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The relaxant effect of adrenomedullin on particular smooth muscles despite a general expression of its mRNA in smooth muscle, endothelial and epithelial cells

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1 By use of the reverse transcription polymerase chain reaction (RT-PCR), we determined the expression of adrenomedullin (AM) mRNA in the various tissues of the pig. To evaluate the significance of the expression of AM mRNA, we also determined the effects of AM on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension development of the porcine smooth muscle strips obtained from the coronary artery, pulmonary vein, trachea, ileum and urinary bladder.

2 AM mRNA was widely expressed in the porcine tissues examined, which included myocardium (left and right ventricle and right atrium), kidney, lung, endothelial cells (aorta and aortic valve), smooth muscles (aorta, main pulmonary artery, pulmonary vein, renal artery and vein, coronary artery, ileum, trachea and urinary bladder) and epithelial cells (trachea and urinary bladder).

3 AM induced a decrease in $[Ca^{2+}]_i$ and tension of the coronary artery, but not the pulmonary vein. AM had no effects on either the $[Ca^{2+}]_i$ or tension of the trachea and urinary bladder strips or on the tension development of strips of ileum.

4 These results indicated that AM has a role as an autocrine and/or paracrine regulator of the coronary arterial tone. AM probably does not have an important role in the regulation of the pulmonary venous, tracheal, ileac and urinary bladder smooth muscle tone, even though AM mRNA is expressed in these tissues; the functional significance of AM in these smooth muscles remains to be determined.

Keywords: Adrenomedullin; mRNA; smooth muscle; coronary artery; pulmonary vein

Introduction

Adrenomedullin (AM) is a potent hypotensive peptide isolated from human phaeochromocytoma cells (Kitamura *et al*., 1993a). It stimulates adenonsine 3′:5′-cyclic monophosphate (cyclic AMP) formation in platelets (Kitamura *et al*., 1993a), rat cultured aortic smooth muscle cells (Ishizuka *et al*., 1994), renal tubular basolateral membranes (Osajima *et al*., 1995) and cultured mesangial cells (Kohno *et al*., 1995b). AM induces a profound degree of hypotension (Kitamura *et al*., 1993a; Nuki *et al*., 1993; Ishiyama *et al*., 1993; Perret *et al*., 1993) and a reduction of the peripheral vascular resistance (Santiago *et al*., 1994; DeWitt *et al*., 1994). Nakamura *et al*., (1995) found that AM induces relaxation of various isolated arteries of the dog. We have recently found that AM decreases $[Ca^{2+}]_i$ and tension of porcine coronary and renal arteries (Kureishi *et al*., 1995; Seguchi *et al*., 1995).

Initially, AM was thought of as a circulating hormone, because it is secreted from the adrenal medulla and present in significant concentrations in plasma (Kitamura *et al*., 1993a). The cDNA cloning of human (Kitamura *et al*., 1993b), rat (Sakata *et al*., 1993) and pig AM (Kitamura *et al*., 1994) revealed that AM mRNA is expressed not only in the adrenal medulla but also in a variety of cells and tissues, including cultured endothelial cells, cultured vascular smooth muscle cells, aorta, renal artery, heart, lung, kidney, stomach, intestine and thyroid gland (Sugo *et al*., 1994a,b; Kitamura *et al*., 1993b; 1994; Sakata *et al*., 1993; Seguchi *et al*., 1995). The expression of AM mRNA in a variety of cell types indicates that AM might have a role not only as a circulating hormone but also as a local autocrine and/or paracrine regulator of various cell functions.

In the present study, using reverse transcription polymerase chain reaction (RT-PCR), we determined the expressions of AM mRNA in various tissues of the pig, including the myocardium (left and right ventricle and right atrium), kidney, lung, endothelial cells (aorta and aortic valve), smooth muscles (aorta, main pulmonary artery, pulmonary vein, renal artery and vein, coronary artery, ileum, trachea and urinary bladder) and epithelial cells (trachea and urinary bladder). We also determined the effects of AM on $[Ca^{2+}]_i$ and tension development of smooth muscle strips obtained from the coronary artery, pulmonary vein, trachea, ileum and urinary bladder of the pig. We have found that, although AM mRNA is expressed in general in the various types of cells examined, not all of the smooth muscle preparations were always relaxed by AM. The limited effects of AM on smooth muscle tone despite the general expression of AM mRNA might indicate the involvement of AM in a cellular function other than the regulation of smooth muscle tone.

Methods

Measurement of AM mRNA by RT-PCR

Total RNA was isolated from the tissue according to the method described by Chomczynski and Sacchi (1987). In order to obtain the endothelial cells or epithelial cells, the surface of the corresponding tissue was scraped by a rubber policeman. To obtain the smooth muscle cells, care was taken not to include the endothelium, epithelium or adventitia during the tissue trimming, as described below. Contaminating genomic DNA, if any, was digested by RNase free DNase. First-strand cDNA was synthesized by use of each type of total RNA for the template. The total RNA (1 μg) was incubated at 37°C with a mixture (total volume = 20 μl) of 200 units of M-MLV

