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Thapsigargin-induced endothelium-dependent triphasic regulation of vascular tone in the porcine renal artery

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1 To elucidate the role of thapsigargin-induced Ca^{2+} entry in endothelial cells in the regulation of vascular tone, changes in Ca^{2+} and force of smooth muscle were simultaneously monitored in fura-2-loaded strips of porcine renal artery.

2 During phenylephrine-induced sustained contraction, thapsigargin caused an endothelium-dependent triphasic response; an initial relaxation, a subsequent transient contraction, and a sustained relaxation. The initial relaxation and the contraction were associated with a decrease and an increase in $[Ca^{2+}]_i$, respectively. There was no apparent $[Ca^{2+}]_i$ decrease during the sustained relaxation. Thapsigargin-induced responses were observed at 10^{-8} M and higher concentrations, with the maximum response observed at 10^{-6} M.

3 The transient contraction was inhibited by a cyclo-oxygenase inhibitor (10^{-5} M indomethacin), a thromboxane A_2 (TXA_2)/prostagrandin H_2 (PGH_2) receptor antagonist (10^{-5} M ONO-3708), and a TXA_2 synthase inhibitor (10^{-5} M OKY-046).

4 During the phenylephrine-induced contraction in the presence of indomethacin, thapsigargin caused an initial, but not a sustained relaxation, in the presence of N^{ω}-nitro-L-arginine methylester (L-NAME). During the contraction induced by phenylephrine plus 40 mM K^+-depolarization in the presence of indomethacin, thapsigargin induced both a transient and a sustained relaxation. However, these relaxations were completely abolished in the presence of L-NAME.

5 Thapsigargin caused a large Ca^{2+} elevation in cultured endothelial cells of the renal artery. The concentration-response relation was thus similar to that for force development in the arterial strips.

6 In conclusion, thapsigargin-induced Ca^{2+} entry in endothelial cells led to triphasic changes in the tone of the porcine renal artery. The endothelium-dependent contraction was mediated mainly by TXA_2. Nitric oxide and hyperpolarizing factor are both involved in the initial relaxation. However, a sustained relaxation was observed which mainly depended on nitric oxide.

Keywords: Renal artery; endothelial cells; thromboxane A_2; endothelium-dependent contraction; endothelium-dependent relaxation

Abbreviations: ANOVA, an analysis of variance; ATP, adenosine 5′-triphosphate; CPA, cyclopiazonic acid; Dil-Ac-LDL, acetylated-low density lipoprotein labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; DMEM, Dulbecco's modified Eagle medium; fura-2/AM, an acetoxymethylester form of fura-2; HBS, HEPES-buffered saline; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid; L-NAME, N^{ω}-nitro-L-arginine methylester; PBS, phosphate buffered saline; PGH_2, prostagrandin H_2; PSS, physiological salt solution; TG, thapsigargin; TXA_2, thromboxane A_2

Introduction

Endothelial cells play an important role in the regulation of vascular tone by producing and releasing relaxing and contracting substances (Katusic & Shepherd, 1991; Rubanyi, 1991; Vanhoutte & Eber, 1991). Nitric oxide, prostacyclin and hyperpolarizing factor are major endothelium-derived relaxing factors (Kelly & Smith, 1996; Shepherd & Katusic, 1991). After the first reports on acetylcholine-induced, endothelium-dependent relaxation in the rabbit aorta, endothelium-dependent relaxations have been reported in various types of vascular tissues (Furchgott & Zawadzki, 1980). Vanhoutte and coworkers showed that endothelial cells produce not only relaxing factors but also contracting factors (De Mey & Vanhoutte, 1983; Miller & Vanhoutte, 1985; Rubanyi & Vanhoutte, 1985). Endothelin was also discovered to be a potent endothelium-derived vasoconstricting substance (Yanagisawa *et al*., 1988). However, reports on endothelium-dependent contraction have been limited to certain types of vascular tissue mostly observed in cerebral, renal and pulmonary arteries. In cerebral artery, nicotine (Shirahase *et al*., 1988a), A23187 (Shirahase *et al*., 1988b), adenosine 5′-triphosphate (ATP) (Shirahase *et al*., 1988c), acetylcholine (Usui *et al*., 1993), somatostatin (Shirahase *et al*., 1993), angiotensin I and angiotensin II (Manabe *et al*., 1989) caused endothelium-dependent contractions, and thromboxane A_2 (TXA_2) was shown to be involved in these contractions. In renal artery, acetylcholine induced endothelium-dependent contractions (Nishimura *et al*., 1995). In the rabbit pulmonary artery, arachidonic acid (Buzzard *et al*., 1993), methacholine (Buzzard *et al*., 1993) and substance P (Shirahase *et al*., 1995) induced endothelium-dependent contractions. However, most studies showed only endothelium-dependent contractions whilst few reports have shown both endothelium-dependent contraction and relaxation in one type of tissue (Nishimura *et al*., 1995).

