of the pellet was counted with a gamma counter (ARC-1000M, Aloka, Tokyo, Japan). The assays were performed in duplicate.

2.3. Assay for cAMP and cGMP

Following each treatment of cardiac myocytes and nonmyocytes with various concentrations of adrenomedullin and CGRP with or without CGRP-(8–37) and adrenomedullin-(22–52) in the presence of 0.5 mM 3-isobutyl-1 methyl-xanthine (IBMX), the medium was removed and the cellular extract was obtained with the use of cold 70% ethanol. The incubation time was 10 min, except for the time-course experiment. Each ethanol sam-

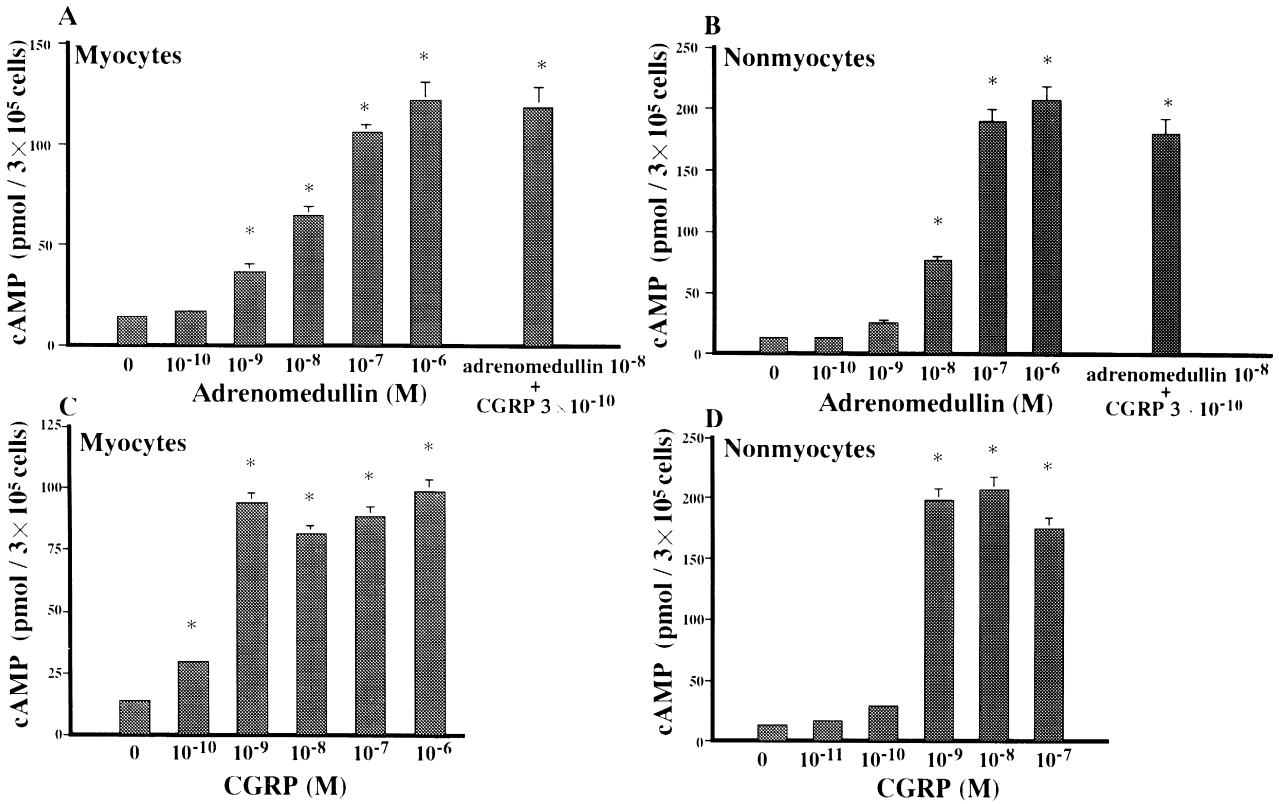

Fig. 2. (A), (B) Bar graph shows the effect of adrenomedullin (adrenomedullin; 10^{-10} to 10^{-6} M) and combined effects of adrenomedullin (10^{-8} mol/l) and calcitonin gene-related peptide (CGRP; 3×10^{-10} mol/l) on the cAMP levels in cardiac myocytes and nonmyocytes. (C), (D) Bar graph shows the effect of CGRP on the cAMP levels in cardiac myocytes and nonmyocytes. Means ± S.D.; $n = 3$–4. * P 0.01 vs. control.

ple was evaporated in a vacuum until dry. The eluate was dissolved in RIA buffer. The RIA for cAMP and cGMP was performed with a RIA kit (cyclic AMP and GMP assay kit; Yamasa Shoyu, Chiba, Japan).