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reverse transcriptase, 1 x RT buffer, 10 mM DTT, 0.5 mM each dNTP (dATP, dCTP, dTTP, dGTP), 20 units of RNase inhibitor and 50 nM of antisense primers (Table 1, RT primer). Thereafter the reaction mixture was incubated 10 min at 90°C to denature the RNA-cDNA hybrid and also to inactivate the reverse transcriptase. For the PCR amplification, an aliquot (1 μl) of RT product was mixed with 0.5 unit of Taq DNA polymerase, 500 nM each of sense and antisense primers (Table 1, PCR primers) in a buffer containing 20 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg ml^{-1} BSA and 200 μM each of dNTP in a 11 μl volume. The thermal cycle profile of the PCR machine (PC 700, Astec, Fukuoka, Japan) used in this study was (1) denaturing for 30 s at 94°C, (2) annealing primers for 60 s at 50°C and (3) extending the primers for 30 s at 72°C. PCR was completed by a final extension step of 10 min at 72°C. The sequences of sense and antisense primers were designed based on the published sequence for pig AM (Kitamura *et al*., 1994) and pig β-actin (obtained from the Genbank database), as shown in Table 1. The expected sizes of the PCR products for AM and β-actin were 290 bp and 241 bp, respectively. PCR was repeated 25 cycles for β-actin and 30 cycles for AM. A portion (10 μl) of the PCR mixture was electrophoresed in a 3% agarose gel in TAE buffer. The gel was stained with ethidium bromide and photographed. The band thickness was quantified by the densitometer (CS-9000, Shimadzu, Tokyo, Japan) with the negative film of these photographs.

In order to assess the extent of the cross contamination of endothelial cells and smooth muscle cells, we also performed RT-PCR for coagulation factor VIII (Factor VIII) and myosin light chain kinase (MLCK). The primers for Factor VIII and MLCK are shown in Table 1. The primers for Factor VIII were designed from the conserved regions between mouse (Elder *et al*., 1993) and human (Truett *et al*., 1985) factor VIII sequences. Those for MLCK were chosen from the conserved regions between the rabbit (Gallagher *et al*., 1991) and bovine MLCK (Kobayashi *et al*., 1992), based on the rabbit sequence. PCR amplification (30 cycles for both Factor VIII and MLCK) was done in the same manner as described about except that the annealing was 90 s at 55°C. The expected sizes of the PCR products for Factor VIII and MLCK were 371 bp and 465 bp, respectively. The sequences of these PCR products were determined by direct sequencing done by Sawady Technology (Tokyo, Japan), by using an autosequencer (373S, ABI, Foster City, CA).

Tissue preparations

The hearts, lungs, urinary bladders, ileums and tracheas of adult pigs were obtained from a local slaughterhouse immediately after the pigs had been killed and were transported to our laboratory in the preaerated ice-cold physiological saline solution (PSS). The coronary arterial and tracheal strips were prepared as previously described (Hirano *et al*., 1990; Kai *et al*., 1993). For the preparation of pulmonary venous strips, intrapulmonary veins were dissected from lungs and cut longitudinally. The muscle sheets were transversely cut into strips 5 mm in length and 1 mm in width. For the strips of urinary bladder, the smooth muscle layers were cut into $2 \times 2 \times 1$ mm strips. The ileac strips were obtained from the circular smooth muscle layers and cut into $2 \times 2 \times 1$ mm strips. In the case of the vascular strips, the endothelium was removed by rubbing the inner surface with a cotton swab and the adventitia was trimmed away (by use of a microscope). All tissue preparations were performed in oxygenated (95% O_2 and 5% CO_2) PSS.

Fura-2 loading

The strips were loaded with fura-2 or fura-PE3 (in case of urinary bladder), in the form of acetoxymethyl ester (fura-2/AM fura-PE3/AM). The strips were incubated in Dulbecco's-modified Eagle's medium containing 25 μM fura-2/AM or fura-PE3/AM dissolved in dimethyl sulphoxide and 5% foetal bovine serum for 4–6 h at 37°C (containing 0.004% Pluronic F-127 in the urinary bladder strips). After loading with fura-2 or fura-PE3, the strips were rinsed with normal PSS to remove the dye in the extracellular space and to equilibrate the strips for at least 60 min at 37°C before starting the measurements.

Measurement of tension development

The strips were mounted vertically in a quartz organ bath and connected to a strain gauge (TB-612T, Nihon Koden, Japan). During the 60 min fura-2 equilibration period, the strips were stimulated with high external K^+-depolarization every 15–20 min, and the resting tension was increased stepwise to get the maximal response of the tension development. The responsiveness of each strip to high external K^+-depolarization was recorded before starting the experimental protocol. The developed tension was expressed as a percentage, assigning the values in normal (5.9 mM K^+) PSS and maximal (steady-state) high external K^+-depolarization (118 mM K^+-depolarization for the coronary strips, 80 mM K^+-deplorization for the pulmonary venous strips, 40 mM K^+-depolarization for the tracheal and ileac strips) to be 0 and 100%, respectively. In the case of the urinary bladder strips, the peak value of the tension induced by 118 mM K^+-depolarization was assigned to be 100%, because we could not obtain a steady state tension with high external K^+-depolarization in this preparation.

Front-surface fluorometry

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were monitored by using a front-surface fluorometer specifically designed by us for fura-2 fluorometry (Hirano *et al*., 1990). In brief, dual wavelength excitation light (340 and 380 nm) was alternately (400 Hz) introduced to the smooth muscle strips through quartz optic fibres. Surface fluorescence (500 nm) of strips was collected by glass optic fibres and in-

