British Journal of Pharmacology (1995) 116, 2040-2047

Mechanism of endothelium-dependent relaxation induced by substance P in the coronary artery of the pig

Mari Kuroiwa, Hiroki Aoki, Sei Kobayashi, Junji Nishimura & [1]Hideo Kanaide

Division of Molecular Cardiology, Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku Fukuoka 812, Japan

1 Using front-surface fluorometry of fura-2-loaded porcine coronary arterial strips with the endothelium intact, we investigated the mechanisms of vasorelaxation induced by substance P (SP). Fura-2 fluorescence signals which indicated the cytosolic Ca^{2+}-concentration ($[Ca^{2+}]_i$), were observed to arise exclusively from the smooth muscle cells in these strips.

2 During the contractions induced by U46619 (100 nM), a thromboxane A_2 analogue, an SP-induced endothelium-dependent, biphasic vasorelaxation was observed, which consisted of an initial rapid relaxation phase followed by a sustained phase, with a transient decrease in $[Ca^{2+}]_i$. Pretreatment with indomethacin (Ind) had no effect on the SP-induced relaxation; however, pretreatment with N^G-nitro-L-arginine (L-NOARG) partially, but significantly inhibited the decrease in both the $[Ca^{2+}]_i$ and tension induced by SP; in addition, the sustained phase of SP-induced relaxation was almost completely abolished. Thus, part of the relaxation was considered to be mediated by L-NOARG-sensitive relaxing factor (endothelium-derived relaxing factor : EDRF).

3 During the 40 mM K^+-depolarization-induced contraction which may eliminate the effects of endothelium-derived hyperpolarizing factor (EDHF), the vasorelaxation induced by SP was completely inhibited by L-NOARG.

4 During the vasorelaxation induced by SP, the $[Ca^{2+}]_i$-tension relationships shifted to the right of the contractions induced by either U46619 or high K^+-depolarization.

5 Using front-surface fluorometry of fura-2 loaded porcine aortic valvular strips, we examined the effects of SP on $[Ca^{2+}]_i$ in endothelial cells *in situ*. SP induced a rapid increase in $[Ca^{2+}]_i$ of endothelial cells *in situ* followed by a small sustained phase in normal PSS (5.9 mM K^+). The increase in extracellular K^+ had no apparent effect on the SP-induced $[Ca^{2+}]_i$ elevation of endothelial cells.

6 We thus conclude that: (1) SP-induced vasorelaxation is mediated by an L-NOARG-sensitive factor (EDRF) and an L-NOARG-resistant factor; and (2) the first, rapid, phase of the relaxation is mediated by both factors while the sustained phase seems to be mediated mainly by EDRF. The underlying mechanisms of L-NOARG-resistant relaxation have yet to be elucidated, but EDHF appears to be a potentially contributing factor.

Keywords: Endothelium-derived relaxing factor; endothelium-derived hyperpolarizing factor; substance P; coronary artery

Introduction

The endothelium plays a vital role in controlling vascular tone. Moncada (1976) reported that prostaglandin I_2 (PGI_2), released from the endothelium, relaxes vascular smooth muscle. In 1980, Furchgott & Zawadzki discovered that the endothelial lining was essential for the acetylcholine (Ach)-induced relaxation of the rabbit isolated aorta; they thus proposed the existence of endothelium-derived relaxing factor (EDRF). Since then, it has been demonstrated that endothelial cells modify the tone of vascular smooth muscle cells by releasing some vasoactive substances (Vane *et al.*, 1990). On the other hand, endothelium-dependent relaxations induced by some agents are accompanied by a concomitant endothelium-dependent hyperpolarization of smooth muscle cells, which is not blocked by inhibitors of EDRF. Chen *et al.* (1988) obtained evidence for the existence of endothelium-derived hyperpolarizing factor (EDHF), a factor distinct from EDRF. Today, endothelium-dependent relaxation is considered to be produced by, at least, three factors: PGI_2, EDRF and EDHF.

Substance P (SP) induces an endothelium-dependent vasorelaxation. There have been reports of SP-induced endothelium-dependent hyperpolarization of smooth muscle cells (Beny *et al.*, 1986; Pacicca *et al.*, 1992). Using front-surface fura-2-fluorometry to determine cytosolic Ca^{2+}-concentrations ($[Ca^{2+}]_i$) in vascular smooth muscle cells and in endothelial cells, we investigated the cellular mechanisms of SP-induced vasorelaxation, with special attention directed to the manner in which PGI_2, EDRF and EDHF contribute to the relaxation.

Methods

Tissue preparation

Coronary arterial strips: fresh pig hearts obtained from a local slaughterhouse were placed in ice-cold normal physiological salt solution (PSS) and brought to the laboratory. The left circumflex coronary arteries (2-3 cm from the origin) were isolated and after removing the adventitia, the segments were cut open, longitudinally. Preparations with an intact endothelium were cut into circular strips (approximately 1 mm wide, 5 mm long, and 0.1 mm thick). The inner surface was rubbed off with a cotton swab to obtain a strip without an endothelium.

Aortic valvular strips: Aortic valves were dissected in a manner so as not to touch their surface. The valve leaflets were cut into strips in an axial direction (approximately 2 mm wide, 5 mm long, and 0.18 mm thick) and used to monitor the $[Ca^{2+}]_i$ in the endothelial cells. The centre of each leaflet, corpus arantii, was not used.

[1] Author for correspondence.

Fura-2 loading

Coronary arterial strips either with or without an endothelium were loaded with $[Ca^{2+}]_i$ indicator dye, fura-2, by incubating in oxygenated (a mixture of 95% O_2 and 5% CO_2) Dulbecco's modified Eagle medium containing 25 μM fura-2/AM (an acetoxymethyl ester form of fura-2) and 5% foetal bovine serum for 4 h at 37°C. The valvular strips were incubated in oxygenated Dulbecco's modified Eagle medium containing 50 μM fura-2/AM, 1 mM probenecid (Di Virgilio *et al.*, 1989) and 5% foetal bovine serum for 1.5 h at 37°C (Aoki *et al.*, 1994). After loading with fura-2, both vascular and valvular strips were incubated in normal PSS for at least 1 h before starting the measurement, in order to remove the dye in the extracellular space and for purposes of equilibration. Loading the vascular strips with fura-2, *per se*, did not affect the contractility, as described (Abe *et al.*, 1990; Hirano *et al.*, 1990).