2.4. Statistical analysis

All data was expressed as the means ± S.D. The multiple comparison was performed with a one-way analysis of variance followed by Scheffe's test. The P values less than 0.05 were considered significant.

3. Results

Fig. 1 shows the time-course of adrenomedullin secretion into the medium from cultured cardiac myocytes and nonmyocytes. Both the myocytes and nonmyocytes secreted adrenomedullin into the medium in a time-dependent manner.

Adrenomedullin increased the cAMP (levels in the myocytes and nonmyocytes in a concentration-dependent manner, with an EC_{50} (50% effective concentration) of 10^{-8} M in the myocytes and 3×10^{-8} M in the nonmyocytes (Fig. 2). In both the myocytes and nonmyocytes, CGRP was more potent than adrenomedullin ($EC_{50} = 5 \times 10^{-10}$ M in the myocytes; 5×10^{-10} M in the nonmyocytes). The maximum cAMP formations by adrenomedullin and CGRP were similar in both the myocytes and nonmyocytes. The effects of EC_{50} concentrations of adrenomedullin and CGRP on cAMP formation were additives in the myocytes and in nonmyocytes.

Fig. 3 shows the time-course of cAMP accumulation produced by adrenomedullin in the cardiac myocytes and nonmyocytes. The cAMP level was significantly increased by adrenomedullin at 2 min and peaked at 10 min in both myocytes and nonmyocytes. Thereafter, the cAMP levels gradually decreased.

The effects of adrenomedullin and CGRP on the cGMP levels in the myocytes and nonmyocytes are shown in Fig. 4. Neither adrenomedullin nor CGRP increased the cGMP levels in the myocytes or in the nonmyocytes.

The effect of CGRP-(8–37) on the cAMP levels in the myocytes produced by adrenomedullin and CGRP are shown in Fig. 5. While CGRP-(8–37) significantly attenuated the adrenomedullin (10^{-8} M)-induced cAMP formation at concentrations greater than 10^{-8} M, CGRP-(8–37) significantly inhibited the CGRP (10^{-9} M)-induced cAMP formation at concentrations greater than 10^{-8} M. In contrast, antagonistic effect of adrenomedullin-(22–52) was considerably less potent in adrenomedullin-stimulated cAMP formation as compared to CGRP-(8–37). Adrenomedullin-(22–52) did not affect the cAMP formation induced by CGRP (10^{-9} M) in myocytes.

The effects of CGRP-(8–37) on the cAMP levels in the nonmyocytes induced by adrenomedullin and CGRP are shown in Fig. 6. The CGRP-(8–37) significantly attenuated the adrenomedullin (10^{-8} M)-induced cAMP levels at concentrations greater than 10^{-9} M and CGRP (10^{-9} M)-induced cAMP levels at concentrations greater than 10^{-9} M, respectively. Adrenomedullin-(22–52) had considerably less potent effect on the cAMP levels induced by adrenomedullin (10^{-8} M) as compared to CGRP-(8–37). Adrenomedullin-(22–52) had no significant effect on the cAMP levels induced by CGRP (10^{-9} M) in nonmyocytes, either.

