British Journal of Pharmacology (2000) 131, 115–123 © 2000 Macmillan Publishers Ltd All rights reserved 0007–1188/00 $15.00
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Mechanisms of the thapsigargin-induced Ca^{2+} entry in *in situ* endothelial cells of the porcine aortic valve and the endothelium-dependent relaxation in the porcine coronary artery

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1 The mechanisms of the thapsigargin (TG)-induced capacitative Ca^{2+} entry in *in situ* endothelial cells and its role in the regulation of arterial tone were investigated using front-surface fluorimetry and fura-2-loaded strips of porcine aortic valve and coronary artery.

2 In the presence of extracellular Ca^{2+}, TG induced an initial rapid and a subsequent sustained elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in valvular strips. In the absence of extracellular Ca^{2+}, TG induced only a transient increase in $[Ca^{2+}]_i$.

3 The TG-induced sustained elevation of $[Ca^{2+}]_i$ in endothelial cells was inhibited completely by 1 mM Ni^{2+} and partly by 10 μM econazole and 30 μM ML-9, but not by 900 ng ml^{-1} pertussis toxin or 100 μM wortmannin. Therefore, cytochrome P450 and protein phosphorylation are suggested to be involved in the TG-induced Ca^{2+} influx in *in situ* endothelial cells.

4 TG induced an endothelium-dependent large relaxation consisting of an initial and a late sustained relaxation in coronary arterial strip precontracted with U46619 (a thromboxane A2 analogue). Indomethacin alone had no effect, while indomethacin plus N^{ω}-nitro-L-arginine (L-NOARG) markedly inhibited the sustained phase and slightly inhibited the initial phase of the TG-induced relaxation.

5 TG induced a smaller but sustained relaxation during the 40 mM K^+-induced precontraction than that seen during the U46619-induced precontraction. This relaxation was completely abolished by the pretreatment with indomethacin plus L-NOARG.

6 In conclusion, both nitric oxide (NO) and endothelium-derived hyperpolarizing factor were suggested to mediate the TG-induced relaxation, while NO plays a major role in the sustained relaxation. The TG-induced sustained $[Ca^{2+}]_i$ elevation in endothelial cells was thus suggested to be mainly linked to the sustained production of NO.
British Journal of Pharmacology (2000) **131,** 115–123

Keywords: Endothelial cells; capacitative Ca^{2+} entry; thapsigargin; endothelium-dependent relaxation

Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CPA, cyclopiazonic acid; DMEM, Dulbecco's modified Eagle medium; EDHF, endothelium-derived hyperpolarizing factor; fura-2/AM, fura-2 acetoxymethyl ester; IAP, islet activating protein, pertussis toxin; L-NOARG, N^{ω}-nitro-L-arginine; MLCK, myosin light chain kinase; NO, nitric oxide; PSS, physiological salt solution; TG, thapsigargin

Introduction

Endothelial cells play an important role in the regulation of vascular tone by producing both vasorelaxing and vasocontracting substances (Furchgott & Vanhoutte, 1989; Katusic & Shepherd, 1991). Endothelium-dependent relaxation has been widely reported in different species and various types of blood vessels. Endothelium-derived relaxing factors include nitric oxide (NO) (Palmer *et al*., 1987), endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al*., 1988; Komori & Vanhoutte, 1990) and prostacyclin. The Ca^{2+} signal in endothelial cells plays a primary role in the production and release of these relaxing factors, and thereby the regulation of vascular tone (Newby & Henderson, 1990). One of the important mechanisms to induce a $[Ca^{2+}]_i$ elevation is the Ca^{2+} influx from the extracellular space. In non-excitable cells such as endothelial cells, where the voltage-operated Ca^{2+} channels are not expressed, one of the dominant Ca^{2+} influx pathways is the capacitative Ca^{2+} entry pathways (Putney & McKay, 1999). However, the functional role of the capacitative Ca^{2+} entry of endothelial cells in the regulation of endothelium-dependent relaxation remains to be elucidated.

Thapsigargin (TG) is an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (Thastrup *et al*., 1990). The inhibition of Ca^{2+}-ATPase by TG is considered to deplete the intracellular Ca^{2+} store and activate the capacitative Ca^{2+} entry (Thastrup *et al*., 1990). In fact, TG and cyclopiazonic acid (CPA), another inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase (Seidler *et al*., 1989), have been shown to induce a sustained elevation of $[Ca^{2+}]_i$ in endothelial cells (Higuchi *et al*., 1996; Kawasaki *et al*., 1999). Since TG induces a large sustained Ca^{2+} influx in endothelial cells (Kawasaki *et al*., 1999), it could serve as a useful tool to investigate the functional role of the capacitative Ca^{2+} entry in the endothelium-dependent regulation of vascular tone.

Since the capacitative Ca^{2+} influx serves as a major Ca^{2+} entry pathway in endothelial cells, it is important to elucidate the mechanism of regulation for the capacitative Ca^{2+} influx. However, the coupling of the depletion of the intracellular

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stores and the activation of the Ca^{2+} entry has yet to be clarified. So far, two mechanisms have been proposed for such coupling; direct coupling and indirect coupling mediated by second messengers (Boulay *et al*., 1999; Holda & Blatter, 1997; Putney, 1999; Putney & McKay, 1999). The candidates for second messengers include tyrosine phosphorylation (Kruse *et al*., 1994; Pfeiffer *et al*., 1995; Sargeant *et al*., 1994), trimeric and small GTP-binding proteins (Bird & Putney, 1993; Sargeant *et al*., 1993), cytochrome P450 (Alvarez *et al*., 1992; Graier *et al*., 1995) and myosin light chain kinase (MLCK) (Watanabe *et al*., 1996). However, the precise intracellular signalling pathway of the capacitative Ca^{2+} entry in *in situ* endothelial cells has not yet been established.

In the present study, we investigated the mechanism of the TG-induced capacitative Ca^{2+} entry in *in situ* endothelial cells using front-surface fluorimetry of fura-2-loaded strips of the porcine aortic valves. We also examined the functional role of the TG-induced Ca^{2+} entry in the regulation of vascular tone by simultaneously monitoring the effect of TG on smooth muscle $[Ca^{2+}]_i$ and tension in fura-2-loaded strips of the porcine coronary artery. In addition, we determined the relative contribution of NO and EDHF to the TG-induced relaxation, and evaluated the correlation between the TG-induced capacitative Ca^{2+} entry and the production of the endothelium-derived relaxing factors.

Methods

Tissue preparation

In order to monitor changes in $[Ca^{2+}]_i$ of *in situ* endothelial cells, strips were prepared from the porcine aortic valve as previously described (Kuroiwa *et al*., 1993). Porcine aortic valves were obtained at a local slaughterhouse and brought to the laboratory in ice-cold normal physiological salt solution (PSS). The aortic valve leaflets were handled with extreme care in order to avoid damaging the endothelial layer. The valve leaflets were cut into strips in an axial direction (approximately 2 mm wide, 5 mm long, and 0.18 mm thick).