Front-surface fluorometry

Experiments on vascular strips with or without an endothelium were carried out at 37°C, as previously described (Hirano *et al.*, 1990). Changes in the fluorescence intensity of the fura-2-Ca^{2+} complex in smooth muscle cells were monitored with a front-surface fluorometer specifically designed for fura-2 fluorometry (CAM-OF2), with the collaboration of the Japan Spectroscopic Co., Tokyo, Japan. In brief, the ratio of the fluorescence (500 nm) intensities at 340 nm excitation to those at 380 nm excitation (ratio) was monitored and expressed as a percentage, while the values at rest in normal (5.9 mM K^+) and 118 mM K^+ PSS were designated as 0% and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ of vascular strips was calculated according to the method of Grynkiewicz *et al.* (1985), With the *K*d (apparent dissociation constant) of the fura-2-Ca^{2+} complex of 225 nM (at 37°C). The $[Ca^{2+}]_i$ levels were determined separately, and 0% and 100% (ratio) were 108 ± 27 and 715 ± 103 nM, respectively (Hirano *et al.*, 1990).

When measuring the fluorescence intensity of vascular strips with an intact endothelium, it is possible that the fura-2 signal arises from either the smooth muscle cells, from the endothelial cells, or both (Sato *et al.*, 1990). The experimental conditions used in the present study, at 37°C with probenecid in the medium, made it feasible to record fura-2 signals derived exclusively from smooth muscle cells in porcine coronary arterial strips with intact endothelial cells (Kuroiwa *et al.*, 1993). These findings are shown in Figure 1. The vascular strips were doubly loaded with fura-2 and acetylated-low density lipoprotein labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL); the latter is specifically taken up by endothelial cells (Nettland *et al.*, 1985). Figures 1a and 1b are photographs of the same cells in which Dil-Ac-LDL and fura-2 are doubly loaded in the absence of probenecid, at 37°C; in similar conditions vascular strips with the endothelium were loaded with fura-2. As shown in Figure 1a (Dil-Ac-LDL observation; excitation 485 nm, emission > 520 nm), there are cells positively stained with Dil-Ac-LDL which have a cobble-stone appearance, thereby indicating the presence of intact endothelial cells. But in Figure 1b (fura-2 observation; excitation 340 nm, emission 500 – 530 nm), which shows the same cells as those in Figure 1a, the endothelial cells are not stained with fura-2, and there are also underlying smooth muscle cells (SMC), running longitudinally and stained with fura-2. Figures 1c and 1d are photographs of the same cells in which Dil-Ac-LDL and fura-2 were loaded in the presence of probenecid, which inhibits the sequestration of fura-2 by cells (Di Virgilio *et al.*, 1989) and was also used to stain the aortic valvular strips in the present study. A cobble-stone appearance of Dil-Ac-LDL-stained cells confirms the presence of intact endothelial cells (Figure 1c, for Dil-Ac-LDL observation). When probenecid was used during loading with fura-2, both endothelial cells (EC) and smooth muscle cells (SMC) were stained with fura-2 (Figure 1d, for fura-2 observation). The cobble-stone appearance of Dil-Ac-LDL-stained cells in Figure 1c corresponds to fura-2-stained cells with a cobble-stone appearance in Figure 1d (labelled EC), but not to cells with a longitudinal running pattern (labelled SMC). Thus, under the present experimental conditions (37°C and without probenecid), the fura-2 signals derived from the endothelial cells of the porcine coronary artery were negligible and the signal was exclusively derived from the smooth muscle cells in the porcine coronary arterial strips with an intact endothelium.

In experiments using aortic valvular strips with an intact endothelium, Ca^{2+} measurements were carried out in a manner similar to that used for vascular strips. However, the temperature during the measurements was 25°C to prevent the leakage of the fluorescent dye, as previously described (Aoki *et al.*, 1991; 1994). Fluorescence-microscopic observations re-

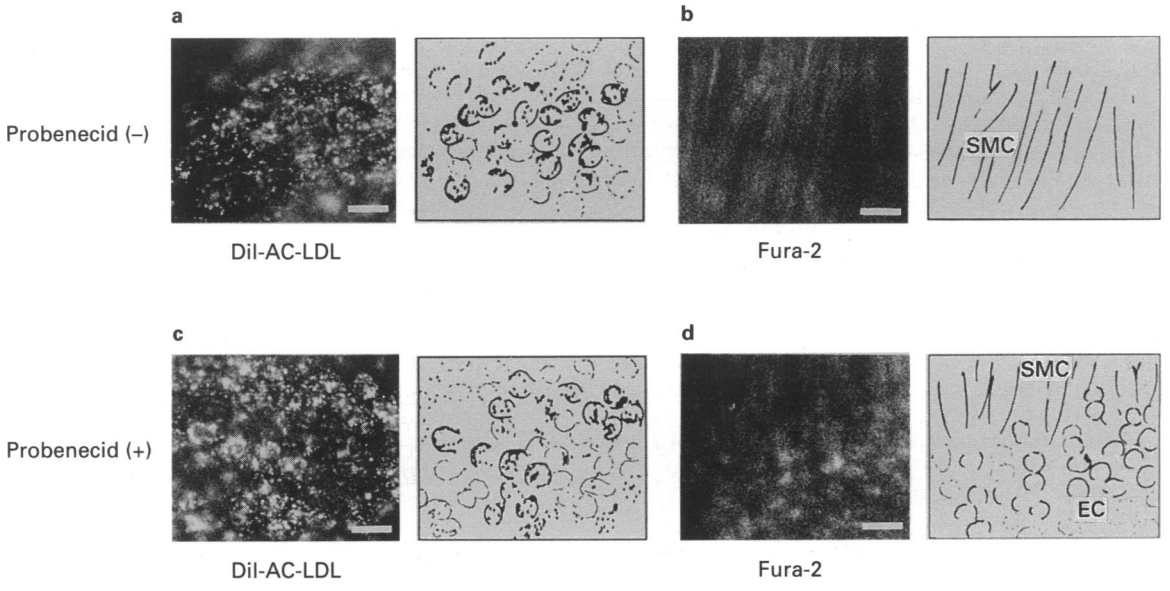


Figure 1 Fluorescence photomicrographs of porcine coronary arterial strips with an intact endothelium. The strips were doubly loaded with Dil-Ac-LDL and fura-2, in the absence (a, b) and presence (c, d) of probenecid, and observed with specific combinations of excitation and emission wavelengths for Dil-Ac-LDL (a, c) and fura-2 (b, d). Dil-Ac-LDL (Ex: 485 nm, Em: > 520 nm), fura-2 (Ex: 340 nm, Em: 500 – 530 nm). SMC; smooth muscle cells, EC; endothelial cells. Bar = 30 μm

vealed that only the endothelial lining is positive for fura-2 fluorescence. Accordingly, the absolute value of $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz *et al.* (1985) with a K_d value of 162 nM (Aoki *et al.*, 1991). The $[Ca^{2+}]_i$ levels of endothelial cells *in situ* at rest and at the peak level of 10 μM ATP-stimulation were designated 0% and 100%, respectively. Separate determinations revealed 0% and 100% to be 63.9 ± 7.4 and 176.7 ± 16.5 nM, respectively ($n = 5$).

