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The mechanism of the decrease in cytosolic Ca^{2+} concentrations induced by angiotensin II in the high K^+-depolarized rabbit femoral artery

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1 Using front-surface fluorometry of fura-2-loaded strips, and measuring the transmembrane $^{45}Ca^{2+}$ fluxes of ring preparations of the rabbit femoral artery, the mechanism underlying a sustained decrease in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by angiotensin II (AT-II) was investigated.

2 The application of AT-II during steady-state 118 mM K^+-induced contractions caused a sustained decrease in $[Ca^{2+}]_i$ following a rapid and transient increase in $[Ca^{2+}]_i$, while the tension was transiently enhanced.

3 When the intracellular Ca^{2+} stores were depleted by thapsigargin, the initial rapid and transient increase in $[Ca^{2+}]_i$ was abolished, however, neither the sustained decrease in $[Ca^{2+}]_i$ nor the enhancement of tension were affected.

4 Depolarization with 118 mM K^+ physiological salt solution containing 1.25 mM Ba^{2+} induced a sustained increase in both the cytosolic Ba^{2+} concentration ($[Ba^{2+}]_i$) level and tension. However, the application of 10^{-6} M AT-II during sustained Ba^{2+}-contractions was found to have no effect on $[Ba^{2+}]_i$, but it did enhance tension.

5 After thapsigargin treatment, AT-II neither decreased nor increased the enhanced Ca^{2+} efflux rate induced by 118 mM K^+-depolarization, whereas AT-II did increase the enhanced $^{45}Ca^{2+}$ influx and the $^{45}Ca^{2+}$ net uptake induced by 118 mM K^+-depolarization.

6 Pretreatment with calphostin-C, partially, but significantly inhibited the decrease in $[Ca^{2+}]_i$ induced by AT-II.

7 These findings therefore suggest that AT-II stimulates Ca^{2+} sequestration into the thapsigargin-insensitive Ca^{2+} stores, and thus induces a decrease in $[Ca^{2+}]_i$ in the high external K^+-stimulated rabbit femoral artery.

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Abbreviations: AT-II, angiotensin-II; $[Ba^{2+}]_i$, cytosolic Ba^{2+} concentration; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; IP_3, inositol 1,4,5-trisphosphate; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PSS, physiological salt solution; SL, sarcolemma; SR, sarcoplasmic reticulum; VOCs, voltage-operated Ca^{2+} channels; VSMCs, vascular smooth muscle cells

Introduction

In vascular smooth muscle cells (VSMCs), angiotensin II (AT-II) induces biphasic increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which consist of an initial rapid increase and a subsequent smaller but sustained increase. The first transient phase is mainly due to the Ca^{2+} release from the intracellular stores mediated by inositol, 1,4,5-trisphosphate (IP_3), and the second sustained phase is due to the Ca^{2+} influx across the plasma membrane (Alexander *et al*., 1985). The Ca^{2+}-signalling pathways activated by AT-II to increase $[Ca^{2+}]_i$ levels have been well studied, however, little is still known about the mechanisms responsible for the decrease of $[Ca^{2+}]_i$ levels, which would be also activated during AT-II-stimulation (Ushio-Fukai *et al*., 1999).

Several investigators reported that some agonists induced a decrease in $[Ca^{2+}]_i$ in 2,5,-di-(tert-butyl)-1,4,-benzohydroquinone-treated rat hepatocytes (Duddy *et al*., 1989), in ionomycin-treated human polymorphonuclear leukocytes (Perianin & Snyderman, 1989), in human platelets (Rink & Sage, 1987) and in thapsigargin-treated smooth muscle cells in culture (Byron & Taylor, 1995). By measuring the $^{45}Ca^{2+}$ efflux, it was concluded that the agonist-induced decreases in $[Ca^{2+}]_i$ might have been due to a stimulation of Ca^{2+} extrusion. In these studies, however, the measurements of $^{45}Ca^{2+}$ efflux were performed either in the absence of extracellular Ca^{2+} or at very low concentrations, if any, of extracellular Ca^{2+} to accelerate the Ca^{2+} efflux, and as a result, mechanisms other than Ca^{2+} extrusion appear to have been largely neglected.

We previously reported the stimulation of the medial strips of the isolated femoral artery with high concentrations of AT-II to cause rapid increases in $[Ca^{2+}]_i$ and tension, which were followed by desensitization of contraction accompanied with a decrease in $[Ca^{2+}]_i$ in the second phase (Ushio-Fukai *et al*., 1999). The decrease in $[Ca^{2+}]_i$ appeared to be due not only to the inhibition of Ca^{2+} influx but also due to other unknown mechanisms. In addition, we found that AT-II induced a

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sustained decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+ (Ushio-Fukai *et al*., 1999). Since 118 mM K^+-depolarization would minimize the effects of AT-II on the membrane potential and, hence, on the Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCs), we hypothesized that the sustained decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+ may be due to the AT-II-induced removal of Ca^{2+} from the cytosol and might partially account for the decrease in $[Ca^{2+}]_i$ during the desensitization. In the present study, to determine the cellular mechanisms involved in the AT-II-induced desensitization accompanied with a reduction of $[Ca^{2+}]_i$, we examined the effects of AT-II on the tension and $[Ca^{2+}]_i$ in the isolated rabbit femoral artery depolarized with 118 mM K^+. We used front-surface fluorometry of fura-2 to monitor the $[Ca^{2+}]_i$ level and tension development simultaneously in medial strips and also measured the $^{45}Ca^{2+}$ influx, $^{45}Ca^{2+}$ efflux and $^{45}Ca^{2+}$ net uptake in the ring preparations. Our data suggest that, in the AT-II-induced decrease in $[Ca^{2+}]_i$ in 118 mM K^+-depolarized vascular strips, the sequestration of Ca^{2+} into thapsigargin-insensitive Ca^{2+} store was found to play a major role, while the sarcolemmal Ca^{2+} pump was observed to play a minor role. The AT-II-induced decrease in $[Ca^{2+}]_i$ is considered to be partially due to the activation of protein kinase C (PKC).

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, body weight 2.5–3.0 kg) were killed with sodium pentobarbital (100 mg kg^{-1} intravenously) and the femoral arteries were immediately excised. The fat and adventitia were dissected out using a binocular microscope. Some of the preparations were longitudinally opened and then cut into approximately 1×3 mm circular strips, 0.2 mm thick for simultaneous measurement of $[Ca^{2+}]_i$ and tension. Other preparations were cut into rings to measure the $^{45}Ca^{2+}$ fluxes. To obtain preparations without the endothelium, the intraluminal surface was rubbed with a cotton swab.

Fura-2 loading

Vascular strips without the endothelium were loaded with $[Ca^{2+}]_i$ indicator dye, fura-2, by incubation in 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 then were equilibrated in normal PSS for at least 1 h before the start of the measurements. The strips thus treated showed a specific fluorescence emission spectrum for the 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 nm and 380 nm, respectively, which were determined using 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), thus suggesting that the contractile responsiveness of the strips were not affected by either Ca^{2+} buffering action of fura-2 or any possible acidification of the cells due to formaldehyde release on the 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 of the strip connected to a force-transducer (strain gauge TB-612T, Nihon Koden, Japan). During a 1 h equilibration period, the strip was 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 be the minimal one (about 350 mg), at which the maximal response was obtained. Tension development was measured at 37°C, and was expressed as a percentage, assuming the values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0 and 100%, respectively.

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

Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex were simultaneously monitored during the tension measurements, using a front-surface fluorometer which had been 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). The 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 (F340) to that at 380 nm excitation (F380) was also recorded and expressed in percentage while assuming the values in normal (5.9 mM K^+) and 118 mM K^+ PSS to be 0 and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ in normal and 118 mM K^+ PSS were determined in separate measurements, using the equation given by Grynkiewicz *et al*. (1985) with minor modifications and the K_d value of 224 nM at 37°C. The mean values of $[Ca^{2+}]_i$ at normal PSS (at rest; 0%) and 118 mM K^+-depolarization (100%) were 113.5 ± 4.1 nM ($n = 10$) and 711.5 ± 4.2 nM ($n = 10$), respectively. To express the $[Ca^{2+}]_i$ levels throughout the experiment, the per cent fluorescence ratio was used, because permeabilization of the cell membrane with ionomycin (25 μM) for calibration at the end of each measurement of various protocols caused considerable deviations in the estimated absolute values of $[Ca^{2+}]_i$.

