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Lack of involvement of endothelin-1 in angiotensin II-induced contraction of the isolated rat tail artery

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1 The contribution of endothelin-1 (ET-1) to angiotensin II (Ang II)-mediated contraction of the isolated rat tail artery was assessed with measurements of tension, and cytosolic calcium ($[Ca^{2+}]_i$). The distribution of the AT_1 receptor was studied with RT–PCR and immunohistochemistry.

2 Ang II induced an endothelium-independent contraction (pEC_{50} 7.95 ± 0.06 and E_{max}: $0.46\ g \pm 0.05$ with endothelium *vs* 7.81 ± 0.02 and $0.41\ g \pm 0.07$ without endothelium; $P > 0.05$). Ang II (0.003–0.3 μM)-induced a non-sustained contraction of endothelium-intact preparations which was not antagonized by BQ-123 (1 μM), but was inhibited by losartan (10 nM). In addition, the maximal contraction induced by ET-1 (0.1 μM) could be further increased by the addition of 0.1 μM Ang II.

3 Ang II (0.001–0.3 μM) elevated $[Ca^{2+}]_i$ in single vascular smooth muscle cells (VSMCs) in a dose-dependent manner (pEC_{50} 9.12 ± 0.26) and the Ang II-induced increases in $[Ca^{2+}]_i$ were not affected by a Ca^{2+}-free solution, but were abolished by pretreatment with caffeine (5 mM). Ang II did not increase $[Ca^{2+}]_i$ in endothelial cells. ET-1 (0.1 μM) increased $[Ca^{2+}]_i$ in single VSMCs in a normal Ca^{2+} containing physiological saline solution (PSS), but not in a Ca^{2+}-free solution.

4 Ang II-induced contraction was insensitive to inhibition by nifedipine (0.1 μM), an antagonist of L-type voltage-gated Ca^{2+} channels, and SK&F96365 (10 μM), which blocks non-selective cation channels, whereas that to ET-1 was inhibited by SK&F69365.

5 RT–PCR data indicate the expression of AT_{1A} and AT_{1B} on both VSMCs and endothelial cells, but immunohistochemical evidence illustrates that the AT_1 is located primarily on VSMCs.

6 These results indicate that endothelium-derived ET-1 is not involved in the Ang II-mediated vasoconstriction of the rat tail artery and that Ang II- and ET-1-mediated VSM contractions utilize distinct pathways.

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Keywords: Angiotensin II; endothelin-1; vasoconstriction; cytosolic free Ca^{2+}; vascular smooth muscle cell; endothelial cell; rat tail artery

Abbreviations: Ang II, angiotensin II; AT_1, angiotensin 1 receptor; BSA, Bovine serum albumin; DMEM, Dubecco's modified essential medium; EGTA, Ethyleneglycol-bis-(β-amino-ethyl ether) n, n′-tetra-acetic acid; ET-1, endothelin-1; ET_A, endothelin A receptor; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; OCT, optimum cutting temperature; PBS, phosphate buffered saline; PE, phenylephrine; PSS, physiological salt solution; RT–PCR, reverse transcription-polymerase chain reaction; TI, soybean trypsin inhibitor; VSMC, vascular smooth muscle cell

Introduction

Angiotensin II (Ang II) exerts a wide range of actions in the kidney, heart, vascular system and central nervous system *via* binding to AT_1 and AT_2 receptor subtypes (Timmermans *et al*., 1993; de Gasparo *et al*., 1995). There are two subtypes of AT_1 receptors, AT_{1A} and AT_{1B}, that are encoded by two different genes in the rat (Lewis *et al*., 1993), but have similar amino acid sequences, pharmacological specificities and signal transduction pathways (Chiu *et al*., 1993). In the vascular system, Ang II is a potent agonist that causes contraction of vascular smooth muscle (VSM) primarily *via* the activation of AT_1 receptors (Timmermans *et al*., 1993). Although an important role for Ang II as an endogenous regulator of VSM tone is well established, the responses to Ang II vary in different vascular beds and among VSM preparations (Bottari *et al*., 1993). The heterogeneity of the tissue response to Ang II may result from the modulatory role of the vascular endothelium (Toda *et al*., 1990; Cortes *et al*., 1996) or from differences in the properties and distribution of Ang II receptor subtypes on vascular endothelium and the underlying VSMCs, as well as species differences (Toda *et al*., 1978; Boulanger *et al*., 1995; Pueyo *et al*., 1997).

Chen *et al*. (1995a,b) have reported that there are endothelium-dependent regional variations in vascular tissue that are sensitive to Ang II and that the vasoconstrictor response to Ang II in the rat tail artery is mediated by Ang II-mediated release of endothelial cell-derived ET-1 that acts on ET_A receptors in the rat tail artery VSM. Ang II can bind to AT_1 receptors on VSMCs and directly activate vasoconstriction (Timmermans *et al*., 1993), and thus it remains controversial whether the vasoconstrictor action of Ang II is entirely due to the release of endothelial cell derived ET-1. The objective of the current study was to determine the role of the endothelium in modulating the response to Ang II-induced smooth muscle contraction and the cellular mechanisms of contraction in the rat tail artery. In addition, we have also

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systematically compared the pharmacological properties and signal transduction pathways for both Ang II- and ET-1-induced vasoconstriction as well as used RT–PCR and immunocytochemistry to study the distribution of AT_1 in the rat tail artery. SK&F96365 was initially introduced as a blocker of receptor-mediated Ca^{2+} influx in a broad sense (Merritt *et al*., 1990). So far the effects of SK&F96365 have been examined in only a limited number of cell types and in all these cells, the drug has been found to block some of the voltage-independent Ca^{2+} channels (Chung *et al*., 1994; Franzius *et al*., 1994; Koch *et al*., 1994; Wayman *et al*., 1996). Some of the data presented in this study have been previously published in abstract form (Jiang *et al*., 1998; 1999).

Methods

Tension measurement

Male Sprague-Dawley rats (400–450 g) were stunned and killed by cervical dislocation following a protocol approved by the Canadian Council of Animal Care. The tail artery was quickly excised and placed in a cold physiological salt solution (PSS) of the following composition (in mM): NaCl 118; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; dextrose 11.1. The pH of the solution after saturation with 95% O_2 + 5% CO_2 gas mixture was 7.4. Adherent connective tissues were cleaned carefully from the artery which was then cut into 5 mm rings. In some experiments, the endothelial cell layer was removed by repeatedly passing a stainless steel wire through the vessel lumen. Each ring was suspended between platinum hooks and mounted in a 20 ml organ bath containing PSS maintained at 37°C and gassed continuously with 95% O_2 + 5% CO_2. The rings were stretched in a stepwise fashion to an optimal tension of 0.8 g. The tissues were equilibrated for 2 h with a PSS change every 20 min. After equilibration, the tissue viability was determined by exposing the tissue to 1 μM phenylephrine (PE), and the presence of a functional endothelium was assessed on the basis of the ability of acetylcholine (10 μM) to induce a greater than 20% relaxation. The absence of a relaxation response to acetylcholine was taken as evidence that the vessel segments were functionally denuded of endothelium. In some protocols, tissues were pretreated for 20 min with nifedipine (1 μM) and caffeine (2 mM) before adding Ang II (1 μM). After ET-1-induced contraction was sustained, SK&F96365 (10 μM) or Ni^{2+} (2 mM) was added to test the involvement of receptor-mediated calcium influx or nonselective cation channel. Isometric tension was recorded with a force displacement transducer (Grass FT 03) coupled to a Grass polygraph model 7E.