Measurement of tension development

Coronary arterial strips mounted vertically in a quartz organ bath were connected to a force-transducer (TB-612T, Nihon Koden, Japan). During the fura-2 equilibration period, the strips were stimulated with 118 mM K^+ PSS every 15 min and the resting tension was increased stepwise. After equilibration, the resting tension was adjusted to 250 mg. The responsiveness of each strip to 118 mM K^+ PSS was recorded before starting the experimental protocol. The developed tension was expressed as a percentage, while the values at rest in normal PSS (5.9 mM K^+) were designated 0%, and those at steady state of contraction in 118 mM K^+ PSS were designated 100%.

Determination of the control $[Ca^{2+}]_i$-tension relationships of coronary arterial strips

To obtain the control $[Ca^{2+}]_i$-tension relation curves of the contraction induced by 118 mM K^+-depolarization of 100 nM U46619 (without SP), the strips were incubated in Ca^{2+}-free PSS containing 2 mM EGTA for 10 min, and then incubated in Ca^{2+}-free PSS without EGTA for 5 min. Subsequently, the tissues were depolarized with 118 mM K^+ or placed in 100 nM U46619 in Ca^{2+}-free PSS, and then extracellular Ca^{2+} was re-added in a cumulative manner (from 0 mM to 1.25 mM) during either 118 mM K^+-depolarization or in the presence of 100 nM U46619. The cumulative application of extracellular Ca^{2+} induced a step-wise increase in $[Ca^{2+}]_i$ and tension (Abe *et al.*, 1990; Hirano *et al.*, 1990). The $[Ca^{2+}]_i$-tension curves thus obtained are control curves for the contraction induced by 118 mM K^+-depolarization or 100 nM U46619. $[Ca^{2+}]_i$ and tension values at sustained phase of SP-induced relaxation during the contraction induced by 118 mM K^+-depolarization or 100 nM U46619 were compared with those of these control curves.

Drugs and solutions

The composition of normal PSS was as follows (mM): 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. High K^+ PSS was prepared by replacing NaCl with equimolar KCl. PSS was bubbled with a mixture of 95% O_2 and 5% CO_2, and resulting pH was 7.4.

Fura-2/AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Indomethacin (Ind) was purchased from Wako (Osaka, Japan), and N^G-nitro-L-arginine (L-NOARG) was from the Aldrich Chemical Company, Inc. (Milwaukee, WIS, U.S.A.). Adenosine 5'-triphosphate (ATP) was purchased from Boehringer Mannheim GmBH (Germany). Probenecid, bovine serum albumin (BSA) and (d)-tubocurarine were purchased from Sigma (St. Louis, MO, U.S.A.). Substance P (SP), charybdotoxin, and iberiotoxin were purchased from the Peptide Institute, Inc. (Osaka, Japan), and acetylated-low density lipoprotein labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) and U46619 were purchased from Funakoshi (Tokyo, Japan). All other chemicals were from Katayama Chemical (Osaka, Japan).

Data analysis

The values are expressed as the mean ± s.e.mean (s.e.mean). Student's *t* test was used to determine statistical significance. *P* values of less than 0.05 were considered to have statistical significance. All data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, Macintosh; Apple Computer, U.S.A.). The significance of the shift of the $[Ca^{2+}]_i$-tension relation from the control curves was determined by analysis of covariance.

Results

Effects of SP on tension and $[Ca^{2+}]_i$ of smooth muscle cells of coronary arterial strips with intact endothelium

Figure 2a shows the representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by 10 nM SP during the contractions with 100 nM U46619 (thromboxane A_2 analogue) of coronary arterial strips with an intact endothelium. When the bathing medium was changed from normal PSS (5.9 mM K^+) to 118 mM K^+ PSS, both $[Ca^{2+}]_i$ and tension rapidly increased and reached the plateau phase within 10 min, while the resting and the plateau phases were designated 0% and 100%, respectively. The application of U46619 induced an abrupt increase in $[Ca^{2+}]_i$, which reached a peak level about 2 min after the application and thereafter slightly decreased to reach a plateau level within 15 min (64.3 ± 0.9%, $n = 5$). The tension also increased rapidly and reached maximum plateau level within 15 min (90.9 ± 8.1%, $n = 5$). Thus, the increase in tension was greater than that for the given increase in $[Ca^{2+}]_i$. SP (10 nM) was applied when the $[Ca^{2+}]_i$ and tension reached the plateau level by U46619. As shown in Figure 2a, SP induced a rapid and transient vasorelaxation (first phase) (to 11.0 ± 2.6% of maximum, $n = 5$) followed by a sustained phase (56.1 ± 8.6%, $n = 5$) in tension of vascular smooth muscle, with a transient decrease in $[Ca^{2+}]_i$ (10.2 ± 2.0%, $n = 5$). When the endothelium was rubbed off, SP did not affect the $[Ca^{2+}]_i$ or tension of the vascular smooth muscle (data not shown), therefore, the SP-induced vasorelaxation was considered to be endothelium-dependent.

Pretreatment with 10 μM indomethacin (Ind), in order to eliminate any effect of PGI_2, had little influence on the SP-induced changes both in $[Ca^{2+}]_i$ and tension in vascular smooth muscle with an endothelium (Figure 2b). Pretreatment with both 10 μM Ind and 100 μM N^G-nitro-L-arginine (L-NOARG), an inhibitor of nitric oxide production, significantly attenuated the decrease of $[Ca^{2+}]_i$ and tension induced by SP, as shown in Figure 2c. Although the first phase of the SP-induced relaxation remained (30.4 ± 3.5%), the sustained phase was almost completely abolished (91.6 ± 4.9%). Figure 2d is a summary of these measurements. SP induced rapid, transient and maximal reductions followed by a sustained reduction of $[Ca^{2+}]_i$ and tension during the contraction induced by U46619. Pretreatment with NLA, but not with Ind, partially inhibited the rapid, transient and maximal reduction and almost completely inhibited the sustained reduction of $[Ca^{2+}]_i$ and tension induced by SP.

