British Journal of Pharmacology (2000) 129, 425–436
www.nature.com/bjp

Changes in the cytosolic Ca^{2+} concentration and Ca^{2+}-sensitivity of the contractile apparatus during angiotensin II-induced desensitization in the rabbit femoral artery

[1]Masuko Ushio-Fukai, [1]Hiromichi Yamamoto, [1]Kazuki Toyofuku, [1]Junji Nishimura, [1]Katsuya Hirano & *,[1]Hideo Kanaide

[1]Division of Molecular Cardiology, Research Institute of Angiocardiology and Center for Research and Practice in Medical Education, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

1 To investigate the underlying mechanism for the angiotensin II-induced desensitization of the contractile response during the prolonged stimulation of the vascular smooth muscle, we determined the effects of angiotensin-II on (1) cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension using fura-2-loaded medial strips of the rabbit femoral artery, (2) $^{45}Ca^{2+}$ influx in ring preparations, and (3) Ca^{2+}-sensitivity of the contractile apparatus in α-toxin permeabilized preparations.

2 In the presence of extracellular Ca^{2+}, high concentrations of angiotensin-II elicited biphasic increases in $[Ca^{2+}]_i$ and tension, which consisted of initial transient and subsequent lower and sustained phases.

3 The $^{45}Ca^{2+}$ influx initially increased after the application of 10^{-6} M angiotensin-II, and thereafter gradually decreased. At 20 min after the application, there was a discrepancy between the level of $[Ca^{2+}]_i$ and the extent of $^{45}Ca^{2+}$ influx.

4 The relationships between $[Ca^{2+}]_i$ and tension suggested that the angiotensin-II-induced increase in the Ca^{2+}-sensitivity of the contractile apparatus was maintained during the desensitization of smooth muscle contraction.

5 When 10^{-6} M angiotensin-II was applied during the sustained phase of contraction induced by 118 mm K^+-depolarization, at 10 min after the application, the $[Ca^{2+}]_i$ levels were significantly lower and the tension levels were significantly higher than those prior to the application of angiotensin-II.

6 In conclusion, the decrease in $[Ca^{2+}]_i$, which is partially due to the inhibition of the Ca^{2+} influx, is mainly responsible for the desensitization evoked by high concentrations of angiotensin-II, and angiotensin-II seems to activate additional mechanisms which inhibit Ca^{2+} signaling during prolonged stimulation.

British Journal of Pharmacology (2000) **129,** 425–436

Keywords: Desensitization; angiotensin-II; cytosolic calcium concentration; vascular smooth muscle

Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PKC, protein kinase C; PSS, physiological salt solution; VOCs, voltage-operated Ca^{2+} channels; VSMCs vascular smooth muscle cells

Introduction

Prolonged treatment with angiotensin-II induces desensitization of the contractile response of smooth muscle, which is defined as an attenuation of contraction in the later phase (Oshiro *et al*., 1989). In cultured intestinal smooth muscle cells, desensitization has been suggested to be caused by the inhibition of Ca^{2+} influx mediated by the activation of protein kinase C (PKC) (Shimuta *et al*., 1990). In vascular smooth muscle cells (VSMCs) the mechanism by which angiotensin-II induces desensitization as well as contraction is still not fully understood. Angiotensin-II has also been suggested to induce the initial activation of phospholipase C to form inositol trisphosphate and diacylglycerol which is rapidly attenuated, and the second prolonged activation of phosphatidylcholine-specific phospholipase D which generates a large amount of phosphatidic acid and secondary diacylglycerol, the latter of which stimulates PKC (Griendling *et al*., 1986; Lasseque *et al*., 1993). These sequential biochemical signaling events induced by angiotensin-II may thus play a part in the mechanisms of contraction and desensitization of smooth muscle.

*Author for correspondence.

The cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) play an important role in the regulation of vascular smooth muscle contraction. In cultured VSMCs, angiotensin-II elicits a transient increase in $[Ca^{2+}]_i$ followed by a rapid decrease to a nearly basal level (Alexander *et al*., 1985; Nabika *et al*., 1985). Smith and Smith (1987) suggested that the rapid decrease in $[Ca^{2+}]_i$ during prolonged stimulation with angiotensin-II was due to an acceleration of the Ca^{2+} efflux accompanied by a decrease in total cell Ca^{2+}. In VSMCs from isolated vascular strips, however, it remains unclear as to how Ca^{2+} homeostasis is regulated during angiotensin-II-induced contraction and desensitization. Only a few studies showed changes in $[Ca^{2+}]_i$ during angiotensin-II-induced contraction by the simultaneous measurement of $[Ca^{2+}]_i$ and tension (Morgan & Morgan, 1982; Shimuta *et al*., 1993).

In vascular smooth muscle strips, some agonists can increase the Ca^{2+}-sensitivity of the contractile apparatus, which is shown by a greater tension development than that expected from a given change in $[Ca^{2+}]_i$, when compared with the contraction induced by membrane depolarization with a high external K^+ solution (Morgan & Morgan, 1984; Rembold & Murphy, 1988; Kodama *et al*., 1989; Abe *et al*., 1990). In

addition, using α-toxin-permeabilized vascular smooth muscle (Nishimura *et al*., 1988; Kitazawa *et al*., 1989), the agonist-induced increase in Ca^{2+} sensitivity was shown to be mediated by receptor-coupled guanosine 5′-triphosphate (GTP)-binding protein (G-protein) and/or by PKC (Nishimura & van Breemen, 1989). Morgan & Morgan (1982) were the first to measure the $[Ca^{2+}]_i$ and tension simultaneously during angiotensin-II-induced contraction, using a Ca^{2+}-sensitive photoprotein, aequorin, and showed an increase in Ca^{2+}-sensitivity of the contractile apparatus. Therefore, in order to determined the underlying intracellular mechanism of contraction and desensitization induced by angiotensin-II in the vascular smooth muscle strips, it seems necessary to simultaneously monitor the changes in $[Ca^{2+}]_i$ and the contraction of the vascular strips.

In the present study, to determine the changes in $[Ca^{2+}]_i$ and Ca^{2+}-sensitivity of the contractile apparatus during antiogensin-II-induced contraction and desensitization, we examined the effects of angiotensin-II on (1) $[Ca^{2+}]_i$ and tension of fura-2-loaded medial strips in the rabbit femoral artery by using front-surface fluorometry, and (2) $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ net uptake in ring preparations, and (3) the Ca^{2+}-sensitivity of the contractile apparatus both in intact and in α-toxin-permeabilized preparations. We thus found angiotensin-II to induce vasoconstriction by releasing Ca^{2+} from intracellular stores, by stimulating Ca^{2+} influx and by increasing Ca^{2+}-sensitivity of the contractile apparatus, while the desensitization of angiotensin-II-induced contraction is attributed to a decrease in $[Ca^{2+}]_i$ in the second component of contraction mainly due to an inhibition of the Ca^{2+} influx, but is not mediated by a decrease in the Ca^{2+}-sensitivity of the contractile apparatus.

Methods

Tissue preparation

The study protocol was approved by the Animal Care Committee of Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University. Japanese white rabbits (male, 16–20 weeks old, bodyweight 2.5–3.0 kg) were killed by the administration of sodium pentobarbital (100 mg kg^{-1} intravenously) and femoral arteries were immediately excised. The fat and adventitia were removed by dissection under a binocular microscope. The preparations were longitudinally opened and then cut into approximately 1×3 mm circular strips, 0.2 mm thick for the simultaneous measurement of $[Ca^{2+}]_i$ and tension. To remove the endothelium, the intraluminal surface was rubbed with a cotton swab.

Fura-2 loading

Vascular strips without the endothelium were loaded with a $[Ca^{2+}]_i$ indicator dye, fura-2, by incubation in a medium containing 50 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 2.5% foetal bovine serum for 3–4 h at 37°C. Subsequently, the strips were washed with physiological salt solution (PSS) containing 1.25 mM Ca^{2+} at 37°C to remove the dye from the extracellular space and were then equilibrated in normal PSS for at least 1 h before initiating the measurements. The strips thus treated showed a fluorescence emission spectrum for fura-2-Ca^{2+} complex with a peak at 500 nm and a specific fluorescence excitation spectrum with a peak and a valley at 340 and 380 nm, respectively, which were determined by use of a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). Loading the vascular strips with fura-2 did not alter either the time course or the maximal levels of force development during 118 mM K^+ depolarization (data not shown), thereby suggesting that the contractile responsiveness of the strips was not affected by either the Ca^{2+} buffering action of fura-2 or any possible acidification of the cells due to formaldehyde release on AM-ester hydrolysis (Hirano *et al*., 1990; Miyagi *et al*., 1995).

Measurement of tension

A strip of the femoral artery was mounted vertically in a quartz organ bath with one end connected to a force-transducer (strain gauge TB-612T, Nihon Koden, Japan). During a 1 h equilibration period, the strips were stimulated with 118 mM K^+-depolarization every 15 min, and the resting tension was increased in a stepwise manner. After the equilibration, the resting tension was adjusted to a minimal one (about 350 mg), at which the maximal response was obtained. Tension development was measured at 37°C and expressed as a percentage, while assuming the values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively.

Measurement of $[Ca^{2+}]_i$

The changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were simultaneously monitored during the measurement of tension, using a front-surface fluorometer specially designed for fura-2 fluorometry (CAM-OF-1) (Abe *et al*., 1990; Hirano *et al*., 1990). In brief, the strips were illuminated by guiding the alternating (400 Hz) 340 and 380 nm excitation light from a Xenon light source through quartz optic fibres arranged in a concentric inner circle (diameter = 3 mm). Surface fluorescence of the strips was collected by glass optic fibres arranged in an outer circle (diameter = 7 mm) and introduced through a 500 nm band-pass filter (full width at half maximum transmission = 10 nm) into a photon-counting photomultiplier. The ratio of the 500 nm fluorescence intensity at 340 nm excitation to that at 380 nm excitation was also recorded and expressed in percentage, while assuming the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0% and 100%, respectively. The mean absolute values of $[Ca^{2+}]_i$ at normal PSS and 118 mM K^+ PSS were 112.5 ± 5.3 nM ($n = 10$) and 710.8 ± 6.2 nM ($n = 10$), respectively, which we determined in separate measurements, using the equation given by Grynkiewicz *et al*. (1985), the values of fluorescence intensity at one wavelength (340 nm) and the K_d value of 224 nM at 37°C. To determine the absolute values of $[Ca^{2+}]_i$, it was necessary to permeabilize the cell membrane with ionomycin (25 μM) in order to calibrate the fluorescence ratio and this procedure caused considerable deviations in the estimated $[Ca^{2+}]_i$ values. We therefore used the % fluorescence ratio to express the $[Ca^{2+}]_i$ levels throughout the experiments. Each protocol in the present study was conducted in different tissue strips to avoid the influence of the first treatment with angiotensin-II on the response to the second treatment.