Cell preparation and intracellular Ca^{2+} measurement

Male Sprague-Dawley rats (400–450 g) were anaesthetized with halothane, and surgical anaesthesia was maintained with methoxyflurane while the lower abdomen was opened to expose the abdominal aorta. The common iliac arteries were located and tied off. The abdominal aorta was cannulated to infuse 10 ml Dubecco's modified essential medium (DMEM) and then the tail was immediately cut off. Following cleaning and removal of the connective tissue, the artery was cut into several pieces and incubated for 30–60 min (according to the activity of collagenase IV) in 1 ml DMEM with collagenase IV (2 mg ml^{-1}), dispase IV (2 mg ml^{-1}), elastase III (20 units ml^{-1}) and collagenolytic proteinase IV (0.085 units ml^{-1}) at 37°C. The enzymatic reactions were stopped by replacing the solution with DMEM supplemented with 2 mM ethyleneglycol-bis-(β-amino-ethylether) n, n'-tetra-acetic acid (EGTA) and 0.1 mg ml^{-1} soybean trypsin inhibitor (DMEM-EGTA-TI) for 5 min and removing the digestion dish to room temperature. Trituration of the vessels was commenced whilst still in the presence of the DMEM-EGTA-TI which was removed and replaced with DMEM plus 5% BSA (Bovine serum albumin) once the cells started to separate from the vessels. The cells were placed on ice before use. The isolated smooth muscle and sheets of endothelial cells were visualized using a microscope, and smooth muscle cells were physically separated with a glass micropipet and identification was verified by immunocytochemisty. The identification of smooth muscle cells was confirmed by positively stained anti-smooth muscle α-actin, whereas endothelial cells were stained by anti-human Von Willibrand factor. Details of the immunochemistry protocols for α-actin and Von Willibrand factor are provided under *Immunocytochemistry*. Cells were loaded with fluorescent dye by incubation in HEPES solution containing 5 μM fura-2-acetoxymethylester for 35 min at room temperature. The cells were then washed and allowed to cleave the dye to the active fura-2 for 15 min. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 and 380 nm were monitored at an emission of 510 nm in single smooth muscle cells or sheets of endothelial cells at room temperature using an SLM-AMINCO 8000 spectrofluorometer (SLM Instruments Inc., U.S.A.) interfaced with a NIKON Diaphot inverted microscope, and the peak ratio of 340 to 380 nm, which was subtracted from the background ratio, was used to indicate intracellular Ca^{2+} changes according to Grynkiewicz *et al*. (1985).

RT–PCR

Rat tail arteries were dissected as described above, and endothelium-intact and -denuded arteries were frozen with liquid nitrogen. Freshly dispersed VSMCs and endothelial cells, prepared as described under *Cell preparation and intracellular Ca^{2+} measurement*, were centrifuged for 3 min at high speed (12,000 × *g*) at room temperature. mRNA was extracted from the arteries and isolated cells using Poly (A) Pure™ MicroPoly (A) Pure™ *mRNA Isolation Kits* from Ambion. RT–PCR was performed by using RETROscript™ *First-Strand Synthesis Kit for RT–PCR* from Ambion. Single-stranded cDNA was synthesized from 2 μg of mRNA in the presence of Moloney murine leukaemia virus reverse transcriptase and the first-strand random primers (provided with the kit). Oligonucleotide primers were chosen from the rat AT_1 genes (Murphy *et al*., 1991); the antisense primer was 5′-GCA CAA TCG CCA TAA TTA TCC-3′ (position 739–719 bp) and the sense primer was 5′-CAC CTA TGT AAG ATC GCT TC-3′ (position 295–314 bp, Murphy *et al*., 1991), which was expected to yield a single fragment of 444 base pairs. Digestion by *Eco*RI was used to differentiate between the AT_1 receptor subtypes: this digestion does not affect the AT_{1B} product, but hydrolyses that of AT_{1A} into two fragments: 269 bp and 175 bp (Pueyo *et al*., 1998). The PCR products were digested by *Eco*RI (17000 u μl^{-1}) for 3 h at 37°C. To visualize the RT–PCR reaction, 20 μl RT–PCR product was run on 1.5% agarose gel in the presence of ethidium bromide (4 μl), and the product was visualized under u.v. light. The undigested band obtained from

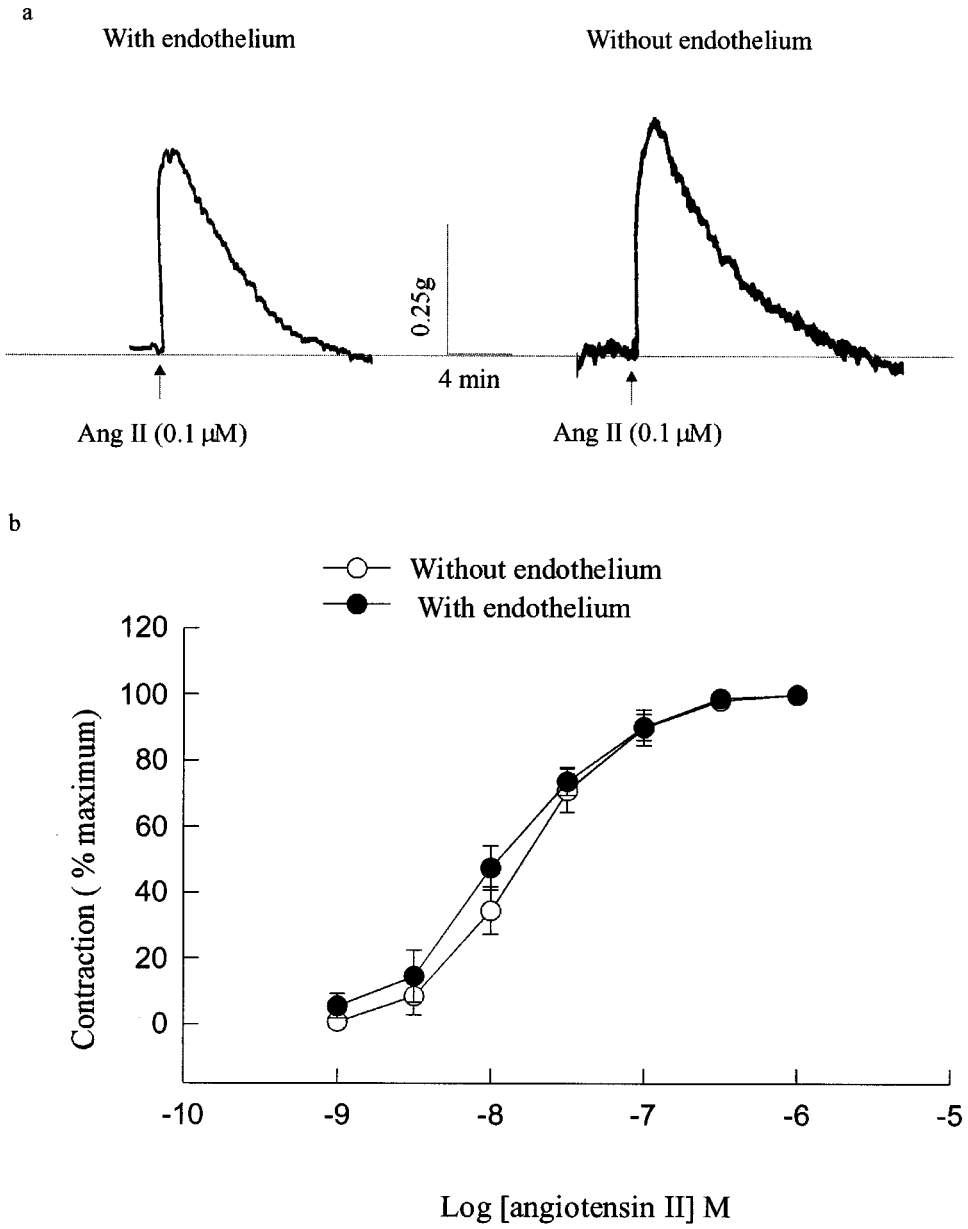


Figure 1 Effect of the endothelium on angiotensin II (Ang II)-induced contraction of the rat tail artery. (a) Representative tension recordings of Ang II-induced contractions of the rat tail artery in the presence and absence of the endothelium. (b) Cumulative concentration-response curves to Ang II in the absence of endothelium or presence of endothelium in the rat tail artery. Each point represents the mean ± s.e.mean, $n = 8-9$.