It has been shown that, in general, endothelium-dependent relaxations are accompanied by a concomitant endothelium-dependent hyperpolarization of smooth muscle cells, and that high-external K^+ inhibits this membrane hyperpolarization during endothelium-dependent relaxation (Nagao & Vanhoutte, 1992; Zhang *et al.*, 1994). Figure 3 shows evidence of the SP-induced relaxation during the contractions induced by high-K^+ (40 mM K^+) PSS, in which the SP-induced membrane hyperpolarization, if any, was eliminated. High-K^+ (40 mM) PSS induced a rapid increase in $[Ca^{2+}]_i$ which reached a plateau level within 15 min (88.0 ± 1.3%, $n = 5$). The tension also rapidly increased and reached a maximum plateau level within 15 min (76.3 ± 0.5%, $n = 5$). SP was applied at this (15 min) plateau phase of 40 K^+ PSS-induced contraction, which induced a rapid and transient vasorelaxation (at maximal reduction; 26.8 ± 1.1%, $n = 5$) followed by a sustained phase (50.7 ± 2.1%, $n = 5$), with a transient decrease in $[Ca^{2+}]_i$ (maximal decrease; 74.5 ± 4.3%, $n = 5$) of vascular smooth muscle. Pretreatment with 10 μM Ind did not affect the SP-

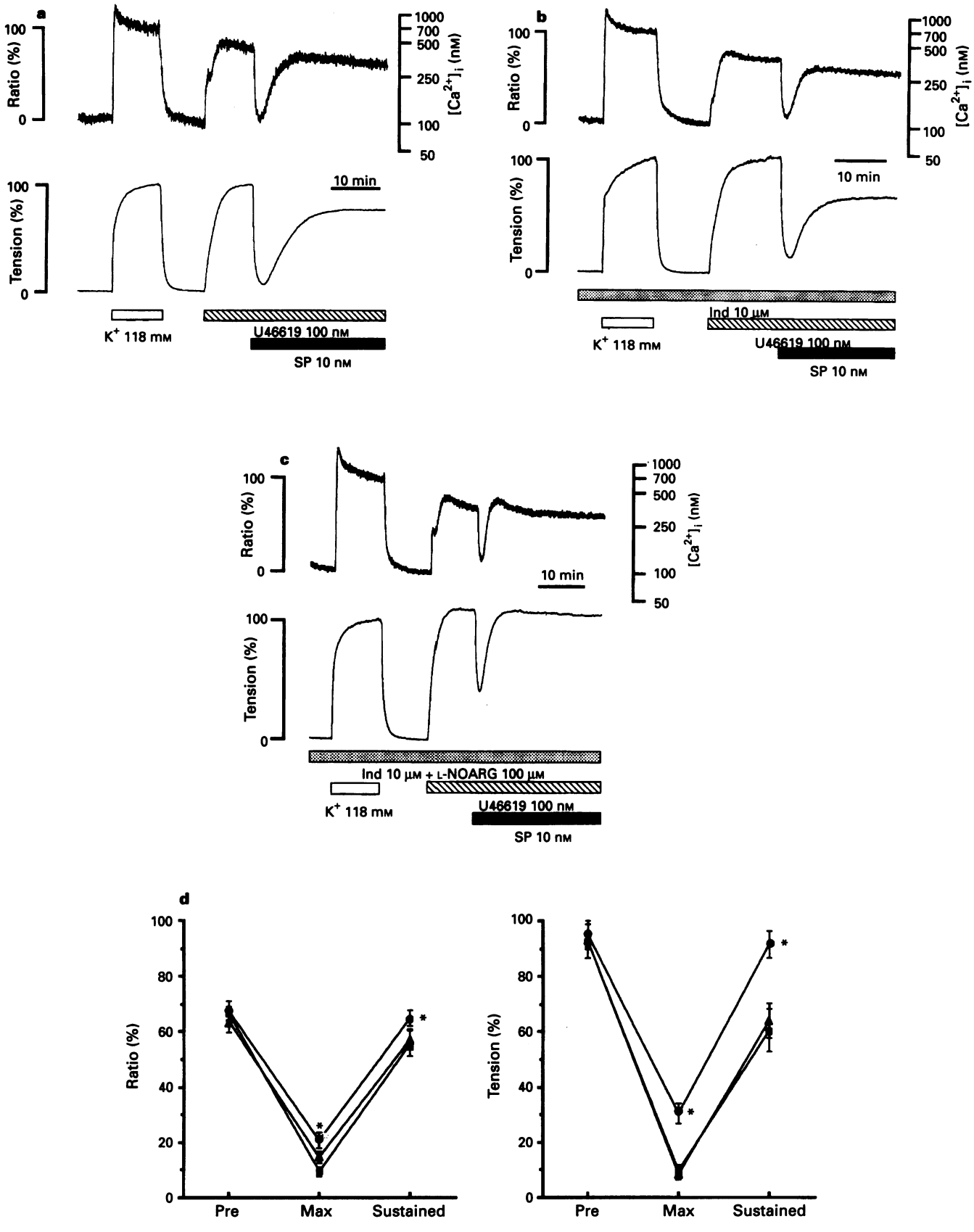


Figure 2 Representative recordings showing the effect of substance P (SP) on $[Ca^{2+}]_i$ and tension in coronary arterial strips with endothelium precontracted with 100 nM U46619. (a) The effect of SP: after the application of 118 mM K^+ depolarization to determine 100% levels of ratio and tension, the strip was precontracted with 100 nM U46619. SP (10 nM) was applied at the plateau phase. (b) The effect of pretreatment with 10 μM indomethacin (Ind) and (c) 10 μM (Ind) and 100 μM N^G-nitro-L-arginine (L-NOARG) on the vasorelaxation induced by 10 μM SP. $[Ca^{2+}]_i$ and tension were expressed as a percentage, while the values in normal (5.9 mM K^+) PSS were designated as 0% and in 118 mM K^+ PSS to be 100%. (d) The effect of Ind and L-NOARG on the rapid phase and the sustained phase of SP-induced relaxation; Pre: the levels of ratio and tension induced by 100 nM U46619 just prior to the application of SP. Max: maximal response (first rapid phase) of SP-induced relaxation. Sustained: the sustained phase of SP-induced relaxation. (■) U46619 only; (▲) U46619 + Ind; (●) U46619 + Ind + L-NOARG, $^*P<0.05$).

induced changes in either the $[Ca^{2+}]_i$ or in tension (Figure 3b). Pretreatment with both 10 μM Ind and 100 μM L-NOARG completely abolished the SP-induced relaxation, the first rapid phase and the following sustained phase, as shown in Figure 3c. Figure 3d shows a summary of these measurements. The changes in $[Ca^{2+}]_i$ and tension induced by SP were completely abolished in the case of pretreatment with Ind and L-NOARG.

Effect of SP on $[Ca^{2+}]_i$-tension relationships

Figures 4a and 4b show representative recordings of the measurement to determine the control $[Ca^{2+}]_i$-tension relation of the contraction in the presence of 100 nM U46619 or during the depolarization with 118 mM K^+, respectively. Figure 4c and 4d show the summary of the changes in $[Ca^{2+}]_i$ and tension, respectively, obtained from the measurements shown in Figures 4a and 4b. The control $[Ca^{2+}]_i$-tension relations, thus obtained, are shown in Figure 4e. The $[Ca^{2+}]_i$-tension relation of the steady state (20 min after the application of SP) of SP-induced relaxation are also shown in Figure 4e. Compared with the control curves, the $[Ca^{2+}]_i$-tension relationships during relaxation by SP shifted to the right, both with 100 nM U46619-contractions and 118 mM K^+-contractions ($P<0.05$). When strips were pretreated with L-NOARG, the $[Ca^{2+}]_i$-tension relationships during SP-induced relaxation reverted to overlap with the control curves, while, on the other hand, Ind did not have such effect.

