Autocrine/paracrine role of adrenomedullin in cultured endothelial and mesangial cells

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Autocrine/paracrine role of adrenomedullin in cultured endothelial and mesangial cells. Adrenomedullin (AM), a potent vasorelaxant and natriuretic peptide isolated from human pheochromocytoma, is present in the kidney and secreted from endothelial cells (EC) and vascular smooth muscle cells (VSMC), but the functional role of AM is still unclear. To clarify the significance of AM as a local regulator, we investigated its secretion and action in cultured cells, and examined the effects of neutralization using a specific monoclonal antibody against AM. The prepared antibody directed against the ring structure showed a high affinity for human and rat AM. Using radioimmunoassay with this antibody, we found significant secretion from cultured rat mesangial cells (MC) of a 6-kDa mature form of AM as seen from EC and VSMC. The addition of AM into cultured cells dose-dependently increased cAMP production and potently inhibited PDGF-stimulated thymidine incorporation. Pretreatment with the monoclonal antibody completely abolished cAMP increase induced by exogenous AM. Moreover, antibody neutralization of endogenously secreted AM in cultured EC, but not in MC or VSMC, markedly (by ~70%) reduced basal cAMP production and significantly (1.7-fold) enhanced DNA synthesis. These results indicate that AM, acting as an autocrine/paracrine regulator, exerts an antiproliferative action on EC and MC, and suggest its role as a local modulator of endothelial and mesangial function.

Adrenomedullin, originally isolated from human pheochromocytoma, is a 52-amino acid peptide with an intramolecular disulfide bond and an amidated C-terminus, and shows structural homology with the potent vasodilator peptide, calcitonin gene-related peptide (CGRP) [1]. Adrenomedullin (AM) elicits a potent, long-lasting hypotensive effect comparable to CGRP [1], mainly by stimulating cAMP production in target tissues via the putative receptors shared with CGRP and those specific for AM [1–6]. Structure-activity relationship studies revealed that the ring structure formed by a disulfide bond and the C-terminal amidation are critical for receptor binding and cAMP production [7]. When administered intrarenally, AM exerts potent natriuretic and vasodilatory actions [8, 9]. Tissue distribution studies have shown that AM is synthesized in various tissues including the adrenal

gland, kidney, lung, and heart [10, 11]. These tissues are rich in AM receptors as well [4], suggesting that AM may act as a local hormone. AM is also present in plasma [12], and using cultured cells, it has been shown to be secreted from endothelial cells (EC) and vascular smooth muscle cells (VSMC) [13, 14]. The functional significance of AM, however, remains to be elucidated.

In the present study, we have developed a monoclonal antibody against the ring structure of AM. Using this antibody, we established a specific and sensitive radioimmunoassay (RIA) for AM, and revealed its secretion not only from endothelial cells (EC) and VSMC but also from mesangial cells (MC). Furthermore, we examined the effects of neutralizing AM endogenously produced by cultured cells, and found that AM, acting as an autocrine regulator, plays an important role in controlling the basal cAMP production and proliferation of the endothelium.

METHODS

Peptides

Fragment peptides of human AM (hAM), hAM (1-15), hAM (15-31), and hAM (27-52) were synthesized by the solid phase method and purified by reverse-phase high performance liquid chromatography. Human adrenomedullin (hAM), rat AM (rAM), hAM (22–52) (an AM receptor antagonist), human calcitonin gene-related peptide (hCGRP)-I, and hCGRP (8-37) (a CGRP$_1$ receptor antagonist) were purchased from Peptide Institute (Osaka, Japan). Platelet-derived growth factor (PDGF)-BB was from Becton Dickinson Labware (Bedford, MA, USA).

Preparation and characterization of monoclonal antibody

Human adrenomedullin (15-31) (3.0 mg) was conjugated to bovine thyroglobulin (10.3 mg; Sigma, St. Louis, MO, USA) using the carbodiimide coupling procedure [15]. Ten BALB/c mice were immunized with subcutaneous injections of the conjugate containing 30 μg of the peptide emulsified in complete Freund's adjuvant (Difco, Detroit, MI, USA) over a period of five months at two to three week intervals. Elevation of antibody titer screened by RIA was observed in four mice, and the one with highest response (final dilution, 1:25,000) was selected for cell fusion. Fusion of spleen cells with mouse myeloma cells X63-Ag8.653 was performed using 50% polyethylene glycol 4000 (Merck, Darmstadt, Germany) as described [16]. Hybridomas were screened for antibody production by the RIA, cloned by limiting dilution and

expanded intraperitoneally in BALB/c mice. Isotyping of the monoclonal antibody was performed by the Western blot technique (Mouse Monoclonal Isotyping Kit; Amersham, Buckinghamshire, UK). Binding affinity was analyzed by a Scatchard plot using the RIA for AM.

