CPA induces a sustained increase in [Ca^<2+>]_i of endothelial cells in situ and relaxes porcine coronary artery

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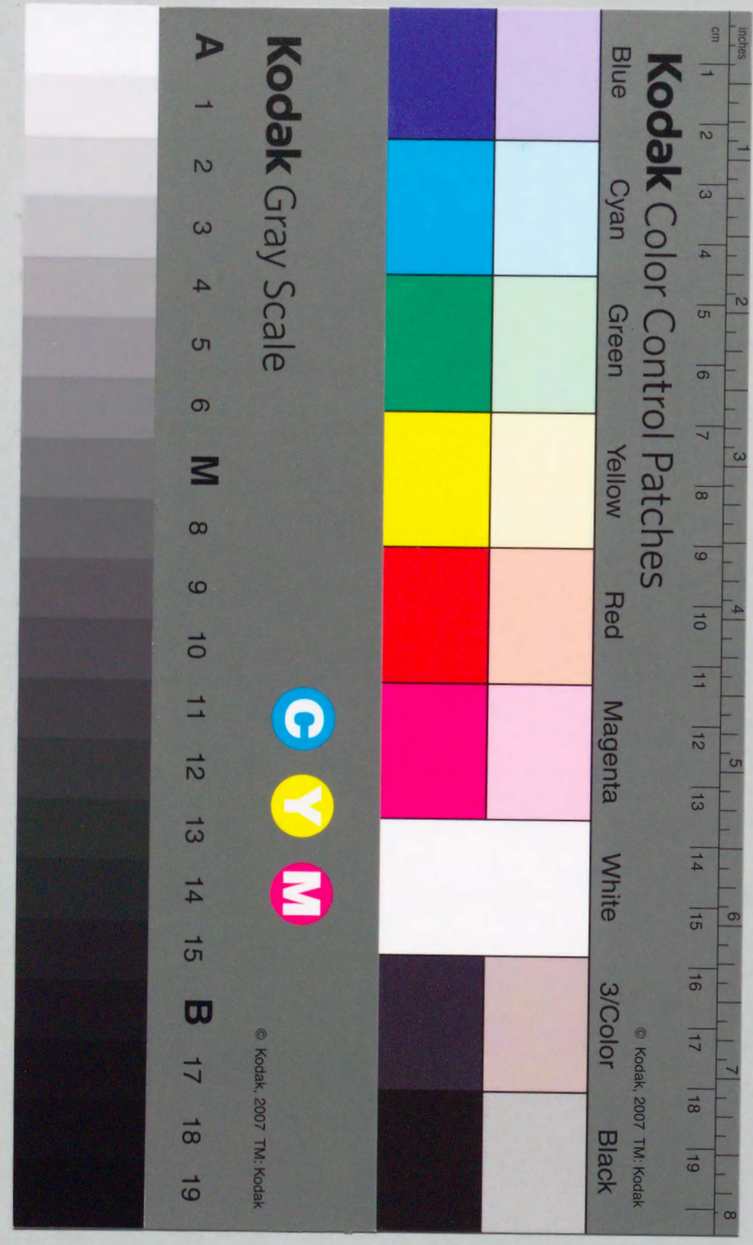

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CPA induces a sustained increase in $[Ca^{2+}]_i$ of endothelial cells *in situ* and relaxes porcine coronary artery

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abbreviated title; The role of Ca^{2+}-ATPase on regulation of $[Ca^{2+}]_i$

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ABSTRACT

Using fura-2 fluorometry, we investigated the effect of cyclopiazonic acid (CPA), an inhibitor of Ca^{2+}pump-ATPase of the endoplasmic reticulum, on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension in porcine aortic valvular endothelial cells and coronary arterial strips with endothelium. In normal PSS, CPA induced a sustained increase in $[Ca^{2+}]_i$ of the valvular strips, while CPA elicited a transient elevation of $[Ca^{2+}]_i$ in Ca^{2+}-free PSS. CPA (30 μM) relaxed coronary strips with endothelium precontracted by 100 nM U46619, which was partially inhibited by N^{ω}-nitro-L-arginine (L-NNA, 100 μM). These results indicate that the CPA-induced increase in $[Ca^{2+}]_i$ depends both on the Ca^{2+} release and the Ca^{2+} influx in the endothelial calls *in situ*, and that the CPA-induced endothelium-dependent decreases in $[Ca^{2+}]_i$ and tension in the smooth muscle is due to the combined effect of L-NNA sensitive and resistant factors.

Index Terms: cyclopiazonic acid; cytosolic Ca^{2+} concentration; porcine aortic valves; endothelium-dependent relaxation; porcine coronary arteries

INTRODUCTION

Vascular endothelial cells play a pivotal role in the regulation of the blood pressure and local blood flow by releasing vasorelaxing factors such as endothelium-derived relaxing factor (EDRF) (10), which is reported to be nitric oxide (NO) or a related substance, prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) (34).

It is generally accepted that in the regulation of the production and the release of these factors, changes in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) play an essential role in the vascular endothelial cells (2, 23). There are two major pathways for the increase in $[Ca^{2+}]_i$; one is the Ca^{2+} release from the intracellular store site, mostly from the endoplasmic reticulum (ER), and the other is the Ca^{2+} influx from the extracellular space. The following reasons indicate that the Ca^{2+} release is more important than the Ca^{2+} influx for Ca^{2+} homeostasis of vascular endothelial cells. Most of the agonists induce a biphasic increase in $[Ca^{2+}]_i$, which is composed of initial transient followed by sustained and steady state increases in $[Ca^{2+}]_i$ (2). The level of $[Ca^{2+}]_i$ in the initial transient increase, which is thought to be due to the intracellular Ca^{2+} release, is much greater than that of the steady state, which is thought to be due to the Ca^{2+} influx from the extracellular space (2). In addition, it was also reported that, in the non-excitable cells, the filling state of ER with Ca^{2+} regulating the Ca^{2+} influx from the extracellular space (5, 26). Recently, Randriamamptia and Tsien (27) reported that the emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx. It appears that, the intracellular store and Ca^{2+} influx pathway are functionally linked and the Ca^{2+} influx pathway is, at least in part, under the control of the intracellular store. Thus, if the ER of endothelial cells are either anatomically or functionally, completely destroyed, then the observed changes in $[Ca^{2+}]_i$ will help to determine the regulatory role of ER in the Ca^{2+} homeostasis of endothelial cells.

Recently, it has become possible to functionally destroy either the ER or the sarcoplasmic reticulum (SR) by using specific inhibitors of intracellular Ca^{2+}-ATPase. Cyclopiazonic acid (CPA) (11, 30) is one of these substances and has been used for investigation of the role of ER Ca^{2+}-ATPase in several cell types, including vascular smooth muscles (33) and cultured endothelial cells (8, 29). But there have been no previous reports concerning the role of the intracellular Ca^{2+}-ATPase on the regulation of $[Ca^{2+}]_i$ in endothelial cells *in situ* or in vivo (not in cultured cells) and smooth muscles. Therefore, we investigated the effects of CPA on $[Ca^{2+}]_i$ of endothelial cells on the porcine aortic valvular strips and smooth muscles in the porcine coronary arterial strips with or without endothelium, using the front-surface fluorometry of fura-2 developed by us (1). In order to assess the effect of CPA on endothelial cell function, we also characterized the CPA-induced endothelium-dependent relaxation in the arterial strips with an intact endothelium by means of measuring the tension development and $[Ca^{2+}]_i$ of the smooth muscles simultaneously, using L-NNA (NO synthase inhibitor), indomethacin (cycloxygenase inhibitor) and depolarization elicited by a high K^+ solution which can inhibit membrane hyperpolarization, and therefore eliminate the effects of EDHF (21).

MATERIALS AND METHODS

Tissue preparation and Fura-2 loading

Coronary arterial strips

Pig hearts obtained from a local slaughterhouse were immediately immersed in ice-cold physiological salt solution (PSS) and transferred to our laboratory. Left coronary arteries were isolated and a longitudinal segment 2-3 cm from the origin were excised. The vessels were cleaned of fat and adhering connective tissue. After removing the adventitia carefully to avoid any stretching, the medial preparations were cut into approximately 1x5 mm circular strips with 0.1 mm thickness under a microscope. During the preparation, PSS was continuously bubbled with 95 % O_2 and 5 % CO_2 under room temperature. When necessary, the endothelium was removed by gently rubbing the lumen surface with a cotton swab, and the absence of an endothelium was confirmed by the observation that the addition of 1 μM bradykinin during contraction induced by 118 mM K^+ did not induce any relaxation.

Both the endothelium-intact and endothelium-removed vascular strips were loaded with the Ca^{2+} indicator dye, fura-2, by incubating them in oxygenated Dullbecco's modified Eagles medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 2.5 % fetal bovine serum for 3-4 h at 37°C. The strips were then washed by normal PSS three times and transferred to oxygenated warm PSS (37°C) for at least 60 min to remove the dye remaining in the extracellular space and to equilibrate the strips before starting the measurements.

Valvular strips

Pig aortic roots were carefully and gently dissected free from the pig hearts promptly after the animals had been killed in the local slaughterhouse, immersed in ice-cold PSS, and then were transferred to our laboratory. The valve leaflets were cut from the aortic root with special care taken not to touch their surface and cut them into 3x5 mm valvular strips. The valvular strips thus obtained were loaded with fura-2 by incubating in oxygenated Dullbecco's modified Eagles medium containing 50 μM fura-2/AM and 5 % fetal bovine serum and 1 mM probenecid (7) for 1.5 h at 37°C. Subsequently, the strips were rinsed with normal PSS three times and left for 1 hour at room temperature for the purpose of equilibration prior to the start of each measurement.

