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Cardiovascular Research 43 (1999) 958–967

Cardiovascular Research

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An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth

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Received 7 May 1998; accepted 2 March 1999

Abstract

Objective: The aim of the present study was to determine the role of adrenomedullin (AM) in cardiac fibroblasts. **Methods:** The production and secretion of AM were examined in cultured neonatal rat cardiac fibroblasts, and the effects of AM on proliferation and protein synthesis of these cells were assessed by [^{3}H]thymidine and [^{3}H]phenylalanine incorporation, respectively. **Results:** Cultured cardiac fibroblasts secreted AM into the medium time-dependently at a rate of 20.3±3.0 fmol/5×10^4 cells/48 h, mean±S.D. Northern blot analysis showed expression of preproAM mRNA of 1.6 kb in these cells. In addition, 10^{-6} mol/l of angiotensin II (Ang II) and endothelin-1 (ET-1) significantly increased the AM secretion by 55 and 48%, respectively. Synthetic AM significantly reduced 10^{-6} mol/l Ang II- or 10^{-7} mol/l ET-1-stimulated [^{3}H]thymidine and [^{3}H]phenylalanine incorporation in a dose-dependent manner, and these effects were attenuated by a calcitonin gene-related peptide (CGRP) type 1 receptor antagonist, CGRP(8-37). Synthetic AM also had a dose-dependent stimulatory effect on cAMP accumulation in these cells, which was significantly attenuated by CGRP(8-37). A cAMP analogue, 8-bromo-cAMP, mimicked the AM effects, inhibiting the Ang II-stimulated [^{3}H]thymidine and [^{3}H]phenylalanine incorporation. Blockage of the effect of endogenous AM by anti-AM monoclonal antibody not only significantly reduced the basal level of intracellular cAMP, but also enhanced the [^{3}H]thymidine and [^{3}H]phenylalanine incorporation into the cells. **Conclusions:** Cultured neonatal rat cardiac fibroblasts produce and secrete AM, and the secreted AM may inhibit proliferation and protein synthesis of these cells. AM may exert these inhibitory effects partly by elevating intracellular cAMP. It is suggested that AM has an important role in modulating the growth of cardiac fibroblasts in an autocrine or a paracrine manner.

Keywords: Adrenomedullin; Fibrosis; Interstitial space; Remodeling; Hormones

1. Introduction

Cardiac fibroblasts constituting the interstitium of the myocardium have an important role in the processes of cardiac hypertrophy and remodeling associated with hypertension and ischemic heart disease [1–4]. The collagen network in the extracellular matrix (ECM) created by the cardiac fibroblasts serves to build up a skeletal support against the intracardiac pressure, but an excessive fibroblast proliferation and increased deposition of ECM proteins, which are synthesized and secreted mainly by cardiac fibroblasts, may result in increased myocardial stiffness, reducing diastolic compliance. It is therefore important to clarify the factors regulating proliferation and ECM protein synthesis of the cardiac fibroblasts. To date, it is known that both a mechanical load and a number of humoral factors, such as angiotensin II (Ang II) and endothelin-1 (ET-1), stimulate the proliferation of cardiac fibroblasts [5–8], while natriuretic peptides and adenosine inhibit the growth of these cells [9,10].

Adrenomedullin (AM) is a bioactive peptide that was discovered in a human pheochromocytoma [11]. Having a slight structural homology with calcitonin gene-related

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Time for primary review 24 days.

0008-6363/99/$ – see front matter
PII: S0008-6363(99)00122-4

peptide (CGRP), AM exerts a potent vasodilator effect via an elevation of the intracellular cyclic AMP (cAMP) level [11]. A specific radioimmunoassay (RIA) revealed that AM circulates in the blood and is present in the adrenal medulla, lung, kidney, cardiac atrium and ventricle of humans and rats [12,13]. Plasma AM concentrations in patients with essential hypertension and acute myocardial infarction were reported to be higher than in controls [14,15]. We found that both AM content and AM mRNA expression are increased in the hypertrophied cardiac ventricle of Dahl salt-sensitive and renovascular hypertensive rats compared to those of the respective controls [16,17]. Cardiac fibroblast proliferation with collagen deposition has been shown to be increased in the interstitium of the heart of these hypertensive rats [2,3]. AM has a broad spectrum of biological actions in addition to the vasodilator effect: diuresis, suppression of aldosterone secretion and inhibition of proliferation of vascular smooth muscle cells [18–20]. It has been shown that such factors as natriuretic peptides and adenosine, which inhibit smooth muscle cell proliferation, have similar inhibitory effects on the cardiac fibroblast growth [8–10,21,22]. Recently, we showed that AM is produced in cultured neonatal rat cardiac myocytes, and that AM attenuates de novo protein synthesis of these cells [23]. Based upon these findings, we hypothesized that AM may be an important factor regulating proliferation and protein synthesis of cardiac fibroblasts.

The purpose of the present study was to clarify the role of AM in the cardiac fibroblasts constituting the interstitium of the heart. In the first part of the study, we looked at the synthesis and secretion of AM from cultured neonatal rat cardiac fibroblasts. In the second part, we examined whether synthetic AM inhibits proliferation and protein synthesis of the fibroblasts, evaluating the role of cAMP as an intracellular second messenger. Finally, the role of endogenous AM secreted from the cells was examined using an anti-AM monoclonal antibody.