Arterial strips with and without endothelium were prepared from the left circumflex branch of porcine coronary arteries as previously described (Kuroiwa *et al*., 1993). Porcine hearts were obtained immediately after the animals had been slaughtered, and brought to the laboratory in ice-cold PSS. A segment of the left circumflex artery 2–3 cm from the origin was excised, and the adventitia of the segment was mechanically removed. The segment was opened longitudinally and cut into circular strips (approximately 1 mm wide, 5 mm long, and 0.1 mm thick). To remove the endothelium, the inner surface was rubbed off with a cotton swab.

Fura-2 loading

The valvular strips were loaded with Ca^{2+} indicator dye, fura-2, by incubating them in oxygenated (a mixture of 95% O_2 and 5% CO_2) Dulbecco's modified Eagle medium (DMEM) containing 50 μM fura-2/AM (an acetoxymethyl ester form of fura-2), 1 mM probenecid and 5% foetal bovine serum for 1.5 h at 37°C (Kanaide, 1999). Probenecid was added to prevent sequestration of fura-2 (Di Virgilio *et al*., 1989). Coronary arterial strips with and without endothelium were loaded with fura-2 in oxygenated DMEM containing 25 μM fura-2/AM and 5% foetal bovine serum for 4 h at 37°C (Kanaide, 1999). After loading with fura-2, both valvular and arterial strips were washed in normal PSS to remove the dye remaining in the extracellular space, and equilibrated in normal PSS for at least 1 h before starting the measurements. Loading the vascular strips with fura-2, *per se*, did not affect the contractility of the arterial strips (Hirano *et al*., 1990).

Front-surface fluorimetry

The changes in $[Ca^{2+}]_i$ of *in situ* endothelial cells were monitored in the fura-2-loaded valvular strips as described previously (Aoki *et al*., 1991; Kuroiwa *et al*., 1995). The measurements were carried out at 25°C to prevent any sequestration of the fluorescence dye, because *in situ* endothelial cells actively sequestrated fura-2 at 37°C (Kuroiwa *et al*., 1993; 1995). In brief, the valvular strips were mounted vertically in the quartz organ bath and changes in fluorescence intensity of the fura-2-Ca^{2+} complex in endothelial cells were monitored with a front-surface fluorimeter specifically designed for fura-2 fluorimetry (CAM-OF2; Japan Spectroscopic Co., Tokyo, Japan). The ratio of the fluorescence intensity (500 nm) at 340 nm excitation to that at 380 nm excitation was monitored as an indicator of $[Ca^{2+}]_i$. The response to 10 μM ATP was recorded as a reference response at the beginning of each experimental protocol. The changes in $[Ca^{2+}]_i$ of *in situ* endothelial cells were expressed in per cent, assigning the level of $[Ca^{2+}]_i$ at rest and that at the peak response to 10 μM ATP to be 0 and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was estimated in the same manner as previously described (Grynkiewicz *et al*., 1985) with an apparent K_d value of 162 nM at 25°C (Aoki *et al*., 1991). The $[Ca^{2+}]_i$ levels of endothelial cells *in situ* at rest (0%) and at the peak level (100%) obtained with 10 μM ATP were determined in separate experiments and found to be 63.9 ± 7.4 and 176.7 ± 16.5 nM, respectively ($n = 5$).

Front-surface fluorimetry of vascular strips was carried out at 37°C, since fura-2 fluorescence was stable in smooth muscle cells for more than 1 h at 37°C (Kanaide, 1999; Kuroiwa *et al*., 1993). The response to 118 mM K^+-depolarization was recorded as a reference response before starting each experimental protocol. Changes in the fluorescence ratio in the smooth muscle cells were expressed in per cent, assigning the value at rest (5.9 mM K^+ PSS) and that obtained in 118 mM K^+ PSS to be 0 and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ of vascular strips was estimated with the K_d of 224 nM at 37°C (Grynkiewicz *et al*., 1985). The $[Ca^{2+}]_i$ levels corresponding to 0 and 100 % fluorescence ratios in smooth muscle were determined in separate experiments and found to be 108 ± 27 and 715 ± 103 nM, respectively (Hirano *et al*., 1990; Kanaide, 1999). We previously demonstrated that the fura-2 signal of the arterial strips arose exclusively from smooth muscle cells even in the presence of an intact endothelium, because endothelial cells of arterial strips excluded fura-2 under the present experimental conditions (37°C and without probenecid) (Kuroiwa *et al*.; 1993; 1995).

Measurement of tension development in the coronary arterial strips

Using a force-transducer TB-612T (Nihon Koden, Tokyo, Japan), the tension development of the fura-2-loaded arterial strip was simultaneously monitored during the fura-2 fluorimetry. During the equilibration period, the strips were stimulated with 118 mM K^+ every 15 min, and the resting load was increased stepwise and then finally adjusted to 250 mg. The developed tension was expressed in per cent, assigning the value at rest (5.9 mM K^+) and that at a steady state of contraction induced by 118 mM K^+ to be 0 and 100%, respectively.

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 aerated with a mixture of 95% O_2 and 5% CO_2, with the resulting pH being 7.4. The Ca^{2+}-free PSS contained no $CaCl_2$ but 2 mM ethylenglycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA). Fura-2/AM and EGTA was purchased from Dojindo (Kumamoto, Japan). Indomethacin was purchased from Wako (Osaka, Japan). N^{ω}-nitro-L-arginine (L-NOARG) was from the Aldrich Chemical Company, Inc. (Milwaukee, WI, U.S.A.). ATP was purchased from Boehringer Mannheim (Germany). Thapsigargin, probenecid, wortmannin and econazole were purchased from Sigma (St. Louis, MO, U.S.A.). Foetal bovine serum was purchased from GIBCO (Rockville, MD, U.S.A.). U46619 (a thromboxane A_2 analogue 9, 11-Dideoxy-11α, 9α-epoxymethano-prostaglandin F2α) was purchased from Funakoshi (Tokyo, Japan). ML-9 (1-(5-Chloronaphthalene-1-sulphonyl)-1*H*-hexahydro-1,4-diazepine) and IAP (pertusis toxin) were purchased from Seikagaku Co. (Tokyo, Japan).

Data analysis

All data were expressed as the mean $\pm$ standard error of the mean (s.e.mean). Student's *t*-test was used to determine statistical significance. *P* values of less than 0.05 were considered to be significant. All data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia, Macintosh; Apple Computer, U.S.A.).