endothelium-intact tissue was extracted from agarose gel using GEL ECLIPSE™ DNA Purification Kit and sequenced with ABI PRISM™ Kit.

Immunohistochemistry and immunocytochemistry

The tail artery was quickly excised and fixed in Modified Zamboni Fixative (100 ml composed of the following compounds: 50 ml of D.D. H_2O, 2 g of paraformaldehyde (2%), to which a few drops of 2 M NaOH were added until the solution became clear, 50 ml of 0.2 M phosphate buffer, and 0.5 ml of 0.5% picric acid, pH 7.4). After washing five times at 5 min intervals with phosphate buffered saline (PBS), the tail artery was placed overnight at 4°C in 20% sucrose in PBS, then embedded in optimium cutting temperature (OCT) compound and frozen prior to being cut into 25 μm thick slices and mounted on slides treated with poly-D-lysine. The slices were skinned by addition of 0.5% Triton X-100 in PBS for 5 min and incubated for 1 h with anti-rabbit serum to decrease nonspecific binding. The slices were treated with the following: (1) polyclonal anti-AT_1 rabbit IgG (diluted 1 : 200) in 3% BSA at 4°C overnight; (2) AT_1 blocking peptide (100 times higher than anti-AT_1 rabbit IgG) for 1 h at room temperature, and then after washing, the slices were incubated with polyclonal anti-AT_1 rabbit IgG (diluted 1 : 200) in 3% BSA at 4°C overnight and (3) PBS alone. After excess antibodies were removed by washing twice with PBS and once with 3% BSA in PBS for 5 min each, the slices were incubated with CY™3-conjugated goat anti-rabbit IgG (diluted 1 : 1000) in 3% BSA in PBS for 1 h in the dark at room temperature. After being washed three times at 5 min intervals with PBS, the slices were mounted in 90% glycerol, and examined with a Nikon Microphoto-FXA fluorescent microscope.

ECs and SMCs were isolated from the rat tail artery as previously described under *Cell preparation and intracellular*

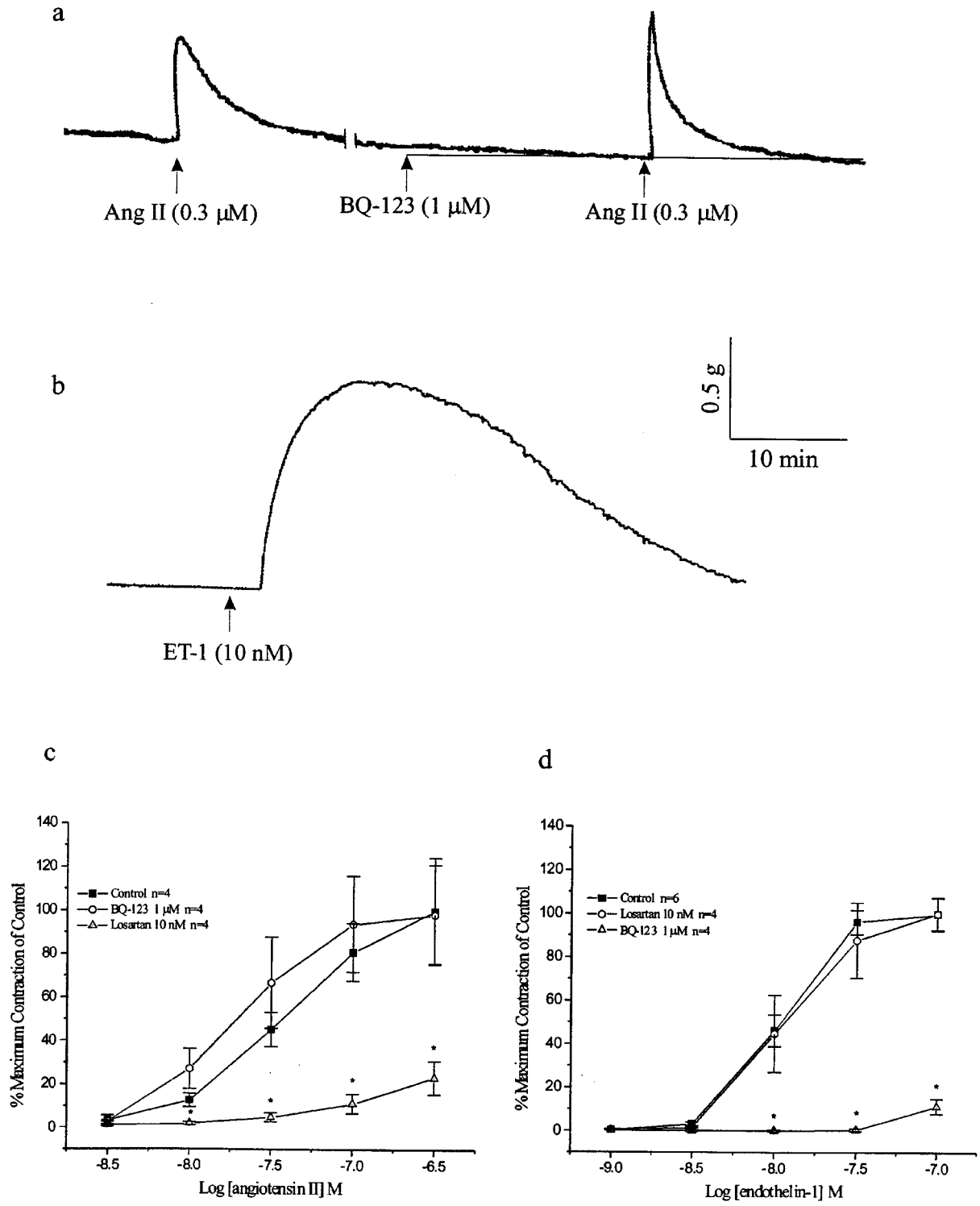


Figure 2 Angiotensin II (Ang II) and endothelin-1 (ET-1) mediated contractions of the rat tail artery. Typical tension tracings of: (a) Ang II-induced phasic contraction, illustrating the lack of effect of the ET_A antagonist BQ-123 (1 μM), and (b) ET-1-induced tonic vasoconstriction. Effects of 20 min pre-treatment with BQ-123 (1 μM), and losartan (10 nM), a selective AT_1 antagonist, on the response to 0.003–0.3 μM Ang II- (c) or 0.001–0.1 μM ET-1 (d) -induced dose-response curves in rat tail artery. Data represent the mean$\pm$s.e.mean ($n=4-6$). *$P<0.05$ compared to control using ANOVA and Student-Newman-Keuls test.