2. Methods

All experiments were performed according to the regulations of the Animal Research Committee of Miyazaki Medical College (1998-037). This investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Reagents

Ang II, ET-1, rat AM, human AM(22-52) and human CGRP(8-37) were purchased from Peptide Institute, Inc. (Osaka, Japan), and methyl-[^{3}H]thymidine, L-[ring-2,3,4,5,6-^{3}H]phenylalanine, [α-^{32}P]deoxycytidine 5′-triphosphate (dCTP), [^{125}I]cyclic AMP from Du Pont–New England Nuclear Co. (Wilmington, DE, USA). Holo-transferrin (human), collagenase type IV, trypsin, insulin (bovine pancreas) and 8-bromo-cAMP were from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Cultured cardiac fibroblasts were isolated from 7 to 14 Wistar rats at 1–3 days of age. After digestion of minced ventricles with 0.12% trypsin and 0.03% collagenase, the cells were collected and plated for 30 min at 37°C to allow fibroblasts to attach to the 10-cm cell culture plates in 10 ml of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). After decantation of the medium, the cardiac fibroblasts were cultured in 10 ml of DMEM with 10% FBS at 37°C in a 95% air/5% CO_2 humidified atmosphere to allow them to reach confluence. Cells were then split into 24-well culture plates at a density of 2.5×10^{4} cells/cm^{2} and grown to confluence in 1.0 ml/well of DMEM supplemented with 10% FBS. To achieve quiescence, the cells were then incubated with serum-free medium containing 10 μg/ml insulin and 10 μg/ml holo-transferrin for 48 h. After the incubation, the cells were treated with the agents or peptides described in fresh serum-free medium. Immunocytochemical examinations showed that all cultured cells exhibited positive staining for vimentin, but negative staining for von Willebrand factor (factor VIII) and α-smooth muscle actin, indicating little contamination of vascular endothelial cells or smooth muscle cells in our cardiac fibroblast culture.

2.3. Measurement of AM in conditioned medium

One millilitre of the conditioned medium of the cardiac fibroblasts was collected and immediately acidified with acetic acid to a final concentration of 1.0 N. The acidified medium was heated at 100°C for 10 min to inactivate proteases, and applied to a Sep-Pak C18 cartridge (Millipore-Waters, Milford, MA, USA). After washing the cartridge with 10% CH_3CN in 0.1% trifluoroacetic acid, the absorbed materials were eluted with 50% CH_3CN in 0.1% trifluoroacetic acid, lyophilized, and stored at −30°C. Recovery of AM by this extraction procedure was 82%, a rate that was apparently constant. AM in the medium extract was measured using a specific RIA for AM as described previously [12]. The antibody used in this RIA recognizes the C-terminal portion of AM with the amide structure, and has no cross-reactivity with CGRP or amylin [12].

2.4. Characterization of secreted AM

To examine the molecular forms of immunoreactive AM (ir-AM), the extracts of conditioned medium were analyzed by reverse phase high performance–liquid chroma-

tography (HPLC) with a column of TSK ODS 120A (Tosoh, Tokyo, Japan). The extracts were eluted from the column with a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid, and the ir-AM in each fraction was measured with the RIA. The recovery of ir-AM in this HPLC was greater than 80%.

2.5. Northern blot analysis

Thirty micrograms of the total RNA extracted from the cultured fibroblasts by the acid guanidinium thiocyanate–phenol–chloroform method was denatured by glyoxal and dimethylsulfoxide. The denatured RNA was electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane (Zeta probe, Bio-Rad). The membrane was hybridized with ^{32}P-labeled rat AM cDNA probe, an *Acc*II–*Nae*I cDNA fragment of rat preproAM of 381 bp [24], at 42°C in 50% formamide, 5× SSPE, 1× Denhardt's, 0.1% SDS and 0.2 mg/ml salmon sperm DNA [24]. Then the hybridized membrane was washed with 0.1× SSC at 50°C and scanned using a Fuji BAS 2000 Bio-imaging analyzer (Fuji Photo Film Co., Tokyo, Japan).

2.6. Measurement of DNA synthesis and protein synthesis

The rates of DNA synthesis and protein synthesis were assayed by measuring [^{3}H]thymidine and [^{3}H]phenylalanine incorporation into the acid-insoluble fraction of the cells, respectively. In brief, after incubation with serum-free medium for 48 h as described above, serum-starved fibroblasts were exposed to 10^{-6} mol/l Ang II or 10^{-7} mol/l ET-1 with or without AM for 24 h. The cells were pulsed with 1.0 μCi/ml of [^{3}H]thymidine or [^{3}H]phenylalanine over the last 4 h of this incubation period. Then the cells were washed twice with cold phosphate-buffered saline (PBS) and incubated with 10% trichloroacetic acid at 4°C for 20 min. After cell residues were rinsed with 95% ethanol, the dried materials were solubilized in 0.5 N NaOH for 2 h. Radioactivity in the solubilized samples was determined using a liquid scintillation counter (LSC-5100, Aloka, Tokyo, Japan). The results are expressed as percentages relative to the mean cpm of control cells for each experiment.

2.7. Intracellular cAMP measurement

The cardiac fibroblasts cultured with serum-free medium for 48 h were washed twice with 1.0 ml/well of assay buffer (Eagle's medium containing 0.05% bovine serum albumin and 25 mmol/l *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesufonic acid], pH 7.2) prior to the experiment. The cells were preincubated with 0.5 ml of assay buffer containing 0.5 mmol/l 3-isobutyl-1-methylxanthine at 37°C. After preincubation for 10 min, peptides were added at the indicated concentrations and the incubation was further continued for 5 min. The reaction was terminated by aspirating the medium and adding 0.5 ml/well of 6% trichloroacetic acid. After scraping the incubated cells, the cell extracts were washed with water-saturated ethyl ether three times. The cell extracts were then lyophilized and stored at −30°C until assayed. cAMP concentrations were measured using a specific RIA as described previously [11].