Results

Effects of TG on $[Ca^{2+}]_i$ in endothelial cells of the porcine aortic valve

In the presence of extracellular Ca^{2+}, TG induced a large sustained increase in $[Ca^{2+}]_i$ in the endothelial cells of aortic valvular strips (Figure 1). As shown in Figure 1a, 1 μM TG induced a rapid increase in $[Ca^{2+}]_i$, which reached a peak (the first component) at 8.4 ± 0.2 min ($n=6$), followed by a sustained increase at a slightly lower level (the second component). The levels of $[Ca^{2+}]_i$ elevation observed at the peak and at 30 min (the second component) were 510.4 ± 31.3 and 389.7 ± 29.1% ($n=6$) of the peak response to 10 μM ATP, respectively. When TG was applied in a cumulative manner (0.001 through 10 μM), the $[Ca^{2+}]_i$ level increased in a concentration-dependent manner (Figure 1c). A significant elevation was observed at concentrations of higher than 0.01 μM TG and the maximum elevation of $[Ca^{2+}]_i$ was obtained at a concentration of 1 μM TG.

When the strips were exposed to the Ca^{2+}-free PSS containing 2 mM EGTA, $[Ca^{2+}]_i$ level gradually decreased reaching a new steady level (-50.8 ± 4.6%, $n=3$) at 10 min (Figure 1b). The endothelial cells were then stimulated with 1 μM TG in the absence of extracellular Ca^{2+}, which caused only a transient $[Ca^{2+}]_i$ elevation with no sustained $[Ca^{2+}]_i$ elevation. The transient elevation of $[Ca^{2+}]_i$ in the Ca^{2+}-free PSS reached its peak slightly earlier (5.1 ± 0.1 min, $n=3$) than that seen in the normal PSS. The peak level obtained with 1 μM TG in the Ca^{2+}-free PSS was 47.3 ± 8.0% ($n=3$) and much lower than that observed in the presence of extracellular Ca^{2+}.

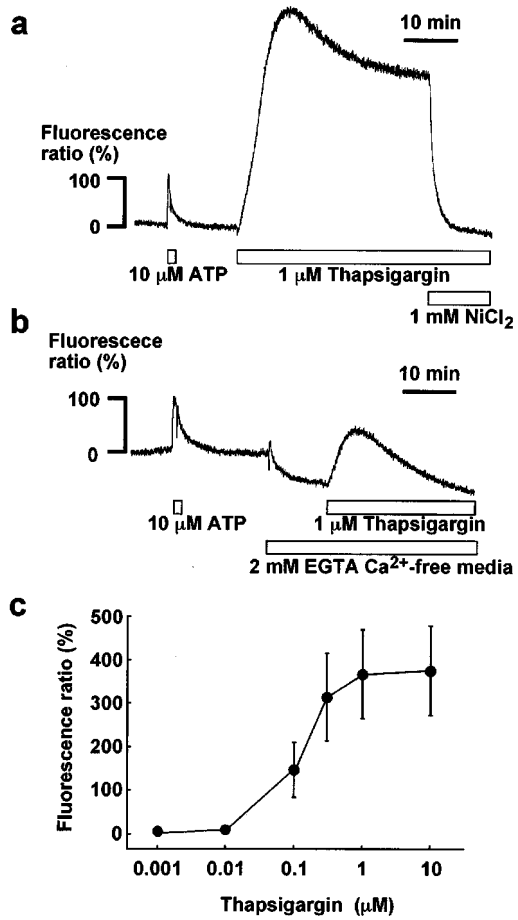

Figure 1 Effect of thapsigargin on $[Ca^{2+}]_i$ in *in situ* endothelial cells of the porcine aortic valve. (a,b) Representative recordings of changes in $[Ca^{2+}]_i$ induced by 10 μM ATP and 1 μM thapsigargin (TG) in endothelial cells in the presence (a) and absence (b) of extracellular Ca^{2+}. In (a), 1 mM $NiCl_2$ was applied during a sustained phase of the TG-induced elevation of $[Ca^{2+}]_i$. In (b), the valvular strip was exposed to the 2 mM EGTA-containing Ca^{2+}-free media for 10 min before and during the application of 1 μM TG. The level of $[Ca^{2+}]_i$ elevation induced by 10 μM ATP was assigned to be 100%. (c) Concentration-dependent effect of TG on $[Ca^{2+}]_i$ in endothelial cells *in situ*. TG was applied in a cumulative manner. Data are the mean $\pm$ s.e.mean ($n=5$).

After $[Ca^{2+}]_i$ returned to the pre-stimulation level, EGTA was removed and the extracellular Ca^{2+} was replenished to a concentration of 1.25 mM, which restored the sustained increase in $[Ca^{2+}]_i$ (data not shown). As a result, the second component of the TG-induced $[Ca^{2+}]_i$ elevation observed in the presence of extracellular Ca^{2+} was dependent on the extracellular Ca^{2+}.

Mechanism of the TG-induced Ca^{2+} influx in endothelial cells of the porcine aortic valve

To elucidate the mechanisms of the TG-induced Ca^{2+} influx in *in situ* endothelial cells, effects of possible inhibitors of Ca^{2+} signalling pathway on the sustained elevation of $[Ca^{2+}]_i$ were investigated. The sustained elevation of $[Ca^{2+}]_i$ was completely inhibited by 1 mM $NiCl_2$ (Figure 1a), while 10 μM diltiazem, an L-type Ca^{2+} channel blocker had no effect (data not shown).

Figure 2 summarizes the effects of other inhibitors on the TG-induced sustained increase in $[Ca^{2+}]_i$. First, the effects of econazole, an inhibitor of cytochrome P450 mono-oxygenase, was examined (Figure 2a). The endothelial cells were pretreated with 10 μM econazole 5 min before and during the application of

TG. This treatment inhibited the elevation of $[Ca^{2+}]_i$ induced by the subsequent application of TG (Figure 2a, pre). Econazole significantly decreased the level of $[Ca^{2+}]_i$ both in the first and second component induced by TG (first component: control, 510.4±31.3% vs econazole, 246.1±14.5%, $n=5$; second component (30 min): control, 389.7±29.1% vs econazole, 183.2±13.0%, $n=5$). When 10 μM econazole were applied after the TG-induced $[Ca^{2+}]_i$ elevation reached the sustained phase, the $[Ca^{2+}]_i$ level gradually decreased to 175.0±14.4% ($n=5$) at 15 min after the application (Figure 2a, post). The effect of the post-treatment with econazole was thus comparable to that of pretreatment.