In the experiment noted in Figure 5, Ca^{2+} in 118 mM K^+-PSS was substituted with Ba^{2+} and changes in the fluorescence ratio (F340/F380) was monitored during 118 mM K^+-depolarization. Since Ba^{2+} enters the cells through VOCs (Yamaguchi *et al*., 1989), can bind to fura-2 with a Kd (780 nM) similar to that for Ca^{2+} and emit fluorescence with a spectrum similar to Ca^{2+}-fura-2 complex, the changes in the fluorescence ratio was thus considered to primarily reflect changes in cytosolic Ba^{2+} concentration ($[Ba^{2+}]_i$) (Schilling *et al*., 1989).

Measurement of the $^{45}Ca^{2+}$ fluxes

The calcium-45 influx, $^{45}Ca^{2+}$ net uptake and $^{45}Ca^{2+}$ efflux 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 in appropriate solutions for various periods and then were incubated in the same solution but with the addition of $^{45}Ca^{2+}$ (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. 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 7 ml liquid scintillation cocktail (ACS II, Amersham Co., U.S.A.), the radioactivity was counted using a liquid scintillation counter (LCS-3500, Aloka Co., Tokyo, Japan). The amount of Ca^{2+} estimated from incorporation of $^{45}Ca^{2+}$ into the samples was expressed as μmoles per kg wet weight per 2 min. For the $^{45}Ca^{2+}$ net uptake, the ring preparations were incubated in normal PSS containing $^{45}Ca^{2+}$ (185 kBq ml^{-1}) for at least 3 h, and then were incubated in $^{45}Ca^{2+}$-labelled experimental solutions for various periods at 37°C. The samples were processed in the same manner as the $^{45}Ca^{2+}$ influx experiments. The $^{45}Ca^{2+}$ net uptake was expressed as μmoles per kg wet weight. For $^{45}Ca^{2+}$ efflux, the samples were incubated in normal PSS containing $^{45}Ca^{2+}$ (740 kBq ml^{-1}) for at least 3 h and then were incubated in 1 ml of appropriate solutions. The solutions were changed with fresh ones every 1 min and trapped in a vial. The radioactivity of each solution and of the samples was measured. The $^{45}Ca^{2+}$ efflux rate was expressed as a fraction lost and plotted as a function of the efflux time.

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 equimolar substitution of KCl for NaCl. Na^+-free PSS was made by equimolar substitution of NaCl and $NaHCO_3$ for N-methyl-D-glucamine or cholineCl. All solutions were gassed with a mixture of 5% CO_2 and 95% O_2 (pH 7.4 at 37°C). Angiotensin-II (human) and calphostin-C were purchased from the Peptide Institute Co. Ltd. (Osaka, Japan) and the Kyowa Medics Co. (Tokyo, Japan), respectively. Thapsigargin, phorbol-12,13-dibutyrate, N-methyl-D-glucamine and cholineCl were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). DUP-753 was obtained from the Taisho Pharmaceutical Co. (Tokyo, Japan). Fura-2/AM was purchased from Dojindo Laboratories (Kumamoto, Japan) and was dissolved in dimethyl sulphoxide (DMSO) as a stock solution and then diluted in the medium just before loading the dye. The final concentration of DMSO was 5%.

Statistical analysis

The values are expressed as the mean ± s.e.mean. Student's *t*-test or an analysis of variance with the multiple comparison test were used to determine statistical significance. A value of $P<0.05$ was considered to be significant.

Results

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

Depolarization with 118 mM K^+ PSS induced rapid increases in the $[Ca^{2+}]_i$ and tension in medial strips of the rabbit femoral artery, which reached steady-state levels within 5 and 10 min, respectively, and were maintained for at least 30 min of observation. Figure 1 shows a summary of the changes in the $[Ca^{2+}]_i$ and tension induced by the various concentrations of AT-II (10^{-9}–10^{-6} M) in the strips pre-contracted for 15 min with 118 mM K^+-depolarization. Both the rapid and transient increase and the subsequent sustained decrease in $[Ca^{2+}]_i$ were observed at concentrations higher than 10^{-9} M AT-II, in a concentration-dependent manner. The higher the concentrations of AT-II, the faster and the greater the transient increase and the sustained decrease in $[Ca^{2+}]_i$. The tension development induced by AT-II was also concentration-dependent. The higher the concentrations of AT-II, the faster and the greater the increase in tension. However, the enhanced tension was not maintained, and the level at 20 min after the application of AT-II was not dependent on the concentration of AT-II. Lower concentrations of AT-II (10^{-10}–10^{-9} M) did not decrease $[Ca^{2+}]_i$, whereas they gradually enhanced the tension development induced by 118 mM K^+ (data not shown).

Figure 2A shows the effects of 10^{-6} M AT-II on the strips depolarized with 118 mM K^+ in the absence of extracellular Na^+, a condition which would eliminate the involvement of the Na^+/Ca^{2+} exchange mechanism for the Ca^{2+} efflux from the cells (Figure 2A). When the external bathing solution was

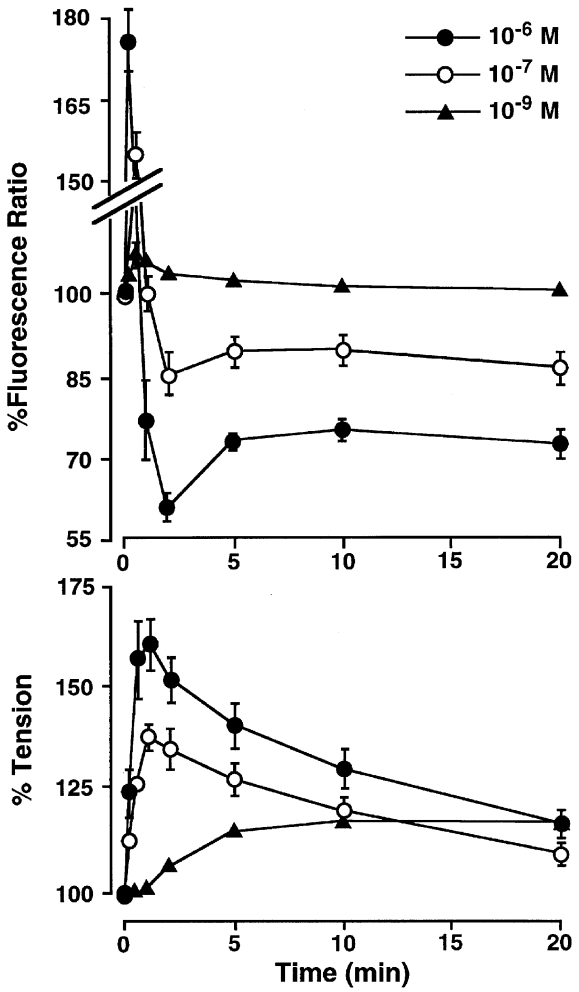

Figure 1 The effects of various concentrations of AT-II on the increases in the fluorescence ratio ($[Ca^{2+}]_i$) and tension induced by 118 mM K^+-depolarization. The abscissa indicates the time after the application of AT-II (min). The ordinate indicates the responses of the fluorescence ratio (upper trace) and tension (lower trace) expressed as a percentage of those obtained by 118 mM K^+-depolarization (100%). The data are the means ± s.e.mean (shown by vertical lines; $n=6$).

changed from normal PSS (5.9 mM K^+) to Na^+-free 118 mM K^+ PSS, the $[Ca^{2+}]_i$ and tension rapidly increased and reached steady-state levels in 5 and 10 min, respectively, and these levels were thereafter maintained for at least 30 min. The maximal levels of the transient increase in $[Ca^{2+}]_i$ ($207.2 \pm 12.6\%$, at 15 s, $n=4$), the sustained decrease in $[Ca^{2+}]_i$ ($66.7 \pm 7.6\%$, at 2 min, $n=4$) and the tension ($156.9 \pm 8.6\%$, at 1 min, $n=4$) induced by 10^{-6} M AT-II in the strips depolarized with Na^+-free 118 mM K^+ PSS were not significantly different from those obtained in normal 118 mM K^+-PSS. The changes in $[Ca^{2+}]_i$ and tension induced by 10^{-6} M AT-II in the strips depolarized with 118 mM K^+ were completely inhibited by 10^{-5} M DUP-753, a specific antagonist of AT-II type 1 receptor (Chiu *et al.*, 1990), thus indicating the effects of AT-II on $[Ca^{2+}]_i$ and tension are mediated by AT-II type 1 receptor (Figure 2B).