2.8. Preparation of anti-AM monoclonal antibody

Synthetic human AM(46-52), a C-terminal fragment of AM, was conjugated to bovine thyroglobulin (Sigma) by the carbodiimide coupling procedure [12]. Five-week-old female BALB/c mice were immunized by subcutaneous injections of the conjugate containing 4.7 μg of the peptide emulsified in Freund's complete adjuvant eight times at intervals of 3 weeks. Fusion of spleen cells of the immunized mice with a mouse myeloma cell line, X63-Ag8.653, was performed at a ratio of 5:1 with 50% polyethylene glycol 4000 (Merck, Darmstadt, Germany) according to the method described by Galfrè et al. [25]. Culture media of the hybridomas were periodically screened for ability to bind ^{125}I-AM. Cells from the well giving the highest titer were cloned by limiting dilution and injected intraperitoneally in BALB/c mice. The monoclonal antibody could be used in an RIA for AM at a final dilution of 1:2 720 000 with K_a value of 7.4×10^{10} M^{-1}. The monoclonal antibody belonged to the immunoglobulin G1 subclass, as determined by the Ouchterlony technique, and cross-reacted equally with rat AM(1-50), but had no cross-reactivity with CGRP or amylin. Prior to use, the monoclonal antibody in ascites fluid was purified by protein A (Amersham), followed by dialysis with PBS at 4°C.

2.9. Statistical analysis

Student's *t*-test was used for comparison between the two variables. Multiple comparison was assessed with one-way ANOVA followed by Scheffè's test. All data were expressed as the means±S.D. of the samples examined, and *p* values less than 0.05 were considered significant. The experiments were repeated by using the cells isolated separately from different groups of neonatal rats. Identical results were obtained from the repeated experiments, and repetition numbers are indicated as *n* in the figure legends.

3. Results

3.1. Secretion and production of AM by cardiac fibroblasts

The cardiac fibroblasts cultured in serum-free medium secreted AM into the medium in a time-dependent manner,

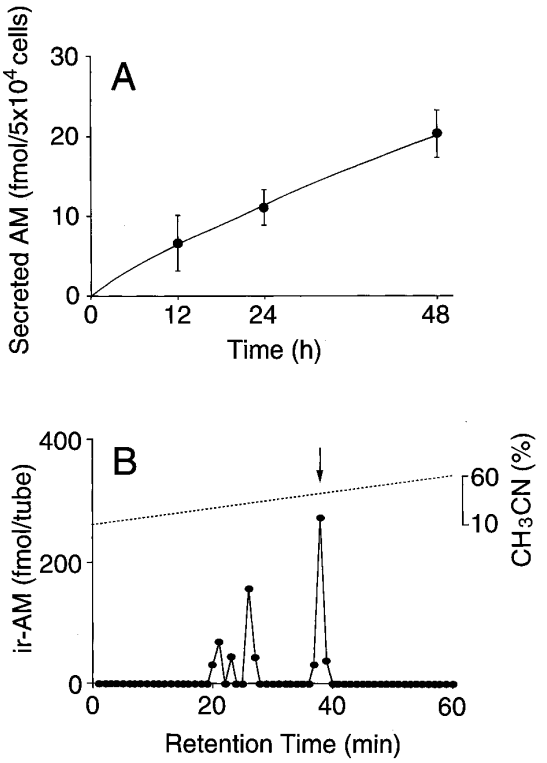

Fig. 1. (A) Time course of AM secretion into the medium from the cardiac fibroblasts. The cells were incubated for the indicated time periods in serum-free medium, and the secreted AM was determined by RIA as described in Methods. Each value represents the mean±S.D. of 12 samples from two separate isolations of the cells (n=2). (B) Reverse phase high performance–liquid chromatography (HPLC) analysis of immunoreactive AM (ir-AM) secreted into the medium. The arrow indicates the elution position of authentic rat AM(1-50)-NH_2.

at a rate of 20.3±3.0 fmol/5×10^4 cells/48 h (Fig. 1A). In addition, 10^{-6} mol/l Ang II and 10^{-6} mol/l ET-1 significantly ($p<0.01$) increased the AM secretion, by 55 and 48%, respectively (Table 1). In analysis by reverse phase HPLC, immunoreactive AM (ir-AM) secreted from the cells was composed of one major peak and several minor peaks, and the major peak appeared at an elution position identical to that of authentic rat AM(1-50)-NH_2, a full-length rat AM (Fig. 1B). Northern blot analysis of 30 μg of total RNA isolated from the cultured cardiac fibroblasts showed a single band at 1.6 kb (Fig. 2), a size identical to that of mRNA for preproAM of other rat tissues [24].

Table 1
AM secretion from cultured cardiac fibroblasts[a]

Treatment	AM (fmol/5×10^4 cells/24 h)
Serum free	11.9±1.60 (18)
10^{-6} mol/l Ang II	18.4±4.38[b] (16)
10^{-6} mol/l ET-1	17.6±5.43[b] (18)

[a] Serum-starved cardiac fibroblasts were treated with 10^{-6} mol/l Ang II or 10^{-6} mol/l ET-1 for 24 h and AM concentrations in the conditioned media were measured as described in Methods. Values are the means±S.D. of samples examined (n=3), and the sample numbers are indicated in parentheses.

[b] $p<0.01$, compared to the cells cultured with serum-free medium.