Next, the effects of the inhibitors of MLCK, ML-9 and wortmannin, were examined (Figure 2b). Pretreatment with ML-9 10 min before and during the application of TG inhibited the elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner. A significant inhibition was seen at 30 μM and higher concentrations (Figure 2b). However, even with 100 μM ML-9, a residual small sustained $[Ca^{2+}]_i$ elevation (122.3±13.6%, $n=5$) was observed. As in the case with econazole, the application of ML-9 during the sustained phase of the TG-induced $[Ca^{2+}]_i$ elevation decreased $[Ca^{2+}]_i$ to a similar level as that observed with pretreatment (data not shown). We also examined the effect of wortmannin, another MLCK inhibitor. In contrast to ML-9, pretreatment with wortmannin for 30 min prior to the application of TG had no significant effect on the TG-induced $[Ca^{2+}]_i$ elevation even at a concentration of 100 μM wortmannin (Figure 2b; control, 389.7±29.1% vs wortmannin, 304.0±90.5%, $n=5$).

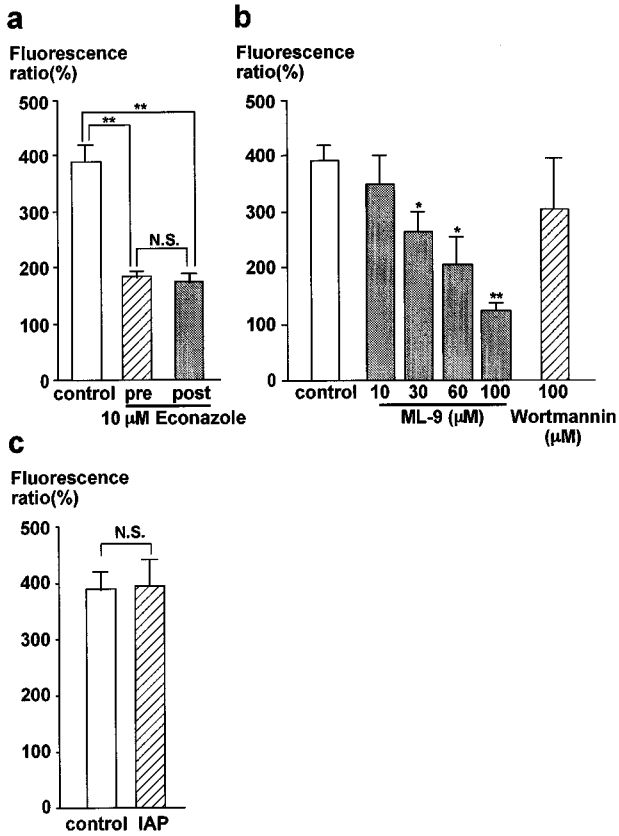

Figure 2 Characterization of the thapsigargin-induced Ca^{2+} entry in *in situ* endothelial cells of the porcine aortic valve. (a) Effect of econazole on the thapsigargin (TG)-induced sustained $[Ca^{2+}]_i$ increase, as evaluated by the $[Ca^{2+}]_i$ level obtained at 30 min after the application of 1 μM TG. Econazole (10 μM) was applied 5 min before the application of TG (pre) or 15 min after the application of TG (post). (b) Effects of pretreatment with myosin light chain kinase inhibitors, ML-9 and wortmannin on the $[Ca^{2+}]_i$ level obtained at 30 min after the application of TG. Inhibitors were applied 10 min before and during the stimulation with 1 μM TG. (c) Effect of pertussis toxin (IAP) on the TG-induced sustained increase in $[Ca^{2+}]_i$. The valvular strips were treated with 900 ng ml^{-1} IAP for 3 h. All data are the mean±s.e.mean ($n=5$). The level of the control $[Ca^{2+}]_i$ elevation induced by 10 μM ATP was considered to be 100%. $*P<0.05$, $**P<0.01$ compared with the control values. N.S., not significantly different.

In our previous study, we showed that the endothelin-1-induced Ca^{2+}-influx in endothelial cells of the porcine aortic valve was inhibited by treatment with 300 ng ml^{-1} IAP for 3 h (Aoki *et al.*, 1994). However, IAP had no significant effect on the TG-induced elevation of $[Ca^{2+}]_i$, even after 3 h treatment at 900 ng ml^{-1} (Figure 2c). The levels of the TG-induced elevation of $[Ca^{2+}]_i$ with and without treatment by IAP were 396.2±46.7% and 389.7±29.1% ($n=5$), respectively.

Effects of TG on smooth muscle $[Ca^{2+}]_i$ and tension of porcine coronary arterial strips

To determine the functional significance of the TG-induced $[Ca^{2+}]_i$ elevation in endothelial cells, the effects of TG on $[Ca^{2+}]_i$ and tension of smooth muscle were simultaneously determined in porcine coronary arterial strips with and without endothelium. In the strips without endothelium, TG gradually elevated $[Ca^{2+}]_i$ in smooth muscle, which reached a sustained phase within 10 min (Figure 3a). The level of $[Ca^{2+}]_i$ elevation induced by TG was 20.0±2.1% of that seen with 118 mM K^+ depolarization ($n=5$). However, this increase in $[Ca^{2+}]_i$ did not produce any tension development (Figure 3a). A thromboxane A_2 analogue, U46619, induced sustained increases in both $[Ca^{2+}]_i$ and tension of smooth muscle in the strips without endothelium (Figure 3b). The level of $[Ca^{2+}]_i$ and tension induced by 100 nM U46619 was 71.4±3.2 and 84.8±1.1% ($n=3$) at 10 min after the application, respectively. When applied during the sustained phase of this contraction, TG induced a slight increase, if any, in $[Ca^{2+}]_i$ and had no effect on the U46619-induced tension development (Figure 3b). As a result, TG had no direct effect on the tension of smooth muscle.

In the strips with an intact endothelium, U46619 also induced a rapid increase in $[Ca^{2+}]_i$ and tension of smooth muscle, which reached the sustained phase within 10 min (Figure 3c). The levels of $[Ca^{2+}]_i$ and tension were 71.5±1.5 and 83.5±5.2% ($n=4$) at 10 min, and 67.8±3.2 and 82.9±3.5% ($n=4$) at 25 min after the induction of contraction, respectively (Figure 5). When 1 μM TG was applied during the sustained contraction induced by U46619, both $[Ca^{2+}]_i$ and tension of smooth muscle decreased rapidly until reaching their lowest levels (the initial relaxation) at 4.8±0.4 min ($n=4$), followed by sustained decreases at a level slightly higher than the resting level (Figure 3c). The levels of $[Ca^{2+}]_i$ and tension decreased to 10.2±1.4 and 3.7±0.8% ($n=4$) at the initial relaxation, and 33.4±8.0 and 8.8±0.9% ($n=4$) at 15 min after the application of TG (25 min after the initiation of the precontraction), respectively (Figure 5).