Effects of AT-II on $^{45}Ca^{2+}$ influx into the strips depolarized with 118 mM K^+

The ring preparations were incubated in normal PSS or 118 mM K^+-PSS for 15 min, and then were incubated with or without 10^{-6} M AT-II in the same solution for an additional 0, 3 and 20 min. The tissue specimens were transferred to the same but $^{45}Ca^{2+}$-labelled solutions, and incubated for 2 min (Figure 3). In normal PSS, AT-II increased $^{45}Ca^{2+}$ influx by 3.0 times (solid bar in PSS + AT-II). Incubation with AT-II for 3 min or 20 min significantly attenuated the increase in the $^{45}Ca^{2+}$ influx induced by AT-II ($P<0.05$, $n=6$, hatched and open bars, respectively, in PSS + AT-II). Depolarization with

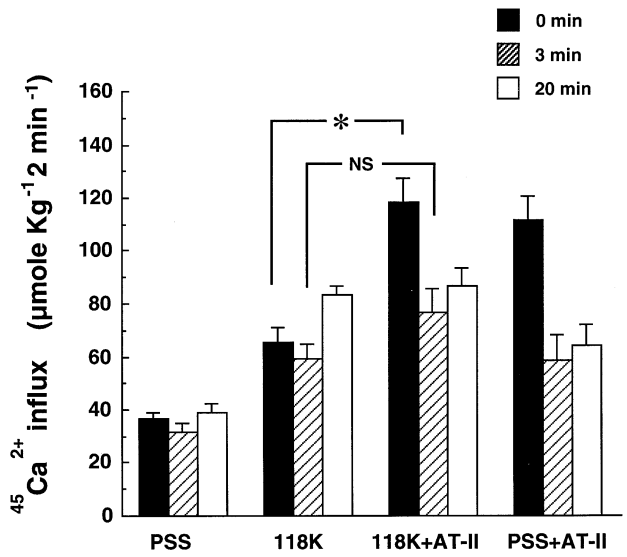

Figure 3 Effects of 118 mM K^+-depolarization and AT-II on the $^{45}Ca^{2+}$ influx into the ring preparations. PSS: $^{45}Ca^{2+}$ influx into the control tissues was measured in normal PSS. 118K: The tissues were incubated in 118 mM K^+-PSS for 15 min, 18 min and 35 min and, then the $^{45}Ca^{2+}$ influx was measured for 2 min in 118 mM K^+-PSS. 118K + AT-II: The tissue specimens were incubated in 118 mM K^+-PSS for 15 min and, then were incubated with 10^{-6} M AT-II for 0, 3 and 20 min. $^{45}Ca^{2+}$ influx was measured for 2 min in 118 mM K^+-PSS containing 10^{-6} M AT-II. PSS + AT-II: The tissue specimens were incubated with AT-II in normal PSS for 0, 3 and 20 min. $^{45}Ca^{2+}$ influx was measured for 2 min in normal PSS containing 10^{-6} M AT-II. $^*P<0.05$. NS; not significant. The data are the means ± s.e.mean (shown by vertical lines, $n=6$).

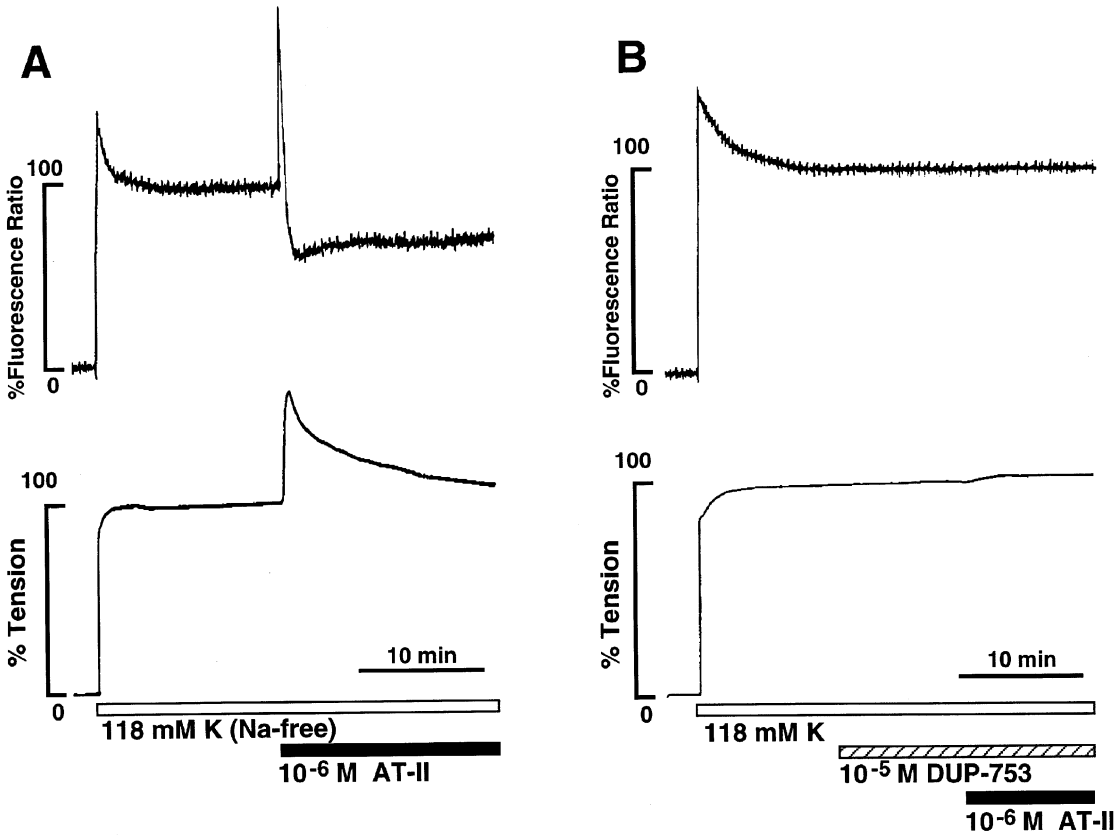

Figure 2 Representative recordings showing the effect of the removal of extracellular Na^+ and pretreatment with DUP-753 on the sustained decrease in $[Ca^{2+}]_i$ and tension induced by AT-II in the strips depolarized with 118 mM K^+. (A) Traces show the changes in the fluorescence ratio ($[Ca^{2+}]_i$, upper trace) and tension development (lower trace) in response to 10^{-6} M AT-II in Na^+-free, 118 mM K^+-PSS. External Na^+ was completely replaced by N-methyl-D-glucamine. (B) DUP-753 (10^{-5} M) was applied to the strips precontracted by 118 mM K^+-depolarization at a steady-state in $[Ca^{2+}]_i$ and tension. AT-II (10^{-6} M) was applied at 10 min after the addition of DUP-753.

118 mM K^+ for 15 min increased the $^{45}Ca^{2+}$ influx by 1.7 times (solid bar in 118K). The application of AT-II to the strips depolarized with 118 mM K^+ for 15 min further increased the $^{45}Ca^{2+}$ influx within 2 min ($P<0.05$, $n=6$) (solid bar in 118K + AT-II). The increase was not additive and was attenuated after the incubation with AT-II for 3 min and 20 min ($P<0.05$, $n=6$, hatched and open bars, respectively, in 118K + AT-II). The $^{45}Ca^{2+}$ influx after incubation with AT-II for 3 min in 118 mM K^+-PSS (hatched bar in 118K + AT-II) was not statistically different from that of the control strips in 118 mM K^+-PSS ($P>0.05$, $n=6$, hatched bar in 118K). Similar results were also obtained in the strips incubated with AT-II for 20 min in 118 mM K^+-PSS (open bar in 118K + AT-II). The $^{45}Ca^{2+}$ influx in the strips depolarized with 118 mM K^+ for 18 min and 35 min ($n=6$, hatched and open bars, respectively, in 118K) were not statistically different from that of the strips depolarized for 15 min.