Radioimmunoassay for adrenomedullin

Human adrenomedullin (15-31) was radioiodinated by the chloramine T method as previously described [15]. The specific activity of ^{125}I-hAM (15–31) ranged from 600 to 900 μCi/μg. The monoclonal antibody named KY-AM-I (final dilution of ascites, $1{:}7.5 \times 10^5$) was incubated with either standard hAM or samples in 200 μl of assay buffer (50 mM phosphate buffer, pH 7.4, containing 0.5% gelatin (Merck), 0.1% Triton X-100, 1 mM Na$_2$EDTA, 0.2 mM L-cystine, and 0.1% NaN$_3$) for 24 hours at 4°C. Then, 50 μl of ^{125}I-hAM (15-31) (approximately 10,000 cpm) was added and the mixture was further incubated for 16 hours at 4°C. Bound and free ligands were separated by adding 1.0 ml of suspension of dextran-coated charcoal consisting of 400 mg of Norit SX Plus (Norit, the Netherlands) and 40 mg of Dextran T-70 (Pharmacia, Uppsala, Sweden) in 100 ml of 50 mM phosphate buffer, pH 7.4, containing 0.1% NaN$_3$ [15].

Cell culture

Bovine aortic EC and rat aortic VSMC were prepared from explants as previously reported [17] and used at passages 10 to 15 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Cells grown to confluence in 100-mm dishes were washed twice with phosphate-buffered saline (PBS) and incubated at 37°C for indicated time in DMEM containing 0.1% bovine serum albumin (BSA). Rat MC were prepared from the glomeruli isolated from 20-week-old male Wistar rats by differential sieving [17, 18] and used at passages 6 to 10 maintained in RPMI 1640 containing 10% FCS. Confluent MC were washed twice with PBS and made quiescent by incubating in DMEM/F-12 supplemented with 10 μg/ml insulin, 10 μg/ml transferrin, 10 ng/ml selenium, and 0.1% BSA [19]. After incubation, conditioned media were condensed using a Sep-Pak C$_{18}$ cartridge as described [13–15], and the dried material was dissolved in the RIA buffer and subjected to RIA for AM.

High-performance gel permeation chromatography

High-performance gel permeation chromatography (HP-GPC) was performed on a TSK-GEL G2000 SW column (7.5 × 600 mm; Toyo Soda, Tokyo, Japan), eluted with 10 mM trifluoroacetic acid containing 0.3 M sodium chloride and 30% acetonitrile as a solvent, as previously described [15]. The flow rate was 0.3 ml/min and the fraction volume was 0.36 ml.

cAMP measurement

Basal and AM-stimulated cAMP production in the cells was measured by RIA as described with slight modification [17, 20]. In brief, cultured EC, VSMC or MC grown to confluence in 24-well plates were washed twice with serum-free DMEM and preincubated for 20 minutes at 37°C in 400 μl DMEM containing 0.1% BSA and 0.5 mM isobutylmethylxanthine (IBMX; Sigma). Rat AM dissolved in 100 μl of the same medium or vehicle was added to the culture (final concentrations, 10^{-11} to 10^{-7} M) and cells were further incubated for 30 minutes at 37°C. After incubation, 1 ml of ice-cold ethanol was added to the culture to disrupt the cells and

the mixture was centrifuged at 15,000 × g for 10 minutes at 4°C. The supernatant was vacuum dried and the pellet dissolved in assay buffer was subjected to RIA for cAMP after succinylation using a commercial kit (Yamasa, Tokyo, Japan).

Neutralization experiment

The monoclonal antibody in ascites was purified with Affi-Gel Protein A MAPS II kit (Bio-Rad, Richmond, CA, USA) [21] and 1 mg of purified monoclonal antibody was dissolved in 1 ml of DMEM/0.1% BSA. Cells grown to confluence in 12-well plates were washed twice with serum-free DMEM and preincubated for 10 minutes at 37°C in 400 μl DMEM/0.1% BSA/0.5 mM IBMX with either 50 μl monoclonal antibody solution (final concentration, 100 μg/ml) or the same concentration of mouse IgG (Sigma). Rat AM dissolved in 50 μl of DMEM/0.1% BSA was added to the medium (final 10^{-8} M) and cells were further incubated for 30 minutes at 37°C. After incubation, cAMP production was determined as above.

Proliferation studies

Cultured EC, VSMC or MC grown to confluence in 24-well plates were washed with DMEM/F-12 and incubated in DMEM/F-12/0.1% BSA with or without rAM (10^{-12} to 10^{-7} M) and PDGF-BB (10 ng/ml) for 12 hours at 37°C. Then, ^{3}H-thymidine (Amersham) was added to the medium (final concentration, 10 μCi/ml) and cells were further incubated for four hours at 37°C. After incubation, cells were washed twice with ice-cold PBS, and the count of ^{3}H-thymidine incorporated into the cells was measured [22].