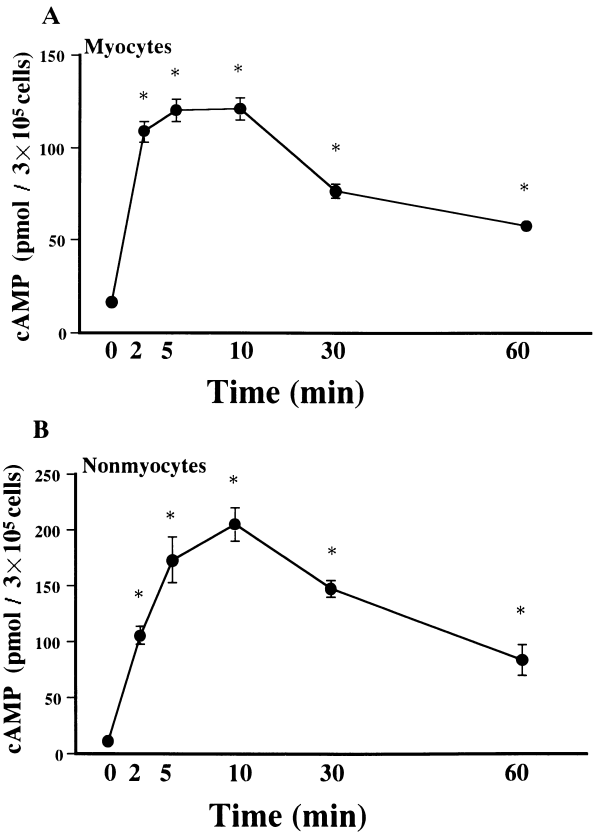

Fig. 3. (A), (B) Line graph shows a time-course of cAMP formation in myocytes and nonmyocytes. Cultured rat cardiac myocytes and nonmyocytes were incubated with 10^{-7} mol/l of adrenomedullin for indicated times at 37°C in the presence of 0.5 mM IBMX. Means ± S.D.; $n = 3$–4. * P 0.01 vs. 0 min.

4. Discussion

We demonstrated in the present study that myocytes and nonmyocytes secreted significant amounts of adrenomedullin into medium, and that adrenomedullin increased not the cGMP levels, but the cAMP levels in both the myocytes and nonmyocytes in a time- and concentration-dependent manner. The CGRP was more potent for cAMP formation than adrenomedullin in both the myocytes and nonmyocytes. The CGRP-(8–37) significantly inhibited the adrenomedullin (10^{-8} M)-induced cAMP

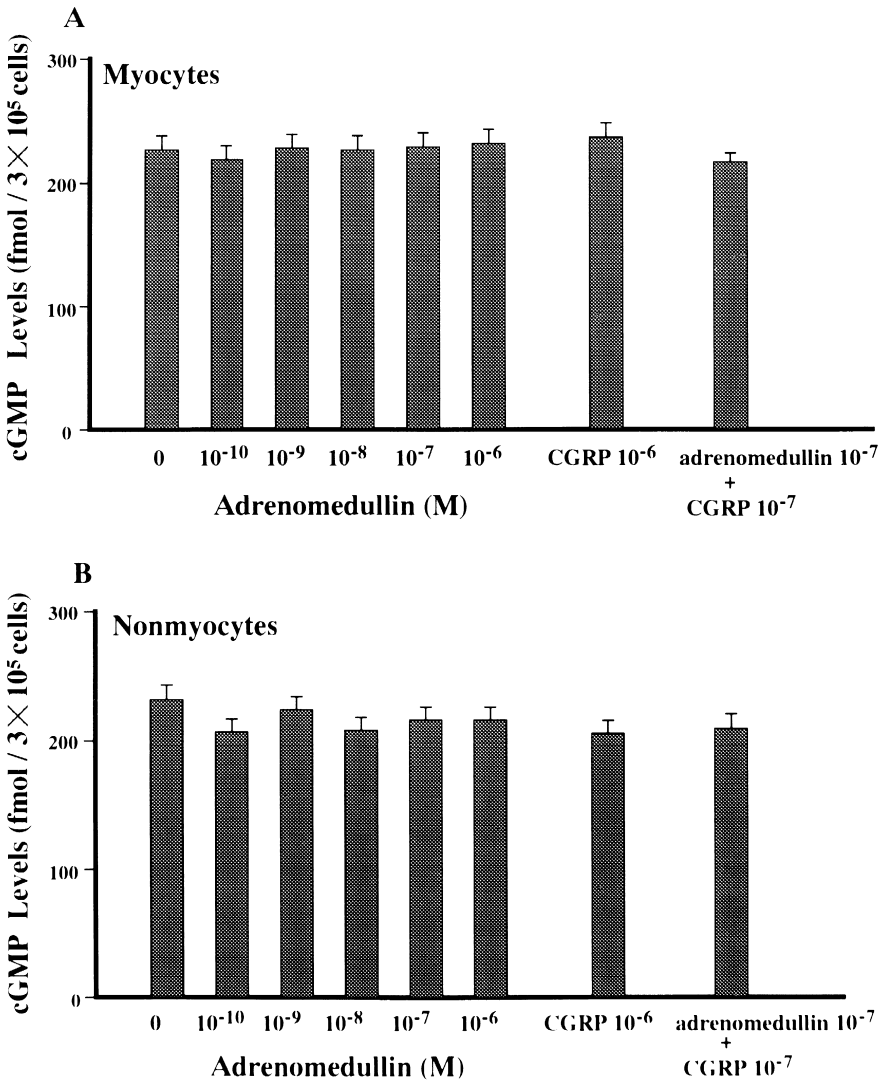

Fig. 4. (A), (B) Bar graph shows the effects of adrenomedullin (10^{-10} to 10^{-6} mol/l), CGRP (10^{-6} mol/l), and the combined effect of adrenomedullin (10^{-7} mol/l) and CGRP (10^{-7} mol/l) on cGMP levels in rat cardiac myocytes and nonmyocytes. Means ± S.D.; $n = 3–4$.