Measurement of tension and $[Ca^{2+}]_i$ of smooth muscles in the porcine coronary artery

The amounts of developed tension and smooth muscular $[Ca^{2+}]_i$ were measured simultaneously in vascular strips with and without an endothelium. The fura-2 loaded strips were mounted vertically to arrange the smooth muscle cells longitudinally between the transducer (top) and clamp (bottom) in a quartz organ bath as the luminal side of the strip was faced to the front of the bath. Tension development was measured using a strain gauge (TB-612T, Nihon Koden, Japan). During a 60 min fura-2 equilibration period, the strips were stimulated with 118 mM K^+-depolarization about every 15 min, and the resting tension was increased stepwise to get the maximum response of tension development to this stimulation. The final resting tension levels ranged from 200 to 300 mg. The developed tension was expressed as a percentage of a control 118 mM K^+-induced tension development measured at 10 min after application, designating the values in normal PSS (5.9 mM K^+) to be 0 % and in 118 mM K^+ to be 100 %.

Changes in the fluorescence intensity of the fura-2/Ca^{2+} complex were monitored with a front-surface fluorometer specially designed for fura-2 fluorometry (CAM-OF1) with the collaborating Japan Spectroscopic Co., Tokyo, Japan. The strips were illuminated by alternating (400 Hz) 340 nm and 380 nm excitation light from xenon lamp through quartz optic fibers. The surface fluorescence of the strips was collected by the glass optic fibers and introduced through a 500 nm bandpass filter into a photomultiplier. The optic fiber was arranged as the excitation light and the emission light pass in a concentric inner circle (diameter, 3 mm) and an outer circle (diameter, 7 mm), respectively. The ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored. The ratio of the fluorescence intensities was expressed as a percentage, designating the values in normal PSS (5.9 mM K^+) and 118 mM K^+ PSS to be 0 % and 100 %, respectively. The absolute value of $[Ca^{2+}]_i$ were calculated from the percent ratio (R), using the following equation given by Grynkiewicz et al. (12):

$$[Ca^{2+}]_i = Kd\,(R\text{-}Rmin) / (Rmax\text{-}R)\,(S_{f2}/S_{b2})$$

where K_d is a dissociation constant and assumed to be 224 nM (12) at 37°C. After permeation of the surface membrane to Ca^{2+}, but not to fura-2, with 25 μM ionomycin, Rmax and Rmin were determined by the addition of normal (1.25 mM Ca^{2+}) and Ca^{2+}-free (2 mM EGTA) PSS, respectively (Rmax=156.3%, Rmin=-76.1%, n=10). S_{f2}/S_{b2} was the ratio of the proportionality coefficients of free dye and Ca^{2+}-bound dye at one wavelength (380nm), and it was around 1.0 in our fluorometry system in the present experimental condition. The $[Ca^{2+}]_i$ levels at rest (0 %) and during 118 mM K^+-depolarization (100 %) were 108.0 ± 27.2 nM and 715.1 ±103.4 nM, respectively (n=10). Since $[Ca^{2+}]_i$ was calculated on the assumption that the Kd value of fura-2 for Ca^{2+} in the cytosol of vascular smooth muscle cells in the present experimental condition to be 224 nM and by use of ionomycin which enlarges the margin of the error of $[Ca^{2+}]_i$ values at the end of the long-term measurement, the $[Ca^{2+}]_i$ value thus obtained is simply an approximation to the true cytosolic $[Ca^{2+}]_i$. The values were shown in the right sided ordinate as an approximation to the figures. On the contrary, since the responses expressed as % fluorescence ratio, to similar stimuli (118 mM K^+-depolarization) remained constant, the statistical analysis of the results were performed by use of these values. The experimental conditions in the present study allowed us to record fura-2 signals exclusively from the smooth muscle cells in the porcine coronary arterial strips with intact endothelial cells, as discussed previously (15). Fluorescence microphotographs in Fig. 1 show that the experimental conditions used in the present study at 37°C without probenecid in the medium, made it feasible to record fura-2 signals derived exclusively from the smooth muscle cells in the coronary artery preparations with intact endothelial cells. It is well known that endothelial cells can be identified by their specific uptake of acetylated-low density lipoprotein labeled with 1,1'-dioctadecyl-

3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) (22). Figure 1 A and B are photographs of the cells in the same field in which DiI-Ac-LDL and fura-2 are doubly loaded in the absence of probenecid at 37°C; this condition (no probenecid) is used for loading fura-2 exclusively in smooth muscle cells in coronary strips with endothelium in the present study. Fluorescence of DiI-Ac-LDL was observed through a 520 nm long-pass filter (LP 520, Zeiss) by illuminating the tissue with an excitation light from xenon light source through a 485 nm band-pass filter (BP 485, Zeiss)(Fig. 1A). Fluorescence of fura-2 was observed through a 500-530 nm band-pass filter (BP 500-530, Zeiss) by illuminating the tissue with an excitation light through a 340 nm band-pass filter (BP 340, Zeiss)(Fig. 1B). As shown in Fig. 1A, endothelial cells are positively stained with DiI-Ac-LDL and show a cobble-stone appearance, thus indicating the presence of intact endothelial cells in the coronary arterial strips. However, in Fig. 1B, endothelial cells are not stained with fura-2, while longitudinally running smooth muscle cells, which underly the endothelial cells, are positively stained with fura-2. Thus, it is apparent that the fluorescent signal is only from smooth muscle cells in the fura-2 loaded coronary arterial strips with endothelium. However, when probenecid was used during loading with fura-2, both endothelial cells and smooth muscle cells were stained with fura-2 (16).

Measurement of [Ca^{2+}] of endothelial cells on the aortic valve

The method to measure the changes in $[Ca^{2+}]_i$ of the aortic valvular endothelial cells *in situ* has been described elsewhere in detail (3). In brief, each strip was mounted vertically in quartz organ bath and changes in $[Ca^{2+}]_i$ of endothelial cells on the surface of the aortic side were monitored using the front-surface fluorometer (CAM-OF-1) which is the same system as that using the vascular strips. The measurements were carried out at 25°C to prevent any leakage of fluorescence dye. We expressed the $[Ca^{2+}]_i$ response as a percentage of the response to 10 μM ATP. The resting level and the peak level of the fluorescence ratio induced by ATP were designated 0 % and 100 %, respectively. The response to 10 μM ATP was recorded as a control response 15 min prior to each experiment. The absolute values of $[Ca^{2+}]_i$ were determined in separate measurements using the same equation described in Ca^{2+} calibration of coronary strips. The apparent Kd at 25°C was 162 nM, determined spectroscopically (3). Rmax and Rmin were 463.9% and -81.0%, respectively (n=10). S_{f2}/S_{b2} was 2.31. The $[Ca^{2+}]_i$ levels at rest (0 %) and peak response of 10 μM ATP (100 %) were 65.3 ± 7.5 nM and 185.9 ± 20.8 nM, respectively (n=10). Because of similar reasons described in the Ca^{2+} calibration of coronary strips, the absolute values $[Ca^{2+}]_i$ scale in the figures are an approximation, and statistical analyses were performed by use of the % fluorescence ratio values.

Drugs and Solutions

The composition of the normal PSS was (in mM); 123 NaCl, 4.7 KCl, 15.5 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.25 $CaCl_2$ and 11.5 D-glucose. The Ca^{2+} free PSS contained 2 mM EGTA (ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid) instead of 1.25 mM $CaCl_2$. High-K^+ PSS was made by an equimolar substitution of KCl for NaCl. All solutions were gassed with a mixture of 5 % CO_2 and 95 % O_2. The solution was maintained at 37°C in the experiment of coronary arterial strips and at 25°C in that of the valvular strips. Cyclopiazonic acid (CPA), probenecid, and BSA were all obtained from SIGMA (St Louis, MO, USA). U46619, N^{ω}-nitro-L-arginine (L-NNA), and indomethacin were purchased from the Aldrich Chemical Co. (USA), the Calbiochem Co (USA), WAKO (Osaka, Japan), respectively. Bradykinin, histamine, serotonin, endothelin-1, and endothelin-2 were purchased from the Peptide Institute Co. Ltd. (Osaka, Japan). Sodium salt of adenosine triphosphate (ATP) was obtained from the Boehringer Mannheim GmbH (Germany). Fura-2 AM and EGTA were purchased from Dojindo Laboratories (Kumamoto, Japan). Acetylated-low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was purchased from Funakoshi (Tokyo, Japan). All other chemicals were from Katayama Chemical (Osaka, Japan). CPA was dissolved in dimetyl sulfoxide (DMSO) and the final concentration of DMSO did not exceed 0.3 %, which had little effect on $[Ca^{2+}]_i$ and the contractile activity in the strips.

Data analysis

All data were collected by a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, Macintosh; Apple Computer, U.S.A.). The collected data were directly printed out from the computer to a laser printer (LaserWriter II NTX-J, Apple Computer, U.S.A.) to make traces for the figures included in this report. The values are expressed are the mean ± standard error (S.E.M.). Student's *t* test was used to determine the statistical significance. $P<0.05$ was considered to be significant. The EC_{50} (or IC_{50}) values, the concentrations that increase (or decrease) the fluorescent ratio and tension to 50 % of the maximum response, were determined from the concentration-response curve fitted according to a four-parameter logistic model (6).