For the neutralization experiment, cells grown to confluence in 24-well plates were washed with DMEM/F-12 and then preincubated for four hours at 37°C in 500 μl DMEM/F-12/0.1% BSA. Then, cells were washed and incubated for 24 hours in the presence of either purified KY-AM-I (1 or 50 μg/ml), mouse IgG (50 μg/ml), hAM (22–52) (10^{-6} M), or hCGRP (8–37) (10^{-6} M). After incubation, cells were labeled with ^{3}H-thymidine for six hours and ^{3}H-thymidine incorporation was measured.

Statistical analysis

Data were expressed as the mean ± SEM. Statistical analysis was performed using analysis of variance followed by Scheffe's test. $P < 0.05$ was considered significant.

RESULTS

Preparation and characterization of monoclonal antibody

After the fusion, three clones among 288 wells of hybridoma gave a positive antibody response. After further culture and cloning, one clone with highest response was selected for expansion and characterization. The established monoclonal antibody, named KY-AM-I, belonged to the IgG$_1$ subclass. Analysis by a Scatchard plot revealed high affinity for AM, with an association constant (K$_a$) of 2.5×10^{10} M^{-1}. Specificity of the monoclonal antibody KY-AM-I in the RIA for AM is shown in Figure 1A. Human adrenomedullin (15-31) showed an equimolar cross-reactivity with standard hAM. There was no cross-reactivity (< 0.01%) with hAM (1-15) or hAM (27-52), indicating that the antibody recognizes the ring structure of AM.

A

B
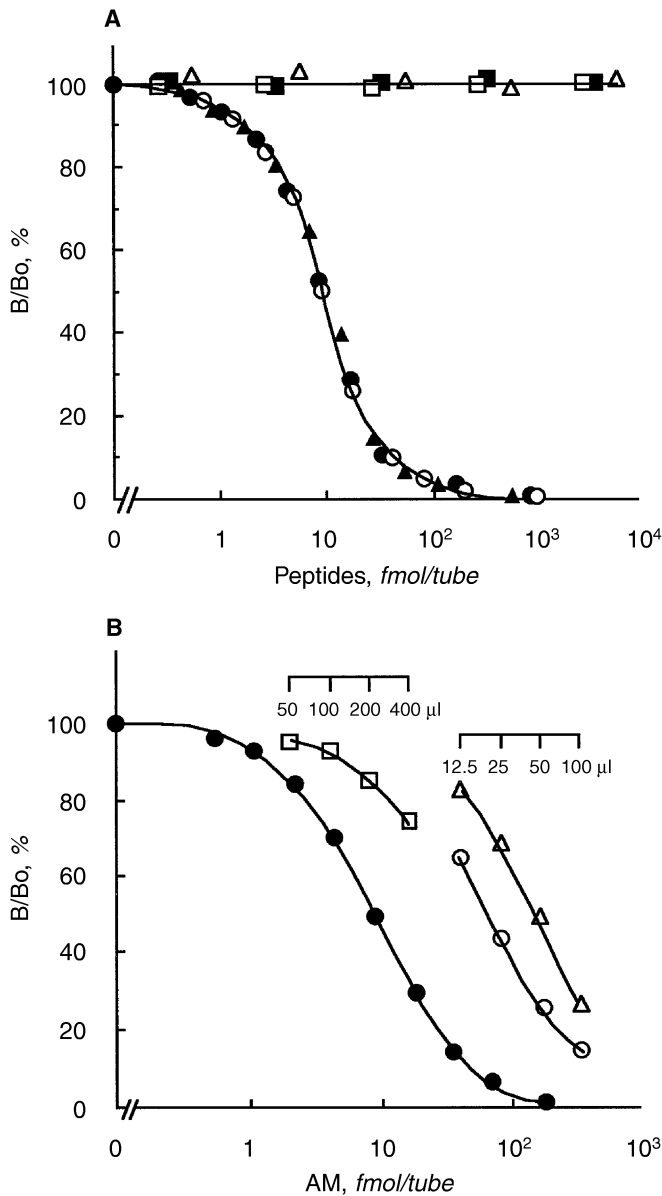
Fig. 1. A typical standard curve of adrenomedullin (AM) and cross-reactivity profiles of its related peptides (*A*) and dilution curves of culture media (*B*) in the radioimmunoassay (RIA) for AM with a monoclonal antibody KY-AM-I. Abbreviations and symbols in A are: (●) hAM, human AM; (○) rAM, rat AM; (▲) hAM (15-31); (△) hAM (1-15); (■) hAM (27-52); (□) hCGRP-I. In B, symbols and abbreviations are: (○) bEC, bovine endothelial cells; (△) rVSMC, rat vascular smooth muscle cells; (□) rMC, rat mesangial cells.