formation at a concentration greater than 10^{-8} M in myocytes and at a concentration greater than 10^{-9} M in nonmyocytes. In contrast, adrenomedullin-(22–52) had a considerably less potent antagonistic effect in adrenomedullin-induced cAMP formation both in myocytes and nonmyocytes. Adrenomedullin-(22–52) had no antagonistic effect in cAMP formation induced by CGRP either in myocytes or nonmyocytes. These results suggest that myocytes and nonmyocytes secrete adrenomedullin and that adrenomedullin increases the cAMP levels possibly via different receptors in myocytes and in nonmyocytes. Adrenomedullin may work as an autocrine and/or paracrine factor in the heart.

Although we found that adrenomedullin stimulated cAMP formation in both myocytes and nonmyocytes in a time- and concentration-dependent manner, there were important differences in both the quantitative and qualitative aspects of adrenomedullin action in the myocytes and nonmyocytes. First, the effect of CGRP on cAMP formation in the myocytes and nonmyocytes was more potent than that of adrenomedullin. Second, CGRP-(8–37) antagonized the adrenomedullin-induced increase in cAMP formation in the myocytes in a concentration-dependent manner. In other systems such as vascular smooth muscle cells, adrenomedullin and CGRP are reported to act on a common receptor while CGRP-(8–37) inhibits the action of both peptides (Eguchi et al., 1994a). The present results suggest that a receptor with equivalent affinity for adrenomedullin and CGRP-(8–37) may exist in myocytes. Third, CGRP-(8–37) also antagonized the adrenomedullin action in the nonmyocytes in a concentration-dependent manner. However, this antagonizing effect of CGRP-(8–37) on adrenomedullin-induced cAMP formation was more potent in nonmyocytes than in myocytes. The CGRP-(8–37) significantly inhibited the adrenomedullin (10^{-8} M)-induced cAMP formation at 10^{-9} M in nonmyocytes, and