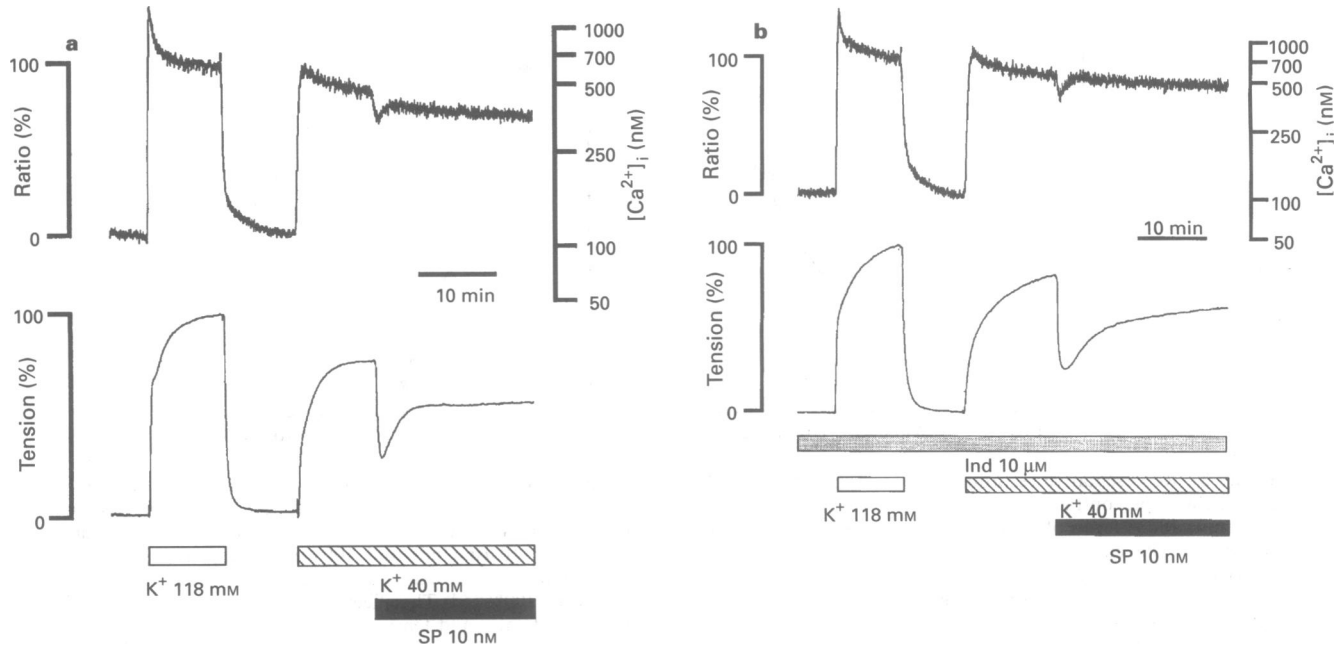
Effect of SP on $[Ca^{2+}]_i$ of endothelial cells in situ

We investigated the effect of SP on $[Ca^{2+}]_i$ of endothelial cells *in situ*, in aortic valvular strips with an intact endothelium. Figure 5a shows a representative recording of changes in $[Ca^{2+}]_i$ of endothelial cells induced by 10 μM ATP (100% response) and then 10 nM SP in normal PSS. SP induced a rapid increase ($97.3 \pm 12.0\%$, $n=4$) of $[Ca^{2+}]_i$ in endothelial cells followed by small sustained phase ($12.8 \pm 3.7\%$, $n=4$). There were no differences in the rapid and the sustained phases of SP-induced $[Ca^{2+}]_i$ elevations between the endothelial cells in normal PSS (5.9 mM K^+) and those in high-K^+ PSS (40 mM K^+) (Figure 5b).

Discussion

In the present study, we examined separately the effects of PGI_2, EDRF, and a possible hyperpolarizing factor (EDHF) on the endothelium-dependent relaxation and found that SP induces an endothelium-dependent biphasic vasorelaxation in the porcine coronary artery; the first phase was mediated by L-NOARG-sensitive relaxing factor (EDRF) and L-NOARG-resistant relaxing factor, while the sustained phase was mediated solely by L-NOARG-sensitive relaxing factor. Some agents which induce endothelium-dependent vasorelaxation are known to induce simultaneously endothelium-dependent membrane hyperpolarization of smooth muscle cells, and there have also been reports discussing whether or not SP induces endothelium-dependent membrane hyperpolarization of smooth muscle cells. SP is known to induce hyperpolarization and relaxation of vascular smooth muscles, including the porcine coronary artery (Beny *et al.*, 1986; Pacicca *et al.*, 1992; Zhang *et al.*, 1994), although in certain types of tissue, SP relaxes the arteries in the absence of any changes in the membrane potential (Bolton & Clapp, 1986). Chen & Suzuki (1989) reported that hyperpolarization is generated by an increase in K^+ conductance of the membrane. In addition, it was also reported that the endothelium-dependent hyperpolarization can be inhibited by an increase of extracellular K^+ concentration (Nagao & Vanhoutte, 1992; Suzuki *et al.*, 1992; Zhang *et al.*, 1994), and they thus suggested the involvement of K^+ channels in the generation of endothelium-dependent hyperpolarization of smooth muscle cells of the porcine coronary artery. In the present work, we used Ind, L-NOARG and high-K^+-depolarizing solution in order to separate the effects of PGI_2, EDRF and hyperpolarizing factor (EDHF), respectively.