Table 1. Accumulation of adrenomedullin (AM)-like immunoreactivity in culture media of bovine endothelial cells (bEC), rat vascular smooth muscle cells (rVSMC), and rat mesangial cells (rMC)

	AM, *fmol/10^5 cells*		
	12 hrs	24 hrs	36 hrs
bEC	10.9 ± 2.3	21.8 ± 4.7	28.3 ± 5.0
rVSMC	3.9 ± 0.8	12.1 ± 2.5	16.4 ± 2.4
rMC	4.8 ± 0.6	6.2 ± 1.2	6.4 ± 0.8

Values are expressed as the mean $\pm$ SEM ($N = 4$).

Adrenomedullin-like immunoreactivity (AM-LI) in culture media

Serial dilution curves of culture media were parallel to the standard curve (Fig. 1B). Table 1 shows time-dependent accumulation of adrenomedullin-like immunoreactivity (AM-LI) in the culture media. A significant amount of AM-LI (10^{-10} to 10^{-11} M) was detected in the culture media conditioned with bovine aortic EC and with rat aortic VSMC, at the level similar to that in previous reports [13, 14]. AM-LI was also detected abundantly in the culture media of rat MC, demonstrating the mesangial secretion of AM at the level comparable to that from EC or VSMC.
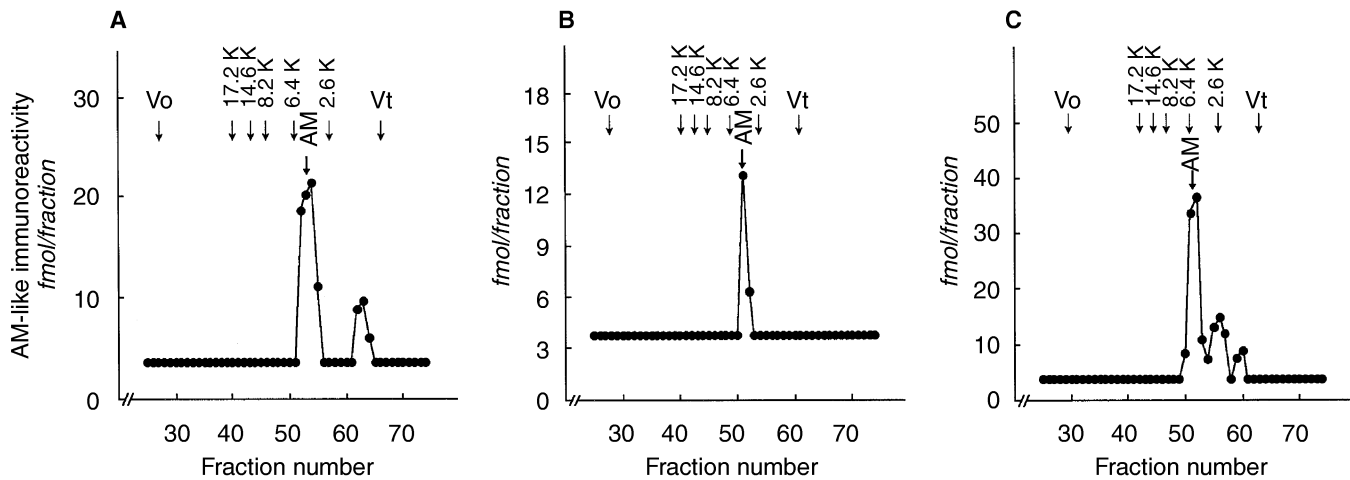
To assess the molecular form of AM-LI secreted from those cells, HP-GPC analysis was performed (Fig. 2). A major component of AM-LI in the culture supernatant of EC and VSMC emerged at the position of 6 kDa corresponding to the elution position of authentic AM (Fig. 2 A, B). A minor peak was detected in the lower molecular weight range, suggesting its degradation product(s). Using culture media of rat MC, an essentially similar pattern was observed (Fig. 2C).