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Cyclopiazonic acid (CPA), which induced a large $[Ca^{2+}]_i$ elevation in endothelial cells, caused only endothelium-dependent relaxation, by releasing nitric oxide, hyperpolarizing factor, and prostacyclin in the porcine coronary artery (Higuchi *et al*., 1996). Stimulations which cause endothelium-dependent contractions have also been reported to increase $[Ca^{2+}]_i$ in endothelial cells (Katusic *et al*., 1987; Shirahase *et al*., 1988b). Capacitative Ca^{2+} influx is a major pathway of Ca^{2+} influx in endothelial cells (Klishin *et al*., 1998). However, it remains to be elucidated, whether or not thapsigargin-induced $[Ca^{2+}]_i$ elevation causes endothelium-dependent contraction in smooth muscle cells.

In the present study, we investigated the role of thapsigargin-induced $[Ca^{2+}]_i$ elevation in endothelial cells in the regulation of vascular tone in the renal artery, by simultaneously monitoring changes in the $[Ca^{2+}]_i$ and force of smooth muscle induced by thapsigargin (TG) and CPA in fura-2-loaded porcine renal artery. To determine the mechanisms of endothelium-dependent relaxation and contraction, we examined the effects of a cyclo-oxygenase inhibitor, a thromboxane A_2 (TXA_2)/prostagrandin H_2 (PGH_2) receptor antagonist, a TXA_2 synthase inhibitor, and a nitric oxide synthase inhibitor on $[Ca^{2+}]_i$ and force of smooth muscle. We also determined the effects of TG and CPA on $[Ca^{2+}]_i$ in cultured endothelial cells of the renal artery using fura-2 microfluorometry.

Methods

Tissue preparation for front-surface fluorometry

Porcine kidneys were freshly obtained from a local slaughterhouse and transported to our laboratory in aerated ice-cold physiological salt solution (PSS). Interlobar arteries were dissected from the kidney and the adventitia was mechanically removed under a binocularscope. The arterial segments thus obtained were opened longitudinally and cut into circular strips (approximately 1 mm wide, 3 mm long, 0.05 mm thick). Care was taken to avoid damaging the endothelium. To obtain strips without an endothelium, the inner surface was rubbed with a cotton swab.

Fura-2 loading of renal arterial strips

Strips with and without endothelium were loaded with fura-2, by incubation in Dulbecco's modified Eagle medium (DMEM) containing 25 μM fura-2/AM (an acetoxymethylester form of fura-2) and 5% foetal bovine serum for 4 h at 37°C, as previously described (Hirano *et al*., 1990). After loading with fura-2, the strips were rinsed with PSS to remove the dye in the extracellular space and then were equilibrated for at least 60 min at 37°C before starting the experimental protocols.