Measurement of tension in α-toxin-permeabilized preparation

Permeabilization by α-toxin was performed as previously described (Nishimura *et al*., 1988) with minor modifications. A small ring (about 200–300 μm in width) from the rabbit femoral artery was sunk in normal PSS and was passed through two tungsten wires. Two tungsten wires were passed through the lumen. One wire was fixed to the chamber and the other was attached to a force transducer. The endothelium in

the inner surface of the arterial wall was then rubbed off gently. After the ring was stretched to an optimal length that resulted in the maximal tension development induced by 85 mM K^+ PSS (85 mM KCl was substituted for 85 mM NaCl in normal PSS), the tissue was treated for 1 h with *Staphylococcus aureus* α-toxin (5000 unit ml^{-1}, BRL) in a Ca^{2+}-free cytoplasmic substitution solution (CSS) containing 2 mM EGTA. The apparent binding constant used for the Ca^{2+}-EGTA was 10^6 M^{-1} (Nishimura *et al*., 1988). After the permeabilization, the tissue strips were stretched by the manipulator, which was connected to the force transducer, to the appropriate resting tension which gives a maximum contraction by 10 mM Ca^{2+}-containing cytoplasmic substitution solution (CSS). The addition of submicromolar concentrations of Ca^{2+} rapidly increased the tension to plateau levels that were well maintained and entirely dependent on an externally supplied high-energy phosphate source. Adding 3×10^{-6} M ionomycin during Ca^{2+}-induced sustained contraction did not cause any significant changes in tension (Nishimura *et al*., 1989), thus indicating that further increase in Ca^{2+} permeability of the plasma membrane did not affect the cytosolic concentration of activating Ca^{2+}. All experiments in alpha-toxin-permeabilized preparations were carried out at 25°C.

Measurement of $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ net uptake

The $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ net uptake were measured according to the method of van Breemen *et al*. (1981) with minor modifications. For the $^{45}Ca^{2+}$ influx, ring preparations of the rabbit femoral artery (length about 3 mm) were incubated with 10^{-8} or 10^{-6} M angiotensin-II in 3 ml normal PSS for various periods and, then, in the same solution but containing $^{45}Ca^{2+}$ (740 kBq ml^{-1}) for 2 min at 37°C. Extracellular $^{45}Ca^{2+}$ was washed out in ice-cold Ca^{2+}-free PSS containing 2 mM EGTA for 15 min. In a preliminary experiment, 15 min was long enough to remove the extracellular $^{45}Ca^{2+}$ in this preparation. The samples were weighed and left overnight in a vial containing 1.5 ml Ca^{2+}-free PSS at room temperature. After the addition of a 7 ml liquid scintillation cocktail (ACS II, Amersham Co., U.S.A.), the radio activity was counted using a liquid scintillation counter (LSC-3500, Aloka Co., Tokyo, Japan). The amount of Ca^{2+} estimated based on the incorporation of $^{45}Ca^{2+}$ into the samples was expressed as micromoles per kilogram wet weight per 2 min. For the $^{45}Ca^{2+}$ net uptake, the samples were incubated in normal PSS containing $^{45}Ca^{2+}$ (185 kBq ml^{-1}) for at least 3 h, and, then, were incubated with 10^{-6} M angiotensin-II in $^{45}Ca^{2+}$-labelled normal PSS for various periods at 37°C. The samples were processed in the same manner as in the $^{45}Ca^{2+}$ influx experiment. The $^{45}Ca^{2+}$ net uptake was expressed as micromoles per kilogram wet weight.

Drugs and solutions

The millimolar composition of the normal physiological salt solution (normal PSS) was: NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. The Ca^{2+}-free solution (Ca^{2+}-free PSS) contained 2 mM EGTA instead of 1.25 mM $CaCl_2$. High K^+ PSS was made by the equimolar substitution of KCl for NaCl. All solutions were gassed with a mixture of 5% CO_2 and 95% O_2 (pH 7.4 at 37°C). CSS for α-toxin-permeabilized tissues contained (in mM): potassium propionate 130, $MgCl_2$ 4.0, Na_2ATP 4.0, Tris-maleate 20, creatine phosphate 10, and 0.1 mg ml^{-1} creatine phosphokinase. The pH was adjusted to 6.8 at 25°C.

Angiotensin II and diltiazem hydrochloride were obtained from the Peptide Institute Co. Ltd. (Osaka, Japan) and Wako Pure Chemicals Co. Ltd. (Osaka, Japan), respectively. Ionomycin was purchased from Sigma (U.S.A.) and Potassium propionate was from Nakarai chemicals (Kyoto, Japan). Guanosine-5′-O-(β-thiodiphosphate)(GDPβS), guanosine-5′-triphosphate (GTP) and adenosine-5′-triphosphate (ATP) were purchased from Boehringer Mannheim (Germany), and *Staphylococcus aureus* α-toxin was from Gibco BRL (Gaithersburg, MO, U.S.A.). Fura-2/AM and EGTA were purchased from Dojindo Laboratories (Kumamoto, Japan), and Fura-2/AM was dissolved in dimethyl sulphoxide (DMSO) as a stock solution and diluted in the medium, just before loading the dye. The final concentration of DMSO was 5%. At this concentration, DMSO had no effect on the contraction of vascular smooth muscle (Hirano *et al*., 1990). $^{45}Ca^{2+}$ was from DuPont/NEN (U.S.A.). All other chemicals were from Katayama Chemical (Osaka, Japan).

Statistical analysis

All values are expressed as the mean ± standard error. Student's *t*-test was used to determine the statistical significance. An analysis of variance (ANOVA) and the multiple comparison test was used for the statistical analysis of the experiments on the $^{45}Ca^{2+}$ fluxes. The statistical analysis of the shift of the $[Ca^{2+}]_i$-tension curves was carried out by an analysis of covariance. *P* values less than 0.05 were considered to be significant.

EC_{50} value, a concentration that increased the fluorescence ratio and tension to 50% of the maximum response, was determined based on the concentration-response curves fitted according to a four-parameter logistic model (De Lean *et al*., 1987).

Results

Effects of AT-II on $[Ca^{2+}]_i$ and tension development in the presence of extracellular Ca^{2+}

Figure 1 shows representative recordings of changes in $[Ca^{2+}]_i$ and tension development in fura-2-loaded femoral arterial strips. When the external bathing solution was changed from normal PSS (5.9 mM K^+) to 118 mM K^+ PSS to determine 0% and 100% levels, respectively, of $[Ca^{2+}]_i$ and tension, $[Ca^{2+}]_i$ and tension rapidly increased and reached steady-state levels (100%) within 5 min and 10 min, respectively, and these levels were maintained for at least 30 min of observation. After the external bathing solution was changed to normal PSS and $[Ca^{2+}]_i$ and the tension returned to the 0% level, 3×10^{-10} M angiotensin-II was applied, which induced gradual and monophasic increases in $[Ca^{2+}]_i$ and tension reaching their plateau levels at 25 min (34.5 ± 4.4%, $n = 6$) and at 30 min (23.2 ± 3.7%, $n = 6$), respectively. These levels were maintained for at least 60 min. When 10^{-9} M angiotensin-II was applied, $[Ca^{2+}]_i$ rapidly rose to reach a peak at 3 min (89.4 ± 1.9%, $n = 6$) and then slightly and gradually declined to a steady-state level at 15 min (72.3 ± 2.6%, $n = 6$), and this level was maintained for at least 30 min. The tension also rapidly developed to reach a maximum at 10 min (97.2 ± 2.2%, $n = 6$) and this level was nearly maintained for at least 60 min. Thus, at the steady state of the contraction (20 min after application), no significant difference was seen in the tension development between 118 mM K^+ and 10^{-9} M angiotensin-II (93.4 ± 2.7%, $n = 6$), while the $[Ca^{2+}]_i$ levels in 118 mM K^+ were much greater than those in 10^{-9} M angiotensin-II. When vascular strips were

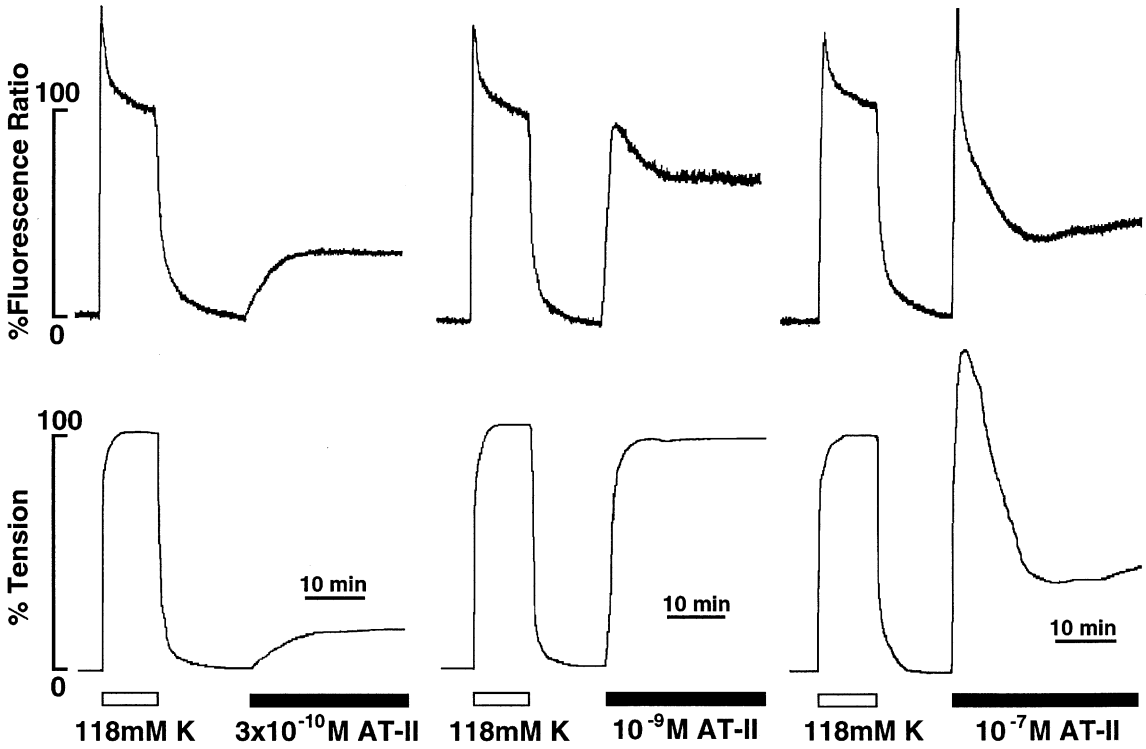

Figure 1 Representative recordings of the changes in the fluorescence ratio ($[Ca^{2+}]_i$, upper traces) and tension development (lower traces) induced by 3×10^{-10}, 10^{-9} and 10^{-7} M angiotensin-II in normal PSS. The responses in the fluorescence ratio and tension to 118 mM K^+-depolarization were recorded before each experiment, as a control (100%).