3.2. Effects of synthetic AM on DNA and protein synthesis

We determined whether synthetic AM affects proliferation of neonatal rat cardiac fibroblasts. As shown in Fig. 3, basal, 10^{-6} mol/l Ang II- and 10^{-7} mol/l ET-1-stimulated [^{3}H]thymidine incorporations were significantly ($p<0.01$) reduced in the presence of 10^{-6} mol/l of synthetic AM, by 60, 38 and 29%, respectively. This AM attenuation of [^{3}H]thymidine incorporation stimulated by Ang II or ET-1 occurred in a concentration-dependent manner (Fig. 4A and B). Next, we examined the effects of two peptide analogues on AM action: one was CGRP(8-37), which is a CGRP type 1 receptor antagonist [26], and

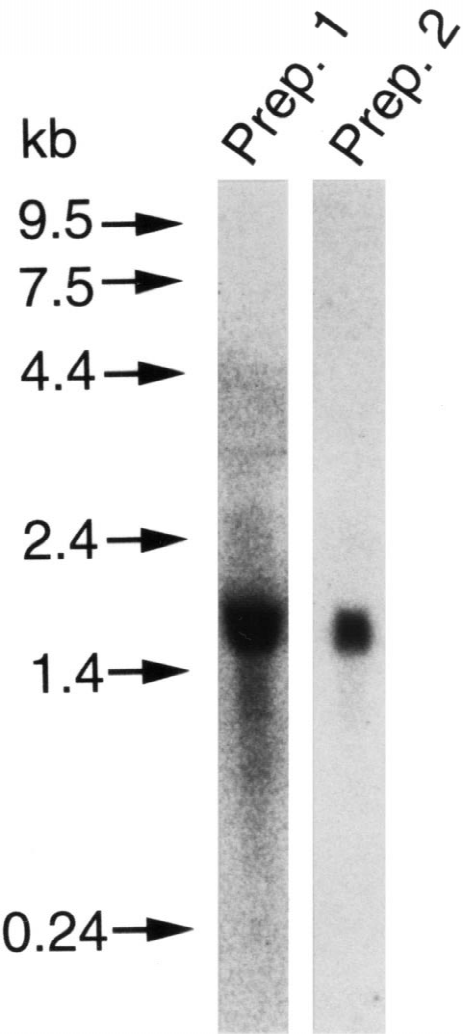

Fig. 2. Expression of AM precursor mRNA in cultured cardiac fibroblasts. Thirty micrograms of total RNA extracted from the cells was analyzed by the Northern blotting as described in Methods. Total RNA of preps. 1 and 2 was separately isolated from different groups of rats.

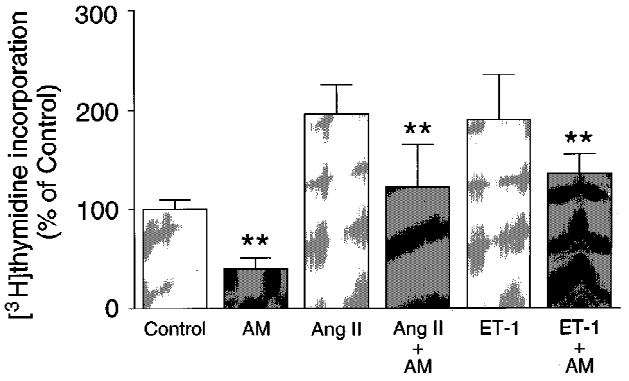

Fig. 3. Inhibitory effect of AM on basal and Ang II- or ET-1-stimulated [^{3}H]thymidine incorporation into the cells. Each values are the means±S.D. of 18 samples (n=3), and radioactivity in control wells was 4317±574 cpm. ** p<0.01, compared to respective controls.

the other was human AM(22-52), an N-terminal-deleted form of AM. As shown in Fig. 5, CGRP(8-37) attenuated the anti-proliferative effect of AM, whereas AM(22-52) had no effect on the AM action. In addition, we examined the effect of AM on protein synthesis in the cardiac fibrobalsts. Synthetic AM significantly (p<0.01) reduced

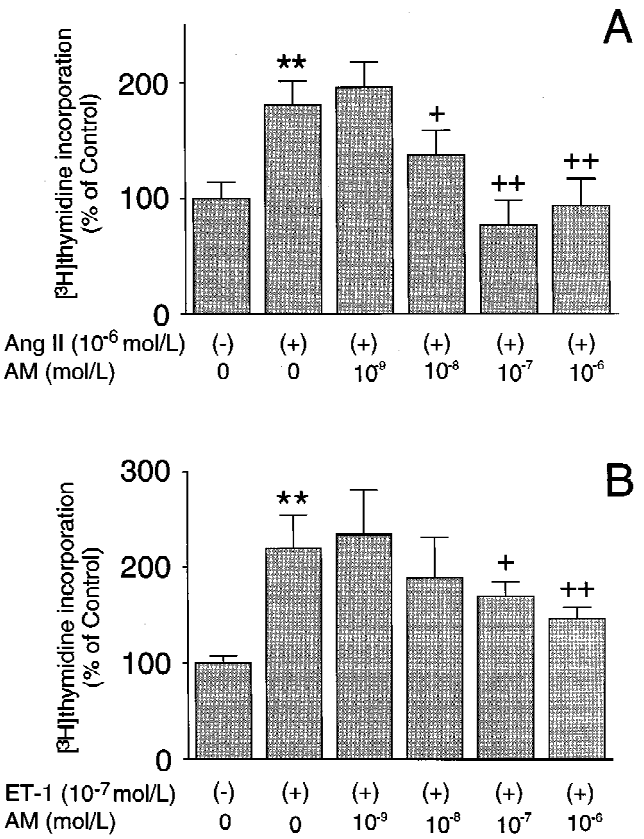

Fig. 4. Dose-dependent inhibition of Ang II- or ET-1-induced proliferation of cardiac fibroblasts by AM. Values are the means±S.D. of 18 samples examined (n=3), and radioactivities in untreated controls for A and B were 3337±607 and 4769±441 cpm, respectively. ** p<0.01, compared to untreated controls; $^{+}$ p<0.05, $^{++}$ p<0.01, compared to the cells incubated with Ang II or ET-1 without AM.