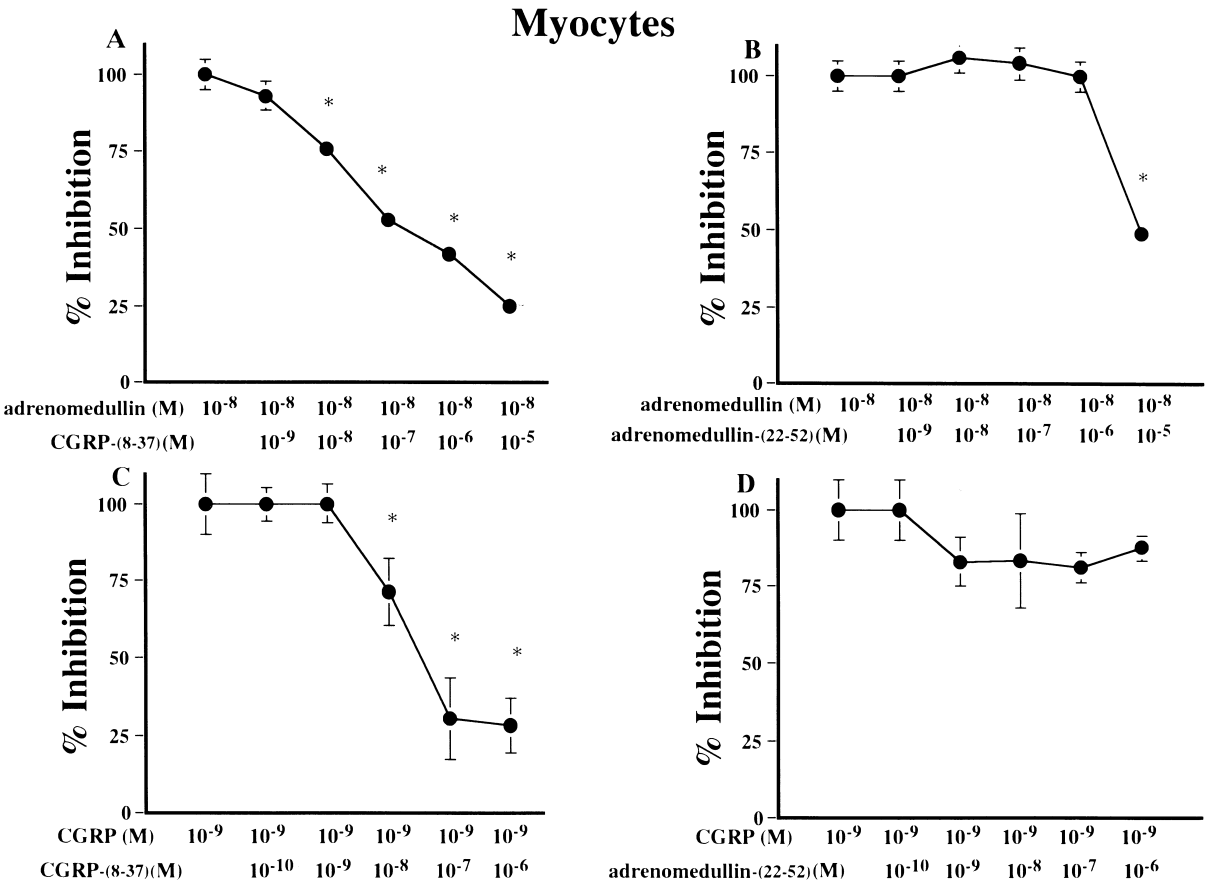

Fig. 5. (A), (B), (C), (D) Line graph shows the antagonistic effects of CGRP-(8–37) and of human adrenomedullin-(22–52) on the cAMP levels stimulated by adrenomedullin (10^{-8} mol/l) or CGRP (10^{-9} mol/l) in myocytes. Cultured rat cardiac myocytes were incubated with CGRP-(8–37) or adrenomedullin-(22–52) in the presence of adrenomedullin (10^{-8} mol/l) or CGRP (10^{-9} mol/l). Means ± S.D.; $n = 3–4$. $*$ P 0.01 vs. control.

inhibited the CGRP (10^{-9} M)-induced cAMP formation at 10^{-9} M. Thus, an adrenomedullin receptor with higher affinity for CGRP-(8–37) and adrenomedullin may be expressed in cultured rat nonmyocytes. Fourth, adrenomedullin-(22–52), which is reported to be a specific receptor antagonist for adrenomedullin (Eguchi et al., 1994b), had a weak effect on the adrenomedullin-induced increases in cAMP, in both the myocytes and nonmyocytes and no effect on the CGRP-induced cAMP formation either in myocytes or nonmyocytes. In other cells, such as mesangial cells, adrenomedullin-(22–52) had a greater inhibitory effect on cAMP formation induced by adrenomedullin than did CGRP-(8–37), indicating that receptors with high affinity for adrenomedullin-(22–52) are preferentially expressed in mesangial cells (Osajima et al., 1996). The present results indicate that an adrenomedullin receptor with low affinity for adrenomedullin-(22–52) may be expressed in cultured rat cardiac myocytes and nonmyocytes. Furthermore, the cAMP production stimulated by these two peptides was additive, suggesting that they elevate cAMP levels by the different pathway. These results suggest that myocytes may have an adrenomedullin receptor which has an equivalent affinity for both adrenomedullin and CGRP-(8–37) and lower affinity for adrenomedullin-(22–52). Nonmyocytes, in contrast, may have an adrenomedullin receptor with a higher affinity for CGRP-(8–37) than for adrenomedullin and lower affinity for adrenomedullin-(22–52). The determination of the structures of both these CGRP and adrenomedullin receptor subtypes by molecular cloning is necessary in a future study to provide further information on the effector systems of these two peptides.

The present experiments clearly demonstrated that adrenomedullin stimulated cAMP formation in myocytes in a concentration-dependent manner. This finding is consistent with those of previous studies using cultured rat neonatal myocytes (Ikeda et al., 1996; Sato et al., 1997). The present study extended our investigation into the effect of adrenomedullin on cAMP levels in nonmyocytes. Adrenomedullin and CGRP also stimulated cAMP levels in nonmyocytes in a concentration-dependent manner. However, a recent report by Ikenouchi et al. revealed that adrenomedullin slightly but significantly increased cGMP levels without affecting cAMP levels in adult rabbit ventricular myocytes (Ikenouchi et al., 1997). Our present result showed that adrenomedullin did not change the cGMP levels in neonatal cultured myocytes or in nonmyocytes. The exact reason for the discrepancy between the