Effects of AT-II on the thapsigargin-pretreated strips depolarized with 118 mM K^+

To elucidate the involvement of Ca^{2+} sequestration into the intracellular stores in the AT-II-induced decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+, we examined the effects of AT-II on the strips in which the intracellularly stored Ca^{2+} was depleted with thapsigargin, an inhibitor of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (Thastrup *et al*., 1989). Figure 4 shows the representative recordings of the effects of pretreatment with 10^{-5} M thapsigargin on the sustained decrease induced by 10^{-6} M AT-II in a strip depolarized with 118 mM K^+. In normal PSS, the application of 10^{-5} M thapsigargin induced a gradual increase in $[Ca^{2+}]_i$, which reached its peak at 3 min (42.5 ± 3.1%, $n=6$), and then, $[Ca^{2+}]_i$ gradually declined, but was maintained at relatively higher level than the resting level for at least 30 min. In contrast, thapsigargin did not increase the tension. Pretreatment with thapsigargin (10^{-5} M) for 20 min abolished the first transient increase in $[Ca^{2+}]_i$ induced by 10^{-6} M AT-II during 118 mM K^+-depolarization without affecting the sustained decrease in $[Ca^{2+}]_i$ and the enhancement of the contraction. The maximal decrease in $[Ca^{2+}]_i$ (51.8 ± 8.5%, at 2 min, $n=6$) and the enhanced contraction (163.9 ± 9.7%, at 1 min, $n=6$) elicited by 10^{-6} M AT-II in thapsigargin-treated strips depolarized with 118 mM K^+-PSS were not significantly different from those in the control experiments (Figure 1; 10^{-6} M AT-II).

Effects of AT-II on the thapsigargin-pretreated strips depolarized with 118 mM K^+-PSS containing Ba^{2+} instead of Ca^{2+}

To elucidate the involvement of the activation of Ca^{2+} pumps in the sustained decrease in $[Ca^{2+}]_i$ induced by AT-II in the strips depolarized with 118 mM K^+, we examined the effects of AT-II on the strips pretreated with 10^{-5} M thapsigargin and depolarized with 118 mM K^+-PSS containing 1.25 mM $BaCl_2$, instead of $CaCl_2$ (Figure 5). After the depletion of the SR Ca^{2+} by pretreatment with 10^{-5} M thapsigargin for 20 min and incubation in Ca^{2+}-free PSS for 10 min, depolarization with 118 mM K^+-PSS containing 1.25 mM Ba^{2+} induced sustained increases in the fluorescence ratio (169.1 ± 5.1%, at 10 min, $n=6$) and tension (56.5 ± 4.2%, at 10 min, $n=6$). At the steady state of the contraction induced by 118 mM K^+-PSS containing 1.25 mM Ba^{2+}, the application of 10^{-6} M AT-II did not change the fluorescence ratio ($[Ba^{2+}]_i$), but did increase the tension. The tension reached a maximum at 1 min (86.8 ± 4.1%, $n=6$) and then declined to a steady state level which was maintained for at least 30 min of observation.

Effects of AT-II on the Ca^{2+} efflux from the strips depolarized with 118 mM K^+

The ring preparations were equilibrated in $^{45}Ca^{2+}$-labelled normal PSS, and then were incubated in normal PSS or 118 mM K^+-PSS for 15 min to monitor the $^{45}Ca^{2+}$ efflux (Figure 6). The $^{45}Ca^{2+}$ efflux rate in 118 mM K^+-PSS at 15 min

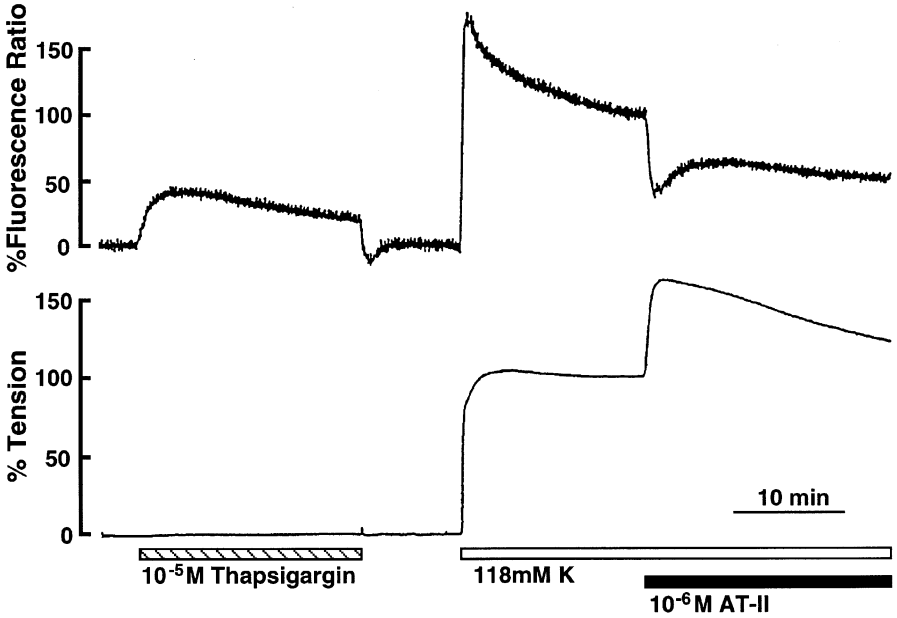

Figure 4 Representative recordings showing the effect of pretreatment with thapsigargin on the sustained decrease in $[Ca^{2+}]_i$ induced by AT-II in the strips depolarized with 118 mM K^+. The upper trace shows the changes in the fluorescence ratio ($[Ca^{2+}]_i$) while the lower trace shows tension development in response to 10^{-6} M AT-II during 118 mM K^+-depolarization after treatment with thapsigargin (10^{-5} M) for 20 min. AT-II (10^{-6} M) was applied 15 min after the initiation of 118 mM K^+-depolarization.

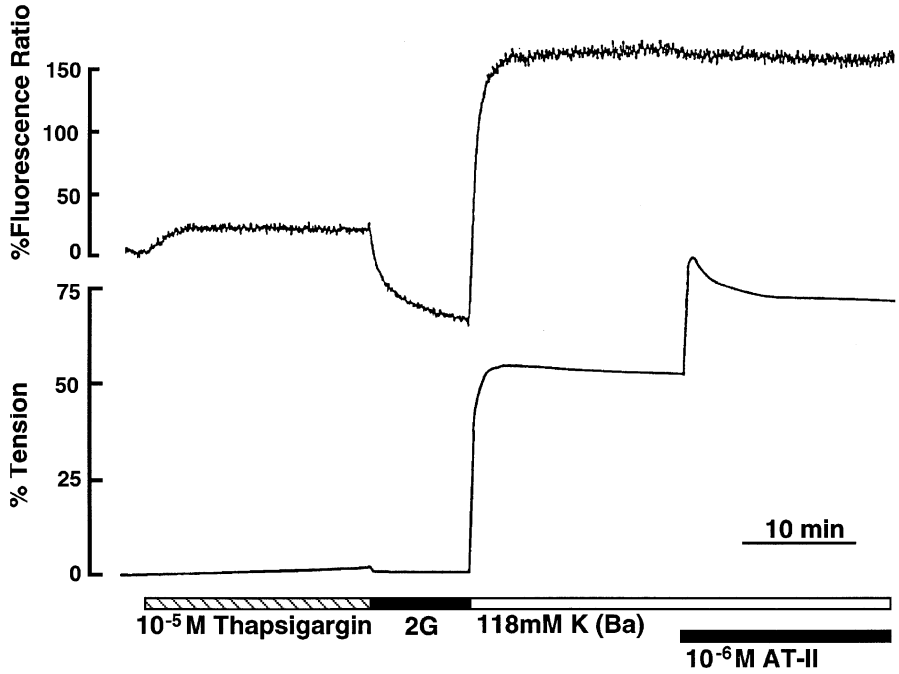

Figure 5 Representative recordings showing the effect of AT-II on the increases in the cytosolic Ba^{2+} concentration ($[Ba^{2+}]_i$) and tension induced by 118 mM K^+-depolarization and substitution of Ca^{2+} for Ba^{2+}. After treatment with thapsigargin for 20 min to deplete the intracellular Ca^{2+} stores and the incubation in Ca^{2+}-free PSS for 10 min (2G), the strips were stimulated with 118 mM K^{+g}-PSS containing 1.25 mM Ba^{2+} instead of Ca^{2+}. AT-II (10^{-6} M) was applied 15 min after the initiation of 118 mM K^+-depolarization containing 1.25 mM Ba^{2+}.