exposed to 10^{-7} M angiotensin-II, biphasic increases occurred in the $[Ca^{2+}]_i$ and tension; the $[Ca^{2+}]_i$ and tension rose abruptly and reached the first transient peaks (the first component) at 30 s ($142.6\pm6.5\%$, $n=6$) and at 2 min ($132.9\pm4.4\%$, $n=6$), respectively, and then declined to fairly lower steady-state levels (the second component) at 15 min ($[Ca^{2+}]_i$, $42.0\pm3.9\%$, $n=6$; and tension $44.6\pm6.9\%$, $n=6$). Therefore, at 20, 30 and 60 min (data not shown), the levels of $[Ca^{2+}]_i$ and tension development induced by 10^{-7} M angiotensin-II were significantly ($P<0.05$) lower than those induced by 10^{-9} M angiotensin-II. After angiotensin-II (10^{-7} M) was washed out with normal PSS for 20 min, the second application of 10^{-7} M angiotensin-II increased $[Ca^{2+}]_i$ and tension to much the same extent as those of the first application, in both the first and the second component ($n=6$; data not shown). Therefore, tachyphylaxis (Miasiro *et al*., 1983) was not induced by 10^{-7} M angiotensin-II under our experimental conditions. Furthermore, these effects were completely inhibited by 10^{-5} M DUP-753 (data not shown), a angiotensin-II type 1 receptor specific antagonist (Chiu *et al*., 1990), thus suggesting them to be mediated by angiotensin-II type 1 receptors.

Figure 2 is a summary of the measurements performed as Figure 1. At low concentrations of angiotensin-II (10^{-10}–10^{-9} M), changes in both $[Ca^{2+}]_i$ and tension were monophasic. However, at concentrations higher than 10^{-9} M, they became biphasic and consisted of the initial transient (the first component) and the subsequent, lower and sustained phases (the second component). The rate of the increase and the peak levels of $[Ca^{2+}]_i$ and tension in the first component as well as the decrease in the second component were dependent on the concentrations of angiotensin-II. This is clearly shown by the concentration-response curves in Figure 3. The peak $[Ca^{2+}]_i$ and tension in the first component (measured at 30 s and at 2 min after angiotensin-II application) increased, in a concentration-dependent manner (10^{-10}–10^{-6} M), and the

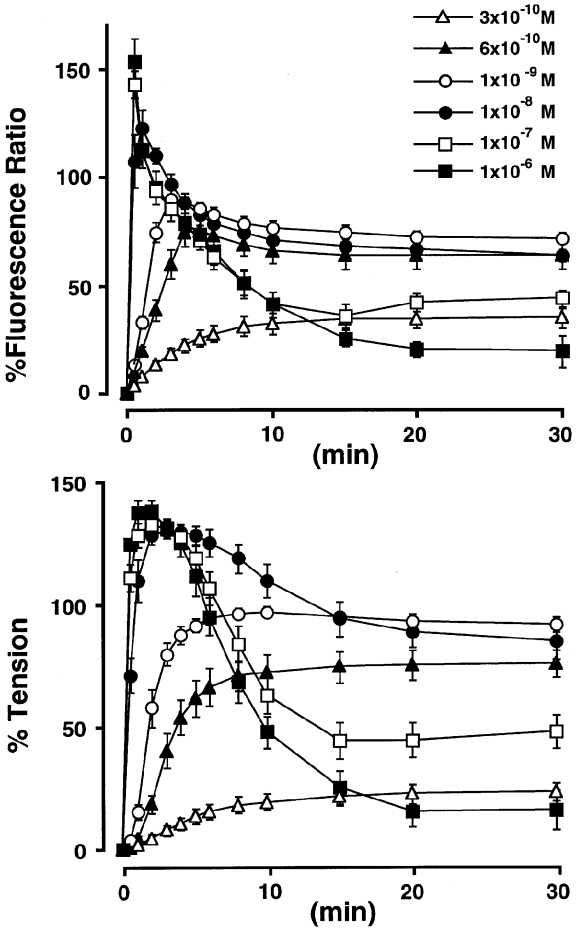

Figure 2 Time courses of the changes in the fluorescence ratio ($[Ca^{2+}]_i$) and tension development induced by 3×10^{-10}, 6×10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M angiotensin-II. The abscissa indicates the time after the application of angiotensin-II. All data are the means ± s.e.mean (shown by vertical lines; $n=6$).

maximal $[Ca^{2+}]_i$ and tension induced by 10^{-6} M angiotensin-II were 153.3±10.7% ($n=6$) and 138.0±4.7% ($n=6$), respectively (Figure 3A). The EC_{50} values for the $[Ca^{2+}]_i$ and tension in the first component were $5.1\pm0.3\times10^{-9}$ M and $1.1\pm0.1\times10^{-9}$ M, respectively. In contrast, $[Ca^{2+}]_i$ and tension in the second component increased at low concentrations of angiotensin-II ($<10^{-9}$ M) and decreased at high concentrations of angiotensin-II ($>10^{-9}$ M), in a concentration-dependent manner (desensitization; Oshiro *et al*., 1989), thus, resulting in bell-shaped concentration-response curves with peaks at 10^{-9} M ($[Ca^{2+}]_i$, 72.3±2.6%, $n=6$; tension, 93.4±2.7%, $n=6$; measured at 20 min after application of angiotensin-II) (Figure 3B).

To investigate possible involvement of voltage-operated Ca^{2+} channels (VOCs) in the increases in $[Ca^{2+}]_i$ and tension induced by angiotensin-II, we used diltiazem, a blocker of VOCs (Figure 4A). To obtain equilibration at its binding sites, diltiazem was applied 10 min before the application of angiotensin-II. The application of diltiazem (10^{-5} M) in normal PSS slightly, but not significantly, decreased the resting $[Ca^{2+}]_i$ levels (−2.2±3.0%, $n=6$), but did not affect the resting tension. In the presence of diltiazem, the second components of the increases in $[Ca^{2+}]_i$ and tension induced by 10^{-7} M angiotensin-II were markedly and significantly ($P<0.05$) lower than those in the absence of diltiazem ($[Ca^{2+}]_i$, 9.8±1.1%, $n=6$; tension, 8.0±0.9%, $n=6$; measured at 20 min after the application), while the first components were slightly, but significantly ($P<0.05$) smaller than that in the absence of diltiazem ($[Ca^{2+}]_i$, 122.0±5.1%, $n=6$; tension, 92.0±7.1%, $n=6$; measured at the peak levels). Diltiazem (10^{-5} M) completely inhibited the increases in $[Ca^{2+}]_i$ and tension induced by 118 mM K^+ (data not shown).

Effects of angiotensin-II on $[Ca^{2+}]_i$ and tension development in the absence of extracellular Ca^{2+}

Figure 4B shows representative recordings of the $[Ca^{2+}]_i$ and tension development induced by 10^{-7} M angiotensin-II in Ca^{2+}-free PSS containing 2 mM EGTA. When the vascular strips were exposed to Ca^{2+}-free PSS, the $[Ca^{2+}]_i$ gradually declined to −20.1±2.0% ($n=6$) in 5 min, while the tension remained unchanged. After 5 min incubation in Ca^{2+}-free PSS, application of 10^{-7} M angiotensin-II induced transient elevations of $[Ca^{2+}]_i$ and tension with peaks at 30 s (83.8±8.9%, $n=6$) and at 1 min (97.5±8.2%, $n=6$), respectively, which were significantly ($P<0.05$) lower than

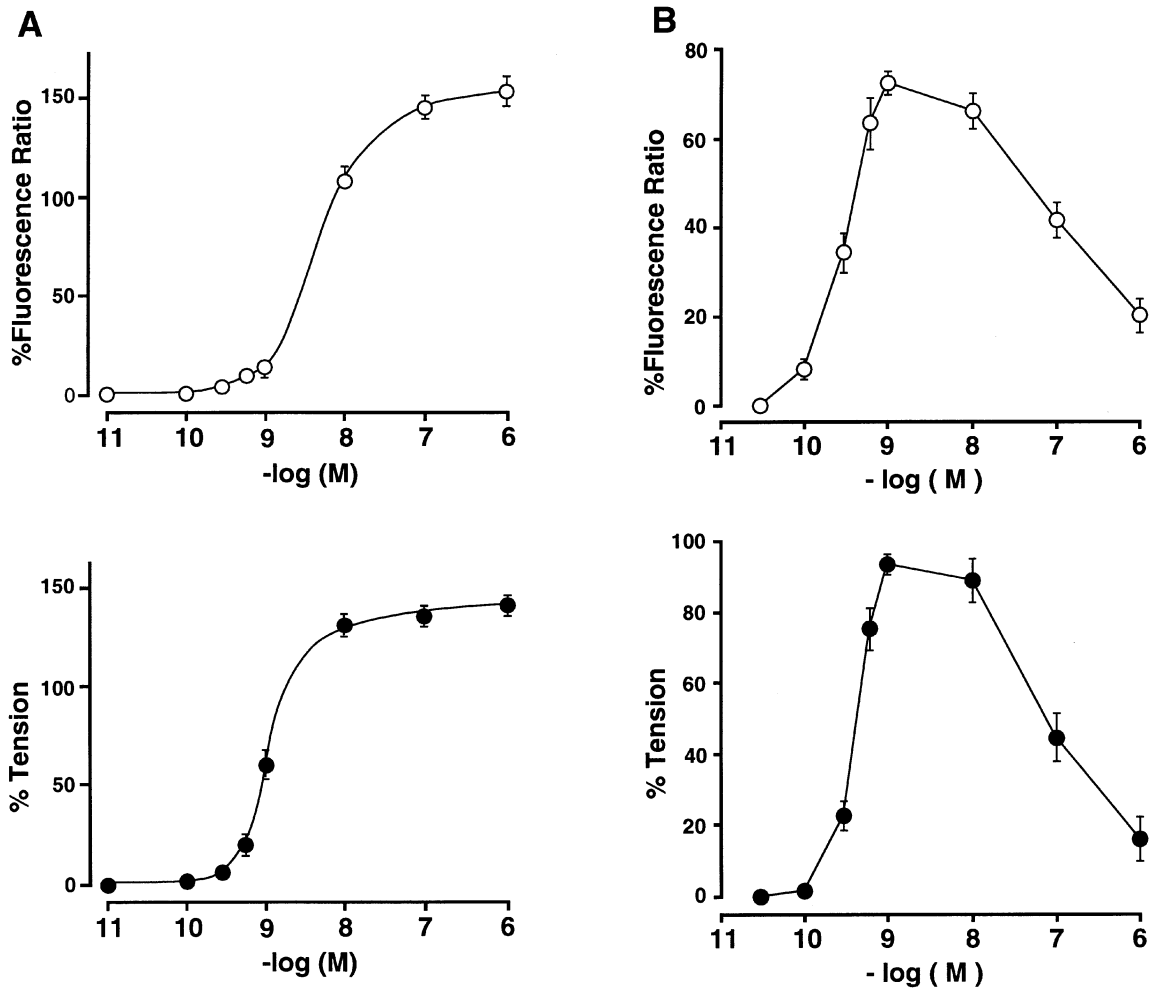

Figure 3 Concentration-response relationships for the increases in the fluorescence ratio ($[Ca^{2+}]_i$ and tension development in the first (A) and the second component (B) during angiotensin-II-induced contraction in normal PSS. (A) The first component. The levels of $[Ca^{2+}]_i$ and tension were measured at 30 s and 2 min after the application of angiotensin-II, respectively. (B) The second component. The levels of $[Ca^{2+}]_i$ and tension were measured at 20 min after the application of angiotensin-II. The abscissa indicates concentration of angiotensin-II. All data are the means±s.e.mean (shown by vertical lines; $n=6$).