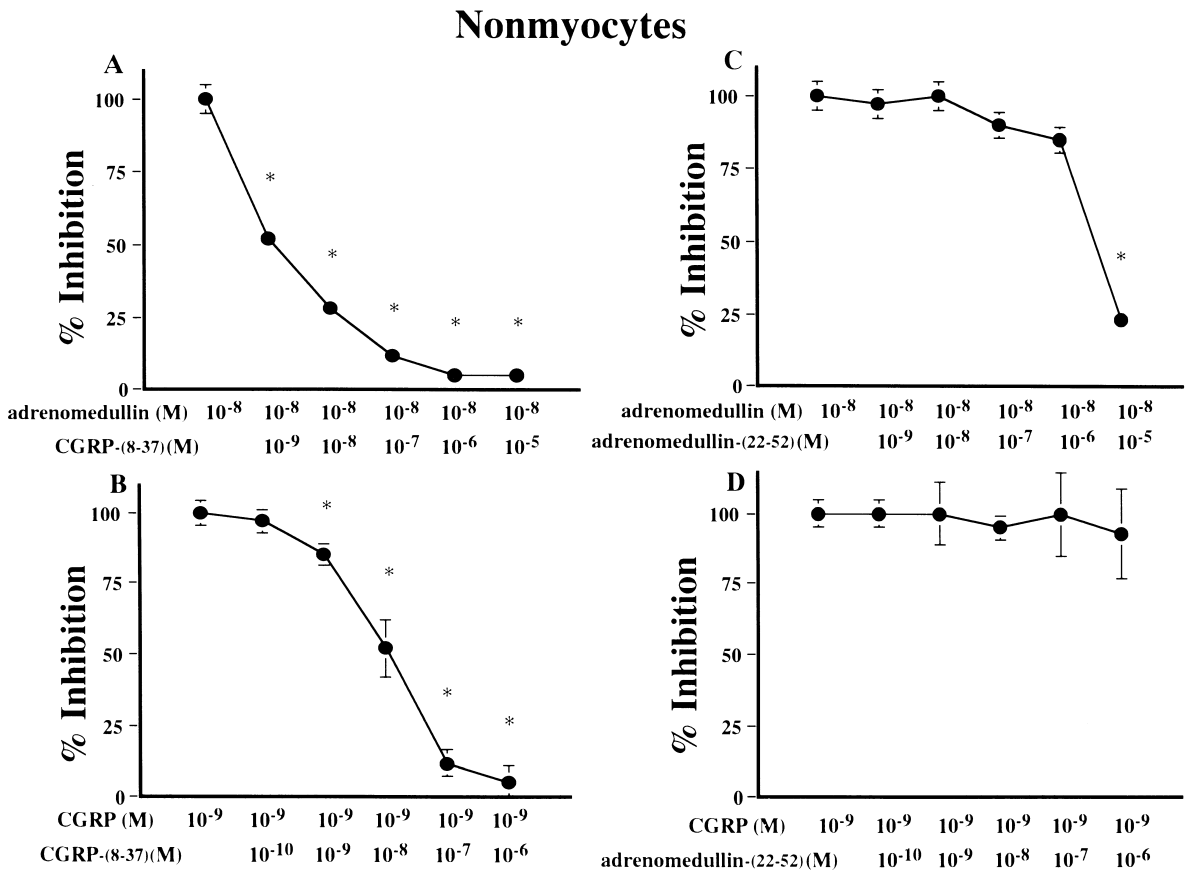

Fig. 6. (A), (B), (C), (D) Line graph shows the antagonistic effects of CGRP-(8–37) or human adrenomedullin-(22–52) on the cAMP levels stimulated by adrenomedullin (10^{-8} mol/l) or CGRP (10^{-9} mol/l) in nonmyocytes. Cultured rat cardiac nonmyocytes were incubated with CGRP-(8–37) or adrenomedullin-(22–52) in the presence of adrenomedullin (10^{-8} mol/l) or CGRP (10^{-9} mol/l). Means ± S.D.; $n = 3–4$. $*$ P 0.01 vs. control.

finding reported by Ikenouchi et al. and ours is unknown; the discrepancy might be due to the difference in the experimental preparation, species, or maturity of cardiac myocytes (neonatal vs. adult).