RESULTS

Effect of CPA on $[Ca^{2+}]_i$ in endothelial cells in situ

Figure 2A shows a representative recording of the effect of the cumulative application of CPA (0.1 - 50 μM) on $[Ca^{2+}]_i$ of the fura-2 loaded endothelial cells on the aortic valvular strips in the presence of extracellular Ca^{2+}. The CPA induced step-wise increases in $[Ca^{2+}]_i$ in a concentration-dependent manner. The maximal response of CPA was obtained at 30 μM and the peak $[Ca^{2+}]_i$ level was 352.3 ± 40.2 % (n=3) (Fig. 2B). The EC_{50} value (concentration of CPA which induces half-maximal $[Ca^{2+}]_i$ elevation) was 5.0 ± 0.6 μM (n=3). As shown in Fig. 3A, in the presence of extracellular 1.25 mM Ca^{2+}, the application of 30 μM CPA induced a rapid elevation of $[Ca^{2+}]_i$ to a peak within 10 min (364.8 ± 32.7 %, n=3) which was followed by a gradual decrease to a sustained level that remained significantly elevated above the resting level (234.0 ± 29.7 %, n=3) as long as the CPA was present. The CPA at a concentration of less than 10 μM caused a gradual and sustained increase in $[Ca^{2+}]_i$ without a peak transient elevation. When CPA was eliminated from the bathing solution, the effect of CPA disappeared as is also shown in Fig. 3A.

Table 1 shows the extent of the increases in $[Ca^{2+}]_i$ of the valvular endothelial cells induced by 30 μM CPA and various receptor agonists such as bradykinin, histamine, serotonin, ATP, endothelin-1 and endothelin-3. These agonists caused biphasic elevations of $[Ca^{2+}]_i$, which consisted of an initial peak transient and followed by a lower and more sustained (steady state) increase in $[Ca^{2+}]_i$. The concentrations of the agonists in this table are enough to obtain the maximal response in both the initial peak and steady state. At the steady state level, the CPA-induced increase in $[Ca^{2+}]_i$ was the most remarkable among the agonists examined.

Since CPA induced a marked increase in $[Ca^{2+}]_i$ especially at the sustained steady-state phase, we postulated that the Ca^{2+} influx from the extracellular space might play a major role in this change in $[Ca^{2+}]_i$. To eliminate the Ca^{2+} influx from the extracellular space, the valvular strips were exposed to Ca^{2+}-free PSS containing 2 mM EGTA. At 10 min in Ca^{2+}-free bath, $[Ca^{2+}]_i$ gradually declined to reach a steady levels, -25.3 ± 4.5 % (n=3), and a subsequent application of 30 μM CPA induced a transient rise in $[Ca^{2+}]_i$ (41.0 ± 5.3 %, n=3) followed by a gradual decrease to reach a pre-stimulation level (Fig. 3B). As shown in Fig. 3C, in the presence of extracellular Ca^{2+}, when 1 mM Ni^{2+} was added to the valvular strips during steady state elevation induced by 30 μM CPA, $[Ca^{2+}]_i$ was rapidly decreased to a basal level (Fig. 3C). These findings were consistent with a previous report on thapsigargin and BHQ (2',5'-di(tert-butyl)-1,4-benzohydroquinone), other kind of Ca^{2+}-ATPase inhibitors, in cultured endothelial cells (8) and indicated that not only Ca^{2+} release but also Ca^{2+} influx from the extracellular space is involved in the CPA-induced increase in $[Ca^{2+}]_i$.

Effects of CPA on $[Ca^{2+}]_i$ and tension of the coronary arterial strips with or without an endothelium

Since CPA induced a huge sustained increase in $[Ca^{2+}]_i$ of the valvular endothelial cells in the present study, and since an increase in $[Ca^{2+}]_i$ should be coupled with the release of vasorelaxing substances from the endothelial cells, we thus tried to answer the following questions: 1) Does CPA also induce an increase in $[Ca^{2+}]_i$ in the smooth muscle cells?, 2) Does CPA induce endothelium-dependent relaxation of the smooth muscle?, 3) What kind of vasorelaxing substances are released from the vascular endothelial cells as a consequence of the increase in $[Ca^{2+}]_i$ by CPA? To answer these questions, we designed the following experiments using simultaneous measurements of smooth muscular $[Ca^{2+}]_i$ and tension of the porcine coronary arterial strips either with or without an endothelium.

When the porcine coronary arterial strips without an endothelium was exposed to 30 μM CPA under resting conditions, $[Ca^{2+}]_i$ rapidly elevated to reach a peak level, and was then sustained around this level (15.6 ± 0.3 %, n=3) without any tension development for at least 30 min (Fig. 4A). In the endothelium intact strip, 30 μM CPA induced only a small transient increase in $[Ca^{2+}]_i$ (8.2 ± 1.2 %, n=3), and then gradually decreased $[Ca^{2+}]_i$ below the resting level (-8.1 ± 0.2 %, n=3). There was also no change in the tension (Fig. 4B). These results indicate that CPA directly induces an increase in $[Ca^{2+}]_i$ in the smooth muscle cells, which is inhibited in the presence of endothelial cells, possibly by the CPA-induced factor(s) from endothelial cells. It should be noted that the levels of $[Ca^{2+}]_i$ at the sustained elevation induced by 30 μM CPA in the smooth muscles was much smaller than that of the endothelial cells. In case the absolute $[Ca^{2+}]_i$ levels were estimated from the values obtained in the separate measurements, as presented in the "Methods", the sustained levels of $[Ca^{2+}]_i$ elevation induced by 30 μM CPA were 146 nM and 542 nM in smooth muscle cells and endothelial cells, respectively.

In the next steps, in order to reveal the vasorelaxing effect of CPA, the coronary arterial strips were precontracted by 100 nM U46619, a thromboxane A_2 analogue, or high K^+-depolarization (40 mM K^+) prior to the application of CPA. When U46619 was applied to the coronary arterial strips with an intact endothelium, $[Ca^{2+}]_i$ and the tension rapidly increased and reached steady state levels within 10 min and both levels were maintained for at least 60 min (Fig. 5A). When CPA was cumulatively applied (0.3 - 30 μM) during the steady state of U46619-induced $[Ca^{2+}]_i$ elevation and contraction, concentration-dependent reductions in $[Ca^{2+}]_i$ and tension were observed (Fig. 5B). CPA at 30 μM induced almost a complete relaxation of the coronary arterial strip. The IC_{50} values (concentration of CPA which induced 50 % of the changes obtained with 30 μM CPA) for $[Ca^{2+}]_i$ and tension were 13.5 ± 6.9 μM (n=4) and 5.2 ± 0.2 μM (n=4), respectively (Fig. 5C), similar to the value of CPA in an increase in $[Ca^{2+}]_i$ in the valvular endothelial cells (Fig. 2B).

When the endothelium was removed, CPA-induced relaxation of the coronary arterial strip was almost completely abolished (Fig. 6A), indicating that CPA-induced relaxation depended on the endothelial cells. In the endothelium intact strips, 30 μM CPA elicited a rapid decrease in tension to more or less the resting level (the first component), and then it was sustained at about this level (12.5 ± 4.3%, n=4) (the second component), although the extent of the decrease in $[Ca^{2+}]_i$ was not so great and $[Ca^{2+}]_i$ was apparently higher than the resting level (32.3 ± 1.9%, n=4) (Figs. 6B and

7). To determine the relative contribution of EDRF and PGI_2 in the CPA-induced endothelium-dependent relaxation, the strips with an intact endothelium were pretreated with indomethacin (a cycloxygenase inhibitor) and L-NNA (a competitive inhibitor of NO synthase) for at least 40 min. L-NNA did not affect the basal tone but the amplitude of contractions induced by 118 mM K^+ and 100 nM U46619 were enhanced by up to 122 % and 127 %, respectively (compared with the amplitude without L-NNA), indicating that there was a basal release of EDRF (4) which was inhibited by L-NNA. As shown in Figs. 6C and 7, when the strips with intact endothelium were pretreated with L-NNA (100 μM) and indomethacin (10 μM), 30 μM CPA induced rapid decrease in $[Ca^{2+}]_i$ and tension, which began to reverse within 1 min and reached to the same level as that obtained from the strip without an endothelium (Figs. 6A and 7). However, the extent of the decrease in tension at the first component was much smaller than that observed in the strips without pretreatment with L-NNA (Figs. 6B, 6C and 7).

Figure 7 summarizes the inhibitory effects of L-NNA (100 μM) and/or indomethacin (10 μM) on the CPA-induced endothelium-dependent relaxation in the U46619 precontracted strips with an intact endothelium. In the presence of L-NNA and indomethacin, the CPA-induced rapid and maximal reduction of $[Ca^{2+}]_i$ and tension (the first component) were markedly, but not completely, inhibited. On the other hand, the $[Ca^{2+}]_i$ and the tension level in the steady state (the second component), which were obtained at 30 min after application of CPA, were completely blocked. Indomethacin alone had no effect on $[Ca^{2+}]_i$ and tension obtained both at the first component and the second component, indicating that PGI_2 may not be involved in the CPA-induced endothelium-dependent relaxation of the porcine coronary artery.

Because CPA-induced endothelium-dependent relaxation was not inhibited by indomethacin, and only partially inhibited by L-NNA, we postulated that some vasorelaxing substance (presumably EDHF), other than EDRF and PGI_2, might be involved in this relaxation. To exclude the effect of EDHF on CPA-induced endothelium-dependent relaxation, the strips either with or without an endothelium were precontracted by high K^+ (40 mM K^+) depolarization, and the effect of CPA on $[Ca^{2+}]_i$ and tension were determined. High K^+ depolarization (40 mM K^+) evoked a sustained elevation of $[Ca^{2+}]_i$ and tension which developed within 10 min and then were maintained throughout the observation period in the porcine coronary artery (Fig. 8A). In the strips with an intact endothelium, CPA induced a slowly developing decrease in tension which reached a maximal relaxation level (14.7 ± 1.5 %, n=4) at about 30 min after application, but the $[Ca^{2+}]_i$ was only slightly, but significantly ($P< 0.05$) decreased to 69.0 ± 1.7 % (n=4) (Figs. 8B and 9). In the presence of L-NNA and indomethacin, the CPA-induced relaxation was completely blocked (Figs. 8C and 9). Figure 9 summarizes the inhibitory effects of L-NNA on the CPA-induced endothelium-dependent relaxation in the strips precontracted by 40 mM K^+, indicating that the CPA-induced endothelium-dependent relaxation was almost completely abolished by 100 μM L-NNA.