Ca^{2+} measurement. The cell suspensions were centrifuged at $500\times g$ for 5 min at room temperature. The cells were fixed with formalin (0.1%) in PBS for 15 min, washed with PBS 3×5 min, and then skinned by 0.5% Triton X-100 for 5 min. The cells were washed with PBS for 5 min and 3% BSA in PBS 2×5 min. The cells were incubated with: (1) Monoclonal anti-smooth muscle α-actin clone 1A4 (diluted 1 : 500); (2) Polyclonal anti-human Von Willebrand factor (diluted 1 : 2500) in 3% BSA for 1 h at room temperature. Excess antibodies were removed by washing with PBS for 2×5 min and 3% BSA in PBS for 5 min. The cells were then incubated with TRITC-conjugated anti-mouse IgG (diluted 1 : 1000), or CY3^{TM}-conjugated goat anti-rabbit IgG (diluted 1 : 1000), respectively, in 3% BSA for 1 h in the dark at room temperature. After washing 3×5 min with PBS, the cells were mounted in 90% glycerol, and

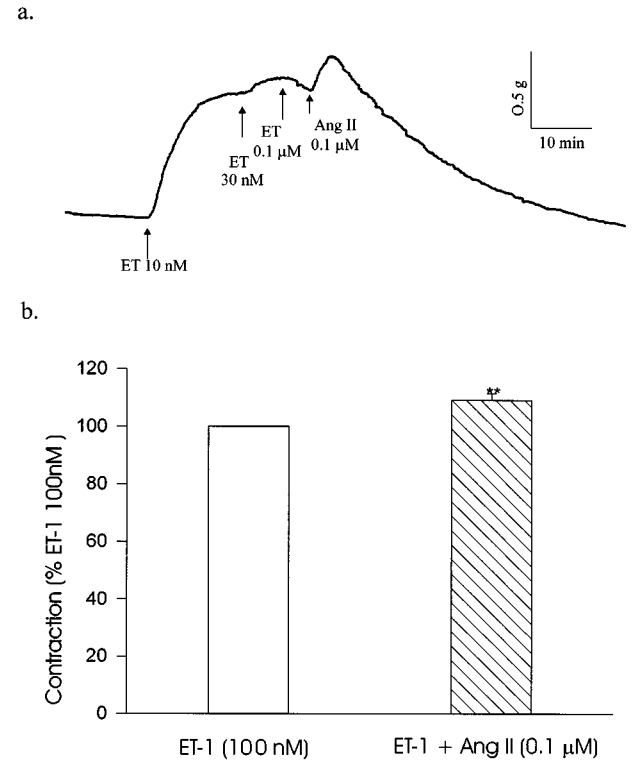


Figure 3 The effect of the addition of Ang II (0.1 μM) to a maximal contraction to ET-1 (0.1 μM). (a) A typical tracing of Ang II-induced contraction on top of maximal response to ET-1. (b) Summary of nine experiments. $^{**}P<0.01$ Ang II plus ET-1 compared to ET-1 using Student's paired *t*-test.

examined with a Nikon Microphoto-FXA fluorescent microscope.

Chemicals and drugs

L-phenylephrine hydrochloride, acetylcholine bromide, angiotensin II, nifedipine, collagenase IV, dispase IV, elastase III, collagenolytic proteinase, primer anti-bodies and secondary anti-bodies were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.); Endothelin-1, BQ-123 sodium, B-788, SK&F96365 were purchased from Research Biochemicals, Inc. (Natick, MA, U.S.A.); caffeine from J.T. Baker Chemical Co. (U.S.A.); fura-2/AM (fura-2-acetoxy methylester) from Molecular Probes Inc. (U.S.A.); MEM Vitamin solution and MEM Amino Acids solution from Gibco BRL., Bovuminar Reagent Pure Powder (Clin. REAG. GRADE BSA) from Intergen Company, and losartan was received as a gift from Merck Frost Canada. Pure™ *mRNA Isolation Kits* and RETROscript™ *First-Strand Synthesis Kit for RT–PCR* were obtained from Ambion. AT_1 receptor antibody and blocking peptide were from Santa Cruz Biotechnology, Inc.

Data analysis

The results are expressed as means ± s.e.mean and the differences between means determined by Student's *t*-test or one way analysis of variance (ANOVA). A *P* value less than 0.05 was considered significant. Data points from individual dose response curve were fitted to a sigmoidal curve using Microcal Origin 5.0. version software. pEC_{50} values were determined from each curve as the negative log molar concentration of drug which produced 50% of the maximal effect.

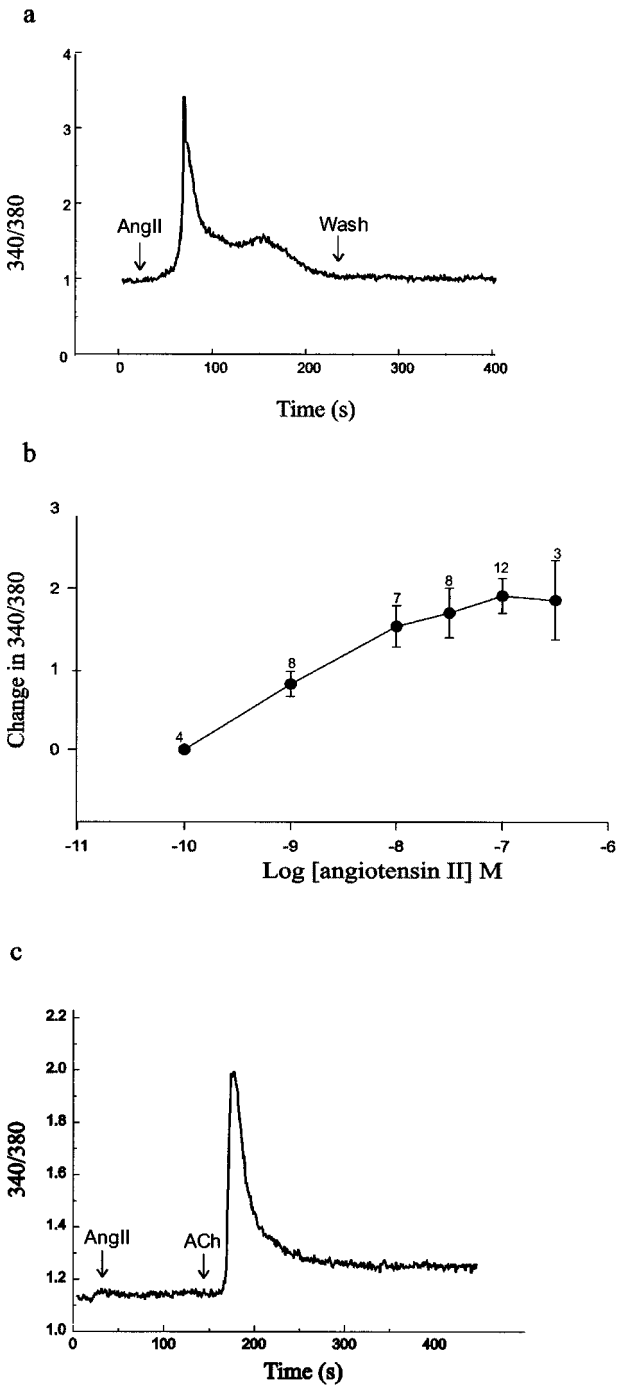


Figure 4 Ang II-induced increase in $[Ca^{2+}]_i$ of freshly dispersed single smooth muscle cells and endothelial cells from rat tail artery. (a) A typical response to Ang II (10 nM) increased $[Ca^{2+}]_i$ in single smooth muscle cells ($n=7$). (b) Concentration response curve for Ang II-induced increase in $[Ca^{2+}]_i$ of single smooth muscle cells. Data represent the mean ± s.e.mean ($n=3-12$). (c) A tracing illustrating the lack of response of endothelial cells to Ang II (0.1 μM), but ability to respond to acetylcholine (ACh, 10 μM). This response was observed in eight experiments.