Table 1 Oligonucleotide primers for RT-PCR

	PCR primers (upper)	*PCR primers (lower)*	*RT primers*
AM*	5′-TGGAATAAGTGGGCTCTAAG-3′ (pig Sense 100–119)	5′-TTGTCCGTGAACTGGTAGAT-3′ (pig Antisense 370–389)	5′GCCGTCCTTGTCTTTGTC-3′ (pig Antisense 385–402)
β-actin**	5′-GTGCGGGACATCAAGGAGAA-3′ (pig Sense 259–278)	5′-TGTCCACGTCGCACTTCAT-3′ (Pig Antisense 481–499)	5′-ATCTGCTGGAAGGTGGACAG-3′ (Pig Antisense 679–698)
Factor† VIII	5′-GTCCTGAAAGAGAATGGTCCAAT-3′ (Human Sense 475–497)	5′-GTGGTGCCCATTCCAATCAC-3′ (Human Antisense 826–845)	5′-AGCAGTAAGGAAAGTTATTG-3′ (Human Antisense 926–945)
MLCK††	5′-GGAAGACACTCAAGACCAC-3′ (rabbit Sense 1402–1420)	5′-GCTGTTCTCCACCTTGAT-3′ (rabbit Antisense 1848–1865)	5′-GCTGTTCTCCACCTTGAT-3′ (rabbit Antisense 1848–1865)

*These primers were designed based on the pig adrenomedullin (AM) sequence described by Kitamura *et al*. (1994). **These primers were designed based on the pig β-actin sequence obtained from Genbank database. †These primers were designed based on human factor VIII sequence described by Truett *et al*. (1985). ††These primers were designed based on the rabbit myosin light chain kinase (MLCK) sequence descibed by Gallagher *et al*. (1991).

troduced into a photomultiplier. The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored and expressed as a percentage, assigning the values at rest in normal (5.9 mM K^+) and during depolarization with high K^+ PSS to be 0% and 100%, respectively, as in the case of tension measurement. All the simultaneous measurements of $[Ca^{2+}]_i$ and tension were carried out at 37°C.

Solutions and drugs

Normal PSS was of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High K^+ PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. PSS was bubbled with 95% O_2 and 5% CO_2, with a resulting pH of 7.4 at 37°C. Synthetic human AM and endothelin-1 were obtained from the Peptide Institute Co. Ltd. (Osaka, Japan). Fura-2/AM, U-46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α) and EGTA (ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid) were purchased from Dojindo (Kumamoto, Japan). Fura-PE3/AM was from Texas Fluorescence Laboratory (Austine, TX, U.S.A.). Pluronic F-127 was from BASF Co. (Parsippany, NJ, U.S.A.). Bovine serum albumin (BSA) was from Sigma (St. Louis, MO, U.S.A.). M-MLV (Moloney murine leukemia virus) reverse transcriptase, 5 × RT buffer, 0.1 M DTT (dithiothreitol) were from BRL (Gaithersburg, MD, U.S.A.). Nusieve 3:1 agarose, dNTPs (dATP, dCTP, dTTP, dGTP) and Hinf I were from TaKaRa (Kyoto, Japan). RNase inhibitor and ØX174/Hinc II digest were purchased from Toyobo (Osaka, Japan). Taq DNA polymerase was from Pharmacia Biotech. (Uppsala, Sweden). RQ1 RNase-free DNase was from Promega (Madison, WI, U.S.A.) All other chemicals were of the highest grade commercially available. The oligonucleotides for primers were synthesized by Sawady Technology (Tokyo, Japan).

Data analysis

All data for the simultaneous measurements of $[Ca^{2+}]_i$ and tension were collected with a computerized data acquisition system (MacLab, Analog. Digital instruments, Castle Hill, Australia: MacIntosh, Apple Computer, Cupertino, CA, U.S.A.). The data for the representative traces shown in this study were directly printed from the computer to a laser printer (LaserWriter II NTX-J, Apple Computer). The measured values were expressed as means ± s.e. (n = number of experiments). For each experiment, a strip from a different animal (3 – 5) was used. For the comparison, unpaired Student's t test was used and P values of less than 0.05 were considered to be significant.

Results

Detection of AM mRNA by RT-PCR

As shown in Figure 1a, the AM bands of the expected size (290 bp) could be detected by RT-PCR with total RNA prepared from the porcine coronary arterial smooth cells and the specific primers for the pig AM only when the cDNA was added. In this experiment, we used 3 coronary arteries from 3