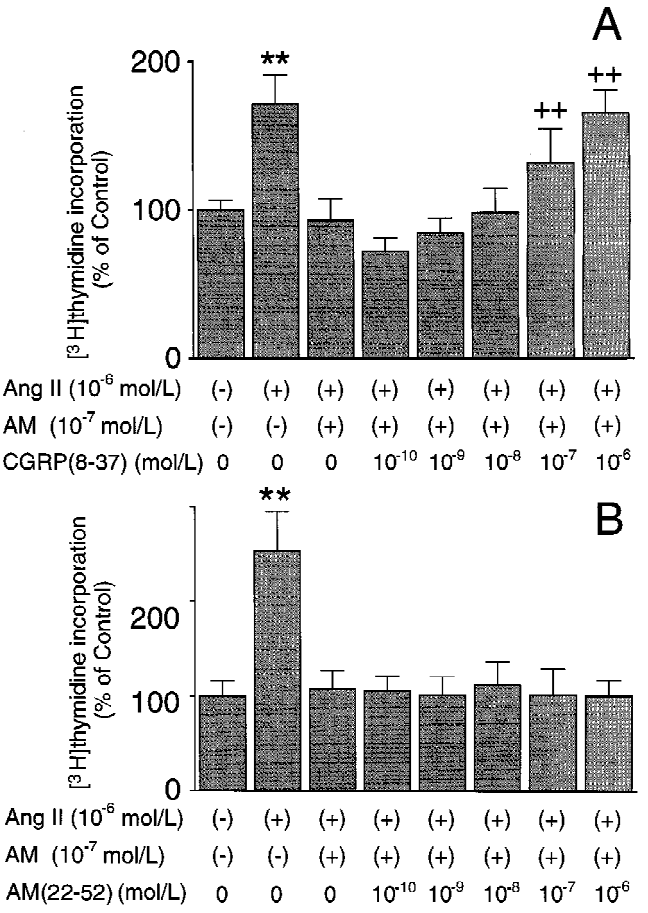

Fig. 5. Effects of CGRP(8-37) (A) and AM(22-52) (B) on the anti-proliferative action of AM in Ang II-treated cardiac fibroblasts. Values are the means±S.D. of 12 samples examined (n=2), and radioactivities in untreated controls for A and B were 5144±538 and 4466±613 cpm, respectively. ** p<0.01, compared to untreated controls; $^{++}$ p<0.01, compared to the cells incubated with Ang II and AM without CGRP(8-37).

the basal level of [^{3}H]phenylalanine incorporation, by 31% (Fig. 6A). Like [^{3}H]thymidine incorporation, 10^{-6} mol/l Ang II-stimulated [^{3}H]phenylalanine incorporation was reduced dose-dependently by synthetic AM (Fig. 6A), and this effect was partially abolished by CGRP(8-37) (Fig. 6B).

3.3. Intracellular cAMP accumulation in cardiac fibroblasts treated with AM

Synthetic AM significantly increased intracellular cAMP in a concentration-dependent manner, whereas neither CGRP(8-37) nor AM(22-52) alone had an effect on the cAMP level (Fig. 7A). As shown in Fig. 7B, CGRP(8-37) inhibited the cAMP accumulation induced by 10^{-6} mol/l AM in a concentration-dependent manner, but AM(22-52) had little effect on the AM-induced cAMP formation. We also tried to measure cAMP concentrations in the medium,

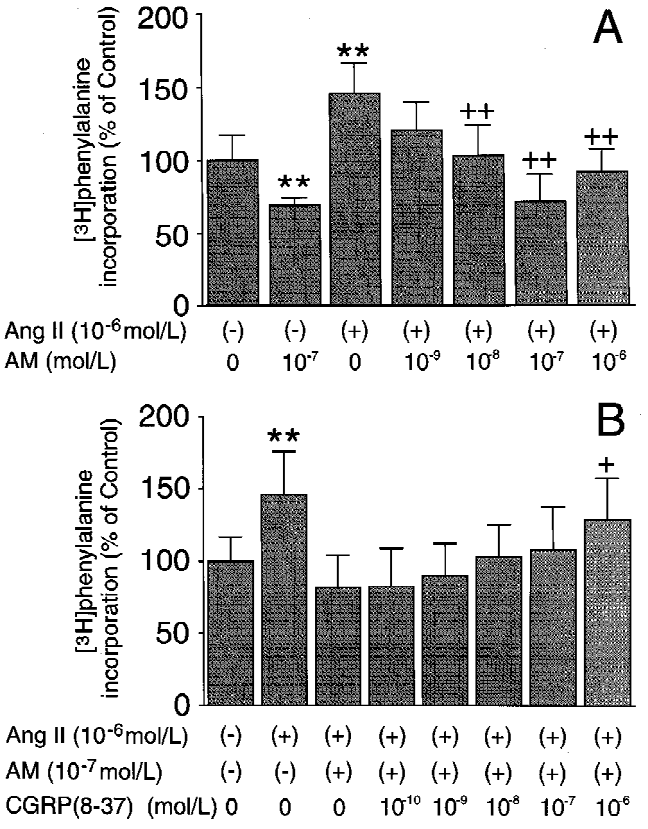

Fig. 6. Inhibitory effect of AM on basal and Ang II-stimulated [^{3}H]phenylalanine incorporation (A) and partial attenuation of the AM effect by CGRP(8-37) (B). Values are the means±S.D. of 10–14 samples examined (n=2), and radioactivities in untreated controls for A and B were 1503±320 and 1672±307 cpm, respectively. ** $p<0.01$, compared to untreated controls; $^{+}p<0.05$, $^{++}p<0.01$, compared to the cells incubated with Ang II without AM (A) or those incubated with Ang II and AM without CGRP(8-37) (B).