Results

Ang II-induced contraction in the presence and absence of the endothelium

In the presence of the endothelium, Ang II elicited a concentration-dependent vasoconstriction of rat tail artery ring preparations (Figure 1). Similar levels of submaximal tension were obtained either by the stepwise cumulative

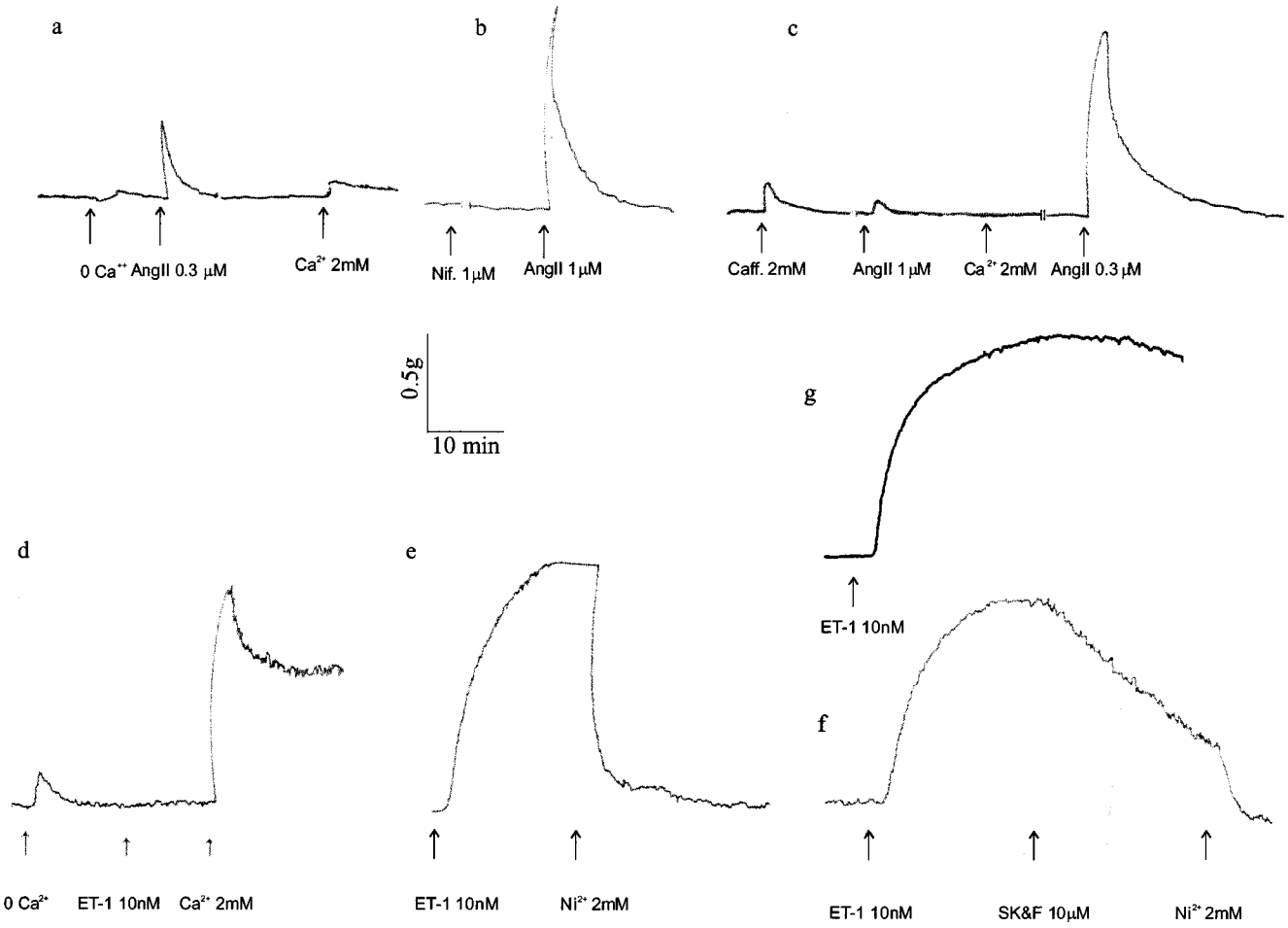


Figure 5 Ca^{2+} sources for Ang II- and ET-1-mediated contractions of rat tail artery. For Ang II the following conditions were used: (a) Ca^{2+}-free solution (Ca^{2+} replaced by 1 mM EGTA in Krebs solution); (b) pretreatment with nifedipine (Nif, 1 μM) for 20 min; (c) pretreatment with 2 mM caffeine (Caff) for 20 min. For ET-1 the following conditions were used: (d). Ca^{2+}-free solution; (e) 2 mM Ni^{2+}; (f) 10 μM SK&F96365; (g) Time course for (e) and (f). For each protocol similar results were observed in 3–7 experiments.

additions of Ang II or by the addition of a single submaximal concentration of Ang II (0.1 μM). Similarly, in the endothelium-denuded preparations, Ang II evoked vasoconstriction of the rat tail artery at either a single concentration or in a concentration-dependent manner (Figure 1a,b). No significant differences were found in either pEC_{50} values (7.95 ± 0.06 *vs* 7.81 ± 0.02, $P > 0.05$) or maximal contraction responses (0.46 ± 0.05 *vs* 0.41 ± 0.07, $P > 0.05$) to Ang II in endothelium-intact ($n = 9$) or endothelium-denuded preparations ($n = 8$), respectively.

Comparison of Ang II- and ET-1-induced contractions

The nature of the Ang II and ET-1 evoked contractile responses was quite different. Ang II (0.3 μM) induced a non-sustained contraction that reached a peak within 5–10 s and then within 8–10 min returned to baseline. In contrast, ET-1 (10 nM) evoked a contraction that reached a plateau over 8–10 min and was maintained for about 10–20 min and then very slowly (over 30–40 min) declined to baseline (Figure 2a,b). The Ang II (0.003–0.3 μM)-induced dose-response curve was not affected by BQ-123 (1 μM), a selective ET_A receptor antagonist, but was significantly inhibited by losartan (10 nM), a selective AT_1 receptor antagonist (Figure 2c). In contrast, the ET-1 (0.001–0.1 μM)-induced dose-response curve was not affected by losartan (10 nM), but was significantly inhibited by BQ-123 (1 μM) (Figure 2d). We also investigated the contractile action of Ang II in tissues maximally contracted with ET-1. As illustrated in Figure 3, ET-1 elicited a concentration-dependent contraction that reached a maximum at 0.1 μM. To further investigate whether Ang II and ET-1 were utilizing the same signalling pathways, 0.1 μM Ang II was added on top of the maximal response to ET-1 and this evoked an additive non-sustained contraction of the rat tail artery (Figure 3).