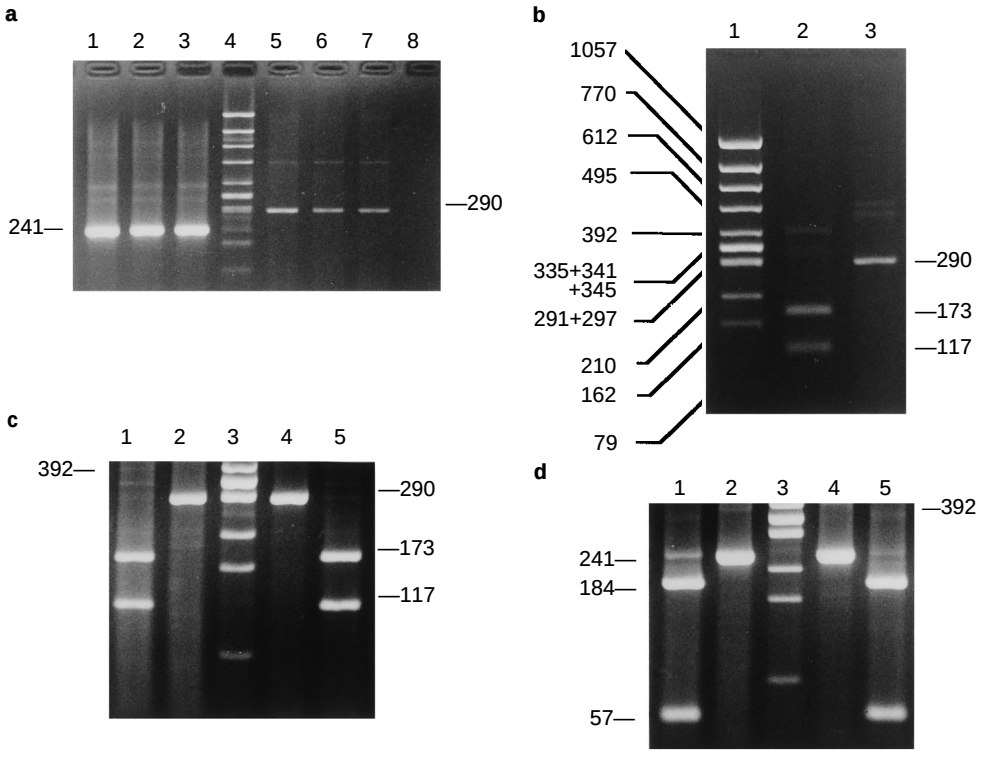

Figure 1 Detection of adrenomedullin (AM) and β-actin mRNAs in the porcine coronary artery (a) by RT-PCR and the identification of the PCR products for AM (b, c) and β-actin (d), using the restriction enzyme, Hinf I. (a) Detection of AM and β-actin mRNAs in the porcine coronary artery by RT-PCR. PCR amplifications for AM (lanes 5 – 7) and β-actin (lanes 1 – 3) were performed 30 and 25 cycles, respectively. The predicted sizes of these PCR amplifications are shown on the left (β-actin) or on the right (AM) of the photograph. The experiments were done with the total RNA from 3 different pigs (lanes 1 and 5 from pig 1, lanes 2 and 6 from pig 2, lanes 3 and 7 from pig 3). Lane 8 is a negative control in which cDNA was omitted during PCR amplification. Lane 4 represents the DNA size marker (ØX174/Hinc II digest). (b) Identification of the PCR products for AM by use of the restriction enzyme, Hinf I. PCR product for AM in the coronary artery (lane 3) was digested into 173 bp and 117 bp fragments (lane 2) by Hinf I, as predicted. Lane 1 represents the DNA size marker (ØX174/Hinc II digest). The size of each band is illustrated on the left side of the photograph. (c) Identification of the PCR products for AM by Hinf I in the porcine tracheal epithelium cells (lane 1, 2) and tracheal smooth muscle cells (lane 4, 5). The specific AM bands were digested into the expected size. (d) Identification of the PCR products for β-actin by Hinf I in the porcine tracheal epithelial cells (lane 1, 2) and tracheal smooth muscle cells (lane 4, 5). The specific β-actin bands were digested into the expected size.

different pigs. All 3 pigs expressed AM mRNA. The RT-PCR for β-actin was also performed to estimate the relative abundance of AM mRNA. The PCR amplifications for AM and β-actin were performed 30 and 25 cycles, respectively. Possible amplifications of the genomic AM sequence could be excluded since the band of the expected size was detected only when reverse transcriptase was added, as shown in Figure 2. To confirm the specificity of the RT-PCR shown in Figures 1a, 2 and 3a, the PCR products for AM and β-actin were digested by a restriction enzyme. As shown in Figure 1b and c, the PCR products for AM from the coronary artery, tracheal smooth muscle cells and tracheal epithelial cells could be digested by Hinf I into the fragments of the predicted size in agarose gel electrophoresis (173 + 117 bp). The PCR products for β-actin from the tracheal smooth muscle cells and tracheal epithelial cells could be digested into 184 + 57 bp fragments by Hinf I, as expected (Figure 1d). Thus, it is apparent that the AM mRNA is present in porcine coronary arterial smooth muscle cells.

AM mRNA was universally expressed in various tissues (Figures 2 and 3). The AM band was barely able to be detected in the renal vein and aortic valvular endothelial cells under the conditions described in Figure 2 legend (30 cycles of amplification). In all the tissues examined, there was no AM band detected when the reverse transcriptase was omitted during RT reaction, indicating that the AM bands shown in Figure 2 were amplified from the AM mRNA, not from genomic DNA.

In order to estimate the relative abundance of AM mRNA to β-actin mRNA in the various tissues, similar experiments to those shown in Figure 1a were carried out with the total RNA preparations from 3–4 different pigs in the various tissues. The ratios (AM mRNA/β-actin mRNA) of the band thickness determined by the densitometer of the negative film of each photograph are shown in Figure 3a.

Figure 3b shows the expressions of MLCK and Factor VIII mRNA in the selected tissues determined by methods similar to that described in Figure 3a. MLCK mRNA was abundantly expressed in smooth muscle preparations (bladder, trachea, ileum, aorta and main pulmonary artery), while it was not detected in epithelial (bladder and trachea) and aortic endothelial cells. In contrast, Factor VIII mRNA was abundantly expressed in aortic endothelial cells. The direct sequencing of the PCR products for Factor VIII revealed that the similarities compared with corresponding region of the human (Truett *et al*., 1985) and mouse (Elder *et al*., 1993) cDNA sequences were 85.1 and 84.8%, respectively. The similarity of the PCR product for MLCK compared with the corresponding region of the rabbit cDNA sequence (Gallagher *et al*., 1991) was 88.7%. These analyses strongly indicated that the PCR product obtained by Factor VIII and the MLCK primers in the present study derived from the pig Factor VIII and MLCK and cDNAs, respectively.