was significantly higher ($P<0.05$) than that in normal PSS (Figure 6A). When 10^{-6} M AT-II was applied in normal PSS, the $^{45}Ca^{2+}$ efflux rate significantly increased in comparison to the control tissues ($P<0.05$, compared at 1 min after application of AT-II). In contrast, 10^{-6} M AT-II did not increase the $^{45}Ca^{2+}$ efflux rate in 118 mM K^+-PSS. Since pretreatment with thapsigargin did not prevent the sustained decrease in $[Ca^{2+}]_i$ induced by AT-II in the strips depolarized with 118 mM K^+ (Figure 4), we therefore examined the effects of AT-II on the $^{45}Ca^{2+}$ efflux in the presence of 10^{-5} M thapsigargin (Figure 6B). In a preliminary study, treatment with 10^{-5} M thapsigargin did not significantly change the $^{45}Ca^{2+}$ efflux rate in normal PSS. Thapsigargin was added to the $^{45}Ca^{2+}$-labelled normal PSS 20 min before the measurement of $^{45}Ca^{2+}$ efflux. The $^{45}Ca^{2+}$ efflux rate in 118 mM K^+-PSS at 15 min was significantly higher ($P<0.05$) than that in normal PSS. In the thapsigargin-treated preparations, the application of 10^{-6} M AT-II in normal PSS increased the $^{45}Ca^{2+}$ efflux rate significantly ($P<0.05$, at 1 min after application of AT-II), however, AT-II did not significantly change the $^{45}Ca^{2+}$ efflux rate in 118 mM K^+-PSS.

Effects of AT-II on the $^{45}Ca^{2+}$ net uptake into the strips depolarized with 118 mM K^+

The effects of AT-II on the $^{45}Ca^{2+}$ net uptake into the rabbit femoral arteries were examined as shown in Figure 7. Depolarization with 118 mM K^+-PSS (solid bar in 118K) for 20 min significantly increased the $^{45}Ca^{2+}$ net uptake in comparison to the control tissues in normal PSS (solid bar in PSS, $P<0.05$). Although the application of 10^{-6} M AT-II in 118 mM K^+-PSS for 5 min to the tissue preparations, which were pre-incubated for 15 min in 118 mM K^+-PSS, induced a sustained decrease in $[Ca^{2+}]_i$ (Figure 1), the $^{45}Ca^{2+}$ net uptake at 5 min after the application of 10^{-6} M AT-II in 118 mM K^+-PSS (solid bar in 118K + AT-II) was significantly greater than that at 20 min after incubation in 118 mM K^+-PSS alone (solid bar in 118K, $P<0.05$). Incubation with 10^{-6} M AT-II for 5 min in normal PSS (solid bar in PSS + AT-II) increased the $^{45}Ca^{2+}$ net uptake to a similar extent as that in 118 mM K^+-PSS alone (solid bar in 118K, $P<0.05$).

When the tissue specimens were pre-incubated for 20 min in normal PSS containing 10^{-5} M thapsigargin, depolarization with 118 mM K^+ for 20 min (hatched bar in 118K) increased the $^{45}Ca^{2+}$ net uptake significantly (vs hatched bar in PSS, $P<0.05$). The $^{45}Ca^{2+}$ net uptake in the thapsigargin-treated and 118 mM K^+-depolarized tissues (hatched bar in 118K) were also significantly smaller than that in the thapsigargin-non-treated tissues (solid bar in 118K, $P<0.05$). The application of 10^{-6} M AT-II for 5 min to the thapsigargin-treated and 118 mM K^+-depolarized tissues induced a sustained decrease in $[Ca^{2+}]_i$ (Figure 4), however, it did not decrease the $^{45}Ca^{2+}$ net uptake (hatched bars; 118K vs 118K + AT-II, $P>0.05$). Similar results were obtained, when the thapsigargin-treated tissues were incubated for 20 min with 10^{-6} M AT-II in 118 mM K^+-PSS after the depolarization of the tissue specimens for 15 min in 118 mM K^+-PSS. Although the $[Ca^{2+}]_i$ level decreased 20 min after the application of 10^{-6} M AT-II (Figure 4), the $^{45}Ca^{2+}$ net uptake did not decrease in comparison to the tissue specimens incubated in 118 mM K^+-PSS alone (open bars; 118K vs 118K + AT-II, $P>0.05$).

Effects of phorbol ester on the strips depolarized with 118 mM K^+ and the effects of AT-II on the calphostin-C-pretreated strips depolarized with 118 mM K^+

In order to investigate the role of PKC in the decrease in $[Ca^{2+}]_i$ and the increase in tension induced by AT-II in the strips depolarized with 118 mM K^+, we examined the effects of phorbol 12,13-dibutyrate (PDBu), a PKC activator, and

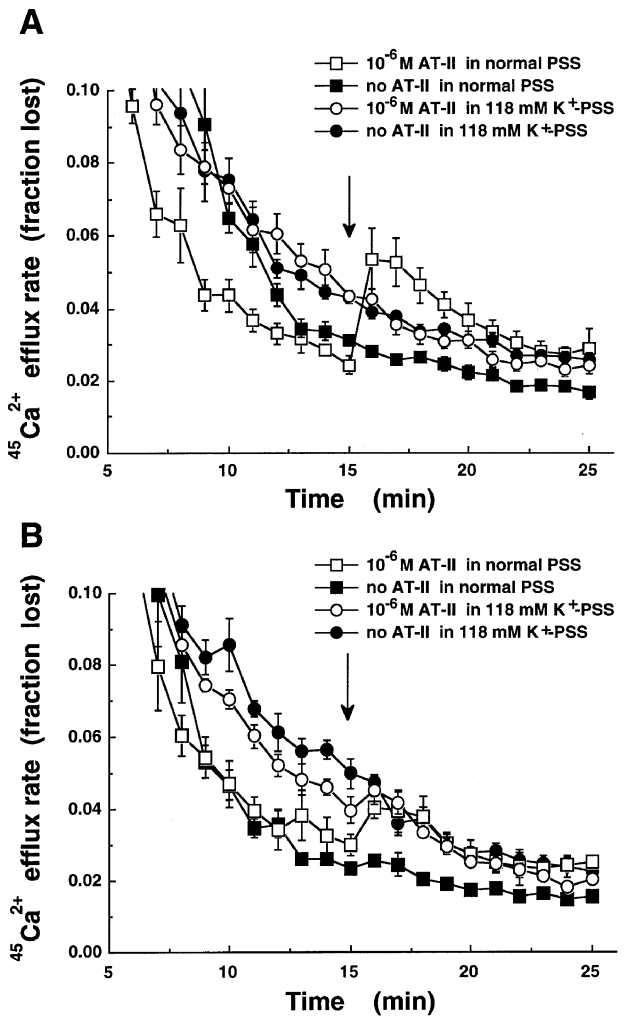

Figure 6 Effects of AT-II on the $^{45}Ca^{2+}$ efflux in normal PSS and in 118 mM K^+-PSS in the absence (A) or the presence (B) of thapsigargin. (A) The ring preparations were equilibrated in $^{45}Ca^{2+}$-labelled PSS for at least 3 h. The $^{45}Ca^{2+}$ efflux was measured in either normal PSS or in 118 mM K^+-PSS. AT-II (10^{-6} M) was applied 15 min after the start of the measurement of the efflux (arrow). $^{45}Ca^{2+}$ efflux rate was expressed as a fraction lost. (B) Similar experiments were conducted but in the presence of 10^{-5} M thapsigargin. Thapsigargin was applied at 20 min before the $^{45}Ca^{2+}$ efflux was measured. The data are the means ± s.e.mean (shown by vertical lines, $n=6$).