A small contribution of PGI_2 was suggested by the finding that Ind had no effect on the SP-induced relaxation (Figure 2b). Pretreatment with L-NOARG partially, but significantly inhibited the first phase only and completely inhibited the sustained phase of the SP-induced relaxation, during the U46619-induced contraction. On the other hand, during high K^+-induced contraction, L-NOARG completely inhibited both the first rapid phase and the sustained phase of SP-induced relaxation. Thus, the transient and rapid vasorelaxation partly depends on the L-NOARG-resistant relaxing factor. This transient time course was similar to that of the membrane



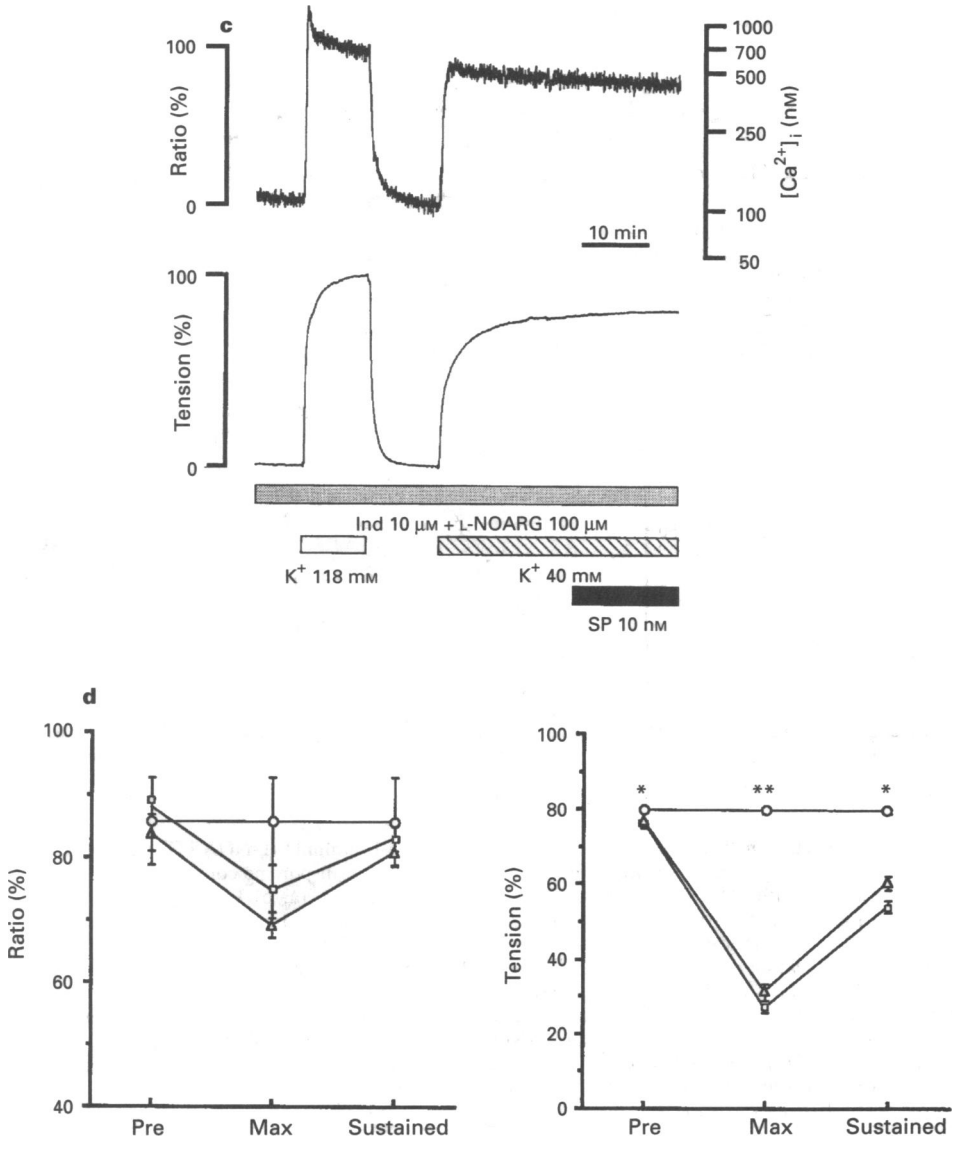


Figure 3 Representative recordings showing the effect of substance P (SP) on $[Ca^{2+}]_i$ and tension in the coronary arterial strips with endothelium precontracted with 40 mM K^+ PSS. (a) The effect of SP: after determination of the 100% levels of ratio and tension by 118 mM K^+ depolarization, the strip was precontracted with 40 m K^+ PSS. SP (10 nM) was applied at the plateau phase. (b) The effect of pretreatment with 10 μM indomethacin (Ind) and (c) 10 μM Ind and 100 μM N^G-nitro-L-arginine (L-NOARG) on the vasorelaxation induced by 10 nM SP. (d) Effect of Ind and L-NOARG on the rapid phase and the sustained phase of SP-induced relaxation; Pre: the levels of ratio and tension induced by 40 mM K^+ PSS just prior to the application of SP. Max: the maximal response (first rapid phase) of SP-induced relaxation. Sustained: the sustained phase of SP-induced relaxation: (□) 40 mM K^+ PSS only. (△) 40 mM K^+ PSS + Ind, (○) 40 mM K^+ PSS + Ind + L-NOARG, $*P<0.05$, $**P<0.001$).

hyperpolarization observed by Nagao & Vanhoutte (1992). We therefore suggest that the first phase of the SP-induced relaxation is mediated by both L-NOARG-sensitive (EDRF) and L-NOARG-resitant relaxing factors while the sustained phase is mainly mediated by L-NOARG-sensitive relaxing factor (EDRF).

In the present study, the $[Ca^{2+}]_i$ and tension relationships of SP-induced relaxation shifted to the right of the control curves obtained with either U46619-induced contractions or high-K^+-induced contractions, and this effect was inhibited by pretreatment with L-NOARG. These findings thus suggest that EDRF decreases the Ca^{2+} sensitivity of the contractile apparatus of vascular smooth muscle. It is known that EDRF increases cyclic GMP in vascular smooth muscle cells, and we reported that nitroglycerin, which also increases cyclic GMP, can decrease the Ca^{2+} sensitivity of the contractile apparatus (Abe *et al.*, 1990).

In endothelial cells of aortic valvular strips, the extent of SP-induced $[Ca^{2+}]_i$ elevation in normal PSS was similar to that in high-K^+ PSS, and the latter medium is known to inhibit membrane hyperpolarization. We suggest that SP increases $[Ca^{2+}]_i$ in endothelial cells, which may thus play a role in producing L-NOARG-sensitive and/or L-NOARG-resistant relaxation.