Ang II-induced $[Ca^{2+}]_i$ changes

Measurement of intracellular Ca^{2+} concentration was made in freshly dispersed single endothelial and smooth muscle cells from the rat tail artery (Figure 4). Ang II induced an increase in $[Ca^{2+}]_i$ of smooth muscle cells in a concentration-dependent manner with a pEC_{50} value of 9.12 ± 0.26 (Figure 4a,b); the increase in $[Ca^{2+}]_i$ occurred simultaneously with contraction of single smooth muscle cells which was observed as a change from spindle to rounded-shape (data not shown). In contrast, a high concentration of 0.1 μM Ang II did not increase the $[Ca^{2+}]_i$ of endothelial cells, whereas, in the same endothelial cells, acetylcholine (10 μM) induced a significant increase in $[Ca^{2+}]_i$ (Figure 4c).

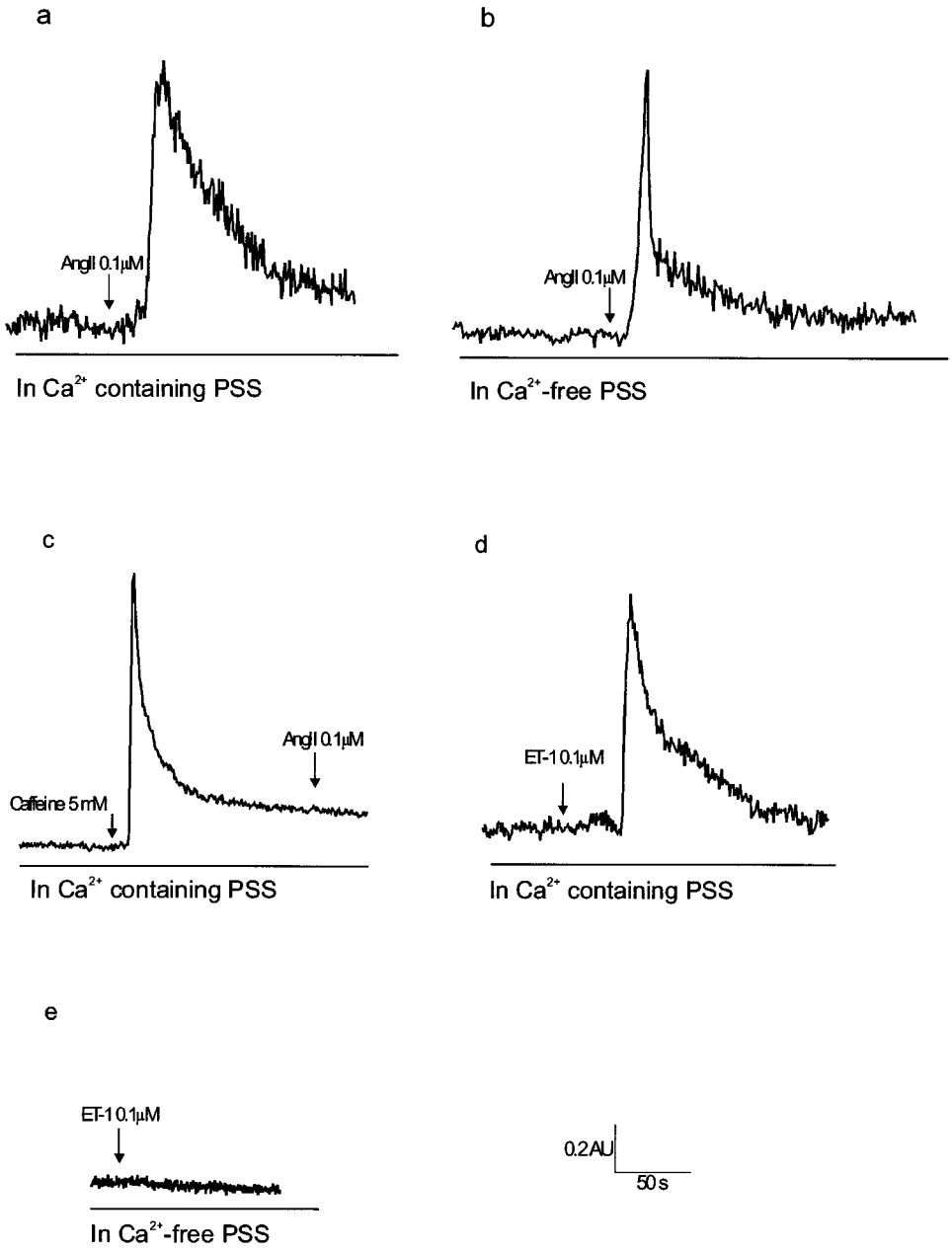


Figure 6 Original tracings illustrate Ang II- and ET-1-mediated increases in $[Ca^{2+}]_i$ of freshly dispersed, single smooth muscle cells from rat tail artery. (a) Ang II (0.1 μM) increased $[Ca^{2+}]_i$ in normal Ca^{2+} solution; (b) in Ca^{2+}-free solution (Ca^{2+} replaced by 1 mM EGTA); (c) 5 mM caffeine (Caff)-induced $[Ca^{2+}]_i$ increase and subsequent addition of Ang II failed to induce $[Ca^{2+}]_i$ increase; (d) ET-1 (0.1 μM) induces $[Ca^{2+}]_i$ increase in normal Ca^{2+} solution; (e) Lack of response to ET-1 in Ca^{2+}-free solution. Each tracing was repeated at least three times. AU represents the change in fluorescence intensity ratio (340 to 380 nm).

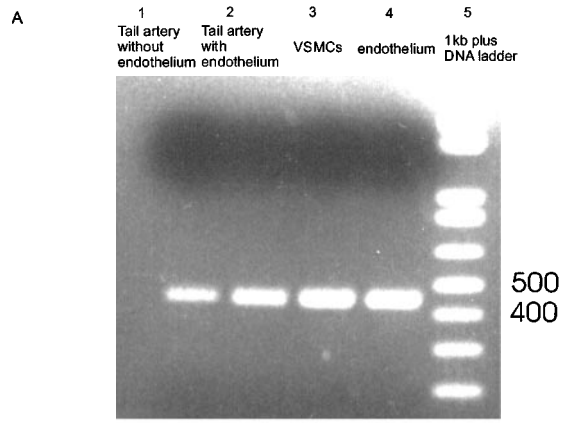
Comparison of Ca^{2+} sources utilized by Ang II and ET-1

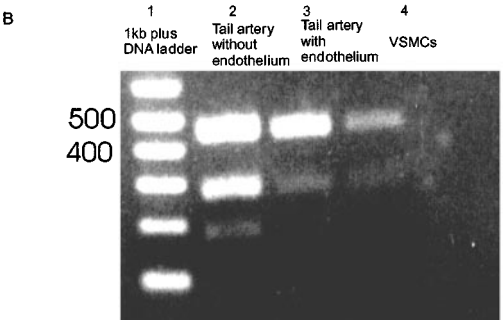
Ang II (0.3 μM) induced a non-sustained contraction in Ca^{2+}-free PSS and the restoration of extracellular Ca^{2+} (2 mM) did not result in a contraction (Figure 5a). In normal PSS, pretreatment with nifedipine (1 μM) (Figure 5b) or SK&F96365 (10 μM) (data not shown) did not significantly inhibit the Ang II-mediated contraction, but pretreatment with caffeine (2 mM) abolished the response (Figure 5c). Conversely, ET-1 (10 nM) did not induce contraction in Ca^{2+}-free PSS, but the restoration of extracellular Ca^{2+} (2 mM) produced a contraction (Figure 5d). Moreover, the ET-1 (10 nM)-mediated contraction was greatly reduced by Ni^{2+} (2 mM) or SK&F96365 (10 μM) (Figure 5e,f), about 30% reduced by nifedipine (1 μM) (not significantly different, data not shown).