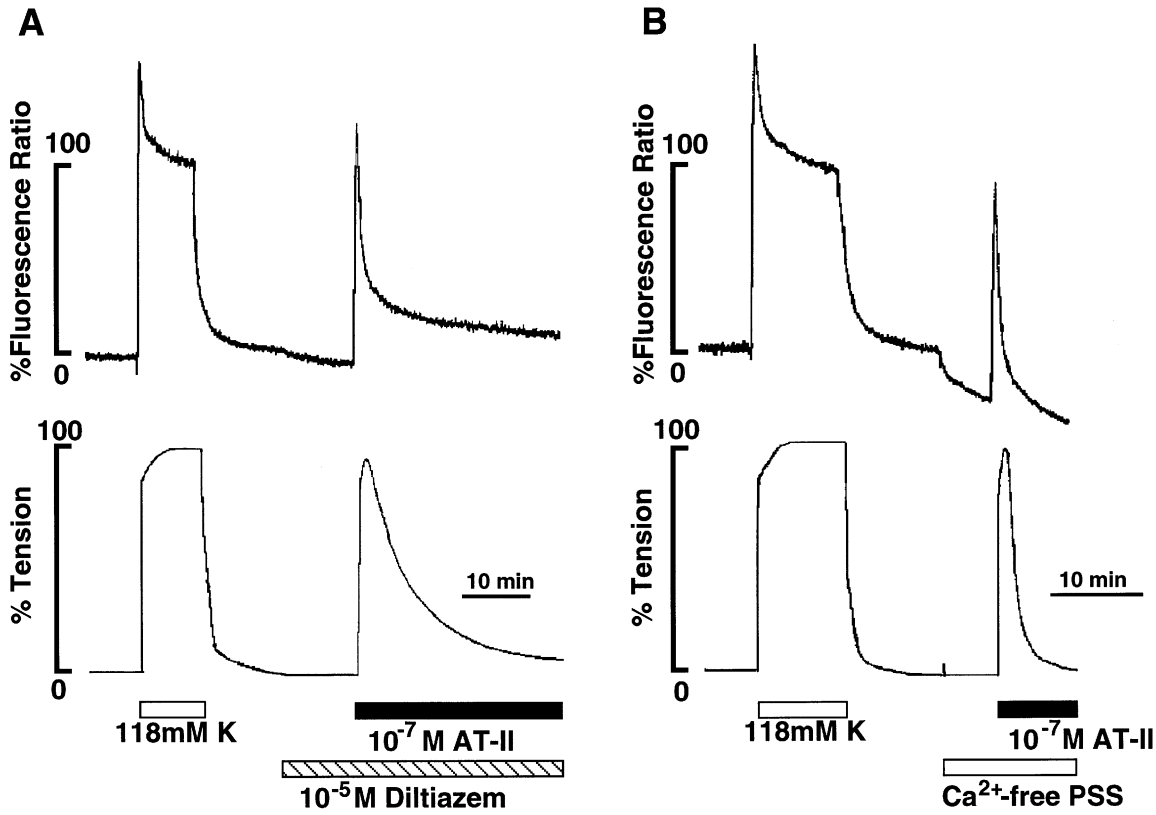

Figure 4 Representative recordings of the effects of diltiazem (A) and the removal of extracellular Ca^{2+} (B) on elevations of the fluorescence ratio ($[Ca^{2+}]_i$, upper traces) ad tension development (lower traces) induced by 10^{-7} M angiotensin-II. (A) Diltiazem (10^{-5} M) was applied 10 min before the application of angiotensin-II. (B) Five min prior to the application of angiotensin-II, normal PSS was exchanged for Ca^{2+}-free PSS containing 2 mM EGTA. The responses of the fluorescence ratio and tension to 118 mM K^+-depolarization were recorded before each experiment, as a control (100%).

those in the first component in normal PSS. The $[Ca^{2+}]_i$ and tension rapidly declined to pre-stimulation levels within 5 min. The peak values in $[Ca^{2+}]_i$ and tension development in Ca^{2+}-free PSS were dependent on the concentrations of angiotensin-II in a range between 10^{-9} and 10^{-6} M (Figure 5). The threshold concentration for angiotensin-II to increase $[Ca^{2+}]_i$ in Ca^{2+}-free PSS was around 10^{-9} M. The EC_{50} values to increase $[Ca^{2+}]_i$ and tension in Ca^{2+}-free PSS were $6.8 \pm 0.2 \times 10^{-9}$ M and $9.3 \pm 1.0 \times 10^{-9}$ M, respectively.

Effects of angiotensin-II on $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$ net uptake

To examine the mechanisms of decrease in $[Ca^{2+}]_i$ in the second component during angiotensin-II-induced desensitization, we measured the $^{45}Ca^{2+}$ influx (Figure 6A) and $^{45}Ca^{2+}$ net uptake (Figure 6B) into the isolated femoral arteries. As shown in Figure 6A, 10^{-6} M angiotensin-II significantly ($P<0.05$, ANOVA) increased $^{45}Ca^{2+}$ influx (133 ± 10 μmole per Kg wet weight per 2 min) within 2 min after applying 3.3 times the control (39 ± 3 μmole per Kg wet weight per 2 min, normal PSS). This increase by 10^{-6} M angiotensin-II was comparable to that induced by 118 mM K^+ (135 ± 10 μmole per Kg wet weight per 2 min). The $^{45}Ca^{2+}$ influx induced by 10^{-6} M angiotensin-II significantly ($P<0.05$, ANOVA) decreased to an intermediate level at 5 min after application and thereafter gradually declined until 20 min. On the other hand, 10^{-8} M angiotensin-II significantly ($P<0.05$, ANOVA) increased $^{45}Ca^{2+}$ influx to a modest degree within 2 min after application which was then maintained at a similar level for 20 min of incubation. As shown in Figure 6B, $^{45}Ca^{2+}$ net uptake was 211 ± 22 μmole per Kg wet weight in normal PSS and significantly ($P<0.05$, ANOVA) increased to 422 ± 48 μmole per Kg wet weight at 30 s after the application of 10^{-6} M angiotensin-II and, thereafter gradually but significantly ($P<0.05$, ANOVA) decreased to 310 ± 30 μmole per Kg wet weight at 20 min.

Effects of angiotensin-II on Ca^{2+}-sensitivity of the contractile apparatus in an intact preparation and an α-toxin-permeabilized preparation

Figure 7A, B and C show summaries of the data on the changes in $[Ca^{2+}]_i$ and tension induced by the cumulative applications of external Ca^{2+} (0–7.5 mM) during depolarization by 118 mM K^+ PSS. Both the $[Ca^{2+}]_i$ and tension increased in a stepwise manner, according to the elevations of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). The $[Ca^{2+}]_i$ increased from $20.1 \pm 2.6\%$ (at 0 mM $[Ca^{2+}]_o$) to $122.6 \pm 11.2\%$ (at 7.5 mM $[Ca^{2+}]_o$), and the tension increased from 0% (at 0 mM $[Ca^{2+}]_o$) to $122.0 \pm 9.9\%$ (at 7.5 mM $[Ca^{2+}]_o$) (Figure 7A, B). A $[Ca^{2+}]_i$ (abscissa)-tension (ordinate) curve was constructed from the data used in Figure 7A, B, and named the 'basic $[Ca^{2+}]_i$-tension relationship' of the Ca^{2+}-induced contractions (Ca^{2+}-contractions) (Figure 7C). The second component of tension development induced by various concentrations of angiotensin-II was also plotted against $[Ca^{2+}]_i$ in Figure 7C together with 'basic $[Ca^{2+}]_i$-tension relationship'. The $[Ca^{2+}]_i$-tension relationship of the concentrations induced by high (10^{-8}–10^{-6} M) as well as low (3×10^{-10}–10^{-9} M) concentrations of angiotensin-II appeared to locate to the left from that of Ca^{2+}-contractions, thus

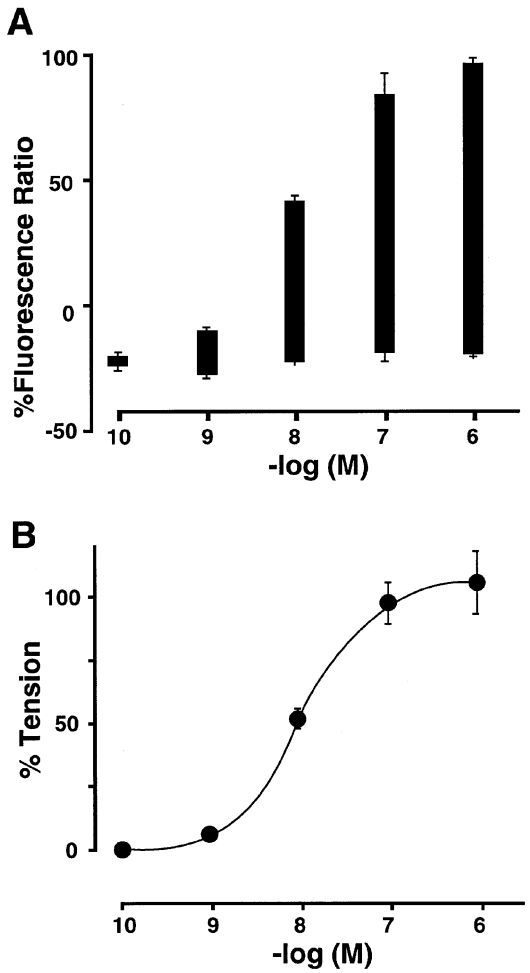

Figure 5 Concentration-response relationships for the effects of various concentrations of angiotensin-II on $[Ca^{2+}]_i$ (A) and on tension development (B) in Ca^{2+}-free PSS containing 2 mM EGTA. Angiotensin-II was applied 5 min after changing normal PSS to Ca^{2+}-free PSS containing 2 mM EGTA. The bottom and top of each column in (A) indicate the $[Ca^{2+}]_i$ just before and at the peak after the application of angiotensin-II, respectively. All data are the means ± s.e.mean (shown by vertical lines; $n=6$).