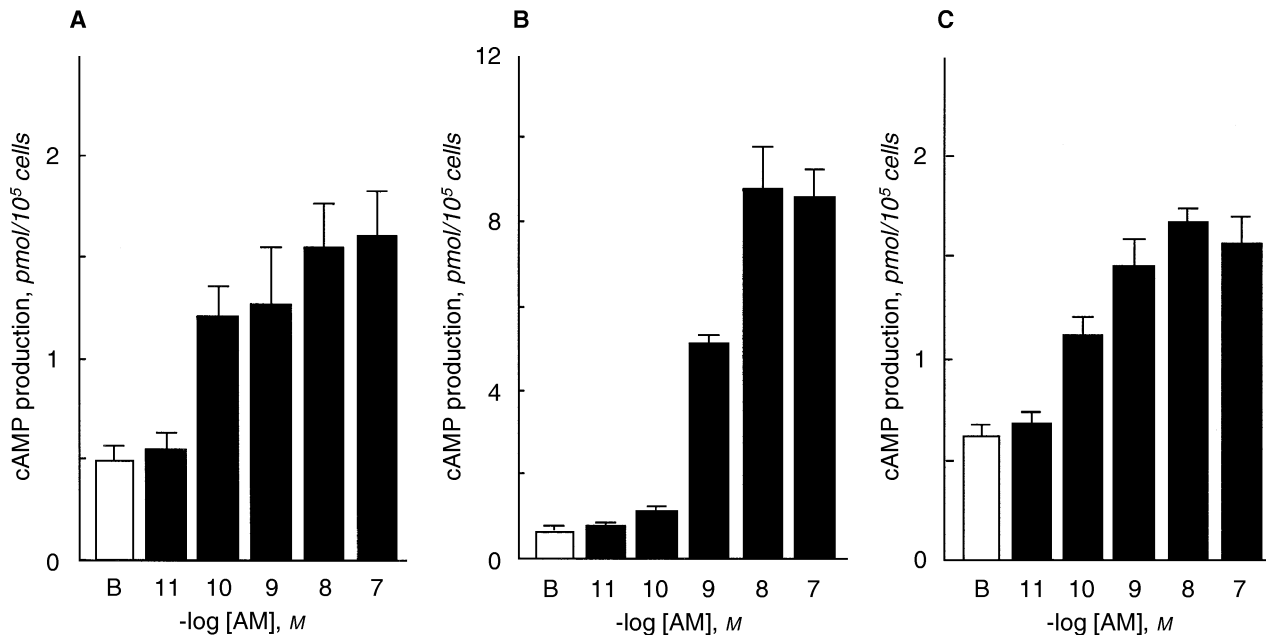### Effect of adrenomedullin on cAMP production

To examine whether AM acts on these cells of AM production, cAMP levels were measured in cultured EC, VSMC and MC after stimulation by exogenous AM. Addition of AM resulted in dose-dependent increases of cAMP production in these cells (Fig. 3), with EC_{50} values of $\sim 10^{-10}$ M in EC and MC, and $\sim 10^{-9}$ M in VSMC.

Neutralization experiment

To further examine the hypothesis that AM acts on these cells in an autocrine fashion, we performed *in vitro* neutralization experiments with the established monoclonal antibody. Figure 4 shows the effects of pretreatment with KY-AM-I on basal and AM-stimulated cAMP production in cultured EC. Addition of rAM (10^{-8} M) into cultured EC showed a threefold increase of cAMP production, and administration of KY-AM-I completely abolished this effect (2.72 ± 0.21 vs. 0.63 ± 0.08 pmol/10^5 cells, $P < 0.001$; Fig. 4). Pretreatment with KY-AM-I in cultured VSMC or MC also effectively abolished cAMP increase induced by exogenous AM (data not shown), indicating that this monoclonal antibody acts as a neutralizing antibody. Under this condition, even in the unstimulated state, neutralization with KY-AM-I of endogenously secreted AM in cultured EC resulted in significant (by $\sim 70\%$) reduction of basal cAMP production (1.05 ± 0.14 vs. 0.35 ± 0.04 pmol/10^5 cells, $P < 0.01$), whereas control IgG did not affect cAMP levels (Fig. 4). These results strongly indicate

Radioimmunoassay for adrenomedullin

In the standard curve of the RIA for AM (Fig. 1), the minimal detectable quantity was 1.0 fmol/tube, and the 50% binding intercept was 8.0 fmol/tube. The cross-reactivities with rAM and hCGRP-I were 100% and < 0.01%, respectively. The intra-assay and interassay coefficients of variation were 4.2% ($N = 8$) and 6.8% ($N = 8$), respectively.

Fig. 2. Typical high performance-gel permeation chromatography (HP-GPC) profiles of adrenomedullin-like immunoreactivity (AM-LI) in the culture supernatant of bovine endothelial cells (EC; *A*), rat vascular smooth muscle cells (VSMC; *B*), and rat mesangial cells (MC; *C*). Arrows denote elution positions of a series of myoglobins of a polypeptide molecular weight calibration kit (Pharmacia), void volume (Vo) and total volume (Vt). The elution position of synthetic AM is also indicated.