Effects of AM on the $[Ca^{2+}]_i$ and tension of the smooth muscle trips

Figure 4 shows representative recordings of the effect of 10 nM and 100 nM AM on increases in $[Ca^{2+}]_i$ and tension of the porcine coronary arterial strips during a contraction induced by 10 nM endothelin-1 (ET-1). Initially, the fluorescence ratio and the tension in response to 118 mM K^+ were recorded as a control (first contraction). The application of 10 nM ET-1 induced rapid increases in $[Ca^{2+}]_i$ and tension, which reached peak levels within 5 min and remained at these levels (steady state) for at least 40 min during the observations. The application of 10 nM and 100 nM AM during the steady-state contraction induced by 10 nM ET-1 decreased both $[Ca^{2+}]_i$ and tension in a concentration-dependent manner. The levels of $[Ca^{2+}]_i$ and tension at the steady state of the contraction induced by ET-1 just before the application of 10 nM AM were $80 \pm 3\%$ and $107 \pm 2\%$ ($n = 5$), respectively. AM (10 nM) significantly reduced both $[Ca^{2+}]_i$ and tension ($60 \pm 3\%$ and $58 \pm 8\%$, $n = 5$, respectively, $P < 0.01$ by Student's t test). Thus, the porcine coronary artery could be relaxed by AM with a decrease in $[Ca^{2+}]_i$.

Figure 5a shows a representative recording of the effect of 100 nM AM on the increase in $[Ca^{2+}]_i$ and tension of the porcine pulmonary venous strips during a contraction induced by 60 nM U46619, a thromboxane A_2 analogue. The application of 100 nM AM during the steady-state contraction induced

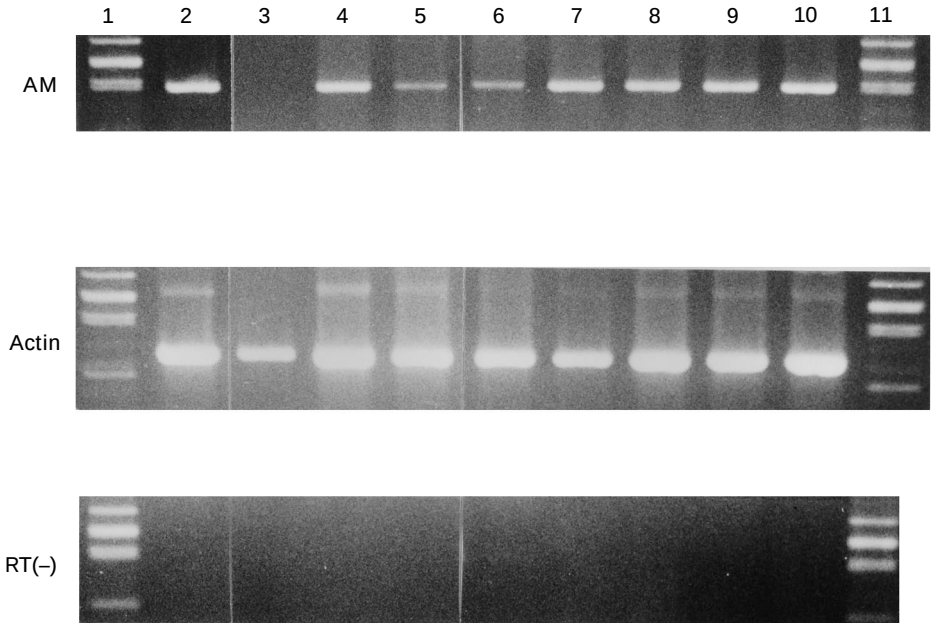

Figure 2 Representative photographs showing the distributions of adrenomedullin (AM) and β-actin mRNA in the various tissues. RT(-) represents the negative control in which the reverse transcriptase was omitted during RT reaction and PCR amplification for AM was performed in the same manner. The tissue used in each lane is as follows: lane 1 and 11, DNA size marker; lane 2, aortic endothelial cells; lane 3, renal vein; lane 4, renal artery; lane 5, coronary artery; lane 6, aorta; lane 7, kidney; lane 8, right atrium; lane 9, right ventricle; lane 10, left ventricle. PCR amplifications for AM and β-actin were performed 30 and 25 cycles, respectively.