The present results showed that nonmyocytes as well as myocytes secrete adrenomedullin and that adrenomedullin stimulates the cAMP production in the nonmyocytes more so than in myocytes. These results suggest that adrenomedullin may modulate nonmyocytes function via a cAMP pathway in an autocrine and/or paracrine manner. Although myocytes occupy about 75% of the structural space of the heart, they constitute only one-third of the total cell population. The nonmyocytes consist mainly of cardiac fibroblasts in the interstitium. An immunocytochemical study revealed that cardiac nonmyocytes are cardiac fibroblasts (Fujisaki et al., 1995). It has been reported that cardiac fibroblasts play an important role through their proliferation with collagen synthesis in the pathophysiology of failing heart or cardiac hypertrophy (Weber and Brilla, 1991; Namba et al., 1997). Therefore, the effect of adrenomedullin on nonmyocytes proliferation is important. It has been reported that adrenomedullin suppresses mesangial cell mitogenesis via a cAMP pathway by reducing the mitogen-activated protein kinase (Chini et al., 1995). Adrenomedullin also attenuated the proliferation of vascular smooth muscle cells stimulated by fetal calf serum or platelet-derived growth factor (Kano et al., 1996). These results indicate that adrenomedullin is an endogenous suppressor of proliferation in these cells. In contrast, adrenomedullin is expressed in a large variety of human tumor cells, and it can function as an autocrine growth factor via a cAMP-dependent mechanism (Martinez et al., 1995; Miller et al., 1996). Adrenomedullin caused a rapid and transient induction of c-fos mRNA expression in rat vascular smooth muscle cells, cardiac myocytes and fibroblasts (Sato and Autelitano, 1995). Thus, the effect of adrenomedullin on cell proliferation or cell growth seems to be dependent on cell type. The question of whether adrenomedullin acts as an autocrine and/or a paracrine growth factor or suppressor on myocytes or nonmyocytes needs further study.

In conclusion, the present study showed that adrenomedullin is secreted by both cardiac myocytes and nonmyocytes. The adrenomedullin produced by myocytes and nonmyocytes may modulate the pathophysiology of failing heart or cardiac hypertrophy in an autocrine and/or paracrine fashion, by increasing the cAMP levels possibly via different receptors.

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References

Chini, E.N., Choi, E., Grande, J.P., Burnett, J.C., Dousa, T.P., 1995. Adrenomedullin suppresses mitogenesis in rat mesangial cells via cAMP pathway. Biochem. Biophys. Res. Commun. 215, 868–873.

Eguchi, S., Hirata, Y., Kano, H., Sato, K., Watanabe, Y., Watanabe, X., Nakajima, K., Sakakibara, S., Marumo, F., 1994a. Specific receptors for adrenomedullin in cultured rat vascular smooth muscle cells. FEBS Lett. 340, 226–230.

Eguchi, S., Hirata, Y., Iwasaki, H., Sato, K., Watanabe, T.X., Inui, T., Nakajima, K., Sakakibara, S., Marumo, F., 1994b. Structure–activity relationship of adrenomedullin, a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. Endocrinology 135, 2454–2458.

Fujisaki, H., Ito, H., Hirata, Y., Tanaka, M., Hata, M., Lin, M., Adachi, S., Akimoto, H., Marumo, F., Hiroe, M., 1995. Natriuretic peptides inhibit angiotensin II-induced proliferation of rat cardiac fibroblasts by blocking endothelium-1 gene expression. J. Clin. Invest. 96, 1059–1065.

Ichiki, Y., Kitamura, K., Kangawa, K., Kawamoto, M., Matsuo, H., Eto, T., 1994. Distribution and characterization of immunoreactive adrenomedullin in human tissue and plasma. FEBS Lett. 338, 6–10.

Ikeda, U., Kanbe, T., Kawahara, Y., Yokoyama, M., Shimada, K., 1996. Adrenomedullin augments inducible nitric oxide synthase expression in cytokine-stimulated cardiac myocytes. Circulation 94, 2560–2565.

Ikenouchi, Y., Kangawa, K., Matsuo, H., Hirata, Y., 1997. Negative inotropic effect of adrenomedullin in isolated adult rabbit cardiac ventricular myocytes. Circulation 95, 2318–2324.

Iwaki, K., Sukhatme, V.P., Shubeita, H.E., Chien, K.R., 1990. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *fos / jun* expression is associated with sarcomere assembly; *Egr*-1 induction is primarily an alpha 1-mediated response. J. Biol. Chem. 265, 13809–13817.

Jougasaki, M., Wei, C., McKinley, L.J., Burnett, J.C., 1995. Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. Circulation 92, 286–289.

Kano, H., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ikeda, M., Minami, M., Hanehira, T., Takeda, T., Yoshikawa, J., 1996. Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. J. Hypertens. 14, 209–213.

Kato, J., Kitamura, K., Kangawa, K., Eto, T., 1995. Receptors for adrenomedullin in human vascular endothelial cells. Eur. J. Pharmacol. 289, 383–385.

Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., Eto, T., 1993a. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem. Biophys. Res. Commun. 192, 553–560.

Kitamura, K., Sakata, J., Kangawa, K., Kojima, M., Matsuo, H., Eto, T., 1993b. Cloning and characterization of cDNA encoding a precursor for human adrenomedullin. Biochem. Biophys. Res. Commun. 194, 720–725.

Martinez, A., Miller, M.J., Unsworth, E.J., Siegfried, J.M., Cuttitta, F., 1995. Expression of adrenomedullin in normal human lung and in pulmonary tumors. Endocrinology 136, 4099–4105.

Miller, M.J., Martinez, A., Unsworth, E.J., Thiele, C.J., Moody, T.W., Elsasser, T., Cuttitta, F., 1996. Adrenomedullin expression in human tumor cell lines. Its potential role as an autocrine growth factor. J. Biol. Chem. 271, 23345–23351.

Namba, T., Tsutsui, H., Tagawa, H., Takahashi, M., Saito, K., Kozai, T., Usui, M., Imanaka-Yoshida, K., Imaizumi, T., Takeshita, A., 1997. Regulation of fibrillar collagen gene expression and protein accumulation in volume-overloaded cardiac hypertrophy. Circulation 95, 2448–2454.

Nishikimi, T., Saito, Y., Kitamura, K., Ishimitsu, T., Eto, T., Kangawa, K., Matsuo, H., Omae, T., Matsuoka, H., 1995. Increased plasma levels of adrenomedullin in patients with heart failure. J. Am. Coll. Cardiol. 26, 1424–1431.

Nishikimi, T., Horio, T., Sasaki, T., Yoshihara, F., Takishita, S., Miyata, A., Matsuo, H., Kangawa, K., 1997. Cardiac production and secretion of adrenomedullin are increased in heart failure. Hypertension 30, 1369–1375.

Osajima, A., Mutoh, Y., Uezono, Y., Kawamura, M., Izumi, F., Takasugi, M., Kuroiwa, A., 1995. Adrenomedullin increases cyclic AMP more potently than CGRP and amylene in rat renal tubular basolateral membranes. Life Sci. 57, 457–462.

Osajima, A., Uezono, Y., Tamura, M., Kitamura, K., Mutoh, Y., Ueta, Y., Kangawa, K., Kawamura, M., Eto, T., Yamashita, H., Izumi, F., Takasugi, M., Kuroiwa, A., 1996. Adrenomedullin-sensitive receptors are preferentially expressed in cultured rat mesangial cells. Eur. J. Pharmacol. 315, 319–325.

Owji, A.A., Smith, D.M., Coppock, H.A., Morgan, D.G.A., Bhogal, R., Ghatei, M.A., Bloom, S.R., 1995. An abundant and specific binding site for the novel vasodilator adrenomedullin in the rat. Endocrinology 136, 2127–2134.

Sakata, J., Shimokubo, T., Kitamura, K., Nakamura, S., Kangawa, K., Matsuo, H., Eto, T., 1993. Molecular cloning and biological activities of rat adrenomedullin, a hypotensive peptide. Biochem. Biophys. Res. Commun. 195, 921–927.

Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Ichiki, Y., Kangawa, K., Matsuo, H., Eto, T., 1994. Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. FEBS Lett. 352, 105–108.

Sato, A., Autelitano, D.J., 1995. Adrenomedullin induces expression of c-*fos* and AP-1 activity in rat vascular smooth muscle cells and cardiac myocytes. Biochem. Biophys. Res. Commun. 217, 211–216.

Sato, A., Canny, B.J., Autelitano, D.J., 1997. Adrenomedullin stimulates cAMP accumulation and inhibits atrial natriuretic peptide gene expression in cardiac myocytes. Biochem. Biophys. Res. Commun. 230, 311–314.

Sumimoto, T., Nishikimi, T., Mukai, K., Matsuzaki, K., Murakami, E., Takishita, S., Miyata, A., Kangawa, K., Matsuo, H., 1997. Plasma adrenomedullin concentrations in patients with essential hypertension. Hypertension 30, 741–745.

Weber, K., Brilla, C.G., 1991. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin–angiotensin–aldosterone system. Circulation 83, 1849–1865.

Zimmermann, U., Fischer, J.A., Frei, K., Fischer, A.H., Reinscheid, R.K., Muff, R., 1996. Identification of adrenomedullin receptors in cultured rat astrocytes and in neuroblastoma × glioma hybrid cells (NG108-15). Brain Res. 724, 238–245.