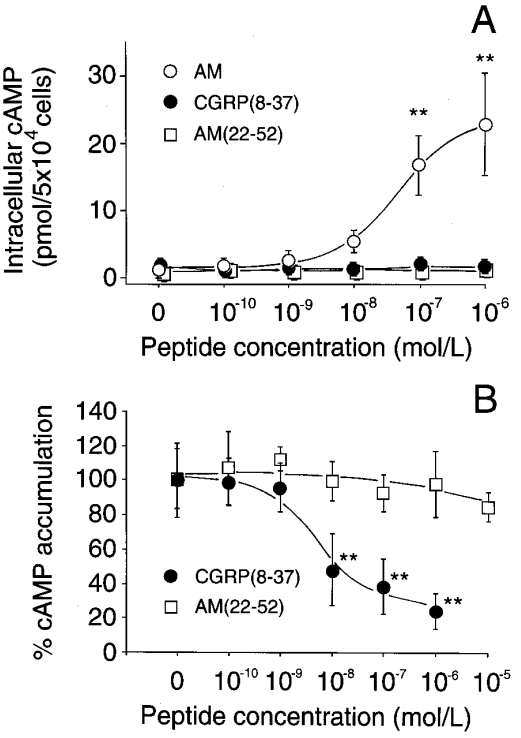

Fig. 7. Stimulatory effect of AM on intracellular cAMP accumulation (A) and effect of CGRP(8-37) or AM(22-52) on AM-stimulated cAMP formation (B) in cardiac fibroblasts. Values are the means±S.D. of 10–12 samples examined (n=3), and the cAMP response to 10^{-6} mol/l AM without CGRP(8-37) or AM (22-52) was 29.5±5.0 pmol/5×10^4 cells in the lower figure (B). ** $p<0.01$, compared to control cells.

but they were too low to be detected even in the cells treated with 10^{-6} mol/l AM.

3.4. Effect of 8-bromo-cAMP on Ang II-stimulated DNA and protein synthesis

To determine whether the inhibitory effects of AM on DNA and protein synthesis are mediated by the increased intracellular cAMP, we examined the effect of 8-bromo-cAMP, a cAMP analogue, on the Ang II-induced increase in [^{3}H]thymidine or [^{3}H]phenylalanine incorporation. As shown in Fig. 8, this cAMP analogue dose-dependently reduced the Ang II-stimulated [^{3}H]thymidine (A) and [^{3}H]phenylalanine (B) incorporation, but its effect on protein synthesis was smaller than that on DNA synthesis.

3.5. Action of endogenous AM

To evaluate the effect of endogenous AM secreted from the cardiac fibroblasts, the cells were incubated with a purified anti-AM monoclonal antibody that specifically binds to the C-terminal structure of AM, a portion important for the biological activity [27]. When incubated for 1 h, 10 μg/ml anti-AM monoclonal antibody significantly ($p<0.01$) reduced the basal levels of intracellular cAMP of the cardiac fibroblasts, by 20% (Fig. 9A). Moreover, incubation with 10 μg/ml of the purified anti-AM monoclonal antibody significantly ($p<0.01$) elevated the basal levels of [^{3}H]thymidine and [^{3}H]phenylalanine incorporations, by 15 and 25%, respectively, compared to those of controls incubated with 10 μg/ml of mouse IgG (B and C).

4. Discussion

AM is present at considerable concentrations in several organs and tissues, such as normal adrenal medulla, lungs, kidneys and cardiac ventricle, where preproAM mRNA was found to be expressed [24,28]. We recently reported that AM is produced and secreted from cultured cardiomyocytes of neonatal rats [23]. In the present study, we found that, similarly to the cardiomyocytes, cultured cardiac fibroblasts synthesized and secreted AM into the medium, and the amount of AM secretion was increased by Ang II and ET-1. Reverse phase HPLC revealed that a

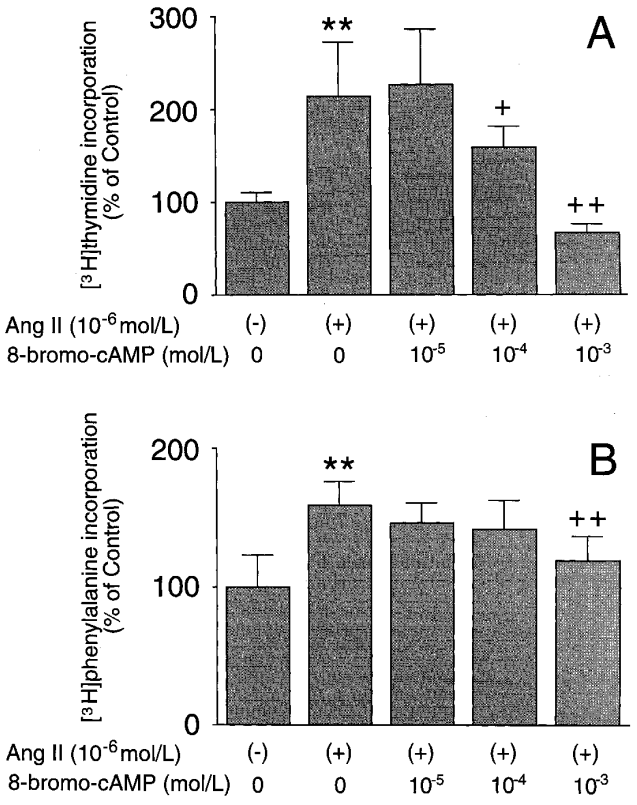

Fig. 8. Inhibitory effects of 8-bromo-cAMP on [^{3}H]thymidine and [^{3}H]phenylalanine incorporation. Serum-starved cardiac fibroblasts were incubated with 10^{-6} mol/l Ang II in the absence or presence of the indicated concentration of 8-bromo-cAMP for 24 h. Values are the means±S.D. of 12 samples examined (n=2), and radioactivities in untreated controls for A and B were 6943±1021 and 1768±306 cpm, respectively. ** $p<0.01$, compared to untreated control cells; $^{+}$ $p<0.05$, $^{++}$ $p<0.01$, compared to cells treated with Ang II without 8-bromo-cAMP.