Fig. 3. Adrenomedullin (AM)-stimulated increase of cAMP production in cultured bovine endothelial cells (EC; *A*), rat vascular smooth muscle cells (VSMC; *B*), and rat mesangial cells (MC; *C*). Values are expressed as the mean ± SEM (*N* = 4). B is basal.

that AM, acting in an autocrine manner, has a significant contribution to maintaining the basal cAMP production in cultured EC. The inhibitory effect of neutralizing AM on basal cAMP production was not observed in cultured VSMC (0.78 ± 0.14 vs. 0.84 ± 0.06 pmol/10^5 cells) or in MC (0.83 ± 0.09 vs. 0.79 ± 0.08 pmol/10^5 cells), suggesting that the contribution of endogenously secreted AM to their basal cAMP levels in these cells was less significant than in cultured EC.

Proliferation studies

To further explore the biological actions exerted by AM, we examined effects on cell proliferation. Addition of AM into cultured cells inhibited PDGF-stimulated DNA synthesis in a dose-dependent manner (Fig. 5). The inhibitory effects of AM at 10^{-10} M were $-84.0 \pm 8.4\%$, $-41.8 \pm 9.7\%$, and $-21.1 \pm 8.3\%$, respectively, in cultured EC, VSMC and MC.

Next, to investigate the possible autocrine role of AM in cell growth, we examined effects of neutralization using the AM monoclonal antibody in these cells. Figure 6 shows the effect of pretreatment with KY-AM-I on DNA synthesis in cultured EC. Neutralization with KY-AM-I of endogenously secreted AM in EC significantly increased basal ^{3}H-thymidine uptake dose-dependently ($142.1 \pm 8.1\%$ and $169.5 \pm 4.0\%$ at 1 and 50 µg/ml KY-AM-I, respectively, of the basal level, $P < 0.01$), whereas
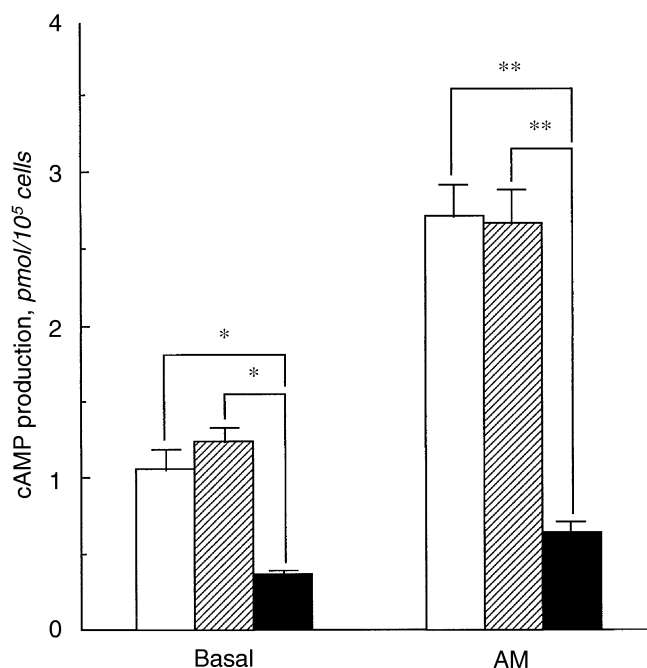
Fig. 4. Effects of pretreatment with the monoclonal antibody KY-AM-I (■) or control IgG (▨) on basal and AM-stimulated cAMP production in cultured bovine endothelial cells (EC; □). Values are expressed as the mean ± SEM ($N = 6$). *$P < 0.01$, **$P < 0.001$.

control IgG showed no effect. Neutralization of AM in cultured MC or VSMC did not affect significantly in their basal DNA synthesis (data not shown). This stimulatory effect of the AM monoclonal antibody on EC growth was mimicked by pretreatment with hAM (22–52), a putative AM receptor antagonist ($167.8 \pm 8.9\%$ at 10^{-6} M) but not hCGRP (8–37), a putative CGRP$_1$ receptor antagonist ($107.1 \pm 4.2\%$ at 10^{-6} M). This suggests that the effects of AM on EC may be mediated mainly via the putative receptor specific for AM.

DISCUSSION

In the present study, we prepared a specific, high-affinity monoclonal antibody against AM. The antibody KY-AM-I is directed against the ring structure of AM, which is essential for exerting its biological activity [7]. The established RIA with this antibody recognizes AM of human, rat and bovine forms with high sensitivity, and detects its native form with the ring structure.

Using this RIA, we detected 6 kDa AM-LI in the culture media conditioned with rat MC, at the level almost comparable to that from EC or VSMC (Table 1) [13, 14]. The minor peaks in HP-GPC (Fig. 2 A, C) may represent natural or artifactual fragments of AM whose nature remains to be characterized [12–14]. Adrenomedullin has so far been demonstrated in the kidney [1, 8, 10–12] and is immunohistochemically localized in glomeruli and tubules [8]; the precise localization of AM production in the kidney, however, has not been clarified. The present study showed that the mesangium, in addition to the vascular endothelium, should be one probable source of AM produced in the kidney. Locally produced AM may act to modulate glomerular function, and indeed, intrarenal administration of AM has been shown to exert potent natriuretic and vasorelaxing activities [8, 9].