In the case of strips with an intact endothelium, there is a possibility that the fura-2 signal could arise from either smooth muscle cells or endothelial cells. However, the contribution of endothelial cells to the fura-2 signal from the strips varied depending on the species and type of tissue (Kuroiwa *et al*., 1993). In the present study, as in coronary artery (Kuroiwa *et al*., 1995), the following observations indicated that the fura-2 signal arose exclusively from smooth muscle in the strips of renal artery with endothelium and that the signal derived from endothelial cells, if any, was negligible; (1) Observation of the fura-2-loaded strips under a fluorescent microscope (Akisiophoto, Zeiss, Germany): The strips either with or without an endothelium 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) as described previously (Kuroiwa *et al*., 1995). In the absence of an endothelium, a longitudinally running pattern of fura-2 fluorescence was observed, thus indicating the smooth muscle cells to be stained with fura-2. In the strip with endothelium, a cobblestone appearance of Dil-Ac-LDL fluorescence staining was observed. However, no such staining pattern was observed with fura-2. The pattern of fura-2 staining was similar to that observed in strips without an endothelium. During equilibration for 60 min at 37°C after loading, fura-2 loaded in smooth muscle cells was kept constant, but that in the endothelial cells was completely lost. Therefore the endothelial cells of renal arterial strips did not produce any fura-2 signal. (2) Carbachol and thrombin induced an endothelium-dependent relaxation in fura-2-loaded renal arterial strips with an endothelium. Only a decrease in the fluorescence ratio was observed, although stimulations to cause endothelium-dependent relaxation were shown to induce $[Ca^{2+}]_i$ elevation in endothelial cells (Rubanyi & Vanhoutte, 1988). (3) Fluorescence signals measured from the adventitial or luminal side of the strips with endothelium and those from the luminal side of the strips without an endothelium were identical.

Simultaneous measurement of $[Ca^{2+}]_i$ and force of arterial strips

Experiments on arterial strips were carried out at 37°C (Hirano *et al*., 1990). The strips were mounted vertically to a force transducer TB-612T (Nihon Koden, Japan) in a quartz organ bath filled with normal PSS. Changes in the level of $[Ca^{2+}]_i$ of the smooth muscle in the arterial strips were monitored as previously described, using a front-surface fluorometer CAM-OF-3 (JASCO, Tokyo, Japan). The strips were illuminated by alternating (400 Hz) 340 nm and 380 nm excitation light from an xenon light source through quartz optic fibres. The surface fluorescence of the strips was collected by glass optic fibres and introduced through a 500 nm band pass filter (full width at half maximum transmission = 10 nm) into a photomultipler. The quartz and glass optic fibres were arranged in a concentric inner circle (3 mm diameter) and an outer circle (7 mm diameter) at one end of the optic fibres facing the strips, respectively. The fluorescence intensities (500 nm) at 340 nm (F340) and 380 nm (F380) excitation and their ratio (Ratio = F340/F380), which indicated $[Ca^{2+}]_i$ of smooth muscle, were continuously monitored.

During the 60 min equilibration period, strips were stimulated with 118 mM K^+ every 10 min, and the resting load was increased in a stepwise manner and finally adjusted to 100 mg. When the renal artery was exposed to 10^{-6} M phenylephrine in normal PSS, $[Ca^{2+}]_i$ and force rose rapidly and reached a peak in 1–3 min, and then declined slightly to reach a sustained level within 15 min (Figure 1a). This level was maintained for more than 30 min. Thereafter, the $[Ca^{2+}]_i$ level and force returned to the resting level after the removal of phenylephrine, the strip was stimulated once with 118 mM K^+ in order to refill the intracellular Ca^{2+} stores and then with 10^{-6} M phenylephrine. The second stimulation by phenylephrine induced a similar contraction to that observed with the first stimulation. The level of $[Ca^{2+}]_i$ and force obtained with the second stimulation was $99.2 \pm 4.6\%$ and $99.7 \pm 4.2\%$ ($n = 6$) of those obtained with the first stimulation, respectively. As a result, the response to 10^{-6} M phenylephrine was used as a reference in the renal artery.

The level of $[Ca^{2+}]_i$ and force at rest and at the sustained phase of the 10^{-6} M phenylephrine-induced contraction were assigned to be 0 and 100%, respectively, in the following measurement. The extent of relaxation and the effects of inhibitors were thus evaluated by how much of the value remained, namely the level of $[Ca^{2+}]_i$ and force obtained under each condition.