When TG was applied in a cumulative manner (0.001 through 10 μM) during the sustained phase of U46619-induced contraction, both $[Ca^{2+}]_i$ and tension decreased in a concentration-dependent manner (data not shown). Significant decreases in both $[Ca^{2+}]_i$ and tension were observed with 0.1 μM TG. The maximum response was obtained with 1 μM TG. The concentrations of TG required to induce the endothelium-dependent relaxation was thus correlated with those required to induce the sustained $[Ca^{2+}]_i$ elevation in valvular strips (Figure 1c).

Mechanisms of the TG-induced endothelium-dependent relaxation

The relative contribution of prostacyclin, NO, and EDHF in the TG-induced relaxation was determined by either using specific inhibitors such as indomethacin and L-NOARG or by using high K^+-depolarization to inhibit hyperpolarization as shown in Figure 4. The results are summarized in Figure 5. Treatment with 10 μM indomethacin had no significant effect on $[Ca^{2+}]_i$ and tension of either the U46619-induced precontraction or the subsequent TG-induced relaxation (data not shown). On the contrary, the treatment with 10 μM indomethacin plus 100 μM L-NOARG significantly attenuated the decreases in $[Ca^{2+}]_i$ and tension, especially in the sustained phase of the TG-induced relaxation (Figure 4a). Under this condition, TG induced only a large transient decrease in $[Ca^{2+}]_i$ without a sustained decrease, while it induced an initial large relaxation followed by a small sustained relaxation (Figure 4a). Since treatment with indomethacin and L-NOARG augmented the 118 mM K^+- and U46619-induced contraction, the level of $[Ca^{2+}]_i$ and tension obtained at rest and by 118 mM K^+ in the presence of indomethacin and L-NOARG were considered to be 0 and 100%, respectively (Figure 5). Based on these criteria, the levels of $[Ca^{2+}]_i$ and tension obtained at 10 min after the initiation of the U46619-

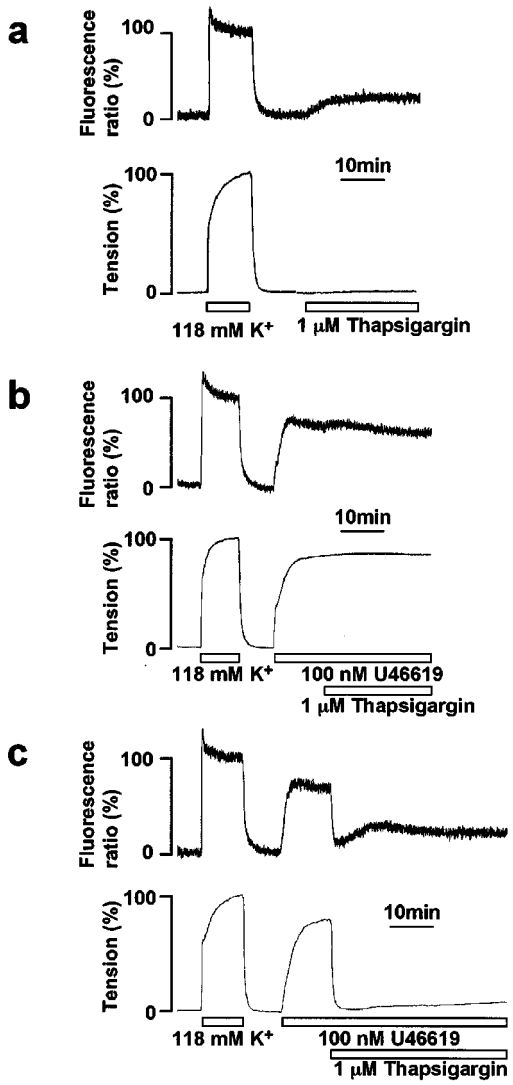

Figure 3 Effect of thapsigargin on smooth muscle $[Ca^{2+}]_i$ and tension in the porcine coronary arterial strips with and without endothelium. (a) A representative recording of the changes in $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) induced by 1 μM thapsigargin (TG) under resting conditions in the strips without endothelium. (b, c) Representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by 1 μM TG during contraction induced by 100 nM U46619 in coronary arterial strips without (b) and with (c) endothelium. TG was applied 10 min after the initiation of the precontraction. The responsiveness to 118 mM K^+-depolarization was recorded at the beginning of each measurement. The levels of $[Ca^{2+}]_i$ and tension seen in normal (5.9 mM K^+) PSS and 118 mM K^+-PSS were designated to be 0 and 100%, respectively.

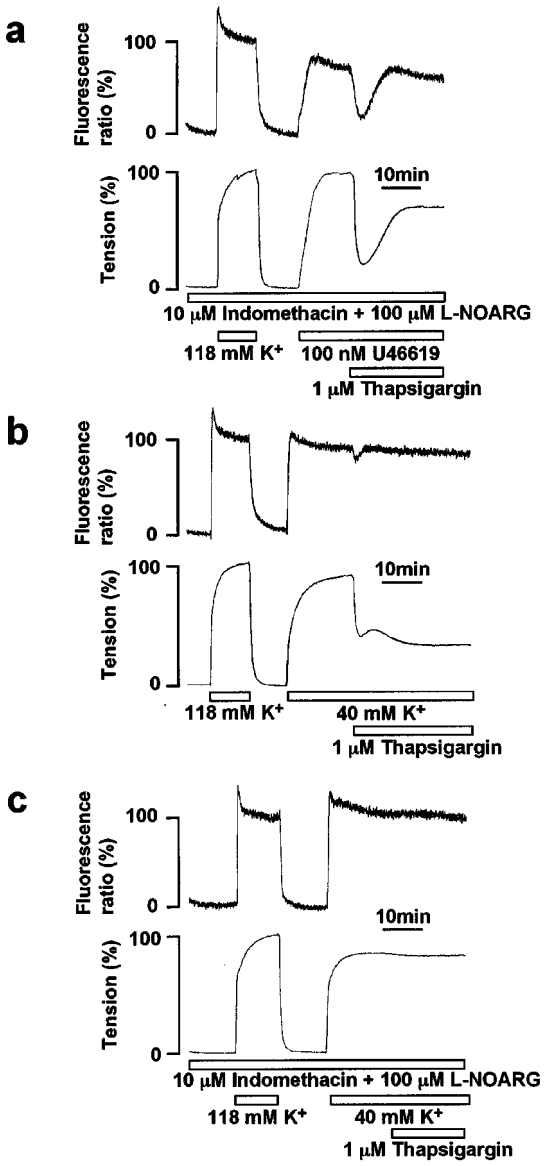

Figure 4 Effect of indomethacin, N^{ω}-nitro-L-arginine (L-NOARG) and high K^+-depolarization on the thapsigargin-induced endothelium-dependent relaxation. (a) A representative recording of the changes in $[Ca^{2+}]_i$ (upper trace) and tension (lower trace) of smooth muscle induced by 1 μM thapsigargin (TG) during contraction induced by 100 nM U46619 in the strips with endothelium that were pretreated with 10 μM indomethacin and 100 μM L-NOARG. TG was applied 10 min after the initiation of the precontraction. (b) A representative recording of changes in $[Ca^{2+}]_i$ and tension of smooth muscle induced by 1 μM TG during the 40 mM K^+-induced contraction in the strip with endothelium. (c) A representative recording of changes in $[Ca^{2+}]_i$ and tension of smooth muscle induced by 1 μM TG during the 40 mM K^+-induced contraction in the presence of 10 μM indomethacin and 100 μM L-NOARG in strips with endothelium. The responsiveness to 118 mM K^+-depolarization was recorded at the beginning of each measurement.