DISCUSSION

In the present study, the role of the ER in the regulation of $[Ca^{2+}]_i$ of the porcine aortic valvular endothelial cells and the coronary smooth muscles were determined by disrupting the ER function with a selective ER Ca^{2+}-ATPase inhibitor, CPA. The mechanism of CPA-induced endothelium-dependent vasorelaxation was also determined by simultaneously measuring smooth muscular $[Ca^{2+}]_i$ and the tension. The reason why we used the valvular strips to investigate the endothelial cells is that cultured endothelial cells have been known to alter their cell surface structures and functions through the procedure of cell culture (3, 17). This method thus provides a better physiological assessment of Ca^{2+} signaling of endothelial cells. The endothelial cells on the cardiac valves also release EDRF(s) which can relax the vascular strips (3, 14), so that this methodology also provides important information on the cell function and pathophysiology of vascular endothelial cells. However, it should be noted that the aortic valvular endothelial cells cannot be a model for coronary aortic endothelial cells. It has been difficult to separate Ca^{2+}/fura-2 signals of smooth muscles from the fura-2 loaded vascular strips with an intact endothelium due to overlap the signals of endothelium except for removing the endothelium partially from the site in which $[Ca^{2+}]_i$ was measured (28). But in this experiment, we are able to measure the $[Ca^{2+}]_i$ exclusively from smooth muscles with an intact endothelium, because the fura-2 readily leaks out from porcine coronary endothelial cells at 37℃, but not from the smooth muscles (15, 16). Therefore, taking advantage of this phenomenon, we can accurately measure the change of $[Ca^{2+}]_i$ in smooth muscles as a result of interaction between the smooth muscles and the adjacent endothelial cells.

The major findings of the present study are as follows : 1) CPA induces a large sustained elevation of $[Ca^{2+}]_i$ in the valvular endothelial cells. 2) This CPA-induced increase in $[Ca^{2+}]_i$ of the endothelial cells is dependent not only on intracellular Ca^{2+} release but also on Ca^{2+} influx from extracellular space. 3) In the arterial strips without endothelium, CPA induced sustained elevation of $[Ca^{2+}]_i$ without any tension development in smooth muscle cells. The extent of the rise in $[Ca^{2+}]_i$ (estimated to be 146 nM) in the smooth muscle cells is much smaller than that in the endothelial cells (estimated to be 542 nM). 4) In the coronary arterial strips with intact endothelium, CPA decreased $[Ca^{2+}]_i$ in smooth muscle cells in endothelium-dependent manner, and then relax the strips. 5) CPA induced endothelium-dependent relaxation mediated by L-NNA sensitive and resistant factors, but not PGI_2. The initial rapid phase of the CPA-induced endothelium-dependent relaxation is due to the release of both L-NNA sensitive and resistant factors, while the prolonged relaxation is mainly due to L-NNA sensitive factors. Since these findings of CPA in this study are also observed in case of thapsigargin (Kuroiwa, personal communication), another structurally different Ca^{2+}-ATPase inhibitor, it appears that these effects are not specific to CPA, but to Ca^{2+}-ATPase inhibitors.

As is clearly shown in Fig. 3B, in the absence of extracellular Ca^{2+}, CPA induced a transient increase in $[Ca^{2+}]_i$, indicating the intracellular Ca^{2+} release in the valvular endothelial cells. The ER membrane is thought to have a Ca^{2+} leak channel, an inositol 1,4,5 trisphosphate (IP_3)-gated Ca^{2+} channel, and an ATP-driven Ca^{2+} pump (Ca^{2+}-ATPase), which are responsible for basal as

well as stimulus-induced Ca^{2+} release and Ca^{2+} re-uptake, respectively (13). Thus, the constant Ca^{2+} release from the internal Ca^{2+} store, which was not visible due to the reuptake by the ER Ca^{2+} pump under normal conditions, may be due to the passive Ca^{2+} leak and/or the basal opening activity of IP_3-gated Ca^{2+} channels. Thus, it is possible to explain how the inhibition of ER Ca^{2+}-ATPase induces a transient increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}.

The mechanism underlying the increase in the Ca^{2+} influx from the extracellular space by CPA is more complicated. As shown in Figs. 2 and 3, CPA induced a large sustained increase in $[Ca^{2+}]_i$ only in the presence of extracellular Ca^{2+}, which was blocked by Ni^{2+}, indicating that CPA induces Ca^{2+} influx from the extracellular space. In recent reports, in several cell types including rat thymic lymphocytes, cultured HL-60 cells and cultured endothelial cells (5, 19, 29), a correlation between the depletion of the intracellular Ca^{2+} store and Ca^{2+} influx across the plasma membrane has been well documented using CPA. This phenomenon has been explained by the so-called "capacitative model" theory, in which the influx of extracellular Ca^{2+} is controlled by the filling state of intracellular Ca^{2+} stores (26). Very recently, in accordance with this signal transduction system from internal Ca^{2+} pools to the plasma membrane, Randriamamptia and Tsien (27) reported that the emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates the Ca^{2+} influx. The results of this study indicate that this mechanism may be operating in *in situ* endothelial cells on the porcine aortic valve. These results is consistent with the findings in cultured endothelial cells essentially, but this is the first report which described that ER Ca^{2+}-ATPase inhibitor induces Ca^{2+} influx in *in situ* (not cultured) endothelial cells. In the cultured endothelial cells, Schilling et al. (29) reported that the $[Ca^{2+}]_i$ levels induced by 10 μM CPA were less than 200 nM and about 1/5 of the initial peak Ca^{2+} transient induced by 50 nM bradykinin. In the present study using *in situ* endothelial cells, the extent of an increase in $[Ca^{2+}]_i$ induced by CPA was much greater than that induced by various receptor agonists, especially in the steady state (Table 1). Thus, it is tempting to speculate that the mechanism for Ca^{2+} influx induced by the depletion of the internal Ca^{2+} stores might be operating more effectively *in situ* than in cultured endothelial cells. Whether this represents a general difference between cultured and *in situ* endothelial cells remains to be investigated, since we have not systematically compared the valvular endothelial $[Ca^{2+}]_i$ regulation in cultured vs. *in situ* preparations. Furthermore, these differences could be due to species and tissue differences; the porcine aortic valvular endothelial cells in our study vs. the calf aortic endothelial cells in Schilling's study (29).

In the present study, CPA (30 μM) induced a sustained but relatively small increase in $[Ca^{2+}]_i$ in the coronary smooth muscles (Fig. 4A). Thus, it is possible to postulate that the mechanism for the Ca^{2+} influx induced by the inhibition of SR Ca^{2+}-ATPase also occurs in smooth muscle. The strongest candidate for this mechanism would also be a "capacitative model". However, the situation may be more complicated in excitable cells, such as smooth muscle cells, because there are numerous kinds of ion channels in the plasma membrane, which might be regulated in a more complex manner. For example, Uyama et al.(33) proposed the hypothesis that the CPA-induced decrease in stored Ca^{2+} due to Ca^{2+}-ATPase inhibition reduces the Ca^{2+}-dependent K^+ current and indirectly enhances Ca^{2+} influx through membrane activity resulting from the increased excitability. Thus, it has yet to be established precisely what kind of mechanism is involved in CPA-induced Ca^{2+} influx of the smooth muscle cells (31). However, it should be noted that the CPA-induced sustained elevation of $[Ca^{2+}]_i$ is considerably less than that in the valvular endothelial cells. At least, it can be concluded that the mechanism for the Ca^{2+} influx induced by emptying internal Ca^{2+} pool may not play a major role by itself in the regulation of smooth muscle contraction, because CPA did not induce any tension development. The reason why CPA did not induce any tension development may be due to the fact that the CPA-induced increase in $[Ca^{2+}]_i$ is not sufficient to induce a contraction of the coronary smooth muscles. This is supported by the finding that the elevation of $[Ca^{2+}]_i$ induced by 15 mM K^+-depolarization, which was comparable to that induced by 30 μM CPA, was not accompanied by any tension development in the same preparation (1). In contrast to the present results, Shima and Blaustein (32) reported that CPA induced a transient rise in the resting tension in both the rat thoracic aorta and small mesenteric artery. At present, it is not easy to explain why there are so many variable responses to CPA at a resting condition. This might be due to the difference in tissues and/or the species used.

Since the CPA-induced endothelium-dependent relaxation was partially inhibited by the treatment with L-NNA in the strips precontracted by U46619, but not affected by indomethacin (Fig. 7), it is suggested that the CPA-induced increase in $[Ca^{2+}]_i$ of the porcine coronary endothelial cells was coupled with the release of L-NNA sensitive and resistant factors, but not PGI_2. Vanhoutte (34) recently reported that endothelium-dependent relaxations, which are not prevented by inhibitors of the L-arginine NO pathway (nitro-L-arginine resistant relaxation) in the presence of indomethacin, are considered to be caused primarily by the effect of EDHF. It has been proposed that EDHF opens K^+ channels, hyperpolarizes the membrane potential, indirectly closes the voltage-dependent Ca^{2+} channels to decrease $[Ca^{2+}]_i$, and thus relaxes the smooth muscle cells (34). Thus, the depolarization elicited by high K^+ solution can inhibit hyperpolarization, and therefore eliminate the effects of EDHF (21). In this experiment, we demonstrated that CPA produced a slowly developing relaxation which was then completely blocked by L-NNA in the strips contracted by high K^+ (40 mM K^+) depolarization. These results provide further evidence in support of EDHF release induced by CPA. This is the first report, to our knowledge, which may indicate the involvement of EDHF in a CPA-induced endothelium-dependent decrease in $[Ca^{2+}]_i$ as well as the tension of the smooth muscle cells. Recently, Moritoki et al. (20) reported that CPA induced endothelium-dependent relaxation in rat aorta mainly due to EDRF/NO, while the EDHF-mediated component of the relaxation is negligible. These differential contributions of EDRF and EDHF to the relaxation may be dependent on tissue and /or species difference.