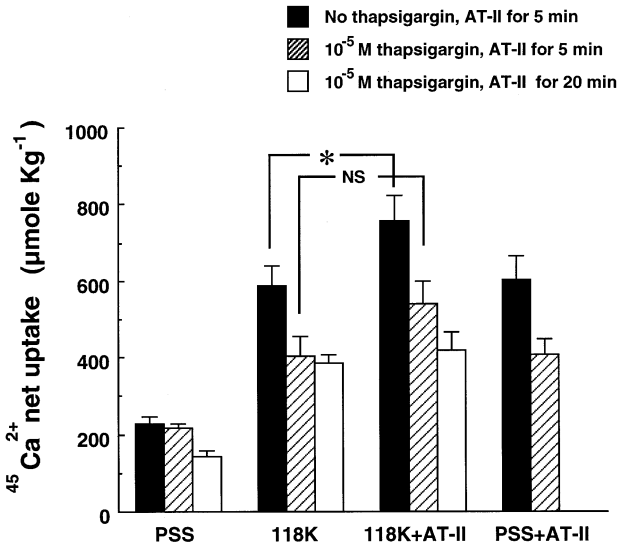

Figure 7 Effects of AT-II on the $^{45}Ca^{2+}$ net uptake in normal PSS and in 118 mM K^+-PSS. The ring preparations were equilibrated in $^{45}Ca^{2+}$-labelled normal PSS for at least 3 h. PSS: $^{45}Ca^{2+}$ net uptake into the control tissues were measured in normal PSS. 118K: The tissue specimens were incubated in the $^{45}Ca^{2+}$-labelled 118 mM K^+-PSS for 20 min in either the absence or the presence of thapsigargin (10^{-5} M), or for 35 min in the presence of thapsigargin (10^{-5} M). Thapsigargin was applied 20 min before starting the incubation in 118 mM K^+-PSS. 118K + AT-II: The tissue specimens were incubated in $^{45}Ca^{2+}$-labelled 118 mM K^+-PSS for 15 min and, thereafter were stimulated by 10^{-6} M AT-II for 5 min in the same solution in the absence or the presence of thapsigargin (10^{-5} M), or for 20 min in the same solution in the presence of thapsigargin (10^{-5} M). PSS + AT-II: The tissue specimens were stimulated by AT-II (10^{-6} M) for 5 min in $^{45}Ca^{2+}$-labelled normal PSS in either the absence or the presence of thapsigargin (10^{-5} M). Thapsigargin was applied 35 min before the application of AT-II. $^*P<0.05$. NS; not significant. The data are the means ± s.e.mean (shown by vertical lines; $n=7$).

calphostin-C, a PKC inhibitor. As shown in Figure 8A, the application of 10^{-8} M PDBu to the strips depolarized with 118 mM K^+ caused a gradual decrease in $[Ca^{2+}]_i$ and an enhancement of contraction. The maximal decrease in $[Ca^{2+}]_i$ (48.3 ± 6.9%, $n=6$) and the maximal increase in tension (151.1 ± 7.4%, $n=6$) were observed at 16 min and 10 min after the application of 10^{-8} M PDBu, respectively. After reaching a maximum, the tension decreased gradually over time, and this level of $[Ca^{2+}]_i$ was maintained at a relatively lower level than that obtained with 118 mM K^+ for at least 30 min.

Pretreatment with calphostin-C (10^{-6} M) for 50 min, partially, but significantly ($P<0.05$) inhibited the first transient increase and the subsequent sustained decrease in $[Ca^{2+}]_i$ provided by 10^{-6} M AT-II, without any significant effects on the AT-II-induced enhancement of tension development, in the strips depolarized with 118 mM K^+ (Figure 8B). The maximal levels of the first transient increase in $[Ca^{2+}]_i$, the subsequent sustained decrease in $[Ca^{2+}]_i$ and the enhancement of contraction induced by 10^{-6} M AT-II in the calphostin-C-treated strips were 117.2 ± 7.2% ($n=6$), 89.6 ± 6.9% ($n=6$) and 149.1 ± 7.5% ($n=6$), respectively. In addition, pretreatment with H-7 (10^{-5} M), another inhibitor of PKC, partially inhibited the AT-II-induced decrease in $[Ca^{2+}]_i$ (data not shown). On the other hand, pretreatment with H-8, an inhibitor of cyclic AMP-dependent protein kinase, and methylene blue, a guanylate cyclase inhibitor, failed to inhibit the AT-II-induced decrease in $[Ca^{2+}]_i$ in the 118 mM K^+-depolarized strips (data not shown).

Discussion

In the present study, the mechanisms involved in the inhibitory effects of AT-II on $[Ca^{2+}]_i$ were determined in the rabbit femoral artery. In medial strips depolarized with 118 mM K^+, AT-II induced a transient increase and subsequently a sustained decrease in $[Ca^{2+}]_i$, while it transiently enhanced the tension. These effects are dependent on the concentrations of AT-II (Figure 1) and completely inhibited by DUP-753 (10^{-5} M), an AT-II type 1 receptor-specific antagonist, thus indicating that they are mediated by AT-II type 1 receptor (Figure 2B). The first transient increase in $[Ca^{2+}]_i$ induced by AT-II is due to an intracellular Ca^{2+} release, because it was abolished by the treatment with thapsigargin, an inhibitor of the SR Ca^{2+}-ATPase (Figure 4) (Thastrup *et al.*, 1989). The enhancement of contraction may have been due to an increase in the Ca^{2+}-sensitivity of the contractile apparatus (Somlyo &

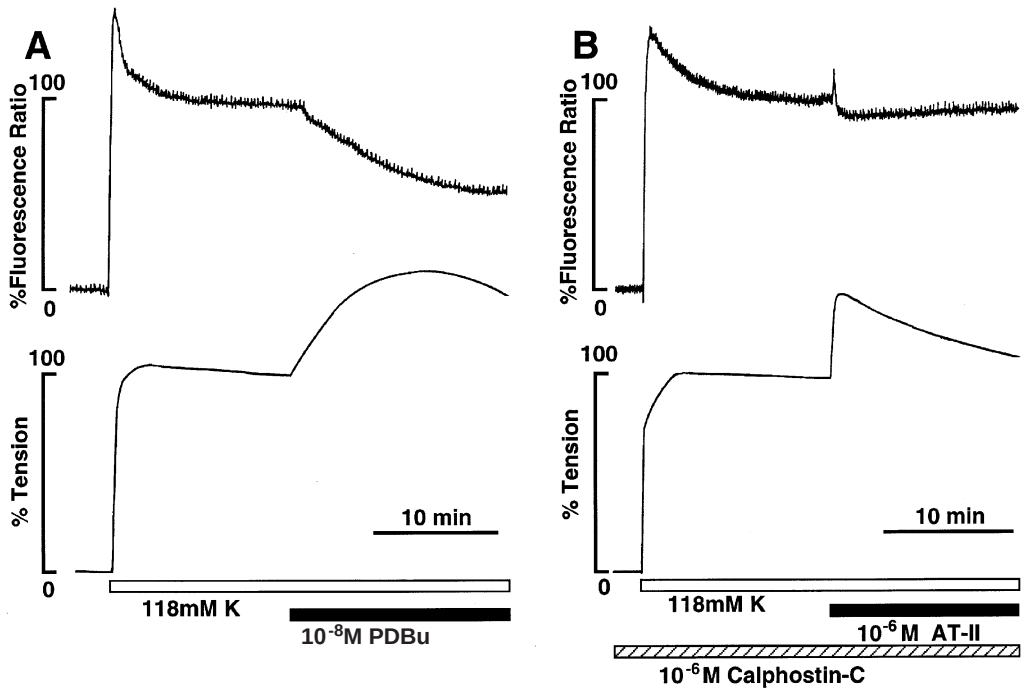

Figure 8 Representative recordings showing the effect of phorbol 12, 13-dibutyrate on the increases in the fluorescence ratio ($[Ca^{2+}]_i$) and tension development induced by 118 mM K^+-depolarization, and the effects of calphostin-C on the sustained decrease in $[Ca^{2+}]_i$ produced by AT-II in the strips depolarized with 118 mM K^+. (A) Phorbol 12, 13-dibutyrate (PDBu, 10^{-8} M) was applied 15 min after the initiation of 118 mM K^+-depolarization. (B) Calphostin-C (10^{-6} M) was applied 35 min before the initiation of 118 mM K^+-depolarization. AT-II (10^{-6} M) was applied 15 min after the initiation of 118 mM K^+-depolarization.