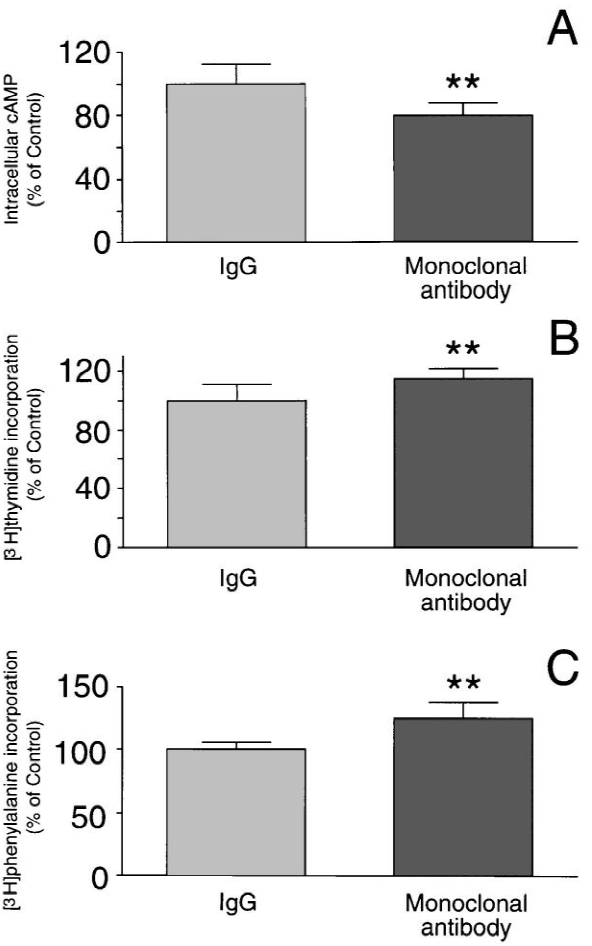

Fig. 9. Effects of anti-AM monoclonal antibody on basal level of intracellular cAMP (A) and [^{3}H]thymidine (B) and [^{3}H]phenylalanine (C) incorporation. (A) Serum-starved cardiac fibroblasts were incubated with either 10 μg/ml of purified anti-AM monoclonal antibody or 10 μg/ml of mouse IgG (ZYMED) in the presence of 0.5 mmol/l 3-isobutyl-1-methylxanthine for 60 min. Values are the means±S.D. of 12 samples examined (n=2), and the basal level of cAMP in the cells incubated with mouse IgG was 1.71±0.79 pmol/5×10^4 cells. (B,C) Serum-starved cardiac fibroblasts were incubated with either 10 μg/ml of purified anti-AM monoclonal antibody or mouse IgG for 24 h. Values are the means±S.D. of 12 samples examined (n=2), and radioactivities in the cells incubated with mouse IgG for B and C were 2131±279 and 1545±283 cpm, respectively. ** $p<0.01$, compared to cells treated with mouse IgG.

large part of AM secreted into the medium appeared at the elution position of authentic rat AM(1-50), a full-length rat AM [24]. The intracellular AM concentration was too low to be detected (data not shown) despite the substantial expression of AM mRNA. This finding is similar to that in cultured cardiomyocytes and appears to accord with the fact that the AM concentration in cardiac ventricles is much lower than in the adrenal medulla in spite of comparable AM mRNA expression in these two tissues [12,13,24,28]. Thus, cardiac fibroblasts seem to secrete AM rapidly after its synthesis, with little intracellular storage.

Both Ang II and ET-1 have been reported to be important factors promoting growth of cardiac fibroblasts as well as hypertrophy of cardiomyocytes [1,2,6–8]. Consistent with this, as shown Figs. 3 and 6, these peptides stimulated [^{3}H]thymidine and [^{3}H]phenylalanine incorporation into the cardiac fibroblasts. The present study showed that synthetic AM reduced not only the basal but also the Ang II- or ET-1-stimulated [^{3}H]thymidine incorporation, and similarly, AM suppressed the basal and Ang II-induced [^{3}H]phenylalanine incorporation. These effects of AM were significantly attenuated in the presence of CGRP(8-37), a CGRP type 1 receptor antagonist [26]. AM was purified and isolated while monitoring cAMP elevation in rat platelets [11]. In accordance with this, it has been reported that many of the AM actions are linked to the elevation of intracellular cAMP [20,27,29]. In this study, synthetic AM increased the intracellular cAMP dose-dependently, and the cAMP accumulation was inhibited by CGRP(8-37), whereas AM(22-52) had little

effect on the cAMP formation induced by AM. These findings are partially in accord with the effects of AM on the [^{3}H]thymidine and [^{3}H]phenylalanine incorporation, and with those of the two peptide analogues. In addition, 8-bromo cAMP, a cAMP analogue, dose-dependently reduced the Ang II-stimulated [^{3}H]thymidine and [^{3}H]phenylalanine incorporation, mimicking the AM effects. Taken together with the antiproliferative effect of cAMP on cardiac fibroblasts reported previously [10], the growth-inhibitory action of AM may partly be mediated by intracellular cAMP.