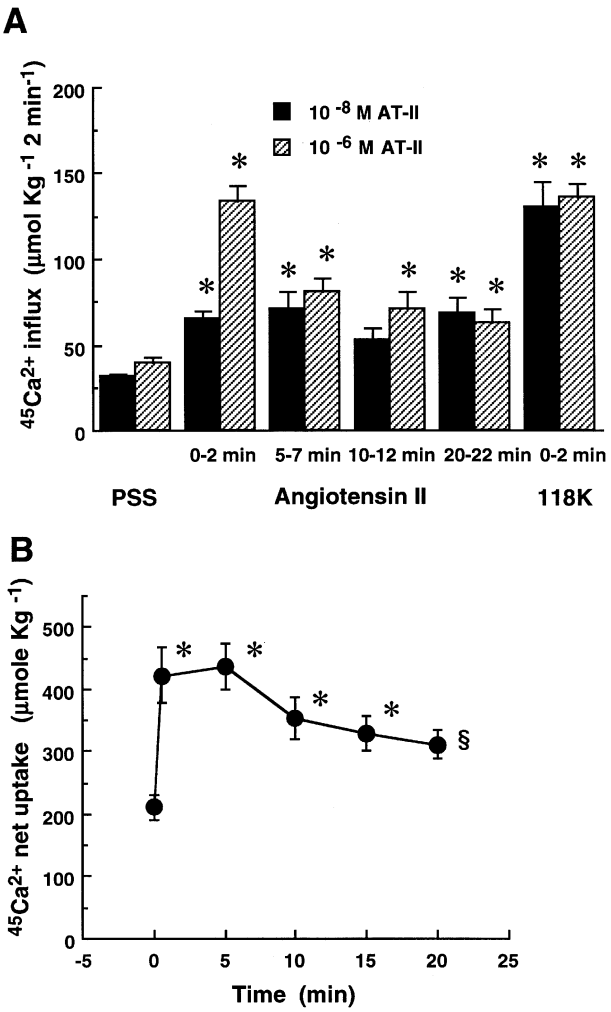

Figure 6 Time courses of the effects of angiotensin-II on $^{45}Ca^{2+}$ influx (A) and $^{45}Ca^{2+}$ net uptake (B). (A) The $^{45}Ca^{2+}$ influx was measured in normal PSS and at the indicated points of time after the application of 10^{-8} and 10^{-6} M angiotensin-II. For comparison purposes, the $^{45}Ca^{2+}$ influx was also measured after the application of 118 mM K^+ PSS. (B) The $^{45}Ca^{2+}$ net uptake was measured in normal PSS (0 min) and at the indicated time points after the application of 10^{-6} M angiotensin-II. *: Significantly different from each control ($P<0.05$, ANOVA). §: Significantly different from the value at 30 s in (B) ($P<0.05$, ANOVA). All data are the means ± s.e.mean (shown by vertical lines; $n=6$).

indicating that the former thus has a significantly ($P<0.05$) higher $[Ca^{2+}]_i$-sensitivity than the latter. Furthermore, the $[Ca^{2+}]_i$-tension relationships of contractions induced by high and low concentrations of angiotensin-II were similar to each other, thus indicating that the $[Ca^{2+}]_i$-sensitivities of the contractions induced by high and low concentrations of angiotensin-II were also similar.

To directly determine whether angiotensin-II increases the Ca^{2+}-sensitivity of the contractile apparatus, we measured the tension development induced by angiotensin-II at a constant $[Ca^{2+}]_i$ which was buffered with 2 mM EGTA, using strips permeabilized with *Staphylococcus aureus* α-toxin (Figure 8). Ca^{2+} itself (up to 10^{-4} M) did not induce a contraction of intact strips in which noradrenaline (10^{-5} M) produced contraction (data not shown). After the permeabilization of the strips with α-toxin, Ca^{2+} (10^{-7}–10^{-4} M) induced contractions, in a concentration-dependent manner (Figure 8A). Since the maximal contraction was obtained by 10^{-4} M Ca^{2+}, the tension development was expressed as a percentage, assuming the value obtained by 10^{-4} M Ca^{2+} to be 100%. In the presence of 10^{-5} M GTP which itself had no effect on the tension, the cumulative applications of angiotensin-II (10^{-10}–10^{-6} M) potentiated the contraction induced by 3×10^{-7} M Ca^{2+}, with a sigmoid-shaped concentration-response relationship (Ca^{2+}-sensitization, Figure 8B,C). The maximum potentiation was obtained by 10^{-8} M angiotensin-II. Higher concentrations ($>10^{-8}$ M) of angiotensin-II neither potentiated further nor attenuated the contraction induced by 3×10^{-7} M Ca^{2+}. This Ca^{2+}-sensitizing effect of angiotensin-II in α-toxin-permeabilized strips required GTP, was abolished by GDPβS, a non-hydrolysable analogue of GDP (data not shown), and thus, appeared to be mediated by G-proteins.

Effects of angiotensin-II on the strips depolarized with 118 mM K^+

To determine whether the attenuation in $[Ca^{2+}]_i$ and tension in the second component of the contractions observed with high concentrations of angiotensin-II was due to either the inhibition of angiotensin-II-induced membrane depolarization or the direct inhibition of Ca^{2+}-movements, we examined effects of 10^{-6} M angiotensin-II on the sustained increases in $[Ca^{2+}]_i$ and tension induced by 118 mM K^+-depolarization. As

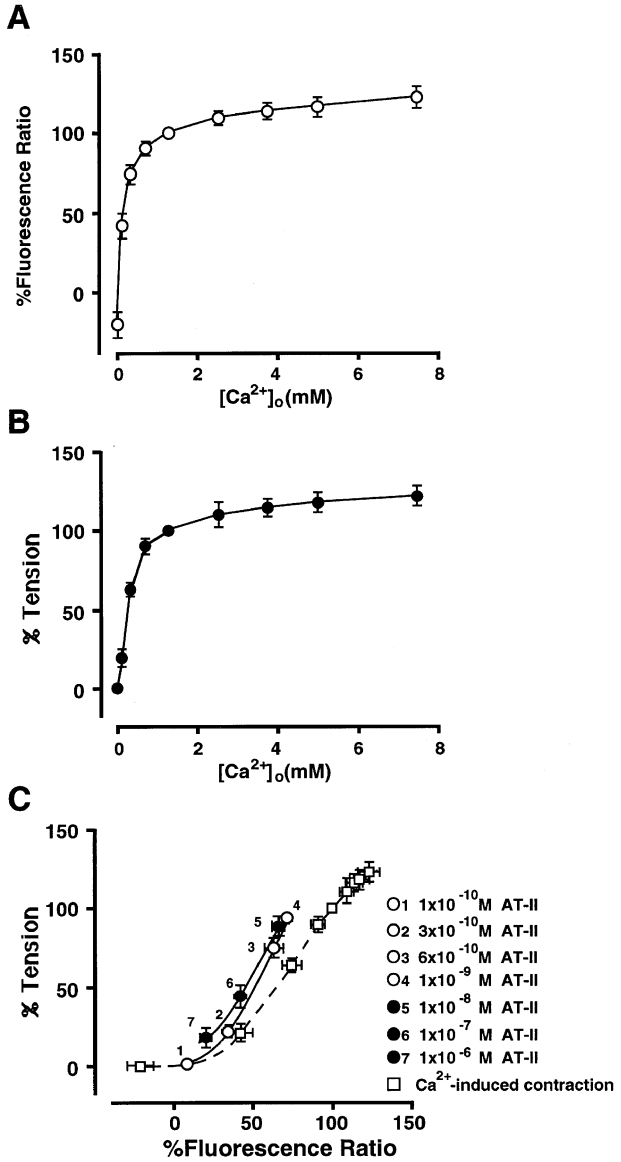

Figure 7 The $[Ca^{2+}]_i$-tension relationships of Ca^{2+}-induced and angiotensin-II-induced contractions. Changes in the fluorescence ratio (A), tension development (B) and the 'basic $[Ca^{2+}]_i$-tension relationship' of Ca^{2+}-induced contraction (C) which were obtained from the data in A and B, in response to the cumulative applications of extracellular Ca^{2+} (0–7.5 mM) during 118 mM K^+-depolarization. The data are obtained at the time of maximal tension development after each application of extracellular Ca^{2+}. All data are the means ± s.e.mean (shown by vertical lines and horizontal lines, $n=10$). In C, the $[Ca^{2+}]_i$-tension relationships in the second component of contractions induced by various concentrations of angiotensin-II were also shown. The individual points represent the $[Ca^{2+}]_i$-tension relationship induced by 10^{-10}, 3×10^{-10}, 6×10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M angiotensin-II. The data are obtained from Figure 3B (measured at 20 min after the application of angiotensin-II). The dashed and the solid lines indicates the $[Ca^{2+}]_i$-tension relationships of Ca^{2+}-induced and angiotensin-II-induced contractions, respectively. All data are the means ± s.e.mean (shown by vertical and horizontal lines; $n=6$).

shown in Figure 9B, when 10^{-6} M angiotensin-II was applied to the strips completely depolarized with 118 mM K^+, a condition which would eliminate additional effects of angiotensin-II on the membrane potential, the $[Ca^{2+}]_i$ abruptly increased to reach a transient peak at 30 s (227.2 ± 12.6%, $n=6$), and thereafter rapidly decreased. The maximal decrease in $[Ca^{2+}]_i$ was observed 2 min after the application of angiotensin-II (61.1 ± 2.6%, $n=6$), and then, the $[Ca^{2+}]_i$ slightly increased but was maintained at a significantly lower level than that obtained by 118 mM K^+ for at least 20 min. In contrast, the tension increased to reach a maximum at 1 min (160.6 ± 6.2%, $n=6$), and thereafter gradually decreased, but remained at a higher level than that of 118 mM K^+ for at least 20 min. At 20 min after the application of 10^{-6} M angiotensin-II, the levels of $[Ca^{2+}]_i$ and tension were 72.2 ± 2.8% ($n=6$) and 115.8 ± 3.9% ($n=6$), respectively. As a result, a marked dissociation of the changes between $[Ca^{2+}]_i$ and tension was observed during the stimulation with 10^{-6} M angiotensin-II of the strips depolarized with 118 mM K^+. In contrast, 10^{-9} M angiotensin-II, which did not show any desensitization of the contractile response in normal PSS, induced a transient and small increase in $[Ca^{2+}]_i$ with a peak at 30 s (106.9 ± 0.5%, $n=6$) without any significant decrease in $[Ca^{2+}]_i$, and a gradual increase in tension reaching its maximum at 10 min (117.4 ± 0.3%, $n=6$, Figure 9A). At 20 min after the application of 10^{-9} M angiotensin-II to the strips depolarized with 118 mM K^+, the levels of $[Ca^{2+}]_i$ and tension were 100.6 ± 1.0% ($n=6$) and 116.8 ± 0.2% ($n=6$), respectively.