Figure 6 illustrates that in freshly dispersed smooth muscle cells from the rat tail artery, Ang II (0.1 μM) induced an increase in $[Ca^{2+}]_i$ in normal PSS (Figure 6a) and Ca^{2+}-free PSS (Figure 6b), but Ang II had no effect on $[Ca^{2+}]_i$ after pretreatment with 5 mM caffeine (Figure 6c). In contrast, ET-1 (0.1 μM) stimulated an increase in $[Ca^{2+}]_i$ in normal PSS (Figure 6d), but not in Ca^{2+}-free PSS (Figure 6e).

AT_1 in VSMC and endothelial cells

mRNA was extracted from tissues and isolated cells. AT_1 mRNA was detected in both endothelial and smooth muscle cells (Figure 7a). Both AT_1 receptor subtypes were identified by RT–PCR analyses using *Eco*RI digestion (Figure 7b) and DNA sequencing (Figure 7c). AT_{1A} and AT_{1B} were detected





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  1  TTTGAATGCC TCGTTTCTTG GCGTGTTCTT TTGAATTTTA TAAGCCTTCT
 51  TTAGGGCTTT CCAAATAAGG GTATAGCTGG TGAGAATGAT AAGGAAAGGG
101  AACACGAAGC CCAGAATGTT CTTGGTTAGG CCCAGCCCTA TGGGGAGCGT
151  TGAGTTCTGA GACTCATAAT GAAACGCGCA CACTGTGATA TTGGTGTTCT
201  CGATGAAATA TACGTTTCGG TGGATGACAG CTGGCAAACT GGCCAAGCCA
251  GCCATTAGCC AGATGATGAT GCAGGTGACT TTGGCTACCA GCATCGTGCG
301  GCGAAGGCGA GACTTCATTG GGTGGACAAT GGCCAGGTAG CGATCGATGC
351  TGAGACACGT GAGAAGGAAC ACACTGGCGT AGAGATTGAA ACTGACGCTG
401  GCCGAAGCGA TCTTACATAG GTGG
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Figure 7 The expression of AT_1 mRNA. mRNA was extracted from rat tail artery tissue with and without endothelium, from freshly dispersed VSMCs and endothelial cells. RT–PCR products were electrophoresed on an agarose gel (1.5%) and visualized by adding ethidium bromide and photographed under u.v. light. (a). Lanes 1–4 contained PCR products from endothelium-denuded tail artery, endothelium-intact tail artery, VSMCs, endothelial cells, respectively, and lane 5 contained 1 kb plus DNA ladder. (b). Lanes 2–4 contained *Eco*RI digest of PCR products from endothelium-denuded tail artery, endothelium-intact tail artery, VSMCs, respectively. Lane 1 contained 1 kb plus DNA ladder. Both (a) and (b) are examples of four similar experiments. (c). The result of one-time DNA sequencing of the *Eco*RI digest-resistant band shown in b.

in both endothelium-intact and -denuded tissues by *Eco*RI (Figure 7b).

Immunohistochemical localization of AT_1 in cross-sections of the rat tail artery was also examined. Figure 8 represents tissue slices cut from the rat tail artery and stained with AT_1 antibody (Figure 8a); AT_1 antibody and AT_1 blocking peptide (Figure 8b); and secondary antibody alone (Figure 8c).

Discussion

In the present study, we have shown that the Ang II-mediated contraction of the rat tail artery is endothelium-independent and that ET-1 released from the endothelium is not involved in the response of the tissue to Ang II. We have also demonstrated that Ang II- and ET-1-mediated contractions of the rat tail artery utilize distinct signalling pathways: (1) Ang II- and ET-1-mediated contractions are antagonized respectively by losartan, the AT_1 selective antagonist and BQ-123, the ET_A selective antagonist and (2) The Ang II-mediated response is mainly dependent on intracellular Ca^{2+} release whereas that of ET-1 is primarily dependent on extracellular Ca^{2+} entry.

Ang II plays an important role in the regulation of blood pressure. It causes vasoconstriction by direct stimulation of Ang II receptors on smooth muscle cells (Peach, 1997; Schultz *et al*., 1981; de Gasparo *et al*., 1995). However, published data also suggest that endothelial cells may be targets for plasma angiotensins and the presence of Ang II receptors on cultured endothelial cells has been reported by several investigators (Patel *et al*., 1989; Danser *et al*., 1995; Vaughan *et al*., 1995; Pueyo *et al*., 1997). Chen *et al*. (1995a,b) have also reported that there are tissue variations in endothelium-dependent sensitivity to Ang II, and that the vasoconstrictor response to Ang II in rat aorta involves activation of AT_1 receptors located on vascular smooth muscle cells, whereas the response in the mesenteric artery involves activation of both vascular and endothelial AT_1 receptors. In contrast, the response to Ang II in the rat tail artery has been described as being entirely dependent on the Ang II-mediated release of ET-1 from the endothelial cells and the subsequent activation of vascular smooth muscle ET_A receptors (Chen *et al*, 1995a,b). In the present study, we have shown that there were no significant differences in pEC_{50} values and maximal contraction response to Ang II in endothelium-intact *vs* endothelium-denuded preparations. Additionally, we have demonstrated that Ang II-mediated vasoconstriction of the endothelium-intact rat tail artery was not sensitive to antagonism by BQ-123, a selective ET_A antagonist, but was blocked by losartan, a selective AT_1 antagonist. These data indicate that Ang II-mediated contraction of the rat tail artery is independent of the release of endothelial cell derived ET-1.

Our findings that ET-1 is not involved in the Ang II-mediated contraction of the rat tail artery are inconsistent with the results reported by Chen *et al*. (1995a,b). The reasons for these discrepancies are unclear, however, the conclusions of Chen *et al*. (1995a,b) were entirely dependent on tension measurements whereas in our investigation, we also measured $[Ca^{2+}]_i$ in single smooth muscle and endothelial cells. In addition, we have studied the expression of AT_1 mRNA using RT–PCR and AT_1 receptor localization using immunohistochemistry. Another consideration is that Chen *et al*. (1995a,b) used Triton X-100 to remove the endothelial cell layer from the rat tail artery preparation and this may have also reduced the sensitivity of the underlying smooth muscle cells to Ang II, thus leading to the conclusion that endothelium-denuded rat tail artery preparation does not respond to Ang II. We have also provided data that indicate that Ang II produced a concentration-dependent increase in $[Ca^{2+}]_i$ with the simultaneous contraction of single smooth muscle cells. Furthermore, the sensitivity of the single cell to Ang II, as reflected by an increase in $[Ca^{2+}]_i$, was greater than that for contraction of the whole tissue (pEC50 values are 9.12 *vs* 7.9). This is in contrast to a previous report from our laboratory comparing the sensitivity of freshly dispersed single cells versus ring preparations of the rat tail artery to α-adrenoceptor agonists (Li *et al*., 1993). In the latter study, isolated cells were less sensitive than the whole tissue to the α-