The analysis of the time course of CPA-induced endothelium-dependent relaxation in the presence and absence of L-NNA indicated that it was composed of a rapid relaxation (the first component) and a subsequent prolonged relaxation (the second component) in the porcine coronary

artery precontracted by U46619 (Fig. 6). L-NNA partially (about 50 % of the maximal responses) inhibited the first component of $[Ca^{2+}]_i$ and tension, but almost completely inhibited the second component (Fig. 7). On the other hand, CPA induced only a slowly developing relaxation when the strips with an intact endothelium were precontracted by 40 mM K^+. Thus, it seems likely that the first component is due to the release of both L-NNA sensitive and resistant factors, while the second component is mainly due to L-NNA sensitive factor. These findings are consistent with previous reports in which EDHF was reported to initiate the endothelium-dependent relaxation observed in giunea-pig basilar arteries (24) and pig carotid arteries (9).

An additional finding of the present study is that EDRF induces a greater decrease in the tension than that expected from the extent of the reduction of $[Ca^{2+}]_i$. This is clearly shown in Figs. 8B and 9 in which the tension decreased by the addition of CPA but only showed a slight decrease in $[Ca^{2+}]_i$. It is well known that EDRF directly activates the soluble isoform of guanylate cyclase and causes an increase in the intracellular concentration of guanosine 3',5'-cyclic monophosphate (cGMP) (18). The increase in cellular cGMP induces not only a reduction of $[Ca^{2+}]_i$ but also a decrease in the Ca^{2+} sensitivity of the contractile apparatus (25). Thus, a large vasorelaxation with little decrease in $[Ca^{2+}]_i$ is a well characterized feature of cGMP-producing vasodilators (1). In summary, we therefore conclude that the ER Ca^{2+}-pump plays a crucial role in the regulation of $[Ca^{2+}]_i$ in endothelial cells *in situ* and thus controls the endothelium-dependent relaxation in the porcine coronary artery by releasing L-NNA sensitive (EDRF) and L-NNA resistant factors (possibly EDHF).

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FIGURE LEGENDS

Figure 1

Fluorescence microphotographs of the same field of a coronary arterial strip with intact endothelium doubly loaded with Dil-Ac-LDL and fura-2.

The same field of the sample was observed with specific combinations of excitation and emission wavelengths for Dil-Ac-LDL (A) and fura-2 (B). Dil-Ac-LDL (Ex: 485 nm, Em: > 520 nm), fura-2 (Ex: 340 nm, Em: 500 - 530 nm). Bar = 20 μm.

Figure 2

The effect of various concentrations of CPA on $[Ca^{2+}]_i$ of the endothelial cells on the fura-2 loaded porcine aortic valve.

A: Representative recording of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and the cumulative application of various concentrations of CPA. ATP (10 μM) was applied for 1 min to induce a 100 % response. CPA (0.1 μM-50 μM) was cumulatively added to the strip at the times indicated by the arrows. The numbers illustrated above these arrows indicate the final concentration (expressed in μM) of CPA.

B: The concentration-response curve of the peak levels of $[Ca^{2+}]_i$ induced by the cumulative application of CPA. The vertical bars indicate the S.E.M. (n=3). The fluorescence ratio was expressed in percent (%), assuming the resting level and the peak response induced by 10 μM ATP to be 0 % and 100 %, respectively.

Figure 3

The representative time course of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and CPA in the presence (A, C) and absence (B) of extracellular Ca^{2+} in the endothelial cells on the porcine aortic valve.

A: Several traces are superimposed. In each trace, 10 μM ATP was applied for 1 min, followed by the addition of CPA (30 μM(a), 10 μM(b), 1 μM(c)) at the time indicated by the arrow. At the time indicated by an arrow with "W", CPA was washed out with normal PSS.

B: Representative recording of $[Ca^{2+}]_i$ induced by 30 μM CPA in the absence of extracellular Ca^{2+}.

C: Ni^{2+}(1 mM) was applied during a sustained elevation of $[Ca^{2+}]_i$ induced by 30 μM CPA.

Figure 4

The effect of 118 mM K^+ and 30 μM CPA on the smooth muscular $[Ca^{2+}]_i$ (the upper panel) and tension (the lower panel) in the fura-2 loaded porcine coronary arterial strips without (A) or with (B) the endothelium under resting conditions.

Figure 5

The effect of various concentrations of CPA on the U46619-induced increase in $[Ca^{2+}]_i$ and tension in the strips with the endothelium.

A: Representative recordings of the changes in $[Ca^{2+}]_i$ and tension induced by 118 mM K^+ and 100 nM U46619 in the coronary arterial strips with the endothelium.

B: Representative recordings of the endothelium-dependent decrease in $[Ca^{2+}]_i$ and tension induced by the cumulatively application of CPA (0.3 μM-30 μM) in the strips precontracted by 100 nM U46619. CPA was added at the times indicated by the arrows. The numbers illustrated either above or below these arrows indicate the final concentrations (expressed in μM) of CPA.

C: Summary of the endothelium-dependent decrease in $[Ca^{2+}]_i$ and tension induced by CPA obtained from 4 independent experiments done in a similar manner as in panel B. The CPA-induced decreases in $[Ca^{2+}]_i$ (○) and tension (●) were expressed as a percentage of the fluorescence ratio and the tension obtained at 10 min after the application of U46619. The vertical bars indicate the S.E.M. (n=4).

Figure 6

The effect of 30 μM CPA on $[Ca^{2+}]_i$ and the tension of coronary arterial strips either without (A) or with (B) an endothelium during contraction induced by 100 nM U46619, and the effect of 100 μM L-NNA and 10 μM indomethacin on the endothelium-dependent relaxation (C).

A: Representative recording of the changes in $[Ca^{2+}]_i$ and the tension induced by 30 μM CPA of the strips without an endothelium during contraction with U46619.

B: Representative recording of the changes in $[Ca^{2+}]_i$ and the tension induced by 30 μM CPA of the strips with an endothelium during contraction with U46619.

C: Representative recording of the changes in $[Ca^{2+}]_i$ and the tension induced by 30 μM CPA during contraction with U46619 of the strips with an endothelium which was pretreated with L-NNA and indomethacin for at least 40 min.

Figure 7

The effect of pretreatment with 100 μM L-NNA and/or 10 μM indomethacin on CPA-induced endothelium-dependent decrease in $[Ca^{2+}]_i$ (A) and tension (B) of the coronary arterial strips precontracted by 100 nM U46619. The graphs shown are a summary of 4 independent experiments done in a similar manner as in Fig. 6. The 4 columns on the left illustrated as "U46619" indicate the steady state levels of $[Ca^{2+}]_i$ and tension at 10 min after the application of U46619. It should be noted that in the case of the strips pretreated with L-NNA and indomethacin (▨), we designated the 118 mM K^+-induced tension enhanced by L-NNA to be 100%. The middle 4 columns illustrated as "U46619 + CPA (first component)" indicate the levels of $[Ca^{2+}]_i$ and tension at maximal relaxation induced by 30 μM CPA. The 4 columns on the right illustrated as "U46619 + CPA (second component)" indicate the levels of $[Ca^{2+}]_i$ and tension at a sustained phase of relaxation which are obtained at 30 min after application of CPA. (■; strips with an endothelium, ▧;strips with an endothelium pretreated by 10 μM indomethacin, ▨; strips with an endothelium pretreated with 100

μM L-NNA and 10 μM indomethacin, □; strips without an endothelium). Vertical bars indicate the S.E.M. (n=4). * P< 0.01 ** P< 0.001 (compared with value without an endothelium (□) at each middle and right column) N.S.; not significant

Figure 8

The effect of 30 μM CPA on $[Ca^{2+}]_i$ and the tension of coronary arterial strips either without (A) or with (B) an endothelium during contraction induced by 40 mM K^+ and the effect of 100 μM L-NNA and 10 μM indomethacin on the endothelium-dependent relaxation (C). The protocol of A, B and C are the same as in Figs. 6 A, B and C, except that the precontraction was induced by 40 mM K^+ instead of U46619.

Figure 9

The effect of pretreatment with 100 μM L-NNA on the CPA-induced endothelium-dependent decrease in $[Ca^{2+}]_i$ (A) and tension (B) of the coronary arterial strips with endothelium precontracted by 40 mM K^+. The graphs shown are a summary of the 4 independent experiments done in a similar manner as in Fig. 8. Left 3 columns illustrated as "40 mM K^+" indicate the steady state level of $[Ca^{2+}]_i$ and tension at 10 min after the application of 40 mM K^+. The 3 columns on the right illustrated as "40 mM K^+ + CPA " indicate the levels of $[Ca^{2+}]_i$ and tension at 30 min after the application of CPA. (■; strips with an endothelium, ▨; strips with an endothelium pretreated with L-NNA, □; strips without an endothelium). The vertical bars indicate the S.E.M. (n=4). * P< 0.05 ** P< 0.001 (compared with the value of that without an endothelium (□)) N.S.; not significant

TABLE

Table 1: Elevation of $[Ca^{2+}]_i$ induced by CPA and various agonists in endothelial cells *in situ*.