Somlyo, 1994), because it was also observed without any elevation of $[Ca^{2+}]_i$ in the thapsigargin-treated strips (Figure 4).

The sustained decrease in $[Ca^{2+}]_i$ induced by AT-II in the strips depolarized with 118 mM K^+ may suggest an unidentified Ca^{2+}-signalling pathway activated by AT-II. The decrease in $[Ca^{2+}]_i$ theoretically occurs *via*: (a) the inhibition of Ca^{2+} influx (Schuhmann & Groschner, 1994; Rane & Dunlap, 1986), (b) the activation of Ca^{2+} extrusion (Smith & Smith, 1987; Vigne, *et al*., 1988; Furukawa *et al*., 1989) and (c) the sequestration of Ca^{2+} into the intracellular stores. As shown in Figure 3, the $^{45}Ca^{2+}$ influx stimulated by 118 mM K^+ was not inhibited by AT-II, thus indicating that the decrease in $[Ca^{2+}]_i$ may not be due to an inhibition of the Ca^{2+} influx. VSMCs have been reported to have two major systems for Ca^{2+} extrusion, namely Na^+/Ca^{2+} exchange (Smith & Smith, 1987) and sarcolemmal Ca^{2+}-pump (Vigne *et al*., 1988; Furukawa *et al*., 1989). In the present study, the AT-II-induced decrease in $[Ca^{2+}]_i$ was not affected by the removal of extracellular Na^+ (Figure 2A). Therefore, the Na^+/Ca^{2+} exchange did not seem to be responsible for the decrease in $[Ca^{2+}]_i$ induced by AT-II. Inconsistent with our results, Smith & Smith (1987) reported that AT-II caused a decrease in the Ca^{2+} content in normal PSS by stimulation of Na^+/Ca^{2+} exchange in cultured VSMCs. We observed that AT-II increased the $^{45}Ca^{2+}$ net uptake into the rabbit femoral arterial strips in normal PSS (Figure 7 and Ushio-Fukai *et al*., 1999). Therefore, regardless of whether the strips are depolarized with high K^+ or not, the Na^+/Ca^{2+} exchange does not seem to be involved in the AT-II-induced decrease in $[Ca^{2+}]_i$ in our preparation. The discrepancy between our data and those of Smith & Smith (1987) could be due to differences in the Ca^{2+} handling between mature cells in isolated strips and cultured cells, or those in the experimental conditions. Furthermore, it should be noted that the effects of AT-II were determined in the strips depolarized with 118 mM K^+ in the present study. This experiment is a good model for investigating the mechanisms involved in the AT-II-induced desensitization, however, $[Ca^{2+}]_i$ levels during the 118 mM K^+ depolarization and just prior to AT-II receptor activation were higher than approximately 800 nM (data not shown), thus resulting in an up/or down regulation of calcium-dependent processes and the depolarization itself and thereby effecting the ionic equilibrium of the muscle.

Pretreatment with thapsigargin which depletes Ca^{2+} accumulated in the IP_3-sensitive and -insensitive stores (Bian *et al*., 1991) and has no effect on either PKC or protein phosphatases (Thastrup *et al*., 1989; 1990) did not affect the sustained decrease in $[Ca^{2+}]_i$ induced by AT-II in the 118 mM K^+-depolarized strips (Figure 4). Since the first transient increase in $[Ca^{2+}]_i$ induced by AT-II was completely abolished by the pretreatment with thapsigargin and the $^{45}Ca^{2+}$ net uptake stimulated with 118 mM K^+ into the thapsigargin-treated strips was significantly less than that into the non-treated strips (Figure 7), it is thus reasonable to assume that the Ca^{2+} uptake into the thapsigargin-sensitive stores was completely inhibited by the pretreatment with thapsigargin under the present conditions (10^{-5} M, 20 min). We thus conclude that the Ca^{2+} uptake into the thapsigargin-sensitive stores may not be involved in the AT-II-induced decrease in $[Ca^{2+}]_i$. Interestingly, the thapsigargin-induced increase in $[Ca^{2+}]_i$ was rapidly reduced following the removal of extracellular Ca^{2+} (Figure 5), thus suggesting the involvement of store-operated Ca^{2+} influx pathway in this response. The AT-II-induced Ca^{2+} release from the thapsigargin-sensitive stores in turn are considered to induce activation of store-operated Ca^{2+} entry which, at least in part, may contribute to the AT-II-stimulated increase in $[Ca^{2+}]_i$ in our preparation.

Since thapsigargin has little or no effect on the Ca^{2+}-ATPase of the plasma membrane (Thastrup *et al*., 1990) and

the thapsigargin-insensitive Ca^{2+} stores exist in various types of cells including smooth muscle cells (Bian *et al*., 1991; Cavallini *et al*., 1995; Hardy *et al*., 1995; Waldron *et al*., 1995), we examined whether the stimulation of Ca^{2+} transport through the Ca^{2+} pumps of the sarcolemma (SL) or of the thapsigargin-insensitive Ca^{2+} stores may be involved in the AT-II-induced decrease in $[Ca^{2+}]_i$. To test this possibility, we replaced Ca^{2+} in the bathing solutions with Ba^{2+}. Ba^{2+} can bind to fura-2 with a similar affinity to Ca^{2+} and also emits fluorescence similar to Ca^{2+} (Schilling *et al*., 1989). Ba^{2+} is permeable to VOCs, ligand-gated channels and leak channels (Hagiwara & Byerly, 1981) and induces contraction, while it cannot substitute for Ca^{2+} in a number of cellular reactions (Schilling *et al*., 1989). As shown in Figure 5, when the thapsigargin-pretreated strips were depolarized with the 118 mM K^+-PSS containing Ba^{2+}, $[Ba^{2+}]_i$ increased and reached a steady state. In the strips depolarized with 118 mM K^+-PSS containing Ca^{2+}, the $^{45}Ca^{2+}$ influx initially increased (135 μmole per kg wet weight per 2 min, Ushio-Fukai *et al*., 1999), then, decreased after incubation for 15 min (65 μmole per kg wet weight per 2 min, Figure 3) but was still greater than that in normal PSS. Then, the Ba^{2+} influx through VOCs during 118 mM K^+-depolarization would also decrease with time but would be still greater than that in normal PSS. If such is the case, Ba^{2+} should be removed from the cytosol through unknown mechanisms during the steady state of $[Ba^{2+}]_i$. When AT-II was applied to the thapsigargin-pretreated strips depolarized with 118 mM K^+, the $[Ba^{2+}]_i$ did not decrease (Figure 5). Because AT-II initially increased but never decreased the $^{45}Ca^{2+}$ influx pre-stimulated by 118 mM K^+-depolarization (Figure 3), AT-II should have stimulated the mechanisms that remove Ca^{2+} from the cytosol, effectively enough to decrease the $[Ca^{2+}]_i$ (Figure 1). Therefore, assuming that AT-II stimulates Ba^{2+} influx through Ca^{2+} channels in the same manner as Ca^{2+} influx, the lack of a change in $[Ba^{2+}]_i$ after the application of AT-II suggests that AT-II may stimulate the unknown mechanisms that remove Ba^{2+} from the cytosol and inhibit an increase in $[Ba^{2+}]_i$ and, secondly, that AT-II may decrease $[Ca^{2+}]_i$ by the transport mechanisms which can not remove Ba^{2+} from the cytosol as effectively as Ca^{2+}. Since Ba^{2+} is not a substrate for the Ca^{2+} pumping ATPases found in the membrane of the SR (Vanderkooi & Martonosi, 1971) and the plasmalemma (Yamaguchi *et al*., 1989), the result of the experiment with Ba^{2+} suggests that the decrease in $[Ca^{2+}]_i$ induced by AT-II might be due to a stimulation of Ca^{2+} extrusion through sarcolemmal Ca^{2+} pumps or of Ca^{2+} sequestration through Ca^{2+} pumps into thapsigargin-insensitive stores.