Primary culture of endothelial cells from the porcine renal artery

Porcine renal artery endothelial cells in primary culture were obtained as previously described (Hirano *et al*., 1993; Mizuno *et al*., 1998). In brief, the same portion of interlobar arteries of kidney was isolated as described above and rinsed with sterilized phosphate buffered saline (PBS). The arteries were opened and the endothelial lining was gently scraped off with a scalpel blade under sterile conditions. The cellular sheets thus obtained were collected by centrifugation at 1000 r.p.m. for 5 min, suspended in growth medium and then mechanically dispersed into smaller clumps by pipetting. These cells were implanted onto sterilized round glass coverslips (diameter 25 mm; Matsunami, Osaka, Japan) in 35 mm culture dishes (Nunc, Copenhagen, Denmark) and cultured in DMEM containing 10% foetal bovine serum (Sanko Junyaku, Tokyo, Japan), 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 50 μg ml^{-1} gentamycin. The growth medium was changed every 2 days. The cells grew into a typical cobblestone monolayer of endothelial cells as observed under a phase-contrast microscopic on days 7–10, when the cells were used for the experiments.

Microfluorometry of endothelial cells in primary culture

Endothelial cells from the renal artery in primary culture on coverslips were loaded with fura-2 by incubation in DMEM containing 5 μM fura-2/AM for 60 min at 37°C, as previously described (Hirano *et al*., 1993). After loading with fura-2, the cells were washed three times with 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES) buffered saline (HBS) at 25°C to remove the dye in the extracellular spaces.

All experiments on cultured endothelial cells were carried out at 25°C to prevent any leakage of fura-2 from the cells (Berthon *et al*., 1984). Fura-2 microfluorometry was performed as previously described (Hirano *et al*., 1993), using an inverted fluorescent microscope (TMD 56, Nikon, Tokyo, Japan) equipped with a spectrophotometer CAM220 (JASCO). The coverslips containing the fura-2-loaded cells were placed in a coverslip holder, which was set on the stage of the microscope. Alternating (400 Hz) excitation light (348 nm and 380 nm) was obtained spectroscopically from an xenon light source. The fluorescent intensities at 348 and 380 nm excitation were measured at 500 nm and their ratio was recorded. Changes in the fluorescent ratio were expressed as a percentage, assuming