Moreover, AM inhibits MC proliferation stimulated by mitogens such as PDGF (Fig. 5C) [23]. Proliferation of MC with mesangial matrix expansion is a characteristic feature of many glomerular diseases, and a variety of growth factors, cytokines and vasoactive substances may mediate this disease process [24]. The synthesis of AM in the mesangium may, as in EC or VSMC [13, 14], be regulated by these hormones and cytokines, thereby potentially modulating mesangial proliferation and function in various pathological conditions. Taken together, it is conceivable that AM may play a role in the regulation of glomerular function through an autocrine/paracrine mechanism.

The present study revealed that AM potently inhibits proliferation of cultured EC (Fig. 5A). This effect is likely to be mediated mainly via the cAMP pathway to inhibit the mitogen-activated protein kinase cascade as reported in MC and VSMC [23, 25, 26]. The antigrowth effect of AM in EC observed here, however, was more potent than in other cell types whereas stimulation of cAMP production was less remarkable (Figs. 3 and 5), suggesting that other intracellular mechanisms such as the nitric oxide pathway may be partly involved in this particular cell type [3, 9]. In other cell types such as tumor cells, AM has been shown to stimulate cell proliferation in a cAMP-dependent manner [27, 28]. The reason for the difference causing these apparently contradictory results on cell proliferation is currently unclear, but the dual function of cAMP has been already known depending upon the relative cellular amounts of two distinct cAMP-dependent protein kinase A isoforms: RI with growth promotion and RII with growth inhibition [29].

In the present study, we demonstrated that neutralization with the AM monoclonal antibody markedly (by ~70%) reduced the basal cAMP production in cultured EC (Fig. 4). Neutralization experiment also revealed the significance of AM as an autocrine regulator of EC growth (Fig. 6). Whether this is true with the *in vivo* situation, and with various pathological conditions in which AM secretion is augmented [30], remain to be clarified. Neutralization of AM in MC or VSMC did not directly affect their cAMP levels or DNA synthesis, but the potent inhibitory effect of exogenous AM on their growth still suggests AM as a paracrine growth regulator in these cells. In general, these cells produce a wide variety of factors capable of promoting or inhibiting growth in an autocrine or paracrine fashion, and it has been proposed that many abnormalities encountered in diabetic renal and vascular complications could be due to the altered EC-MC and EC-VSMC interactions [31, 32]. Furthermore, we have recently demonstrated that shear stress, one of the potent modifiers of vascular remodeling, augments AM expression in cultured EC [33]. From these results, it seems likely that AM, together with C-type natriuretic peptide [34], could represent one of the major peptide-type endothelium-derived relaxing factors, participating in the modulation of vascular and mesangial function through paracrine interactions.

In conclusion, we developed a high-affinity, neutralizing monoclonal antibody against AM, which should provide a useful tool to further explore the functional role of AM. Moreover, the findings on the autocrine role of AM in modulating endothelial growth, together with the mesangial secretion of AM, point toward the need for investigative studies to determine its significance in various pathological conditions, such as diabetic, atherosclerotic, and hypertensive renal and vascular complications.
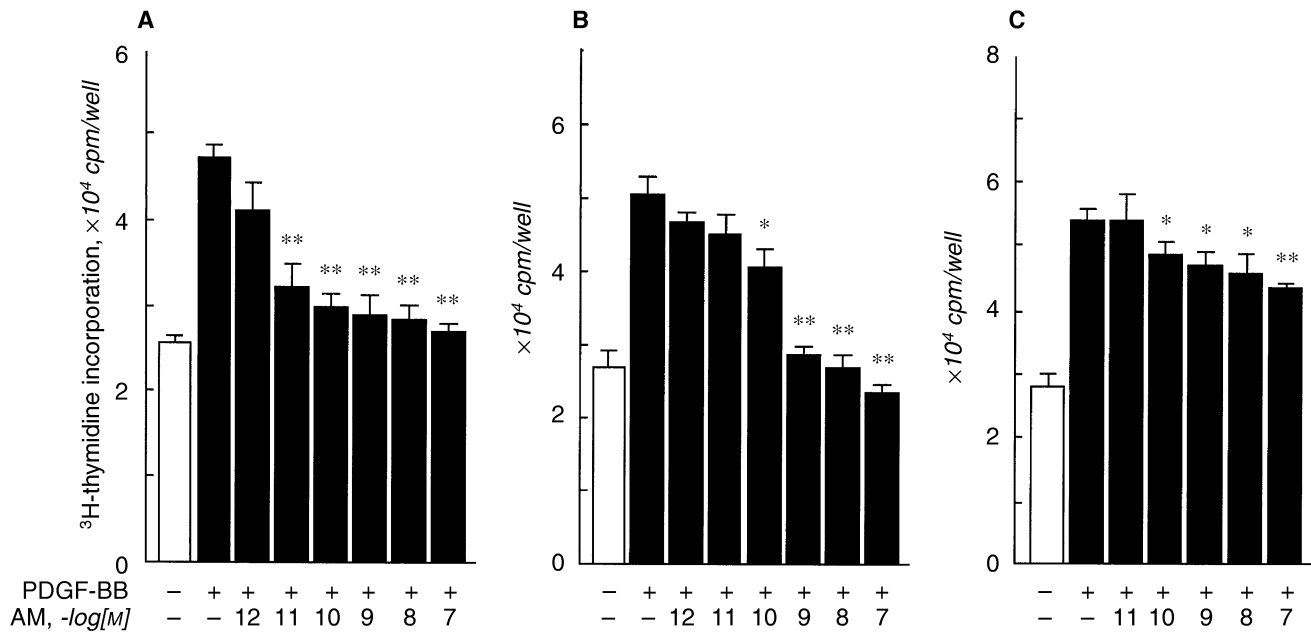
A