Despite the fact that the AT-II induced sustained decrease in $[Ca^{2+}]_i$ (Figures 1 and 4), AT-II did not increase the $^{45}Ca^{2+}$ efflux rate and did not decrease the $^{45}Ca^{2+}$ net uptake in both the thapsigargin-treated and non-treated strips depolarized with 118 mM K^+ (Figures 6 and 7). These results suggest that Ca^{2+} sequestration into thapsigargin-insensitive stores rather than Ca^{2+} extrusion from cells may therefore be responsible for the AT-II-induced decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+. Although the nature and the physiological role of the thapsigargin-insensitive Ca^{2+} stores were not known, some studies suggested that the mitochondria accumulate Ca^{2+} in the presence of thapsigargin in lacrimal acinar cells (Bird *et al*., 1992), hepatocytes (Hoek *et al*., 1997) and T lymphocytes (Hoth *et al*., 1997). However, as already discussed, the Ba^{2+} study suggested that AT-II may stimulate the sarcolemmal Ca^{2+} pumps. Because Ca^{2+} efflux rate is presumed to be dependent on $[Ca^{2+}]_i$, it is possible that the increase in $[Ca^{2+}]_i$ by 118 mM K^+-depolarization could stimulate the $^{45}Ca^{2+}$ efflux (Figure 6) and the decrease in $[Ca^{2+}]_i$ produced by AT-II would thus inhibit the 118 mM K^+-stimulated $^{45}Ca^{2+}$ efflux. Therefore, even if AT-II does stimulate the sarcolemmal Ca^{2+} pumps directly, the AT-II-induced decrease in $[Ca^{2+}]_i$ itself might mask the effect, and thus resulting in an apparent lack of an increase in the $^{45}Ca^{2+}$ efflux rate. Furthermore, the AT-II-induced transient increase in $[Ca^{2+}]_i$ in 118 mM K^+-depolarized strips (Figure 1) was not reflected in the changes in the $^{45}Ca^{2+}$ efflux rate, while the $^{45}Ca^{2+}$ efflux rate rapidly increased after the addition of AT-II in normal PSS (5.9 mM K^+) (Figure 6). It is possible that, in 118 mM K^+-PSS containing 1.25 mM Ca^{2+}, a condition in which $[Ca^{2+}]_i$ was highly elevated and Ca^{2+} extrusion mechanisms were presumed to be activated to a large extent, the sensitivity of the $^{45}Ca^{2+}$ efflux measurement used in the present study may be too low to detect any small and transient changes. It was reported that agonists stimulated the $^{45}Ca^{2+}$ efflux in low extracellular Ca^{2+} (Smith & Smith, 1987) or Ca^{2+}-free solutions (Rink & Sage, 1987), which would decrease the Ca^{2+} gradient between the cytoplasm and the extracellular space and accelerate Ca^{2+} efflux. However, when AT-II induced a decrease in $[Ca^{2+}]_i$ from the level obtained by 118 mM K^+-depolarization, the $^{45}Ca^{2+}$ net uptake did increase, thus suggesting that the contribution of the AT-II-stimulated $^{45}Ca^{2+}$ efflux by the activation of SL Ca^{2+} pumps may thus be small.

The next question was what kind of mediators are involved in the AT-II-induced sustained decrease in $[Ca^{2+}]_i$ in the strips depolarized with 118 mM K^+. It has been reported that the AT-II type 1 receptor-mediated signalling pathways consist of the initial phase mainly due to phospholipase C-mediated transient IP_3 production (Alexander *et al*., 1985) and the subsequent second phase due to phospholipase D-mediated long-lasting diacylglycerol (DAG) production in VSMCs (Lassegue *et al*., 1993). Since PKC activated by DAG plays an important role in various vascular effects of AT-II (for review, Griendling & Alexander, 1990), we examined whether PKC mediates the AT-II-induced decrease in $[Ca^{2+}]_i$. As shown in Figure 8B, pretreatment with 10^{-6} M calphostin-C which completely inhibits the PKC activity *in vitro*, partially but significantly, inhibited the decrease in $[Ca^{2+}]_i$ induced by AT-II. Furthermore, the application of phorbol-12, 13-dibutyrate, an activator of PKC, at the steady-state to the strips precontracted by 118 mM K^+-depolarization caused a sustained decrease in $[Ca^{2+}]_i$ and transiently enhanced the tension development, as did AT-II (Figure 8A). These results suggest that the AT-II-induced decrease in $[Ca^{2+}]_i$ may be partially mediated by PKC. Consistent with our results, it was reported that phorbol ester induced a sustained decrease in $[Ca^{2+}]_i$ and also enhanced the contraction in 118 mM K^+-depolarized intestinal smooth muscle (Mitsui & Karaki, 1993). However, there were kinetic differences between the AT-II-induced and PDBu-induced changes in $[Ca^{2+}]_i$ and tension. It has been hypothesized that the activation of AT_1 receptors by AT-II causes the assembly of signalling complexes and the movement of the receptors to specific signalling domains from which prolonged signals are generated (Griendling *et al*., 1997; Ishizaka *et al*., 1998). In contrast, PDBu, an exogenous activator of PKC, does not induce such specialized spatial distribution of receptors and therefore acts upon the signalling molecules that have not been formed into receptor complexes. Therefore, the kinetic aspects of the signalling by PDBu would be different from those activated by AT-II. In Figure 8B, if the $[Ca^{2+}]_i$-tension relationship is constant, then the AT-II-induced decrease in $[Ca^{2+}]_i$ would be inhibited by calphostin-C which would thus enhance the level of tension induced by

AT-II. However, the maximum level of the contraction induced by AT-II was apparently unaffected by the pretreatment with calphostin-C. It is possible that the increase in Ca^{2+}-sensitivity of the contractile apparatus induced by AT-II was also inhibited by pretreatment with calphostin-C and, as a result, a similar level of tension was observed. Since the inhibition by calphostin-C was partial, there is a possibility that other mediators, such as cyclic AMP and cyclic GMP, may also be involved in the AT-II-induced decrease in $[Ca^{2+}]_i$ in the present study. However, this hypothesis does not seem likely, because cyclic AMP induces relaxation without decreasing $[Ca^{2+}]_i$ in the vascular strips depolarized with 118 mM K^+ (Ushio-Fukai *et al*., 1993), and because H-8, an inhibitor of cyclic AMP-dependent protein kinase, and methylene blue, a guanylate cyclase inhibitor, failed to inhibit the AT-II-induced decrease in $[Ca^{2+}]_i$ in our preparation (data not shown).

We previously reported that the tension development and desensitization induced by AT-II reflect the changes in $[Ca^{2+}]_i$ and that AT-II increases Ca^{2+}-sensitivity of the contractile apparatus (Ushio-Fukai *et al*., 1999). In the present study, however, in spite of the decrease in $[Ca^{2+}]_i$, AT-II enhanced the tension development in the thapsigargin-treated and 118 mM K^+-depolarized strips (Figure 1). In addition, after the application of AT-II, the Ba^{2+}-contraction was enhanced and sustained without an increase in $[Ba^{2+}]_i$ (Figure 5). PDBu enhanced the tension while it decreased $[Ca^{2+}]_i$ in the 118 mM K^+-depolarized strips (Figure 8). These results suggest that the relationships between $[Ca^{2+}]_i$ or $[Ba^{2+}]_i$ and tension are not always parallel, and the latter is regulated by unknown mechanisms including Ca^{2+} sensitivity of the contractile apparatus of smooth muscle.

In conclusion, the findings of this study suggested that Ca^{2+} sequestration into the thapsigargin-insensitive Ca^{2+} stores plays a major role while the SL Ca^{2+} pumps play a minor role in the AT-II-induced decrease in $[Ca^{2+}]_i$ in the 118 mM K^+-depolarized vascular strips. We separately reported that the desensitization of the contractile response evoked by prolonged stimulation with high concentrations of AT-II is considered to be due to a decrease in $[Ca^{2+}]_i$ in the second component, but is not correlated with any change in Ca^{2+}-sensitivity of the contractile apparatus (Ushio-Fukai *et al*., 1999). Taking all these findings together, we propose that the observed desensitization is due to the long-lasting inhibitory effects of AT-II on Ca^{2+} signalling which includes not only an inhibition of the Ca^{2+} influx but also an activation of Ca^{2+} sequestration which are partially mediated by PKC.

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