In the present study, we demonstrated that in the presence of both Ind and L-NOARG, SP can still induce a transient endothelium-dependent vasorelaxation of the strips precontracted with U46619 (Figure 2c). Thus, it is suggested that a part of the endothelium-dependent relaxation induced by SP is probably due to neither PGI_2 nor EDRF. This Ind- and L-NOARG-resistant vasorelaxation was abolished in high K^+-solution (Figure 3c). In the electrophysiological measurements, Zhang *et al.* (1994) demonstrated that SP-induced hyperpolarization is completely inhibited in high K^+-solution. Taken together, these results suggest that membrane hyperpolarization may play an important role in the Ind- and L-NOARG-resistant endothelium-dependent vasorelaxation induced by SP. Since to date the EDHF is the only known endothelium-

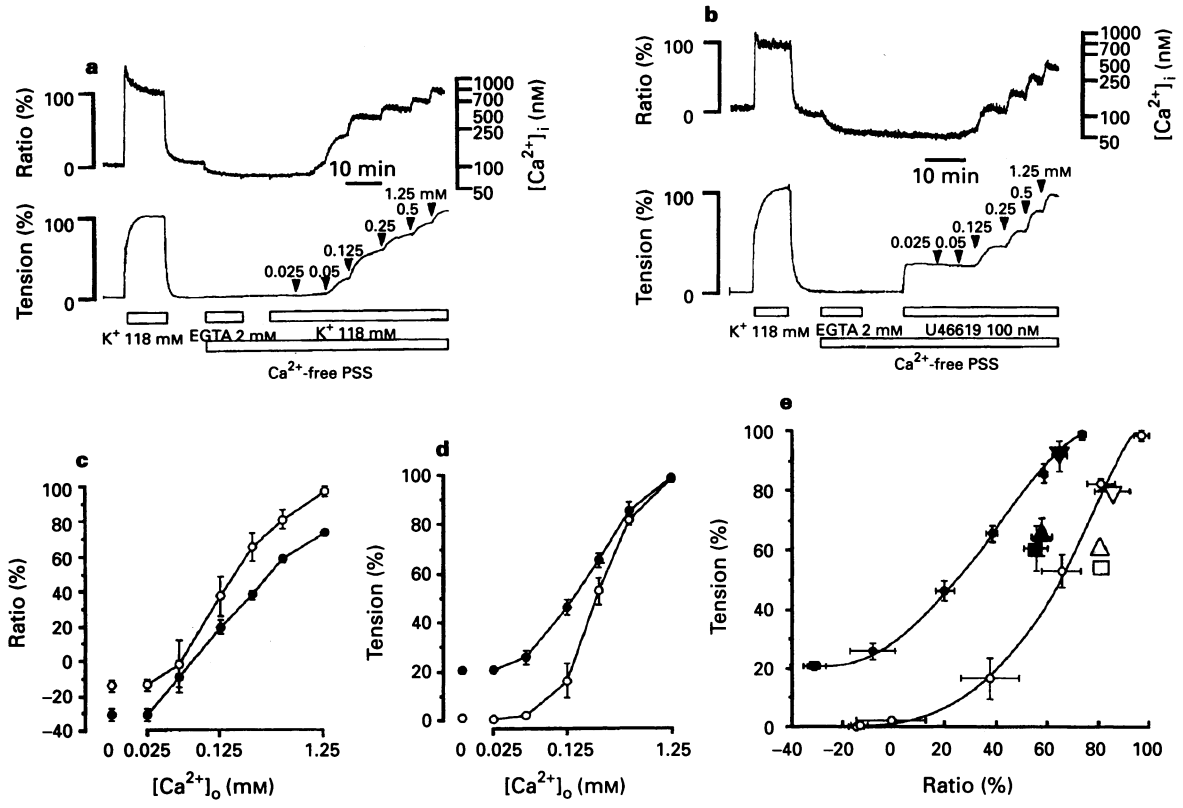


Figure 4 The effect of indomethacin (Ind) treatment or Ind and N^G-nitro-L-arginine (L-NOARG) treatment on $[Ca^{2+}]_i$-tension relationships obtained at sustained phases of substance P (SP)-induced relaxation in porcine coronary arterial strips, with an intact endothelium and precontracted with 100 nM U46619 or 40 mM K^+ depolarization. (a, b) Representative recordings showing the effects of the cumulative application of extracellular Ca^{2+} (0 mM – 1.25 mM) on $[Ca^{2+}]_i$ and tension in coronary arterial strips in the presence of 100 nM U46619 (a) or 118 mM K^+ depolarization (b) in Ca^{2+}-free PSS. (c, d) Changes in $[Ca^{2+}]_i$ (c) and tension (d) induced by the cumulative application of extracellular Ca^{2+} (0 mM – 1.25 mM) in the presence of 100 nM U46619 (●) or 118 mM K^+ deplolarization (○) in Ca^{2+}-free PSS ($n=4$). (e); $[Ca^{2+}]_i$-tension relationships obtained at sustained phases of SP-induced relaxation in porcine coronary arterial strips, with an intact endothelium and precontracted with 100 nM U46619 or 40 mM K^+ depolarization: (■) U46619 only; (▲) U46619 + Ind; (▼) U46619 + Ind + L-NOARG (□) 40 mM K^+ PSS only, (△) 40 mM K^+ PSS + Ind; (▽) 40 mM K^+ PSS + Ind + -L-NOARG). The control $[Ca^{2+}]_i$-tension curves (without SP) were obtained at plateau levels of contractions induced by the cumulative applications of extracellular Ca^{2+} (0 mM – 1.25 mM) in the presence of 100 nM U46619 (●) or 118 mM K^+ depolarization (○) in Ca^{2+}-free PSS as shown in (c) and (d).

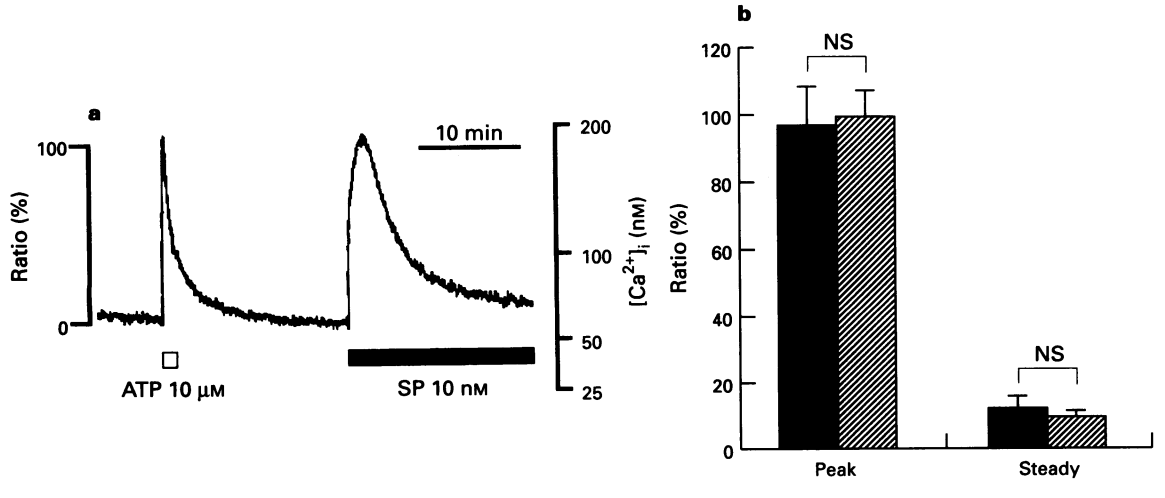


Figure 5 The effect of substance P (SP) on $[Ca^{2+}]_i$ of endothelial cells *in situ*. (a) The representative recording of changes in $[Ca^{2+}]_i$ induced by the successive application of 10 μM ATP and 10 nM SP in endothelial cells in normal (5.9 mM K^+) PSS. SP induced a rapid rise (peak) and sustained-type $[Ca^{2+}]_i$ elevation, (b) effect of SP on $[Ca^{2+}]_i$ in endothelial cells *in situ* in normal PSS and in 40 mM K^+ PSS. The $[Ca^{2+}]_i$ levels at the peak and sustained phase are compared. The fluorescence ratios of the resting level and the peak response induced by 10 μM ATP, recorded prior to each measurement, were designated 0 and 100%, respectively. Means with s.e.mean are shown: solid column, 5.9K^+ + SP; hatched columns, 40K^+ + SP.