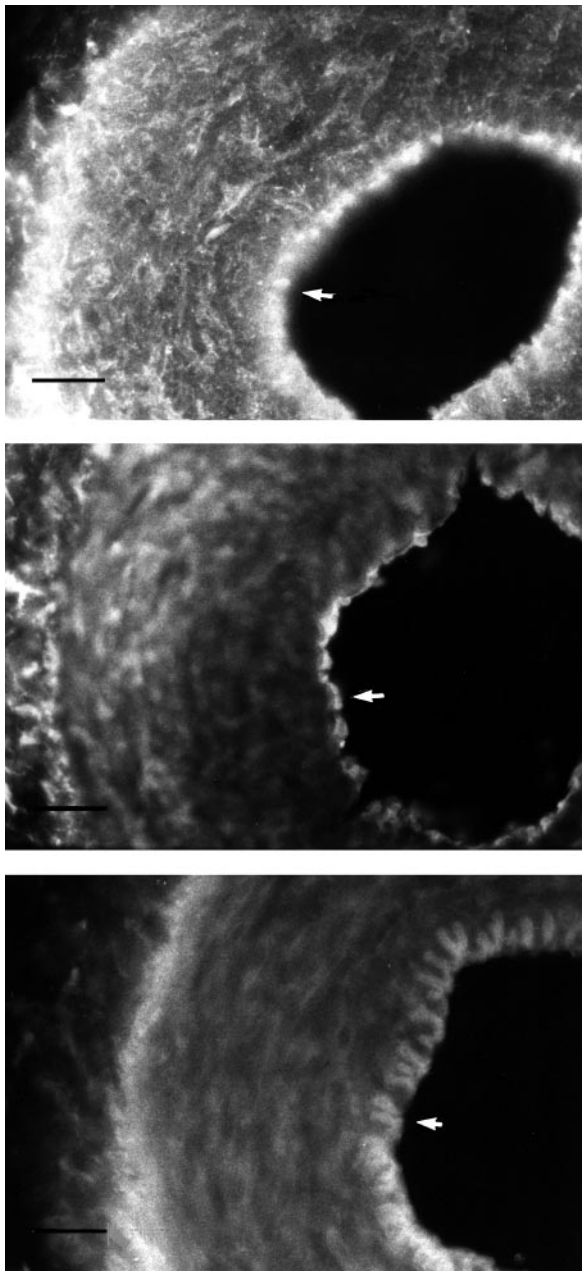

Figure 8 Immunohistochemistry to localize the AT_1 receptors in the cross-section of the rat tail artery. Cross-sections of rat tail artery stained with rabbit anti-human AT_1 IgG and secondary antibody as described in Methods. An arrow indicates the vascular lumen. (a). Treated with rabbit anti-human AT_1 IgG; (b). Treated with rabbit anti-human AT_1 IgG and blocking peptide; (c). Treated with secondary antibody only. (Scale bar: 30 μm). Photos are representative of three experiments.

agonist, cirazoline. These differences may reflect changes in the technique used for cell isolation. In our 1993 study we had included papain in the cell isolation PSS, and it is conceivable that papain may alter agonist-receptor interaction and reduce the sensitivity of single smooth muscle cells. The super-sensitivity of isolated smooth muscle cells to agonists has been demonstrated by others. For instance, Collins & Crankshaw (1986) reported a high sensitivity of single smooth muscle cells isolated from the canine stomach to muscarinic agonists and suggested that enzyme treatment may expose a population of high affinity binding sites. Neither Ang II nor ET_1 produced an increase in $[Ca^{2+}]_i$ in endothelial cells even though the same cells responded to acetylcholine with an increase in $[Ca^{2+}]_i$. These data provide direct evidence that Ang II binds to AT_1 receptors on smooth muscle cells and elicits contraction *via* an increase in free $[Ca^{2+}]_i$. Our results which indicate that Ang II did not increase $[Ca^{2+}]_i$ in endothelial cells are at variance with those reported by Wang *et al*. (1995) and Pueyo *et al*. (1998) who studied, respectively, cultures of rat tail artery endothelial cells isolated from spontaneously hypertensive rats and also rat aortic endothelial cells. Strain differences and changes in the cell phenotype under culture conditions may explain the differences between the data that we report and that from Wang *et al*. (1995) and Pueyo *et al*. (1998). For instance, Pueyo *et al*. (1996) used subcultures of rat tail artery endothelial cells that had been passaged three times. Furthermore, although our immunohistochemistry data illustrate that rat tail artery endothelial cells possess AT_1 receptors, albeit at a lower level than in the VSMCs, the failure of the cells to respond to Ang II suggests that they are not coupled to calcium entry/mobilization.

The RT–PCR data indicate bands corresponding to AT_1 receptor (444 bp) in both intact-tissue and endothelium-denuded tissue as well as in freshly dispersed smooth muscle cells and endothelial cells. Three interpretations can be offered: (1) The bands observed in endothelial cells were due to contamination with smooth muscle cells; (2) There is a low level of AT_1 distribution, but no functional AT_1 receptors, in endothelial cells and (3) There is mRNA, but no receptor protein expression in endothelial cells. Collectively, the data from our tissue bath, single VSMC and endothelial cells studies as well as RT–PCR and immunohistochemistry suggest that rat tail artery endothelial cells do not possess functional AT_1 receptors.

In the present study, we have found that Ang II- and ET-1-mediated contractions of the rat tail artery have diverse pharmacological properties: Ang II produced a non-sustained contraction, whereas ET-1 produced a sustained contraction of the rat tail artery. This observation implies that Ang II and ET-1 utilize distinct Ca^{2+} sources to induce contraction. Our data also indicate that the Ang II-mediated response is mainly dependent on intracellular Ca^{2+} release whereas that of ET-1 is primarily dependent on extracellular Ca^{2+} entry. Our results are consistent with those reported by others (Huang *et al*., 1991; Morel *et al*., 1996). Furthermore, Ang II evokes an additive contraction on top of the maximal response of the rat tail artery to ET-1, supporting our conclusion that Ang II and ET-1 utilize distinct signalling pathways. Taken together, these data provide further evidence against the involvement of ET-1 in mediating the Ang II-induced contraction of the rat tail artery.

We have also demonstrated that ET-1-mediated contraction is less sensitive to the blockade of voltage-gated L-type Ca^{2+} channels by nifedipine and verapamil, but very sensitive to Ni^{2+}, a nonselective blocker of cation channels, and SK&F96365, a putative receptor-operated Ca^{2+} channel blocker (Uchida *et al*., 1998a,b). These findings suggest that voltage-gated L-type Ca^{2+} channels only play a marginal role in ET-1-mediated contraction of the rat tail artery. Considerable evidence exists that ET-1 stimulates Ca^{2+} influx into VSMC *via* non-dihydropyridine-sensitive Ca^{2+} channels (Rubanyi & Polokoff, 1994; Iwamuro *et al*., 1998), however, our data provide evidence that nonselective cation channels and receptor-operated Ca^{2+} channels are also involved in ET-1-mediated contractions of the rat tail artery.

In conclusion, we have provided evidence against the involvement of ET-1 in the Ang II-mediated vasoconstriction

of the rat tail artery. Ang II produced an endothelium-independent contraction *via* a signalling pathway distinct from that utilized by ET-1 in the rat tail artery. The Ang II-mediated response is primarily dependent on intracellular Ca^{2+} release *via* a caffeine-sensitive pathway, whereas that of ET-1 is primarily dependent on extracellular Ca^{2+} entry *via* nonselective cation channels and/or receptor-operated Ca^{2+} channels.

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