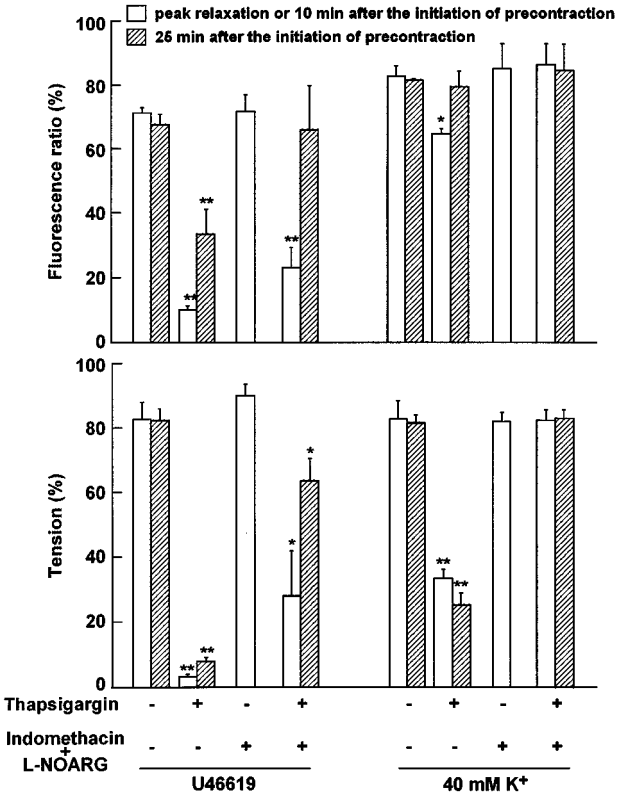

Figure 5 Summary of the effect of thapsigargin on $[Ca^{2+}]_i$ and tension during the contraction induced by U46619 or 40 mM K^+, either in the presence or absence of indomethacin and L-NOARG. The levels of $[Ca^{2+}]_i$ (upper panel) and force (lower panel) were obtained at peak relaxation and at 25 min (sustained phase of relaxation) after the initiation of precontraction by U46619 and 40 mM K^+, either in the presence or absence of 10 μM indomethacin + 100 μM L-NOARG. During the control precontraction obtained in the absence of indomethacin and L-NOARG (thapsigargin (−), indomethacin + L-NOARG (−)), the level of $[Ca^{2+}]_i$ and force sustained for more than 25 min. For the precontraction obtained in the presence of indomethacin and L-NOARG (thapsigargin (−), indomethacin + L-NOARG (+)), therefore, only the data obtained at 10 min after the initiation of precontraction are shown. The levels of $[Ca^{2+}]_i$ and tension seen in normal (5.9 mM K^+) PSS and 118 mM K^+-PSS were considered to be 0 and 100 %, respectively. When the effects of indomethacin and L-NOARG were evaluated, the 0 and 100% levels were determined in their presence. The data are the mean ± s.e.mean (n = 3–4). *P < 0.05, **P < 0.01 compared with the value obtained at the corresponding time point without thapsigargin.

induced precontraction were 71.9 ± 5.4 and 90.6 ± 3.1% (n = 3), respectively (Figure 5). In the presence of indomethacin and L-NOARG, the levels of $[Ca^{2+}]_i$ at the initial phase of the TG-induced relaxation and at 15 min after the application of TG (25 min after the initiation of precontraction) were 23.4 ± 6.2 and 66.1 ± 14.0% (n = 3), respectively (Figure 5). The $[Ca^{2+}]_i$ level seen at 15 min in the presence of indomethacin and L-NOARG did not differ significantly from that obtained during the precontraction induced by 100 mM U46619 (67.8 ± 3.2%, n = 3) (Figure 5). The levels of tension seen at the initial relaxation and at 15 min after the application of TG were 28.5 ± 13.8 and 64.3 ± 6.7% (n = 3), respectively (Figure 5). Both levels were significantly higher than those obtained with TG in the absence of inhibitors (control relaxation), and significantly lower than those obtained during the precontraction (Figure 5). Treatment with L-NOARG alone had the same effect as that seen with the combination of indomethacin and L-NOARG (data not shown).

High extracellular K^+ has been shown to inhibit hyperpolarization of the membrane potential induced by EDHF in smooth muscle cells (Nagao & Vanhoutte, 1992). We previously showed that the extracellular K^+ concentration of 40 mM was high enough to inhibit the effects of EDHF induced by substance P in the porcine coronary artery (Kuroiwa *et al*., 1995) and that induced by TG in porcine renal artery (Ihara *et al*., 1999). Accordingly, to clarify the involvement of EDHF, the relaxation induced by TG was investigated during the contraction induced by 40 mM K^+-depolarization. Stimulation with 40 mM K^+-depolarization caused rapid increases in $[Ca^{2+}]_i$ and tension, followed by the sustained increases. The levels of $[Ca^{2+}]_i$ and tension during the sustained contraction were 83.0 ± 3.3 and 83.0 ± 5.5% at 10 min, and 81.6 ± 0.5 and 82.0 ± 2.7 % at 25 min, respectively (n = 3, Figure 5). The level of tension obtained with 40 mM K^+ was thus similar to that obtained with 100 nM U46619, while the level of $[Ca^{2+}]_i$ was higher than that obtained with U46619 (Figure 5). When 1 μM TG was applied during the 40 mM K^+-induced sustained contraction, it induced only a small transient decrease in $[Ca^{2+}]_i$ and a sustained decrease in tension (Figure 4b). As a result, the sustained decrease in tension was not accompanied by a decrease in $[Ca^{2+}]_i$ (Figure 4b). The levels of $[Ca^{2+}]_i$ and tension at the initial relaxation were 64.8 ± 1.8 and 34.0 ± 2.7% (n = 3), respectively (Figure 5). The levels of $[Ca^{2+}]_i$ and tension obtained at 15 min after the application of TG were 79.6 ± 4.8 and 25.7 ± 3.7% (n = 3), respectively (Figure 5). The $[Ca^{2+}]_i$ level obtained at 15 min after the application of TG did not significantly differ from that obtained during the 40 mM K^+-induced precontraction, while the level of tension was significantly lower than the precontraction level. Treatment with 10 μM indomethacin had no effect on the relaxation induced by TG during 40 mM K^+-depolarization. However, the combination of indomethacin and L-NOARG completely abolished the decreases in both $[Ca^{2+}]_i$ and tension (Figure 4c). The levels of $[Ca^{2+}]_i$ and tension obtained with TG, indomethacin and L-NOARG during the 40 mM K^+-induced contraction did not differ significantly from those seen during the control contraction induced by 40 mM K^+ (Figure 5).