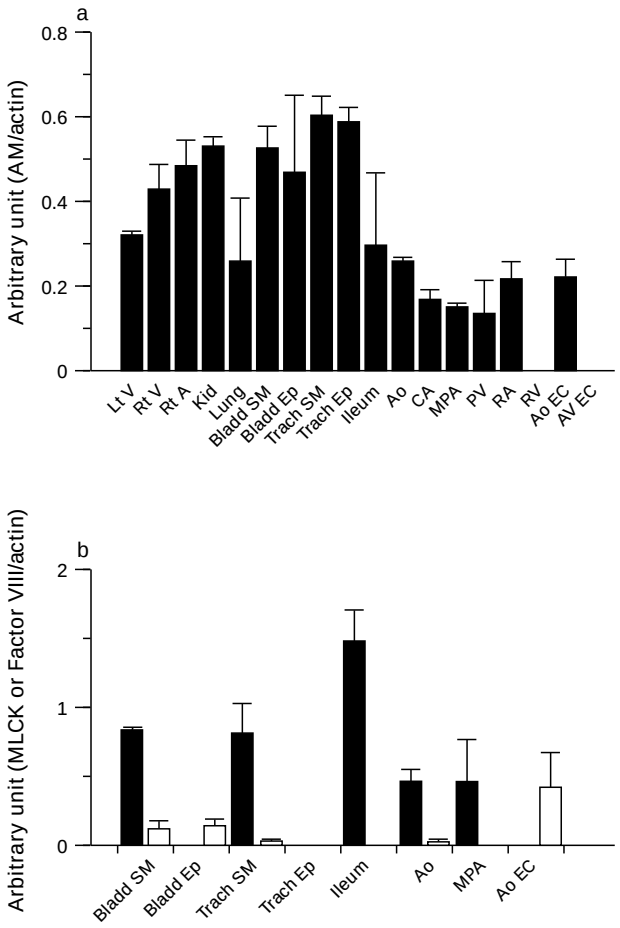

Figure 3 Summary of the expressions of adrenomedullin (AM) (a), myosin light chain kinase (MLCK) and Factor VIII (b) mRNAs in the various porcine tissues. (a) Similar experiments to those shown in Figure 1a were carried out with total RNA preparations from 3–4 different pigs and the ratios of AM band density/β-actin band density were plotted. PCR amplifications for AM and β-actin were performed for 30 and 25 cycles, respectively. Abbreviations used are: Lt V, left ventricle; Rt V, right ventricle; Rt A, right atrium; Kid, kidney; Bladd SM, urinary bladder smooth muscle; Bladd Ep, urinary bladder epithelium; Trach SM, tracheal smooth muscle; Trach Ep, tracheal epithelium; CA, coronary artery; MPA, main pulmonary artery; RA, renal artery; RV, renal vein; Ao EC, aortic endothelial cells; AV EC, aortic valvular endothelial cells. (b) Similar experiments to those shown in Figure 1a for MLCK (solid columns) and Factor VIII (open columns) were carried out with total RNA preparations from 3 different pigs and the ratios of MLCK or Factor VIII band density/β-actin band density were plotted. PCR amplifications for MLCK and Factor VIII were performed for 30 cycles.

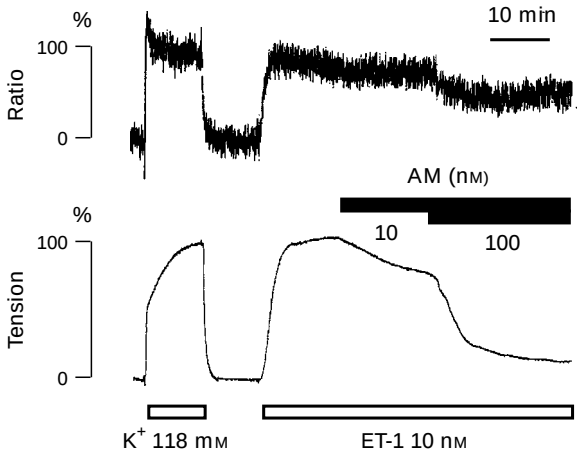

Figure 4 Representative recordings of the effect of 10 nM and 100 nM adrenomedullin (AM) on the increases in $[Ca^{2+}]_i$ and tension of the coronary strips during a contraction induced by 10 nM endothelin-1 (ET-1). First, the response, in terms of fluorescence ratio and the tension, to 118 mM K^+ was recorded as control (the first contraction). ET-1 (10 nM) induced sustained increases in $[Ca^{2+}]_i$ and tension and 10 nM and subsequently 100 nM AM added during this steady state contraction induced by ET-1 decreased $[Ca^{2+}{}_i]$ and tension.

by U46619 had no effect on either $[Ca^{2+}]_i$ or tension. The traces shown are representative of 3 similar independent experiments. Thus, in contrast to the coronary strips, the relaxing effect of AM could not be detected in the pulmonary venous strips. Figure 5b shows a representative time course of the effect of various concentrations of AM on the increase in $[Ca^{2+}]_i$ and tension of the tracheal strips during a contraction induced by 100 nM carbachol. The application of AM had no effect on either $[Ca^{2+}]_i$ or tension. The traces shown are representative of 3 similar independent experiments. As shown in Figure 6a 100 nM AM had no effect on the increase in $[Ca^{2+}]_i$ or tension of urinary bladder strips during a contraction induced by 10 nM ET-1. The traces shown are representative of 4 similar independent experiments. AM (100 nM) had no effect on the tension development of porcine ileac strips during a contraction induced by 300 nM carbachol (Figure 6b). The traces shown are representative of 5 similar independent experiments.

Discussion

In the present study, we have examined the hypothesis that AM is an autocrine and/or paracrine mediator of smooth muscle tone. The data obtained indicate that (1) AM mRNA is generally expressed in various tissues, including smooth muscle and neighbouring endothelial and epithelial cells, (2) AM induces relaxation of porcine coronary artery, but not pulmonary vein, (3) AM has no effect on the smooth muscle tone of the porcine trachea, ileum or urinary bladder.

There have been several studies which have shown the general expression of AM mRNA (Sugo *et al*., 1994a, b; Kitamura *et al*., 1993b; 1994; Sakata *et al*., 1993; Seguchi *et al*., 1995). In the present study, we extended this observation using several cell types, some of which have not been examined before. As shown in Figure 1a, AM mRNA was expressed in the porcine coronary artery. The specificity of the RT-PCR was confirmed by the experiment shown in Figures 1b and c, and 2. This is the first time that AM mRNA has been shown to be expressed in the coronary artery, main pulmonary artery, pulmonary vein, urinary bladder smooth muscle cells and epithelial cells, ileac smooth muscle cells (not whole ileum) and *in situ* (not cultured) endothelial cells (Figure 3a).

In order to assess the extent of the cross contamination of endothelial cells and smooth muscle cells in smooth muscle and non-smooth muscle preparations, respectively, we performed RT-PCR for Factor VIII, one of the oldest markers of endothelial cells (Burgdord *et al*., 1981; Reinders *et al*., 1988), and MLCK, a smooth muscle marker. MLCK mRNA was predominantly expressed in smooth muscle preparations and Factor VIII was predominantly expressed in aortic endothelial cells (Figure 3b), indicating that any cross contamination was not significant.