Agonists	Concentration (μM)	% Fluorescence Ratio (initial peak)	% Fluorescence Ratio (steady state)	(n)
CPA	30	364.8 ± 32.7 %	234.0 ± 29.7 %	3
BK	0.1	191.2 ± 31.9 %	12.5 ± 3.7 %	5
HIS	100	122.5 ± 5.8 %	15.3 ± 1.2 %	3
5-HT	10	95.1 ± 1.9 %	27.9 ± 0.6 %	3
ATP	1,000	256.1 ± 26.0 %	30.2 ± 0.3 %	3
ET-1	1	234 ± 27 %	28 ± 8 %	3
ET-3	1	133 ± 17 %	21 ± 2 %	3

The ATP (10 μM)-induced changes in the fluorescence ratio were taken to be 100% in each experiment. The concentrations of the agonists are enough to obtain the maximal response in both the initial peak and steady state. The values are given as the mean ± S.E.M. from the number (n) of experiments. BK; bradykinin, HIS; histamine, 5-HT; serotonin, ATP; adenosine triphosphate, ET-1; endothelin-1, ET-3; endothelin-3.

Figure 2

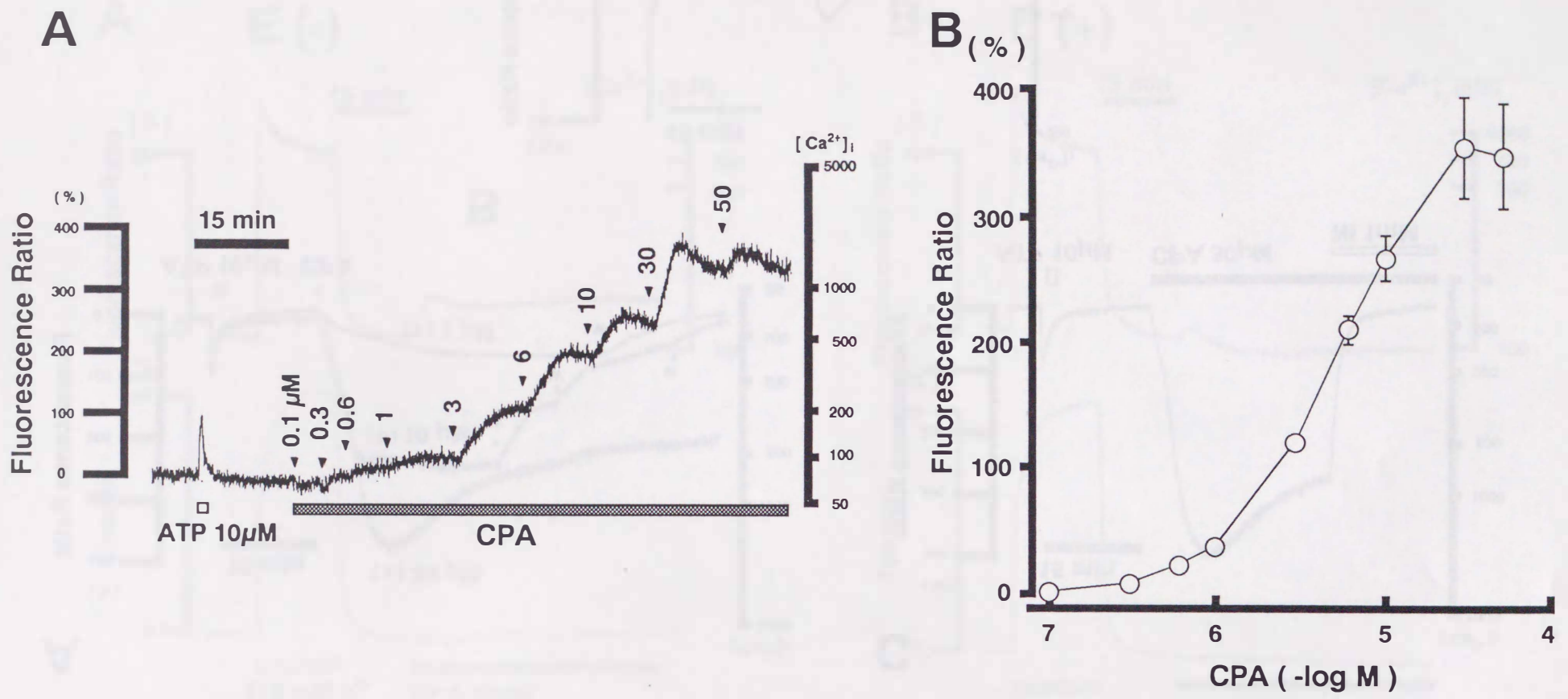


Figure 1

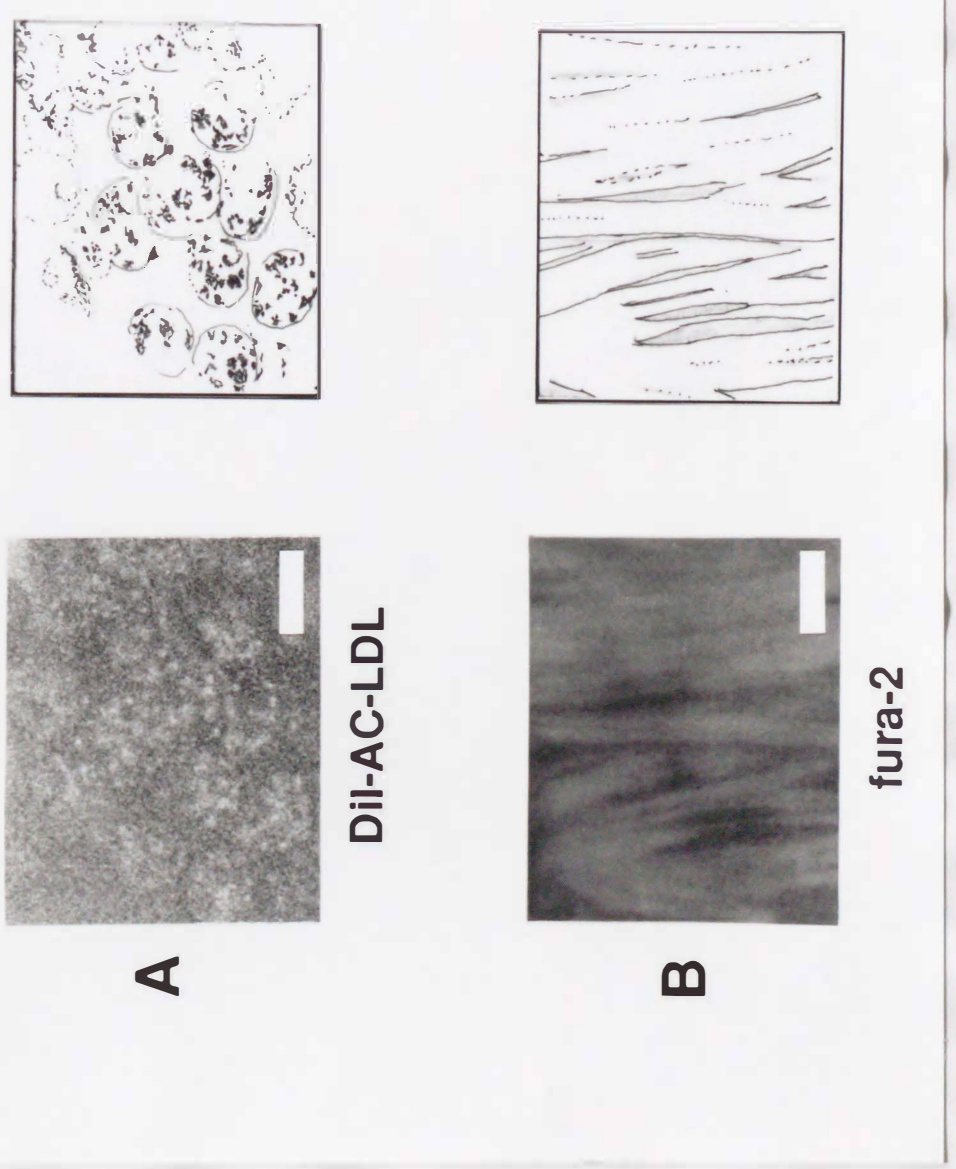


Figure 4

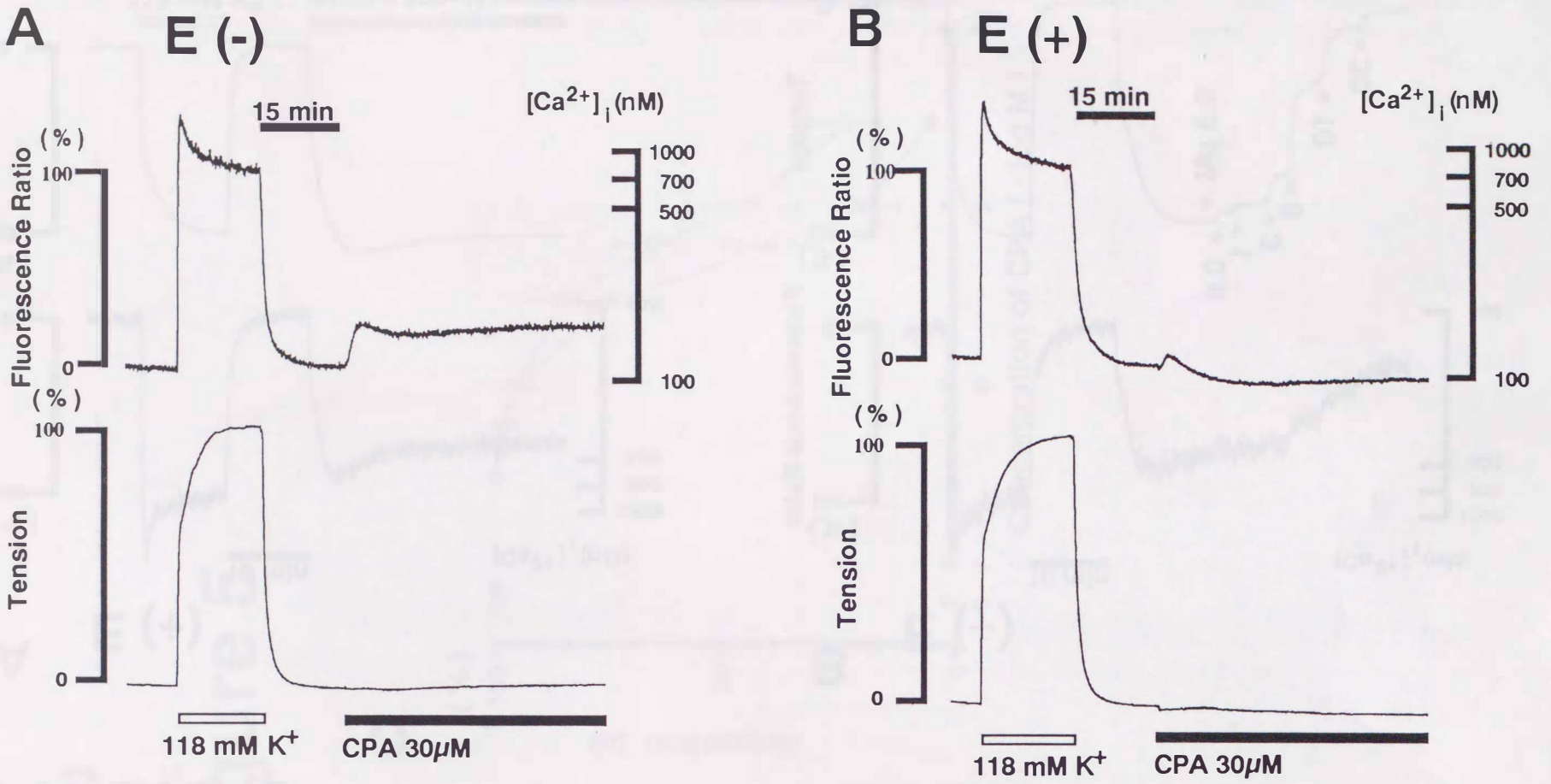
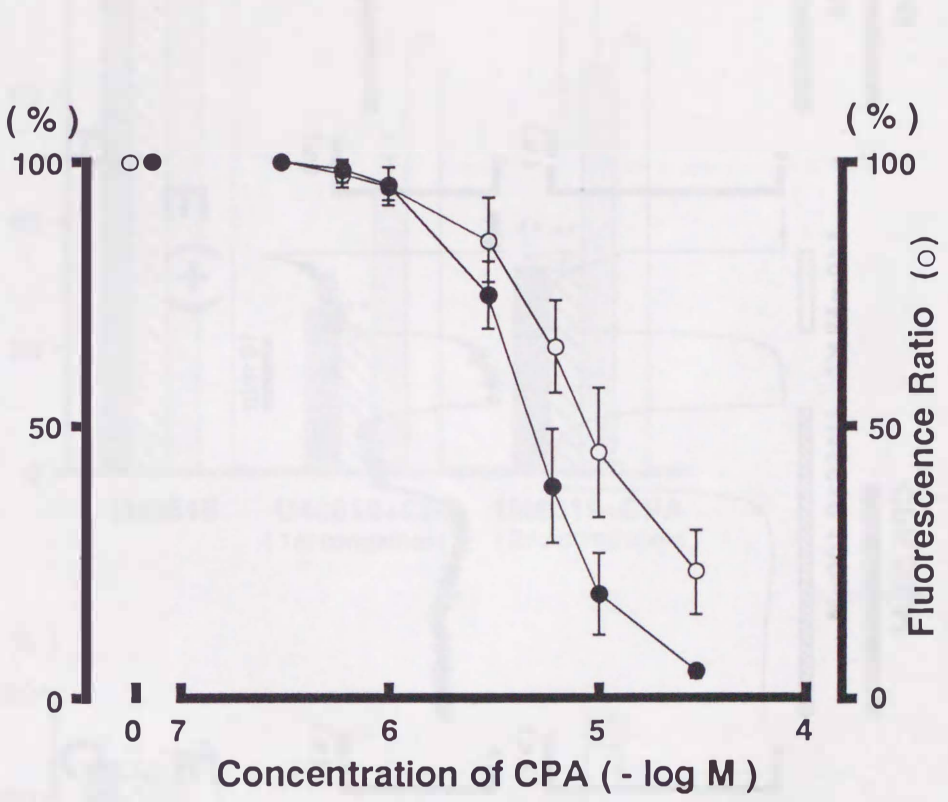
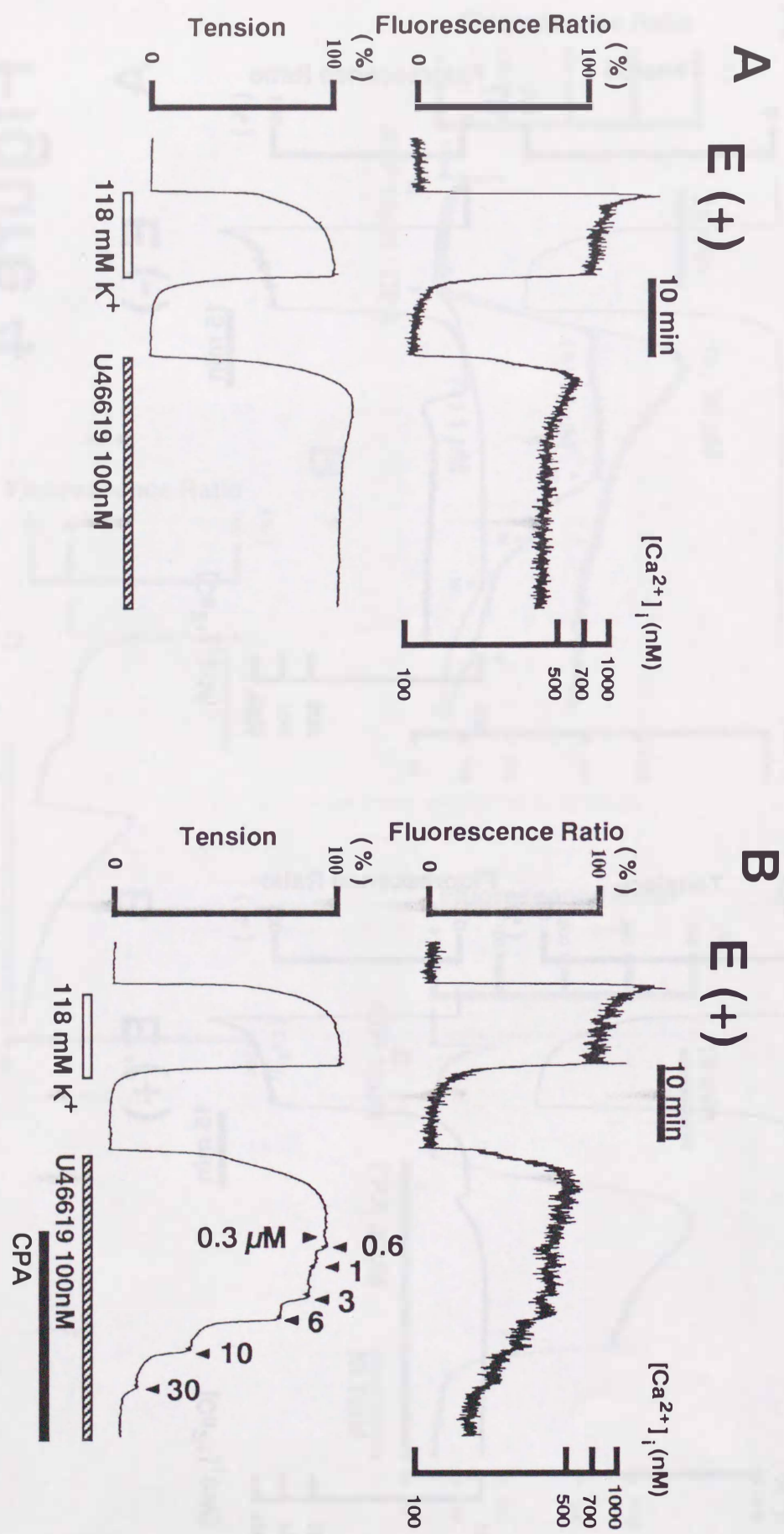


Figure 3

Figure 5


C
(%)
100
50
0
Relaxation (●)
○●
Fluorescence Ratio (○)
0 7 6 5 4
Concentration of CPA (- log M)


A
E (+)
Tension
Fluorescence Ratio
(%)
100
0
118 mM K+
U46619 100nM
10 min
[Ca2+]i (nM)
1000
700
500
100
B
E (+)
0.3 μM
0.6
1
3
6
10
30
CPA