Discussion

The present study demonstrated that TG induced a large sustained elevation of $[Ca^{2+}]_i$ in the *in situ* endothelial cells of the porcine aortic valve, and an endothelium-dependent large sustained relaxation in porcine coronary arterial strips. One of the most intriguing observations is that the TG-induced relaxation continued to be sustained as long as the arterial strips were exposed to TG. The endothelium-dependent relaxations induced by such receptor agonists as acetylcholine, bradykinin, endothelin-1 or substance P were originally shown to be relatively transient (Furchgott & Zawadzki, 1980; Gryglewski *et al*., 1986). Among the reasons for the transient nature of the endothelium-dependent relaxation are the short half life of NO (Palmer *et al*., 1987) and the transient nature of the $[Ca^{2+}]_i$ elevation induced by agonists (Aoki *et al*., 1991; Himmel *et al*., 1993; Hirano & Kanaide, 1993; Kuroiwa *et al*., 1995). The TG-induced sustained elevation of $[Ca^{2+}]_i$ in endothelial cells and the sustained endothelium-dependent relaxation in arterial strips were thus considered to be unique in this respect.

The TG-induced elevation of $[Ca^{2+}]_i$ in the valvular endothelial cells consisted of the rapid peak (the first component) and the following sustained increase (the second

component). In Ca^{2+}-free PSS, the first component was partially inhibited, while the second one was completely abolished. The second component was also completely abolished by Ni^{2+}. These findings suggested that the second component of the TG-induced $[Ca^{2+}]_i$ elevation was due to an influx of extracellular Ca^{2+}, and that the first component was partly due to the Ca^{2+} release from the intracellular store sites and partly due to the Ca^{2+} influx. In both the present study and a previous study (Kawasaki *et al*., 1999), a Ca^{2+} channel blocker, diltiazem, had no effect on TG-induced sustained $[Ca^{2+}]_i$ elevation, thus indicating that L-type Ca^{2+} channel is not involved in the TG-induced Ca^{2+} influx in endothelial cells *in situ*. This observation is consistent with reports showing no activity of L-type Ca^{2+} channel in either freshly isolated (Busse *et al*., 1988) or cultured endothelial cells (Colden-Stanfield *et al*., 1987). On the other hand, a similar large sustained $[Ca^{2+}]_i$ elevation was observed with CPA in porcine aortic valvular strips (Higuchi *et al*., 1996). Both TG and CPA are inhibitors of the Ca^{2+} pump of the intracellular Ca^{2+} stores (Seidler *et al*., 1989; Thastrup *et al*., 1990). The inhibition of the Ca^{2+} pump was considered to deplete Ca^{2+} in store sites and thereby induce Ca^{2+} entry, which is called capacitative Ca^{2+} entry (Putney & McKay, 1999). The Ca^{2+} influx induced by TG was thus suggested to be due to the capacitative Ca^{2+} entry, and the transient $[Ca^{2+}]_i$ elevation seen in the absence of extracellular Ca^{2+} reflected the release and the resultant depletion of Ca^{2+} in the store sites.

The mechanism of activation and regulation of capacitative Ca^{2+} entry have yet to be determined in *in situ* endothelial cells. In general, two mechanisms have been proposed for coupling between the store depletion and the activation of Ca^{2+} entry; the direct coupling mediated by direct interaction between the inositol 1,4,5- trisphosphate receptor and Ca^{2+} entry channel (Boulay *et al*., 1999; Putney, 1999) or that mediated by cytoskeletal structures such as actin filaments (Holda & Blatter, 1997), and the indirect coupling mediated by second messengers. The candidates proposed for the second messengers include Ca^{2+} influx factor, cyclic GMP, cytochrome P450 metabolites of arachidonic acids, heterotrimeric GTP-binding proteins, small GTP-binding proteins, tyrosine kinase, myosin light chain kinase and protein phosphatases (for review, see Parekh & Penner, (1997)).

In the present study, econazole was used as a cytochrome P450 inhibitor (Graier *et al*., 1995; Mason *et al*., 1993; Vostal & Fratantoni, 1993), and we found that it significantly inhibited the TG-induced Ca^{2+} influx. This finding suggested cytochrome P450 to be involved in the regulation of the TG-induced Ca^{2+} influx in *in situ* endothelial cells as well as in cultured endothelial cells (Graier *et al*., 1995). MLCK was suggested to be involved in the capacitative Ca^{2+} influx in cultured porcine aortic endothelial cells (Watanabe *et al*., 1996). In the present study, ML-9 inhibited the TG-induced $[Ca^{2+}]_i$ elevation in a concentration-dependent manner. However, wortmannin had no significant effect on TG-induced $[Ca^{2+}]_i$ elevation. The concentration of wortmannin used in the present study (100 μM) was high enough to inhibit MLCK in addition to phosphatidylinositol 3-kinase (Yano *et al*., 1993). Based on our results, MLCK was not suggested to play an important role in the TG-induced Ca^{2+} influx in *in situ* endothelial cells. The inhibition of the TG-induced Ca^{2+} influx by ML-9 may be due to an inhibition of some kinases other than MLCK. Since both ML-9 and wortmannin inhibited the TG-induced Ca^{2+} influx in cultured endothelial cells (Watanabe *et al*., 1996), the kinase involved in the TG-induced Ca^{2+} influx might thus have been altered by culture conditions. Several authors reported GTP-binding proteins to be involved in the activation of capacitative Ca^{2+} entry (Bird & Putney, 1993; Sargeant *et al*., 1993). We previously reported that the IAP-sensitive GTP-binding protein was involved in the endothelin-1-induced Ca^{2+} influx but not Ca^{2+} release in *in situ* endothelial cells (Aoki *et al*., 1994). However, IAP had no effect on the TG-induced $[Ca^{2+}]_i$ elevation, thus suggesting that the IAP-sensitive GTP-binding protein did not play a role in the TG-induced Ca^{2+} influx in *in situ* endothelial cells. Collectively, the present study suggested that cytochrome P450 and ML-9-sensitive protein phosphorylation were involved in the TG-induced Ca^{2+} influx in *in situ* endothelial cells of porcine aortic valve. The mechanisms of how these two factors contribute to the TG-induced Ca^{2+} influx remain to be elucidated.