Discussion

It has long been recognized that the renin-angiotensin system plays a major role in regulating arterial blood pressure, and modest changes in the plasma concentration of angiotensin-II acutely increase blood pressure. When a single moderate dose of angiotensin-II is applied to VSMCs in vascular strips, then the tension rapidly rises. Prolonged treatment with angiotensin-II, however, induces a desensitization of this contractile response of VSMCs, which is defined as an attenuation of the contraction (Oshiro *et al*., 1989). The modulation of blood pressure *in vivo*, which is relevant to the desensitization of the contractile apparatus of VSMCs *in vitro*, has yet to be clarified. Although changes in $[Ca^{2+}]_i$ play an important role in the regulation of contraction in VSMCs, it remains unclear as to how Ca^{2+} homeostasis is regulated during angiotensin-II-induced contraction and desensitization. In the present study, we demonstrated that the changes in $[Ca^{2+}]_i$ and tension during contractions induced by high concentrations of angiotensin-II (10^{-8}–10^{-6} M) are biphasic, while those induced by lower concentrations of angiotensin-II (10^{-10}–10^{-9} M) are monophasic in the rabbit femoral artery (Figures 1 and 2). These effects were mediated by angiotensin-II type1 receptor which has been demonstrated to be coupled to $G\alpha_{q/11}$ and $G\alpha_{12}$ proteins in VSMCs (Kai *et al*., 1996; Ushio-Fukai *et al*., 1998). Our results were consistent with previous reports on cultured VSMCs (Nabika *et al*., 1985; Dostal *et al*., 1990). The biphasic changes consist of the first transient (the first component) and the subsequent lower sustained increases in $[Ca^{2+}]_i$ and tension (the second component). Since an application of diltiazem or the removal of extracellular Ca^{2+} only slightly, but significantly ($P<0.05$), attenuated the increase in $[Ca^{2+}]_i$ in the first component induced by 10^{-7} M angiotensin-II (Figure 4A, B) as compared with that obtained in normal PSS (Figure 2) and completely abolished the second component, the first component is thus suggested to be mainly due to the intracellular Ca^{2+} release, and only in part due to the extracellular Ca^{2+} influx, whereas the second component is solely due to the extracellular Ca^{2+} influx (Deth & van Breemen, 1974).

In the present study, the changes in both $[Ca^{2+}]_i$ and tension in the second component induced by angiotensin-II clearly

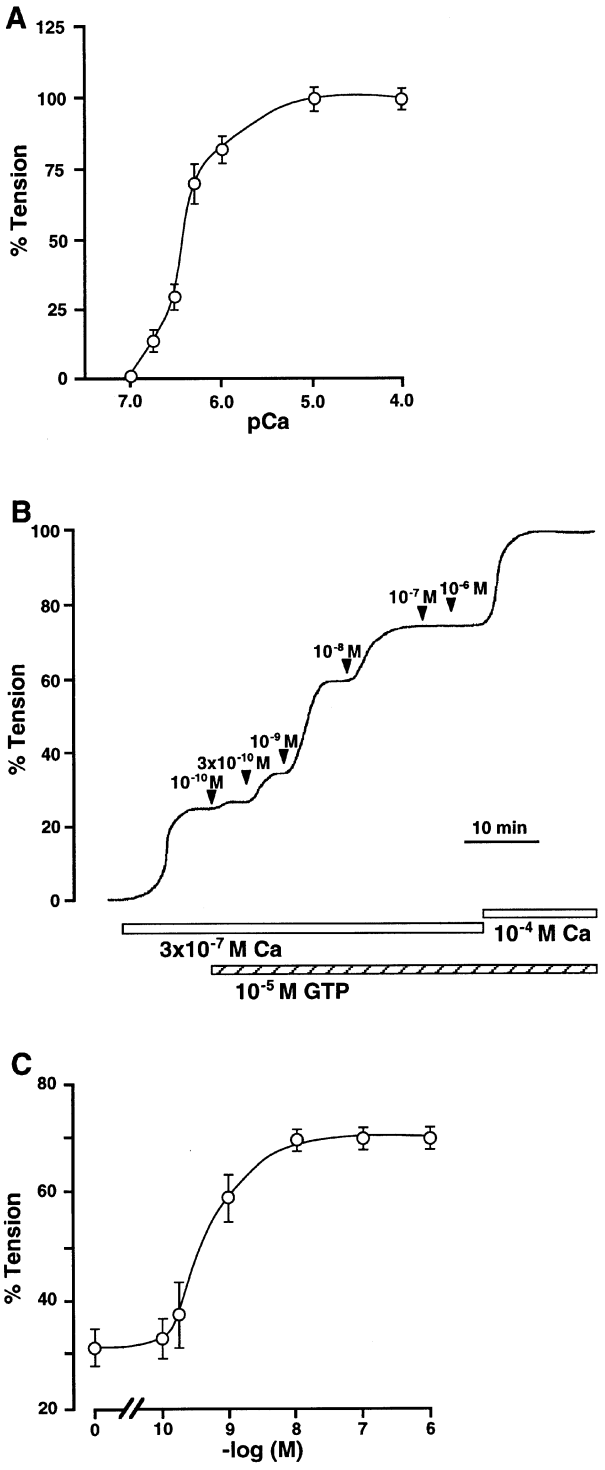

Figure 8 Effects of the cumulative applications of angiotensin-II on the Ca^{2+}-induced contraction in the α-toxin-permeabilized rabbit femoral artery. (A) A control pCa-tension curve in the α-toxin-permeabilized rabbit femoral artery. The abscissa indicates the concentrations of extracellular Ca^{2+} (pCa). All data are the means ± s.e.mean (shown by vertical lines; $n = 10$). (B) Representative recordings of the effects of the cumulative applications of various concentrations of angiotensin-II (10^{-10}–10^{-6} M) on Ca^{2+}-induced contraction (pCa = 7.3) in the presence of 10^{-5} M GTP. The response to the 10^{-4} M Ca^{2+} solution at the end of the experiment illustrates the extent of the maximal Ca^{2+}-induced contraction (100%). The concentrations of angiotensin-II are indicated above the trace. (C) A summary of the data obtained in the measurements as B. All data are the means ± s.e.mean (shown by vertical lines; $n = 7$). In each panel, the ordinate indicates the tension development expressed as a percentage of the maximal Ca^{2+}-induced contraction (pCa = 4).

demonstrated the bell-shaped concentration-response relationship with peaks at 10^{-9} M and ranging from 10^{-10}–10^{-6} M (Figure 3B), while those in the first component showed the sigmoid concentration-response relationship (Figure 3A). This phenomenon seems to be peculiar to angiotensin-II-induced contraction, because the concentration-response relationships in both the first and the second component of the increases in $[Ca^{2+}]_i$ and tension induced by some other agonists such as noradrenaline and serotonin showed sigmoid concentration-response curves in the rabbit femoral artery (Fukuizumi *et al.*, 1995). The decline in the contraction during prolonged stimulation with angiotensin-II was called 'desensitization' by Oshiro *et al.* (1989). Therefore, in contrast to noradrenaline and serotonin, it is likely that high concentrations of angiotensin-II ($> 10^{-9}$ M) may activate the signaling pathways, thus causing decreases in $[Ca^{2+}]_i$ and tension in the second component, and thereby inducing desensitization. The angiotensin-II-stimulated changes in signal transduction in VSMCs have been reported to be biphasic, and secondary diacylglycerol produced by sustained activation of phosphatidylcholine-specific phospholipase D may activate PKC (Griendling *et al.*, 1986; Lasseque *et al.*, 1993). It is therefore possible that the long-lasting activation of the secondary signaling pathways, probably PKC activation, may be involved in the decrease in $[Ca^{2+}]_i$ and tension in the second component. Further supporting this view, there has been a report suggesting that PKC is involved in the desensitization of angiotensin-II-induced contraction in the guinea-pig ileum (Shimuta *et al.*, 1990).

The following three mechanisms might play a role in the decrease of $[Ca^{2+}]_i$ in the second component: (1) an inhibition of Ca^{2+} influx, (2) an acceleration of Ca^{2+} efflux from the cytosol, and (3) the degradation of angiotensin-II or inhibition of the generation of second messengers which maintain both $[Ca^{2+}]_i$ and tension at high levels. (1) and (2): In the present study, when 10^{-6} M angiotensin-II was applied, the $^{45}Ca^{2+}$ influx immediately increased within 2 min, and thereafter rapidly decreased after 5 min of incubation (Figure 6), which also paralleled a decrease in $[Ca^{2+}]_i$ in the second component. A similar decrease in the $^{45}Ca^{2+}$ influx during prolonged treatment with angiotensin-II was also demonstrated in cultured intestinal SMCs as the mechanism of desensitization in the guinea-pig ileum (Shimuta *et al.*, 1990). It is therefore suggested that the decrease in $[Ca^{2+}]_i$ during angiotensin-II-induced desensitization is caused by the inhibition of the Ca^{2+} influx. In the present study, the increase in $[Ca^{2+}]_i$ in the second component was sensitive to diltiazem (Figure 4A), thus suggesting the Ca^{2+} influx stimulated by angiotensin-II to be mainly through VOCs in the rabbit femoral artery. However, the inhibition of the angiotensin-II-induced Ca^{2+} influx by diltiazem was not complete, thus raising the possibility that additional mechanisms, such as the activation of receptor-operated Ca^{2+} channels (Capponi *et al.*, 1985) and/or inhibition of Ca^{2+}-activated K^+ channels (Minami *et al.*, 1995) may also be involved in the second component. Regarding the mechanisms by which angiotensin-II opens VOCs, there have been several reports describing: (a) an indirect inhibition of the K^+ channels (Brauneis *et al.*, 1991) which depolarizes the cell membrane and induces VOCs opening in cultured aortic SMCs (Zelcer & Sperelakis, 1981), (b) the potentiation of the Ca^{2+} current through VOCs *via* pertussis toxin-sensitive G-protein (Hescheler *et al.*, 1988), and (c) the direct activation of VOCs *via* the activation of PKC (Lang & Vallotton, 1987). However, prolonged treatment with 10^{-6} M angiotensin-II did not inhibit the