As to the expression of AM mRNA in the lung, Martinez *et al*. (1995) showed that AM was expressed in the columnar epithelium, some glands, neurones of the pulmonary parasympathetic nervous system, endothelial cells, chondrocytes, alveolar macrophages and smooth muscle cells of the human lung. We thus confirmed their results, since we also found that AM mRNA was expressed in tracheal smooth muscle and epithelial cells. These observations indicate that AM might be one of the epithelium derived-relaxing factors, which were first described by Flavahan *et al*. (1985). However, this possibility

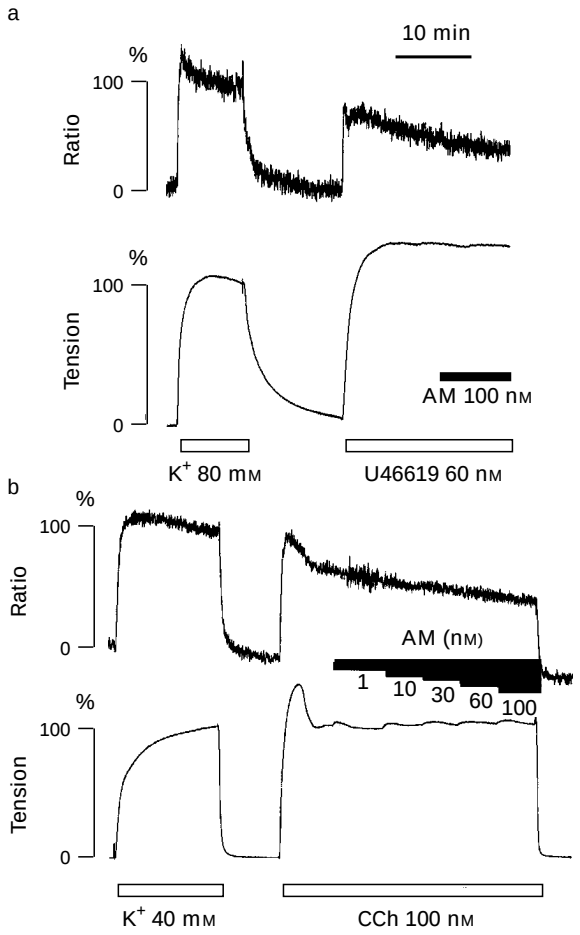

Figure 5 Effect of adrenomedullin (AM) on the $[Ca^{2+}]_i$ and tension of the (a) pulmonary venous and (b) tracheal strips. (a) Representative recordings of the effect of 100 nM AM on the increases in $[Ca^{2+}]_i$ and tension of the pulmonary venous strip during the contraction induced by 60 nM U46619, a thromboxane A_2 analogue. The application of 100 nM AM had no effect on the $[Ca^{2+}]_i$ and tension. The traces shown are representative of 3 similar experiments. (b) Representative recordings of the effect of AM on the increases in $[Ca^{2+}]_i$ and tension of the tracheal strip during the contraction induced by 100 nM carbachol (CCh). The cumulative applications of the various concentrations of AM had no effect on the $[Ca^{2+}]_i$ and tension. The traces shown are representative of 3 similar experiments.

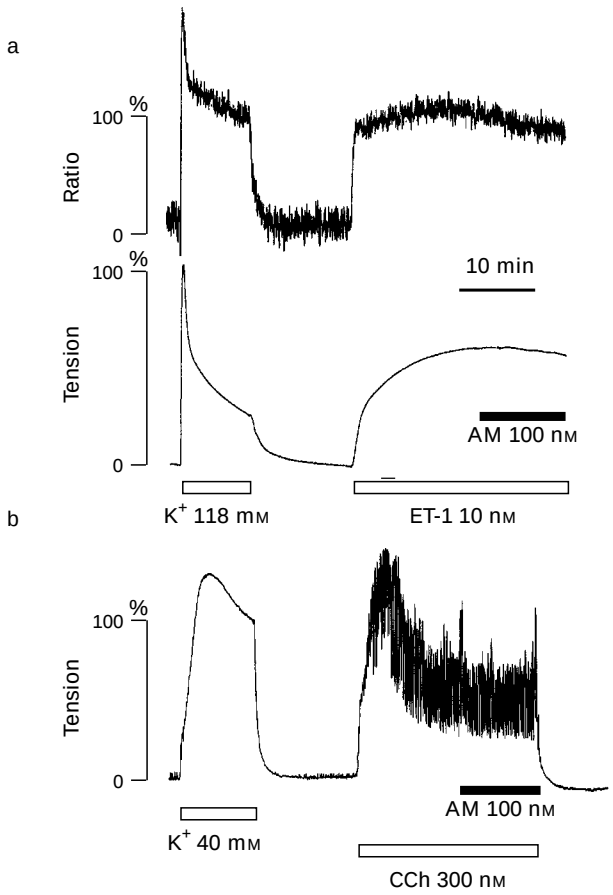

Figure 6 Effect of adrenomedullin (AM) on (a) the $[Ca^{2+}]_i$ and tension of the urinary bladder strip and (b) on the tension of the porcine ileac strip. (a) Representative recordings of the effect of 100 nM AM on the increases in $[Ca^{2+}]_i$ and tension of the urinary bladder strip during the contraction induced by 10 nM ET-1. The application of 100 nM had no effect on the $[Ca^{2+}]_i$ and tension. The traces shown are representative of 4 similar experiments. (b) Representative recordings of the effect of AM on the tension development of the ileum strip during the contraction induced by 300 nM carbachol (CCh). The strip showed an oscillatory contraction. The application of 100 nM AM had no effect on the tension. The traces shown are representative of 5 similar experiments.

may not be valid, at least in trachea, since AM had no effect on the tracheal smooth muscle tone (Figure 6). On the other hand, Kanazawa *et al*., (1994) showed that AM has a bronchodilator action *in vivo*. Thus, it is still not clear whether or not AM is an epithelium derived-relaxing factor in the bronchus or bronchioles.