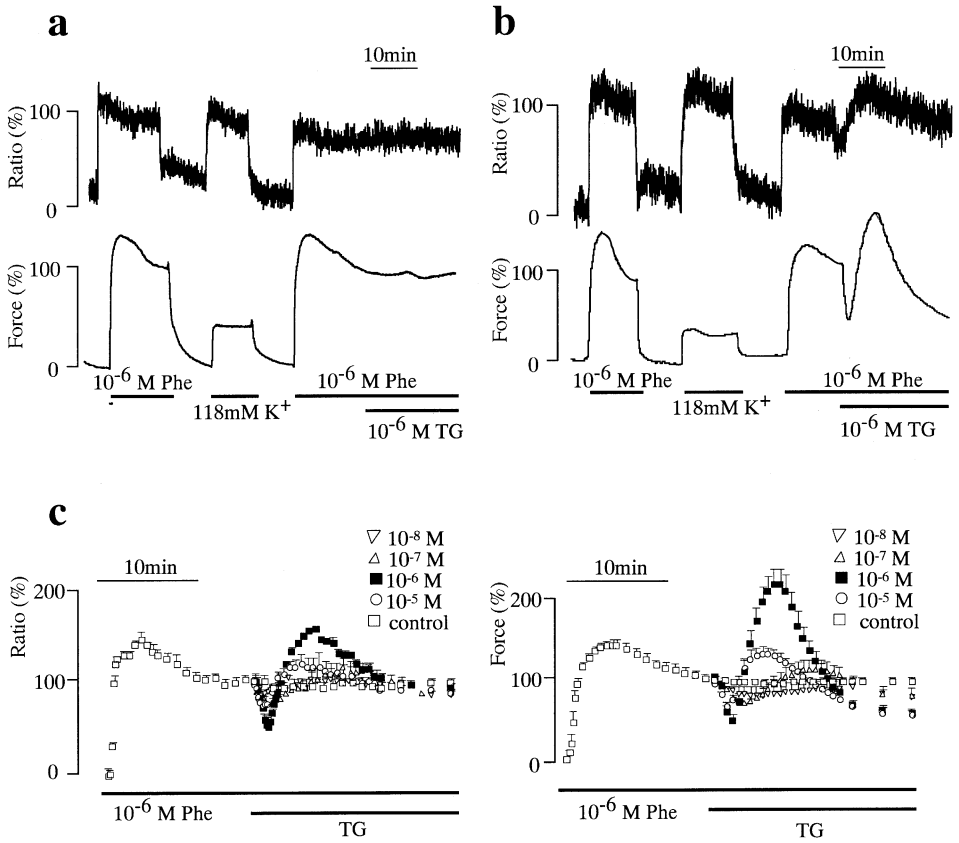

Figure 1 Thapsigargin (TG)-induced endothelium-dependent triphasic changes in $[Ca^{2+}]_i$ and force during phenylephrine (Phe)-induced sustained contraction in porcine renal artery. (a) and (b) The representative recordings of changes in $[Ca^{2+}]_i$ and force induced by 10^{-6} M TG in the strips without (a) and with (b) endothelium. After the reference response to 10^{-6} M Phe was recorded and the strip was stimulated once with 118 mM K^+ depolarization, the precontraction was initiated with 10^{-6} M Phe. TG (10^{-6} M) was applied at 15 min during the sustained contraction induced by Phe. (c) Summary of the time courses for the endothelium-dependent changes in $[Ca^{2+}]_i$ and force induced by 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M TG, and the control. The levels of $[Ca^{2+}]_i$ and force at rest and during Phe-induced sustained contraction were designated as 0 and 100%, respectively. All data are the mean ± s.e.mean. ($n = 6$).

the values obtained in normal HBS and with 10^{-5} M ATP stimulation to be 0 and 100%, respectively.

Drugs and solution

The composition of normal PSS was (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. PSS was aerated with 95% O_2 and 5% CO_2, with the resulting pH to be 7.4. PSS containing high K^+ was prepared by replacing NaCl with equimolar KCl. HBS was composed of (mM): NaCl 135, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, glucose 5.5 and HEPES (pH 7.4) 10, respectively. PBS was composed of (mM): NaCl 136.9, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.47.

Fura-2/AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Indomethacin was purchased from Wako (Osaka, Japan). DMEM was purchased from GIBCO (Grand Island, NY, U.S.A.). ONO-3708, a TXA_2/PGH_2 receptor antagonist and OKY-046, a TXA_2 synthase inhibitor, were kindly donated by Ono Pharmaceutical Co. (Osaka, Japan). N^{ω}-nitro-L-arginine methyl ester (L-NAME), thapsigargin (TG), probenecid, and phenylephrine were purchased from Sigma (St. Louis, MO, U.S.A.). Dil-Ac-LDL was purchased from Biomedical Technologies Inc. (Stoughton, MA, U.S.A.). ATP was purchased from Boehringer Mannheim (Germany). PGH_2 was purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.).