derived factor that hyperpolarizes the vascular smooth muscles in the presence of Ind plus L-NOARG, it thus seems plausible to postulate the involvement of EDHF. However, the precise mechanism for the Ind- and L-NOARG-resistant vasorelaxation induced by SP could not be determined in the present study. Since the involvement of the K^+ channels has already been proposed in the endothelium-dependent hyperpolarization (Standen *et al.*, 1989), we have investigated the effects of K^+ channel blockers on the L-NOARG-resistant relaxation. However, charybdotoxin (50 nM, $n=3$ strips), iberiotoxin (1 μM, $n=3$ strips), and (d)-tubocurarine (1 mM, $n=5$ strips) had no effect (data not shown). In regard to the effect of K^+ channel blockers on the endothelium-dependent and L-NOARG-resistant hyperpolarization, controversial results have also been obtained in the pig coronary artery (Von der Weid & Bény, 1992).

In conclusion, endothelium-dependent relaxation induced by SP in the pig coronary artery appears to be mediated by L-NOARG-sensitive (EDRF) and L-NOARG-resistant relaxing factors; the initial rapid phase is mediated by both EDRF and L-NOARG-resistant relaxing factors while the sustained phase is solely mediated by EDRF. This sustained relaxation is accompanied by a decrease in Ca^{2+}-sensitivity of the contractile apparatus. The underlying mechanisms of L-NOARG-resistant relaxation have yet to be clarified but EDHF appears to be a potentially contributory factor.

We thank M. Ohara and B. Quinn for helpful comments. This study was supported in part by Grants-in-Aid for Developmental Scientific Research (No. 03557043) and for General Scientific Research (No. 04454268, 05837015, 05837016) from the Ministry of Education, Science and Culture, Japan, and Grants from the Uehara Memorial Foundation, from Yokoyama Rinshouyakuri, from the Japan Heart Foundation, from the Ichiro Kanehara Foundation, from the Kaibara Morikazu Medical Science Promotion Foundation, from the Mochida Memorial Foundation, and from The Tokyo Biochemical Research Foundation.

References

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

AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1994). Sensitivity of G-protein involved in endothelin-1-induced Ca^{2+} influx to pertussis toxin in porcine endothelial cells *in situ*. *Br. J. Pharmacol.*, **111**, 989–996.

AOKI, H., KOBAYASHI, S., NISHIMURA, J., YAMAMOTO, H. & KANAIDE, H. (1991). Endothelin induces the Ca^{2+}-transient in endothelial cells *in situ*. *Biochem. Biophys. Res. Commun.*, **181**, 1352–1357.

BENY, J.-L., BRUNET, P.C. & HYGGEL, H. (1986). Effect of mechanical stimulation, substance P and vasoactive intestinal polypeptide on the electrical and mechanical activities of circular smooth muscles from pig coronary arteries contracted with acetylcholine: role of endothelium. *Pharmacology*, **33**, 61–68.

BOLTON, T.B. & CLAPP, L.H. (1986). Endothelial-dependent relaxant actions of carbachol and substance P in arterial smooth muscle. *Br. J. Pharmacol.*, **87**, 713–723.

CHEN, G. & SUZUKI, H. (1989). Some electrical properties of the endothelium-dependent hyperpolarization recorded from rat arterial smooth muscle cells. *J. Physiol.*, **410**, 91–106.

CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarizing factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.

DI VIRGILIO, F., STEINBERG, T.H. & SILVERSTEIN, S.C. (1989). Organic-anion transport inhibitors to facilitate measurement of cytosolic free Ca^{2+} with fura-2. In *Methods in Cell Biology*. ed. Tartakoff, A.M. pp. 453–462. San Diego: Academic Press.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation on arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

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

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

KUROIWA, M., AOKI, H., KOBAYASHI, S., NISHIMURA, J. & KANAIDE, H. (1993). Role of GTP-protein and endothelium in contraction induced by ethanol in pig coronary artery. *J. Physiol.*, **470**, 521–537.

MONCADA, S., GRYGLEWSKI, R., BUNTING, S. & VANEM, J.R. (1976). An enzyme isolated from arteries transform prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263**, 663–665.

NAGAO, H. & VANHOUTTE, P.M. (1992). Hyperpolarization as a mechanism for endothelium-dependent relaxations in the porcine coronary artery. *J. Physiol.*, **445**, 355–367.

NETTLAND, P.A., ZETTER, B.R., VIA, D.P. & VOYTA, J.C. (1985). In situ labelling of vascular endothelium with fluorescent acetylated low density lipoprotein. *Histochem. J.*, **17**, 1309–1320.

PACICCA, C., VON DER WEID, P.-Y. & BÉNY, J.-L. (1992). Effect of nitro-L-arginine on endothelium-dependent hyperpolarizations and relaxations of pig coronary arteries. *J. Physiol.*, **457**, 247–256.

SATO, K., OZAKI, H. & KARAKI, H. (1990). Differential effects of calbacol on cytosolic calcium levels in vascular endothelium and smooth muscle. *J. Pharmacol. Exp. Ther.*, **255**, 114–119.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

SUZUKI, H., CHEN, G. & YAMAMOTO, Y. (1992). Endothelium-derived hyperpolarizing factor (EDHF). *Jpn. Circ. J.*, **56**, 170–174.

VANE, J.R., ANGGARD, E.F. & BOTTING, R.M. (1990). Regulatory functions of the vascular endothelium. *New Engl. J. Med.*, **327**, 27–36.

VON DER WEID, P.-Y. & BÉNY, J.-L. (1992). Effect of Ca^{2+} ionophores on membrane potential of pig coronary artery endothelial cells. *Am. J. Physiol.*, **262**, H1823–H1831.

ZHANG, G., YAMAMOTO, Y., MIWA, K. & SUZUKI, H. (1994). Vasodilation induced by substance P in guinea pig carotid arteries. *Am. J. Physiol.*, **266**, H1132–H1137.

(Received April 3, 1995
Accepted June 14, 1995)