B

C

Fig. 5. Inhibitory effects of adrenomedullin (AM) on platelet-derived growth factor (PDGF)-stimulated ³H-thymidine incorporation into cultured bovine endothelial cells (EC; *A*), rat vascular smooth muscle cells (VSMC; *B*), and rat mesangial cells (MC; *C*). Values are expressed as the mean ± SEM ($N = 6$). *$P < 0.05$, **$P < 0.01$, as compared to the levels with PDGF alone.
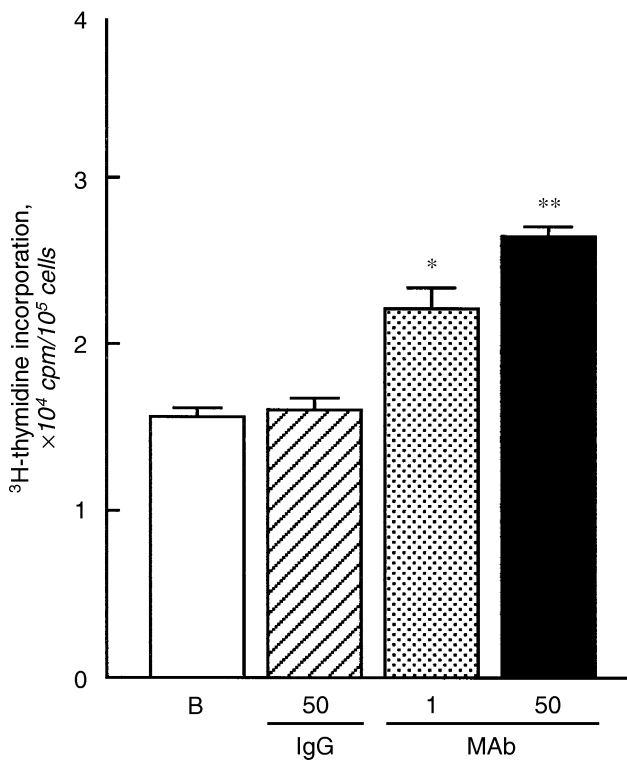

Fig. 6. Effects of pretreatment with the monoclonal antibody (MAb) on basal ³H-thymidine incorporation into cultured endothelial cells (EC). Symbols are: (□) basal level (B) with vehicle; (▨) mouse IgG (50 μg/ml); (▦) low dose of MAb KY-AM-I (1 μg/ml); (■) high dose of KY-AM-I (50 μg/ml). Values are expressed as the mean ± SEM ($N = 6$). *$P < 0.01$, **$P < 0.001$, as compared to basal.

ACKNOWLEDGMENTS

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APPENDIX

Abbreviations used in this article are: AM, adrenomedullin; AM-LI, adrenomedullin-like immunoreactivity; bEC, bovine endothelial cells; BSA, bovine serum albumin; CGRP, calcitonin gene-related peptide; DMEM, Dulbecco's modified Eagle's medium; EC, endothelial cells; FCS, fetal calf serum; hAM, human adrenomedullin; hCGRP-I, human calcitonin gene-related peptide; HP-GPC, high-performance gel permeation chromatography; IBMX, isobutylmethylxanthine; MC, mesangial cells; PBS, phosphate buffered saline; PDGF, platelet-derived growth factor; rAM, rat AM; RIA, radioimmunoassay; rMC, rat mesangial cells; rVSMC, rat vascular smooth muscle cells; VSMC, vascular smooth muscle cells.

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