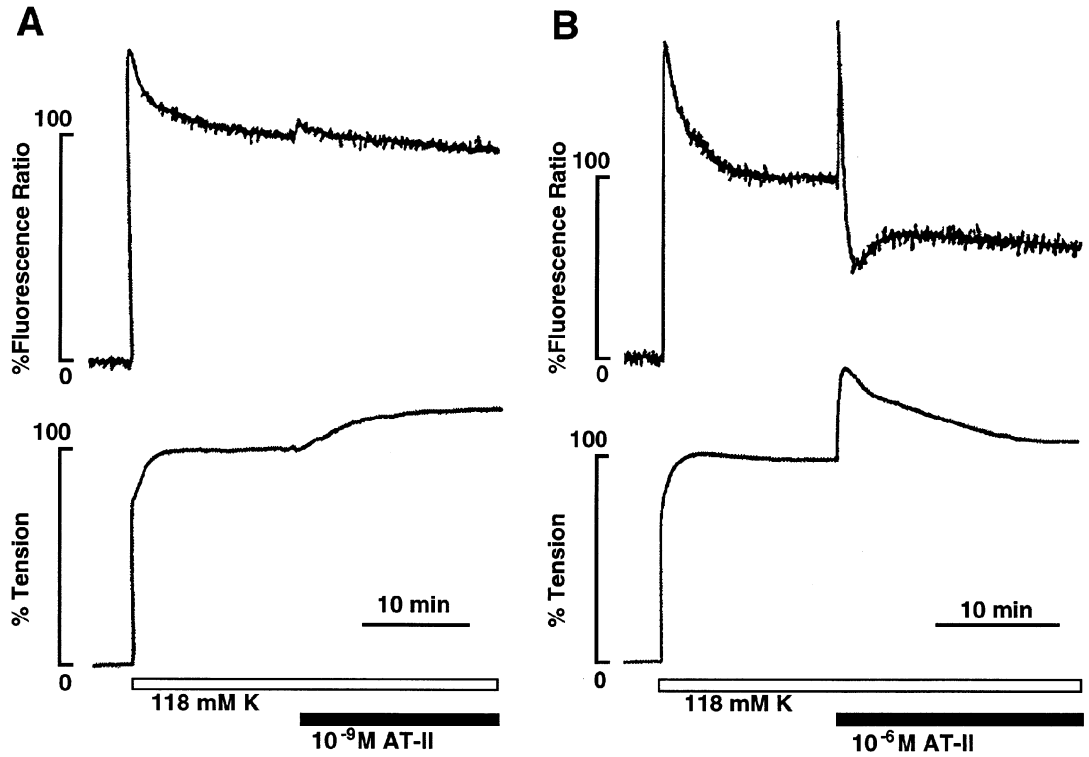

Figure 9 Representative recordings of the effects of angiotensin-II on the increases in the fluorescence ratio ($[Ca^{2+}]_i$) and tension development in the strips precontracted by depolarization with 118 mM K^+. Angiotensin-II (A: 10^{-9} M, B: 10^{-6} M) was applied 15 min after changing normal PSS to 118 mM K^+-PSS.

$^{45}Ca^{2+}$ influx stimulated by 118 mM K^+ depolarization (Ushio-Fukai *et al*., 1999), thus suggesting that angiotensin-II does not directly inhibit VOCs, but rather inhibits the process from the receptor activation to the membrane depolarization, and thus, decreases $[Ca^{2+}]_i$. Furthermore, the inhibition of Ca^{2+} influx alone may be not sufficient to explain the whole decrease in $[Ca^{2+}]_i$, because the $[Ca^{2+}]_i$ at 20 min after the application of 10^{-6} M angiotensin-II was obviously lower than that induced by 10^{-8} M angiotensin-II ($P<0.05$) (Figure 3B), although there was no significant difference in the extent of $^{45}Ca^{2+}$ influx between 10^{-6} M angiotensin-II and 10^{-8} M angiotensin-II stimulations (Figure 6A). In cultured VSMCs, it was reported that Ca^{2+} efflux *via* Na^+/Ca^{2+} exchange might contribute to the rapid decrease in $[Ca^{2+}]_i$ during angiotensin-II stimulation, which was accompanied with a decrease in total cell Ca^{2+} from the basal level (Smith & Smith, 1987). In the present study, however, such a decrease in the total cell Ca^{2+} from the basal level was not observed during angiotensin-II-stimulation (Figure 6B), and furthermore, the rate of relaxation as well as the decrease in $[Ca^{2+}]_i$ in the second component during angiotensin-II-induced desensitization in the absence of extracellular Na^+ were similar to those in the presence of extracellular Na^+ (data not shown). These findings would thus eliminate the possible involvement of Na^+/Ca^{2+} exchange mechanism (Blaustein *et al*., 1986) for the decrease in $[Ca^{2+}]_i$. Other Ca^{2+} transport systems such as Ca^{2+} extrusion through the plasma membrane Ca^{2+} pump and/or Ca^{2+} sequestration into the intracellular stores remained to be examined in the mechanisms of angiotensin-II-induced decrease in $[Ca^{2+}]_i$. (3): It is unlikely that the decrease in $[Ca^{2+}]_i$ induced by 10^{-6} M angiotensin-II was due to the degradation of angiotensin-II or to a decrease in signal generation, because 10^{-9} M angiotensin-II was able to maintain $[Ca^{2+}]_i$ in the second component at a higher level than that by 10^{-6} M angiotensin-II (Figure 2), and because the prolonged stimulation with angiotensin-II caused sustained production of diacylglycerol, in a concentration-dependent manner, and ranging from $10^{-9}-10^{-6}$ M in cultured VSMCs (Griendling *et al*., 1986).

When 10^{-6} M angiotensin-II was applied at the steady-state of contraction induced by 118 mM K^+-depolarization, $[Ca^{2+}]_i$ transiently increased, and then, decreased to reach a sustained level, which was lower than that prior to angiotensin-II application (Figure 9). Since 118 mM K^+-depolarization almost completely eliminates the effects of angiotensin-II on the membrane potential and, hence, on the Ca^{2+} influx through VOCs and since 10^{-6} M angiotensin-II did not inhibit the $^{45}Ca^{2+}$ influx stimulated by 118 mM K^+-depolarization (Ushio-Fukai *et al*., 1999), these results indicate a novel inhibitory effect of angiotensin-II on $[Ca^{2+}]_i$ through mechanisms other than the inhibition of Ca^{2+} influx through VOCs. We thus speculate that the mechanism underlying the inhibitory effect of angiotensin-II on $[Ca^{2+}]_i$ in 118 mM K^+-depolarized strips may thus play an important role in the decrease in $[Ca^{2+}]_i$ during angiotensin-II-induced desensitization in normal PSS. This notion is also supported by our observations that both the decrease in $[Ca^{2+}]_i$ during desensitization in normal PSS and that in the 118 mM K^+-depolarized strips were elicited by the same concentrations of angiotensin-II ($>10^{-9}$ M), in a concentration-dependent manner (Ushio-Fukai *et al*., 1999), and that 10^{-9} M angiotensin-II (Figure 9A) as well as noradrenaline and serotonin (data not shown), which do not induce desensitization in normal PSS, did not induce a decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+.

The contraction of smooth muscle is regulated not only by $[Ca^{2+}]_i$ but also by Ca^{2+}-sensitivity of the contractile

apparatus, which is also regulated by the intracellular signaling systems. In the present study, high concentrations ($>10^{-9}$ M) of angiotensin-II increased Ca^{2+}-sensitivity of the contractile apparatus in the intact strips, which was expressed as the leftward shift of the $[Ca^{2+}]_i$-tension relationship in the second component of the angiotensin-II-induced contraction from that for the Ca^{2+}-contraction during 118 mM K^+-depolarization (Figure 7C). In the α-toxin-permeabilized preparation (Figure 8) in which the intracellular Ca^{2+} concentration is kept constant and the receptor-coupled signal transduction systems are preserved intact (Nishimura *et al*., 1988; Kitazawa *et al*., 1989), angiotensin-II increased tension, in a concentration-dependent manner, in a range from $10^{-10}-10^{-6}$ M, in the presence of GTP, but not in the presence of GDPβS, thus indicating that the G-protein-mediated Ca^{2+}-sensitization did not decrease at high concentrations of angiotensin-II. Therefore, a decrease in Ca^{2+}-sensitivity is not suggested to contribute to the desensitization of angiotensin-II-induced contraction. Since the experiments in the permeabilized preparation were carried out at 25°C compared to those in the intact preparation at 37°C, it is possible that a lower temperature might inhibit the angiotensin-II-induced desensitization. However, this possibility can be ruled out, because even at 25°C, prolonged stimulation with angiotensin-II induced desensitization accompanying a decrease in $[Ca^{2+}]_i$ in the second component (data not shown). Therefore, a change in Ca^{2+}-sensitivity of the contractile apparatus does not appear to play a role in the occurrence of desensitization during prolonged stimulation with high concentrations of angiotensin-II.

In summary, our above findings suggest that: (1) angiotensin-II elicits vasoconstriction by stimulating Ca^{2+} influx from the extracellular space, by releasing Ca^{2+} from the intracellular stores, and by increasing the Ca^{2+} sensitivity of the contractile apparatus. (2) Desensitization of the contractile response evoked by prolonged stimulation with high concentrations of angiotensin-II is attributed to a decrease in $[Ca^{2+}]_i$ in the second component, but is not correlated with a change in Ca^{2+}-sensitivity of the contractile apparatus. (3) The decrease in $[Ca^{2+}]_i$ in the second component may be due not to an inhibition of the Ca^{2+} influx, but also is possibly due to the activation of Ca^{2+} extrusion and/or Ca^{2+} sequestration. (4) High concentrations of angiotensin-II induce a sustained decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+. Angiotensin-II thus has not only a stimulatory but also an inhibitory effect on Ca^{2+}-signaling, and the long-lasting inhibitory effects on $[Ca^{2+}]_i$, but not the changes in Ca^{2+}-sensitivity of the contractile apparatus, may therefore play a major role in the angiotensin-II-induced desensitization of the contractile response in isolated vascular smooth muscle. It will be necessary to investigate whether or not angiotensin-II activates Ca^{2+} sequestration and/or extrusion, thereby decreasing $[Ca^{2+}]_i$ during desensitization in the future.