Using a variety of types of cultured cells, some research groups reported that AM inhibits cell proliferation [20,30], but others showed a stimulation by AM. For example, AM was shown to stimulate the proliferation of Swiss 3T3 fibroblasts [31,32]. It is known that cAMP exerts differential effects on cell mitogenesis, depending upon the cell type. Recently, Cass et al. reported that the activation of pp70 S6 kinase is an important step for cAMP-mediated control of cellular proliferation [33]. They showed that the activation occurred in response to cAMP in Swiss 3T3 fibroblasts, but cAMP failed to activate this kinase in other types of fibroblasts whose proliferation was not stimulated by cAMP. Thus, intracellular pathways responding to cAMP seem to differ from cell to cell. In the meantime, the previous reports inferred that there are intracellular signaling systems for AM other than cAMP, such as nitric oxide (NO)-cGMP or Ca^{2+}-calmodulin pathways [34–36]. In fact, it appears partially impossible to explain the AM effects observed in the present study only by elevation of the intracellular cAMP. For example, 10^{-8} mol/l CGRP(8-37) significantly suppressed the 10^{-6} mol/l AM-stimulated cAMP formation, while this dose of CGRP(8-37) failed to attenuate the inhibitory effects of 10^{-6} mol/l AM on [^{3}H]thymidine and [^{3}H]phenylalanine incorporation. Additionally, compared to the level of endogenous cAMP stimulated by AM, much higher concentrations of 8-bromo-cAMP were needed to significantly suppress the incorporations.

For further understanding the effects of AM on cardiac fibroblasts, it will be important to determine the receptor on which AM acts, but the receptor subtype of AM or CGRP is a very controversial subject [37–42]. In the present study, the AM actions were attenuated by CGRP(8-37), but not by AM(22-52), which has been shown to inhibit the AM action in cultured smooth muscle cells [27]. Recently, McLatchie et al. discovered receptor-activity-modifying proteins (RAMPs) which regulate the ligand specificity of the calcitonin-receptor-like receptor for AM or CGRP [43]. These differences may partially be explained by subtypes of RAMPs expressed; however, further experiments are needed to clarify the receptor-mediated signaling systems through which AM exerts the growth-inhibitory effect on cardiac fibroblasts.

It should be noted that the concentration of synthetic AM which significantly inhibited the cellular growth and stimulated the cAMP production in this study was much higher than that found in the conditioned media. Therefore, in the last part of the experiments, we examined the action of endogenous AM secreted from the cardiac fibroblasts by using purified anti-AM monoclonal antibody. When the action of the endogenous AM secreted from the cardiac fibroblasts was neutralized by the anti-AM monoclonal antibody, the basal level of intracellular cAMP was significantly lowered, and both the [^{3}H]thymidine and [^{3}H]phenylalanine incorporations were increased compared to the controls. These results suggest a possible role of the endogenous AM secreted from the cells in modulating proliferation, protein synthesis and the intracellular cAMP level of the cardiac fibroblasts.

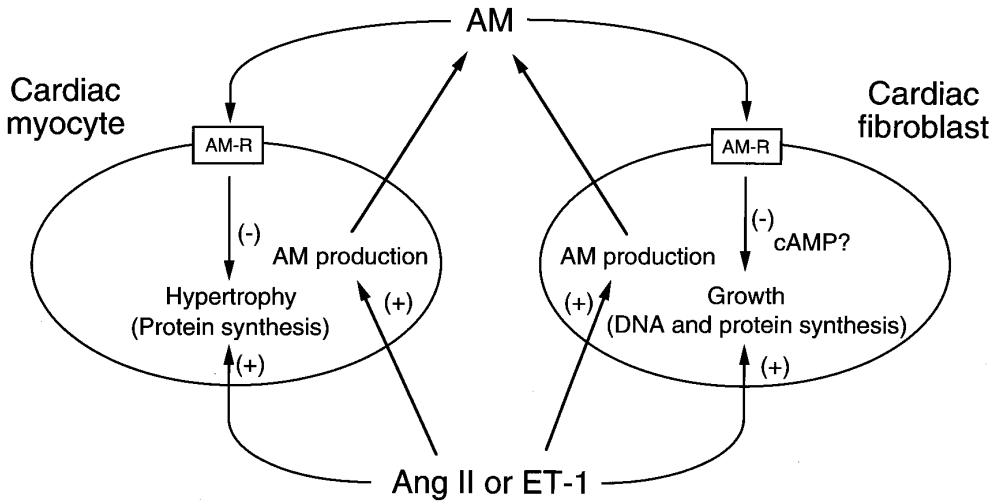

Fig. 10. Possible role of AM as an autocrine or a paracrine factor in cardiac myocytes and fibroblasts. (+) stimulation; (−) inhibition; AM-R, AM receptor.

Fig. 10 is a schematic representation of our hypothesis for the role of AM in the myocardium. As reported previously and shown in this study, AM is produced and secreted from both cultured cardiac myocytes and fibroblasts, and the secretion is augmented by either Ang II or ET-1. The secreted AM may act on the myocytes to inhibit hypertrophy and on the fibroblasts to inhibit growth. We previously reported that plasma AM concentrations in patients with hypertension, myocardial infarction or heart failure secondary to ischemic heart disease are increased compared to controls, suggesting a role of plasma AM in acting against a further worsening of the diseases through its potent vasodilator and diuretic action [14,15,44]. Taken together with the present results, AM may function not only systemically as a circulating hormone but also locally in the cardiac ventricle as an autocrine or a paracrine factor which modulates growth of cardiac fibroblasts as well as hypertrophy of cardiomyocytes.

In summary, AM is synthesized and secreted from cultured neonatal rat cardiac fibroblasts, and the secreted AM may inhibit proliferation and protein synthesis of the cardiac fibroblasts. Thus, AM may modulate growth of cardiac fibroblasts in an autocrine or a paracrine manner.

Acknowledgements

We are grateful to Drs Kosuke Marutsuka and Yujiro Asada, First Department of Pathology, Miyazaki Medical College, for their technical advice on histochemical staining of the cultured cells. This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education in Japan.

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