We previously reported that CPA induced a similar endothelium-dependent relaxation in the porcine coronary artery, and induced a large sustained $[Ca^{2+}]_i$ elevation in *in situ* endothelial cells (Higuchi *et al*., 1996). Both TG and CPA have been reported to induce a sustained elevation of $[Ca^{2+}]_i$ due to the activation of capacitative Ca^{2+} entry (Putney & McKay, 1999). It is therefore conceivable that the capacitative Ca^{2+} entry induced by TG or CPA was functionally linked to the sustained production of endothelium-derived relaxing factors in the porcine coronary artery.

In the present study, we evaluated the relative contribution of NO, EDHF and prostacyclin in the TG-induced relaxation by using specific inhibitors. Indomethacin did not alter the extent of the TG-induced relaxation at all, thus suggesting that prostacyclin played a negligible role in the relaxation. L-NOARG significantly but only partially inhibited the TG-induced relaxation, thus suggesting that not only NO but also other relaxing factors contributed to the relaxation. The elevation of the external K^+ concentration also partially inhibited the relaxation induced by TG, but the combination of L-NOARG and high K^+ completely abolished it. These observations suggested that NO and EDHF were major factors contributing to the TG-induced relaxation. L-NOARG inhibited the sustained relaxation more potently than high K^+, although the $[Ca^{2+}]_i$ decrease in smooth muscle cells during the sustained relaxation was completely inhibited by either L-NOARG or high K^+. On the contrary, the initial phase of the TG-induced decreases in tension was similarly inhibited by high K^+ and L-NOARG. The findings of the present study suggest that NO was the major mediator of the sustained phase of the TG-induced relaxation, while both NO and EDHF contributed to the initial phase of the relaxation. Such a time-dependent alteration of the relative contribution of NO and EDHF in the TG-induced relaxation is similar to that observed in the CPA-induced relaxation in the porcine coronary artery (Higuchi *et al*., 1996). Furthermore, examining the $[Ca^{2+}]_i$-force relationship during relaxation indicates that the Ca^{2+}-sensitivity of the contractile apparatus decreased during sustained relaxation. The decrease in the myofilament Ca^{2+}-sensitivity was obvious during the sustained relaxation seen with the 40 mM K^+-induced precontraction, when the contribution of EDHF was blocked and NO was the only mediator of the relaxation. These observations are consistent with the involvement of NO in the late sustained relaxation, because NO was shown to increase the cytosolic cyclic GMP level (Ignarro *et al*., 1987), which was shown to decrease the Ca^{2+} sensitivity of the contractile apparatus (Nishimura & van Breemen, 1989). It could thus be concluded that the sustained $[Ca^{2+}]_i$ elevation due to the Ca^{2+} entry induced by TG in endothelial cells was most closely linked to the production of NO.

The link between the $[Ca^{2+}]_i$ elevation and NO production in endothelial cells is consistent with a biochemical study showing NO synthase to be a Ca^{2+}/calmodulin-dependent enzyme (Fleming *et al*., 1997). However, NO synthase activity is considered to be regulated both in a Ca^{2+}-dependent and Ca^{2+}-independent manner, and the alteration of the Ca^{2+}-sensitivity of the NO production process is considered to play an important regulatory role (Fleming *et al*., 1997). We previously reported that the relationship between the $[Ca^{2+}]_i$ elevation and NO production in endothelial cells varies with the type of stimulation (Mizuno *et al*., 1998; 2000). The phosphorylation of NO synthase by Akt/protein kinase B was reported to activate NO synthase in a Ca^{2+}-independent manner (Dimmeler *et al*., 1999; Fulton *et al*., 1999). The findings of the present study, using thapsigargin as the endothelial stimulus, emphasize the importance of the Ca^{2+} signal as a primary determinant of the NO synthase activity.

In contrast to endothelial cells, TG induced a small sustained elevation of $[Ca^{2+}]_i$ in smooth muscle cells of the porcine coronary artery. Furthermore, such $[Ca^{2+}]_i$ elevation was not linked to the force development. It is therefore possible that TG decreased the Ca^{2+}-sensitivity of contractile apparatus, and therefore did not induce any force development despite of $[Ca^{2+}]_i$ elevation. However, this is not the case because TG had almost a negligible effect on the U46619-induced contraction. On the other hand, it is possible that the level of $[Ca^{2+}]_i$ elevation was not high enough to cause a contraction. We reported that 15 mM K^+ induced a $[Ca^{2+}]_i$ elevation ($\sim$20% of the 118 mM K^+-induced elevation) without any force development, but higher concentrations of external K^+ induced both a $[Ca^{2+}]_i$ elevation and force development (Abe *et al*., 1990). Therefore, the capacitative Ca^{2+} entry pathway is suggested to play a greater role as a major Ca^{2+} influx pathway in endothelial cells than it plays in smooth muscle cells.

In conclusion, TG induced a large sustained elevation of $[Ca^{2+}]_i$ consisting of an initial rapid phase and a sustained phase in *in situ* endothelial cells. The initial rapid $[Ca^{2+}]_i$ elevation was due to both the Ca^{2+} release from the intracellular store sites and capacitative Ca^{2+} entry, while the sustained $[Ca^{2+}]_i$ elevation was due to the Ca^{2+} entry. Cytochrome P450 and ML-9-sensitive protein phosphorylation were suggested to be involved in the TG-induced Ca^{2+} influx. The TG-induced Ca^{2+} influx was shown to be functionally linked to the production of endothelium-derived relaxing factors and thereby induce relaxation in the porcine coronary artery. This relaxation was composed of two components; an initial rapid relaxation due to NO and EDHF and a late sustained relaxation mainly due to NO. Thus, the TG-induced sustained $[Ca^{2+}]_i$ elevation due to capacitative Ca^{2+} entry in endothelial cells most closely correlated with the production of NO. During the treatment with TG, the sustained Ca^{2+} elevation might therefore help to maintain a high activity of NO synthase thereby causing a sustained production of NO, which in turn induces the sustained relaxation of coronary artery despite the short half life of NO.

We thank Mr Brian Quinn for comments and help with the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 10557072, 11838013, 11670687), for the Encouragement of Young Scientists (No. 10770308) from the Ministry of Education, Science, Sports and Culture, Japan, by the Research Grant for Cardiovascular Diseases (11C-1) from the Ministry of Health and Welfare, Japan, and by grants from the Vehicle Racing Commemorative Foundation, the Foundation for the Promotion of Clinical Medicine, the Suzuken Memorial Foundation and KANZAWA Medical Research Foundation.

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(Received February 9, 2000
Revised May 4, 2000
Accepted June 26, 2000)