We thank Mr Brian Quinn for reading the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (A) (Nos.06557045 and 07407022) and (C) (Nos. 05837017 and 0783308) and Grants-in-Aid for Creative Basic Research Studies of Intracellular Signaling Network from the Ministry of Education, Science, Sports and Culture, Japan, and by The Vehicle Racing Commemorative Foundation.

References

ABE, S., KANAIDE, H. & NAKAMURA, M. (1990). Front-surface fluorometry with fura-2 and effects of nitroglycerin on cytosolic calcium concentrations and tension in the coronary artery of the pig. *Br. J. Pharmacol.,* **101,** 545 – 552.

ALEXANDER, R.W., BROCK, T.A., GIMBRONE, Jr, M.A. & RITTENHOUSE, S.E. (1985). Angiotensin II increases inositol trisphosphate and calcium in vascular smooth muscle. *Hypertension,* **7,** 447 – 451.

BLAUSTEIN, M.P., ASHIDA, T., GOLDMAN, W.F., WIER, W.G. & HAMLYN, J.M. (1986). Sodium/calcium exchange in vascular smooth muscle: a link between sodium metabolism and hypertension. *Ann. N.Y. Acad. Sci. U.S.A.,* **488,** 199 – 216.

BRAUNEIS, V., VASSILEV, P., QUINN, S.J., WILLIAMS, G.H. & TILLOTSON, D.L. (1991). Angiotensin II blocks potassium currents in zona glomerulosa cells from rat, bovine, and human adrenals. *Am. J. Physiol.,* **260,** E772 – E779.

CAPPONI, A.M., LEW, P.D., VALLOTTON, M.B. (1985). Cytosolic free calcium levels in monolayers of cultured rat aortic smooth muscle cells. Effects of angiotensin II and vasopressin. *J. Biol. Chem.,* **260,** 7836 – 7842.

CHIU, A.T., MCCALL, D.E., PRICE, W.A., WONG, P.C., CARINI, D.J., DUNCIA, J.V., WEXLER, R.R., YOO, S.E., JOHNSON, A.L. & TIMMERMANS, P.B. (1990). Nonpeptide angiotensin II receptor antagonists. VII. Cellular and biochemical pharmacology of DuP 753, an orally active antihypertensive agent. *J. Pharmacol. Exp. Ther.,* **252,** 711 – 718.

DE LEAN, A., MUNSON, P.J. & RODBARD, D. (1987). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol,.* **235,** E97 – E102.

DETH, R. & VAN BREEMEN, C. (1974). Relative contributions of Ca^{2+} influx and cellular Ca^{2+} release during drug induced activation of the rabbit aorta. *Pflügers Archiv.,* **348,** 13 – 22.

DOSTAL, D.E., MURAHASHI, T. & PEACH, M.J. (1990). Regulation of cytosolic calcium by angiotensins in vascular smooth muscle. *Hypertension,* **15,** 815 – 822.

FUKUIZUMI, Y., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1995). Cytosolic calcium concentration-force relation during contractions in the rabbit femoral artery: Time-dependency and stimulus specificity. *Br. J. Pharmacol.,* **114,** 329 – 338.

GRIENDLING, K.K., RITTENHOUSE, S.E., BROCK, T.A., EKSTEIN, L.S., GIMBRONE, Jr, M.A. & ALEXANDER, R.W. (1986). Sustained diacylglycerol formation from inositol phospholipids in angiotensin II-stimulated vascular smooth muscle cells. *J. Biol. Chem.,* **261,** 5901 – 5906.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440 – 3450.

HESCHELER, J., RESENTHAL, W., HINSCH, K.D., WULFEN, M., TRAUTWIEN, W. & SCHULTZ, G. (1988). Angiotensin II-induced stimulation of voltage-dependent Ca^{2+} currents in an adrenal cortical cell line. *EMBO J.,* **7,** 619 – 624.

HIRANO, K., KANAIDE, H., ABE, S. & NAKAMURA, M. (1990). Effects of diltiazem on calcium concentrations in the cytosol and on force of contractions in porcine coronary arterial strips. *Br. J. Pharmacol.,* **101,** 273 – 280.

HIRANO, K., KANAIDE, H., ABE, S. & NAKAMURA, M. (1991). Temporal changes in the calcium-force relation during histamine-induced contractions of the strips of the coronary artery of the pig. *Br. J. Pharmacol.,* **102,** 27 – 34.

KAI, H., FUKUI, T., LASSÉGUE, B., SHAH, A., MINIERI, C.A. & GRIENDLING, K.K. (1996). Prolonged exposure to agonist results in a reduction in the levels of the G_q/G_{11} α-subunits in cultured vascular smooth muscle cells. *Mol. Pharmacol.,* **49,** 96 – 104.

KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A.P. & SOMLYO, A.V. (1989). Receptor-coupled, permeabilized smooth muscle: Role of phosphatidylinositol cascade, G-proteins and modulation of the contractile response to Ca^{2+}. *J. Biol. Chem.,* **264,** 5339 – 5342.

KODAMA, M., KANAIDE, H., ABE, S., HIRANO, K., KAI, H. & NAKAMURA, M. (1989). Endothelin-induced Ca^{2+}-independent contraction of the porcine coronary artery. *Biochem. Biophys. Res. Commun.,* **160,** 1302–1308.

LANG, U. & VALLOTTON, M.B. (1987). Angiotensin II but not potassium induces subcellular redistribution of protein kinase C in bovine adrenal glomerulosa cells. *J. Biol. Chem.,* **262,** 8047–8050.

LASSEQUE, B., ALEXANDER, R.W., CLARK, M., AKERS, M. & GRIENDLING, K.K. (1993). Phosphatidylcholine is a major source of phosphatidic acid and diacylglycerol in angiotensin II-stimulated vascular smooth-muscle cells. *Biochem. J.,* **292,** 509–517.

MIASIRO, N., OSHIRO, M.E.M., PAIVA, T.B. & PAIVA, A.C.M. (1983). Role of the two N-terminal residues of angiotensin II in production of tacyphylaxis. *Eur. J. Pharmacol.,* **87,** 397–406.

MINAMI, K., HIRATA, Y., TOKUMURA, A., NAKAYA, Y. & FUKUZAWA, K. (1995). Protein kinase C-independent inhibition of the Ca(2+)-activated K^+ channel by angiotensin II and endothelin-1. *Biochem. Pharmacol.,* **49,** 1051–1056.

MIYAGI, Y., KOBAYASHI, S., NISHIMURA, J., FUKUI, M. & KANAIDE, H. (1995). Resting load regulates vascular sensitivity by a cytosolic Ca^{2+}-insensitive mechanism. *Am. J. Physiol.,* **268,** C1332–C1341.

MORGAN, K.G. & MORGAN, J.P. (1982). Vascular smooth muscle: The first recorded Ca^{2+} transients. *Pflügers Arch.,* **395,** 75–77.

MORGAN, K.G. & MORGAN, J.P. (1984). Stimulus-specific patterns of intracellular calcium levels in smooth muscle of ferret portal vein. *J. Physiol.,* **351,** 155–167.

NABIKA, T., VELLETRI, P.A., LOVENBERG, W. & BEAVEN, M.A. (1985). Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and [Arg]-vasopressin in vascular smooth muscle cells. *J. Biol. Chem.,* **260,** 4661–4670.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP-γS increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochem. Biophys. Res. Commun.,* **157,** 677–683.

NISHIMURA, J. & VAN BREEMEN, C. (1989). Direct regulation of smooth muscle contractile elements by second messengers. *Biochem. Biophys. Res. Commun.,* **163,** 929–935.

OSHIRO, M.E.M., SIMUTA, S.I., PAIVA, T.B. & PAIVA, A.C.M. (1989). Evidence for a regulation site in the angiotensin II receptor of smooth muscle. *Eur. J. Pharmacol.,* **166,** 411–417.

REMBOLD, C.M. & MURPHY, R.A. (1988). Myoplasmic $[Ca^{2+}]_i$ determines myosin phosphorylation in agonist-stimulated swine arterial smooth muscle. *Circ. Res.,* **63,** 593–603.

SHIMUTA, S.I., KANASHIRO, C.A., FERREIRA, A.T., OSHIRO, M.E.M., PAIVA, T.B. & PAIVA, A.C.M. (1993). Role of Na^+ and protein kinase C in angiotensin desensitization and tachyphylaxis in the guinea-pig ileum. *Naunyn-Schmiedberg's Archiv. Pharmacol.,* **347,** 425–431.

SHIMUTA, S.I., KANASHIRO, C.A., OSHIRO, M.E.M., PAIVA, T.B. & PAIVA, A.C.M. (1990). Angiotensin II desensitization and Ca^{++} and Na^+ fluxes in cultured intestinal smooth muscle cells. *J. Pharmacol. Exp. Ther.,* **253,** 1215–1221.

SMITH, J.B. & SMITH, L. (1987). Extracellular Na^+ dependence of changes in free Ca^{2+}, $^{45}Ca^{2+}$ efflux, and total cell Ca^{2+} produced by angiotensin II in cultured arterial muscle cells. *J. Biol. Chem.,* **262,** 17455–17460.

USHIO-FUKAI, M., ABE, S., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1993). Effects of isoprenaline on cytosolic calcium concentrations and on tension in the porcine coronary artery. *J. Physiol.,* **462,** 679–696.

USHIO-FUKAI, M., GRIENDLING, K.K., AKERS, M., LYONS, R. & ALEXANDER, R.W. (1998). Temporal dispersion of activation of phospholipase C-β1 and -γ isoforms by angiotensin II in vascular smooth muscle cells: Role of $\alpha_{q/11}$, α_{12}, and $\beta\gamma$ G protein subunits. *J. Biol. Chem.,* **273,** 19772–19777.

USHIO-FUKAI, M., YAMAMOTO, H., NISHIMURA, J., HIRANO, K. & KANAIDE, H. (1999). The mechanism of the decrease in cytosolic Ca^{2+} concentrations induced by angiotensin II in the high K^+-depolarized rabbit femoral artery. *Br. J. Pharmacol.,* **129,** 437–447.

VAN BREEMEN, C., HWANG, O. & MEISHERI, K. (1981). The mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility. *J. Pharmacol. Exp. Ther.,* **218,** 459–463.

ZELCER, E. & SPERELAKIS, N. (1981). Angiotensin induction of active responses in cultured reaggregates of rat aortic smooth muscle cells. *Blood Vessels,* **18,** 263–279.

(Received June 1, 1999
Revised October 22, 1999
Accepted November 9, 1999)