Similarly, Sugo *et al*. (1994a) have proposed that AM may be one of the endothelium derived-relaxing factors. They observed 20–40 times higher expression of AM mRNA in cultured endothelial cells than in the adrenal gland. We also observed the expression of AM mRNA in the porcine aortic endothelial scrape (Figure 1). However, the expression level was not so high as that observed by Sugo *et al*. (1994a). This could be explained by differences in the endothelial preparation, namely, cultured endothelial cells vs. *in situ* endothelial cells. Furthermore, there might be a difference in AM mRNA expression between endothelial cells from different sites, because the expression of AM mRNA in endothelial cells of the aortic valve was much lower than that in aortic endothelial cells in the present study (Figure 3a).

Relaxation induced by AM was observed in the porcine coronary artery (Figure 4 and Kureishi *et al*., 1995) and renal artery (Seguchi *et al*., 1995). Thus, we deduced that smooth muscle cells of the coronary and renal arteries have receptors which are responsible for this AM-mediated relaxation, presumably AM receptors. However, at present, there is controversy about a receptor(s) responsible for AM-induced vasorelaxation. Nuki *et al*. (1993) first proposed that calcitonin gene-related peptide (CGRP) receptors are involved in AM-induced vasorelaxation, since AM-induced vasorelaxation in the rat perfused mesenteric vascular bed could be inhibited by a CGRP receptor antagonist, CGRP [8-37]. Whereas, Kato *et al*. (1995) demonstrated that the cultured endothelial cells of human umbilical vein possess specific AM receptors coupled with adenylate cyclase activity that may have little affinity with CGRP. These controversial opinions can be classified into three groups: (1) AM receptors are the same as CGRP receptors (Nuki *et al*., 1993; Ishizaka *et al*., 1994; Baskaya *et al*., (1995), (2) AM receptors are different from CGRP receptors (Kato *et al*., 1995; Nakamura *et al*., (1995), (3) AM has its own specific receptors with which CGRP interacts (Eguchi *et al*., 1994; Osajima *et al*., 1995). Furthermore, Owji *et al*. (1995) have described two types of AM binding sites which have different molecular weights and pharmacological properties, of which one is sensitive to CGRP and the other is not. A full explanation for this controversy needs to await the cloning of AM receptors. However, it is obvious that there are receptors

which are responsible for AM mediated relaxation of the vascular smooth muscle, even if they are the same as CGRP receptors. Thus, it appears that AM may be an autocrine and/or paracrine regulator of coronary and renal arterial tone, because AM mRNA was expressed in the coronary artery, renal artery and endothelial cells (Figure 1).

However, in the pulmonary vein, we could not detect any effect of AM on the contractile tone (Figure 5a), even though AM mRNA was detected in this vein (Figure 3a). Thus, it is concluded that AM may not play a major role as an autocrine and/or paracrine transmitter in the regulation of the pulmonary venous tone. However, there is still a possibility that AM might operate as an autocrine and/or paracrine transmitter for the regulation of the smooth muscle cell proliferation, because AM might have an effect on the cell cycle progression of vascular smooth muscle cells (Kobayashi *et al*., 1995). Recently, Sato and Autelitano (1995) showed that AM induces *c-fos* mRNA in rat vascular smooth muscle cells and cardiomyocytes. Interestingly, Satoh *et al*. (1995) and Martinez *et al*. (1995) found that AM is expressed in tumour cells and, hence, it might be involved in carcinogenesis. These data support the idea that the function of AM in smooth muscle might be on the regulation of cell proliferation rather than relaxation.

Since the results shown above indicated that AM induces relaxation of some vascular smooth muscle strips, we wanted to determine whether similar autocrine and/or paracrine systems might be operating in the regulation of smooth muscle tone in muscles other than vascular smooth muscle. As shown in Figure 3a, AM mRNA was expressed in tracheal smooth muscle cells, tracheal epithelial cells, urinary bladder smooth muscle cells, urinary bladder epithelial cells and ileac smooth muscle cells. However, AM had almost no effects on the contractions of these smooth muscles (Figures 5b and 6(a and b)). Although we tried various concentrations of different kinds of agonists for the precontraction, we could not observe any AM-induced relaxation of these strips. Thus, these observations indicate that the autocrine and/or paracrine regulation of the smooth muscle tone may not operate in the trachea, urinary bladder or ileum.

However, the effect of AM is not limited to the relaxation of smooth muscle. In addition to a vasorelaxing effect AM has also been found to have other functions such as a natriuretic action (Jougasaki *et al*., 1995), an inhibitory action on cytokine-induced neutrophil chemoattractants from alveolar macrophages (Kamoi *et al*., 1995); a bronchodilator effect *in vivo* (Kanazawa *et al*., 1994); to induce relaxation of hepatic pericytes (Kawada & Ione, 1994); an inhibitory action on endothelin-1 production (Kohno *et al*., 1995a), pituitary adrenocorticotropin release (Samson *et al*., 1995) and aldosterone secretion from adrenal zona glomerulosa cells (Yamaguchi *et al*., 1994). Thus, there is a possibility that AM might be involved in functions other than the smooth muscle tone of the trachea, ileum and urinary bladder as a local autocrine and/or paracrine regulator. Overall, the limited effects of AM on smooth muscle tone, despite the general expression of the AM mRNA, might indicate the possible involvement of AM in cellular functions other than the regulation of tone in smooth muscle.

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