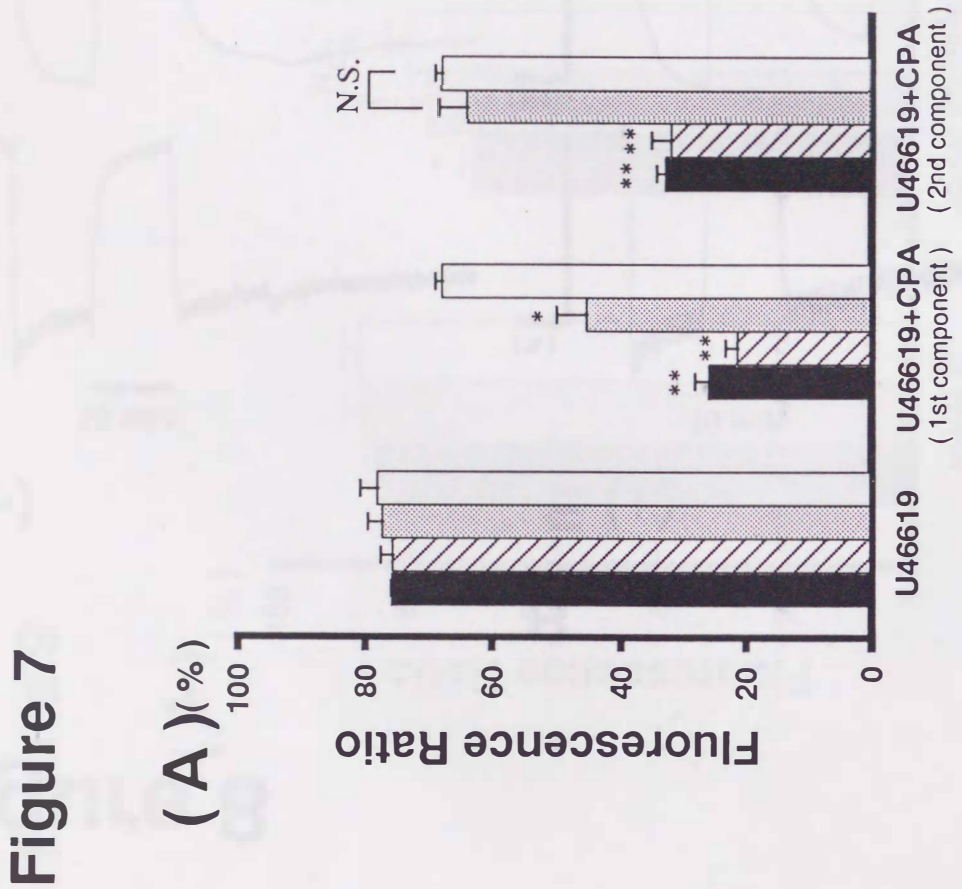
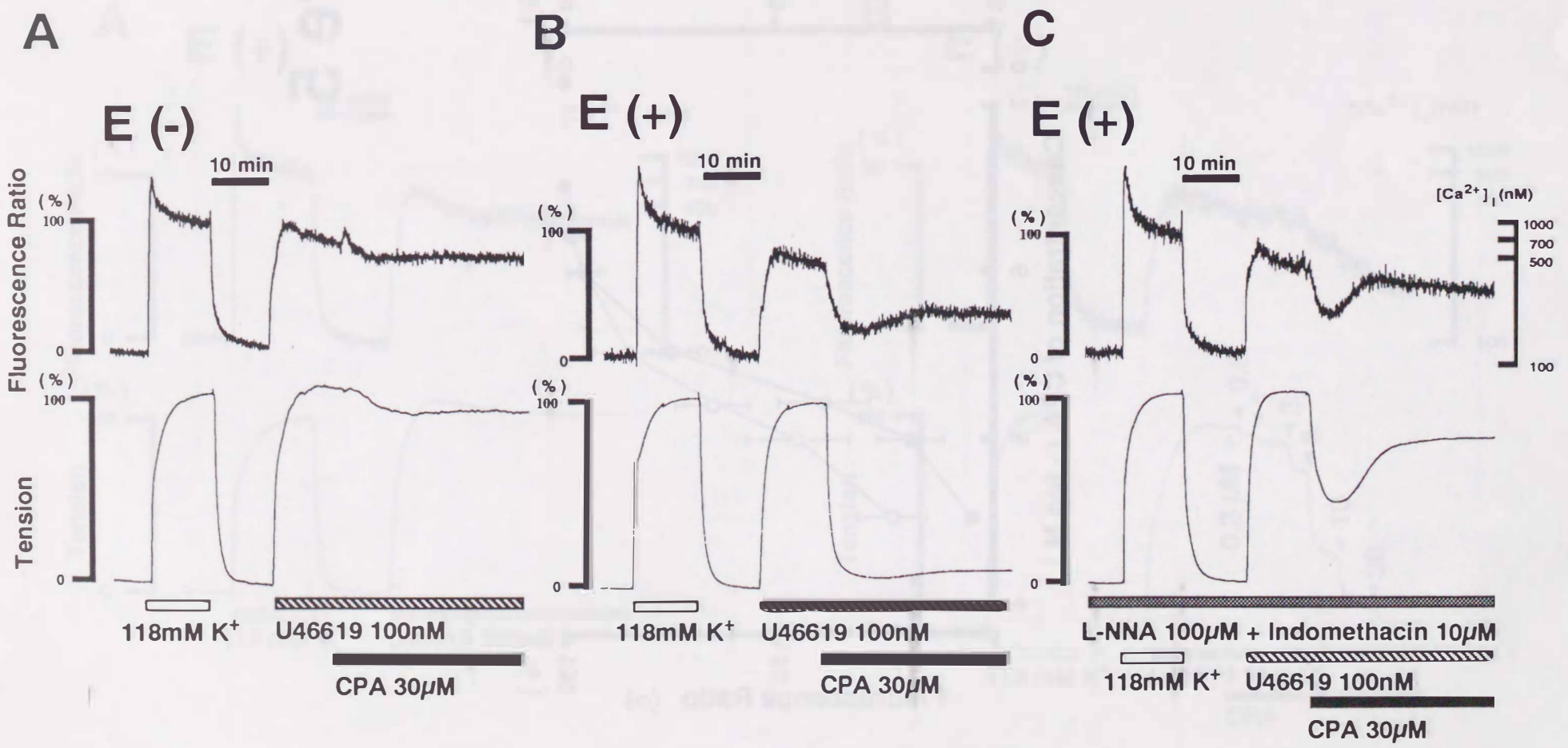


Figure 7
(A)
Fluorescence Ratio
(%)
100
80
60
40
20
0
N.S.
**
*
**
***
U46619
U46619+CPA
(1st component)
U46619+CPA
(2nd component)
(B)
Tension
(%)
120
100
80
60
40
20
0
N.S.
**
**
***
U46619
U46619+CPA
(1st component)
U46619+CPA
(2nd component)

Figure 6


A
E (-)
B
E (+)
C
E (+)
10 min
10 min
10 min
Fluorescence Ratio
(%)
100
0
Tension
(%)
100
0
[Ca2+]i (nM)
1000
700
500
100
118mM K+
U46619 100nM
CPA 30μM
118mM K+
U46619 100nM
CPA 30μM
L-NNA 100μM + Indomethacin 10μM
118mM K+
U46619 100nM
CPA 30μM

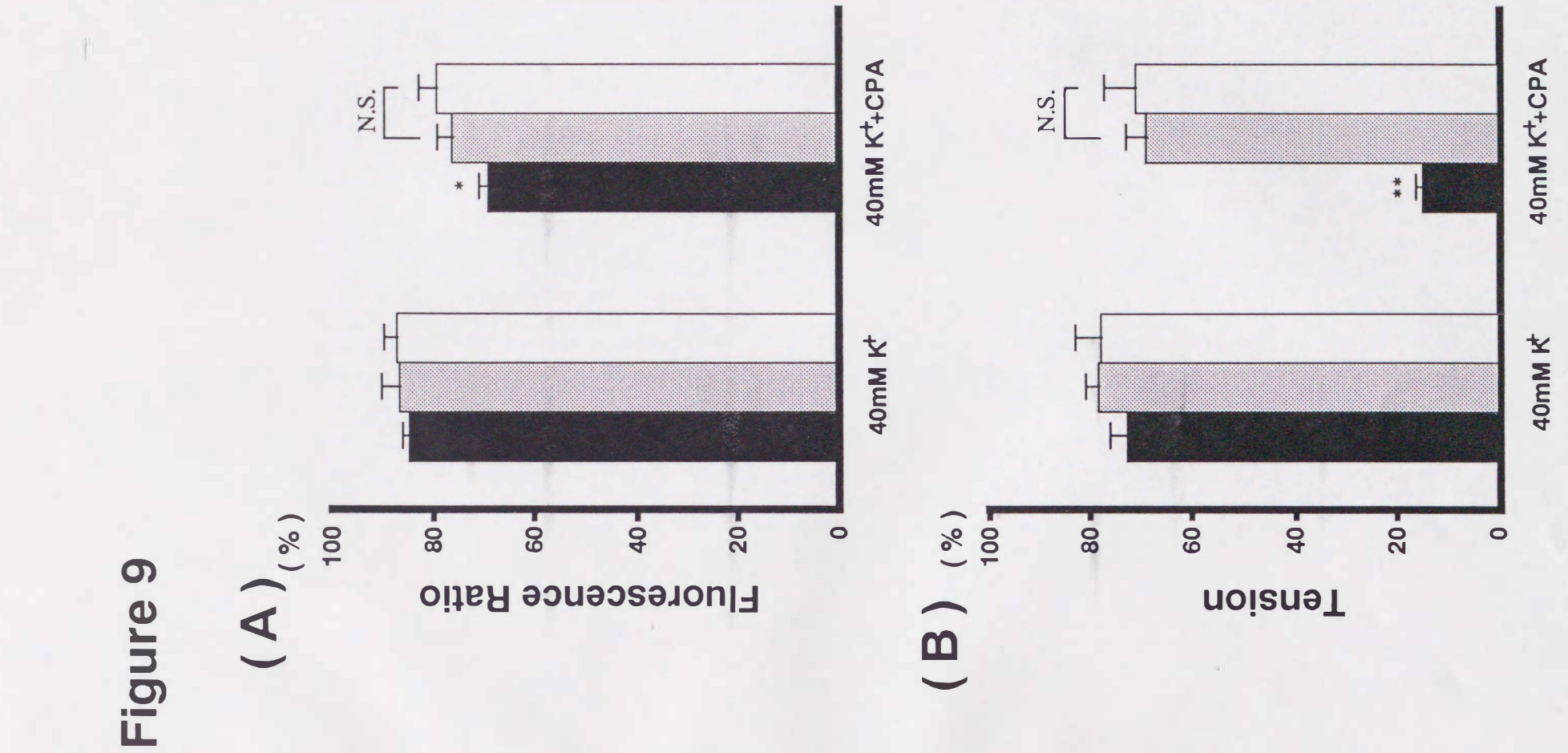


Figure 9

Figure 8