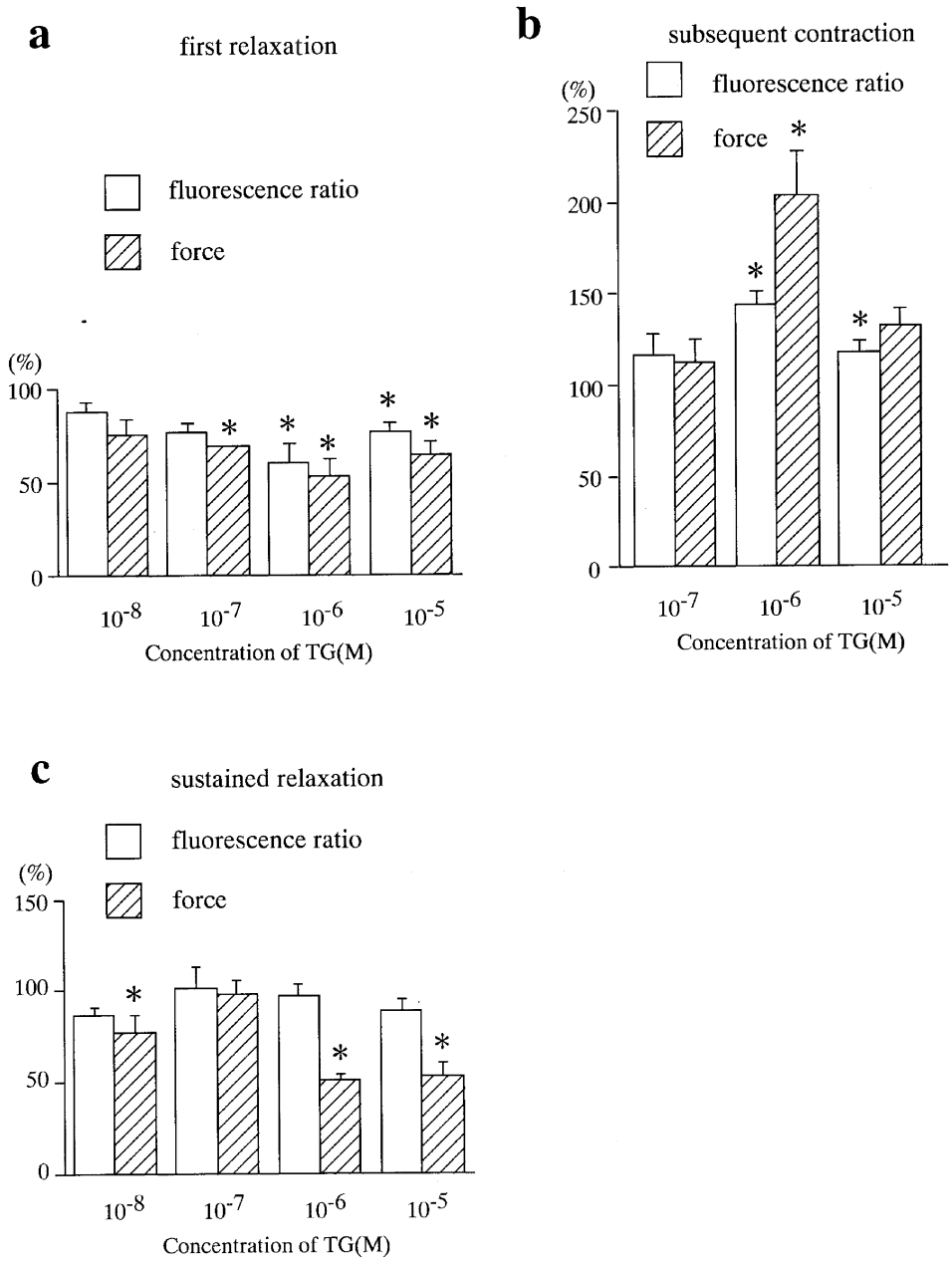

Figure 2 Concentration-dependency of triphasic changes in $[Ca^{2+}]_i$ and force induced by thapsigargin (TG) during phenylephrine (phe)-induced sustained contraction in the porcine renal artery. The levels of $[Ca^{2+}]_i$ and force at (a) maximum initial relaxation, at (b) the peak of transient contraction and at (c) 30 min after application of TG during the sustained relaxation are shown. The levels of $[Ca^{2+}]_i$ and force at rest and during Phe-induced sustained contraction were designated as 0 and 100%, respectively. All data are the mean ± s.e.mean ($n=6$). *Significantly different from the levels of $[Ca^{2+}]_i$ and force seen during the sustained phase of control contraction induced by 10^{-6} M Phe ($P<0.05$).

Statistical analysis

All data are expressed as the mean ± s.e.mean. One strip obtained from one animal was used for each experiment, therefore the number of experiments (n value) indicates the number of animals. Statistically significant differences were determined by an analysis of variance (ANOVA) followed by Bonferroni/Dunn test. The $P<0.005$ in Bonferroni/Dunn test (equivalent to Probability, $P<0.05$) 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

Effect of TG on the $[Ca^{2+}]_i$ level and force of smooth muscle in renal arterial strips with an intact endothelium

Figure 1 shows representative recordings of changes in the $[Ca^{2+}]_i$ level and force following the application of 10^{-6} M TG during sustained contraction induced by 10^{-6} M phenylephrine. In the absence of an endothelium, 10^{-6} M TG had no effect on either the $[Ca^{2+}]_i$ level or force of the phenylephrine-induced contraction (Figure 1a). On the other hand, in the presence of an endothelium (Figure 1b), 10^{-6} M TG produced

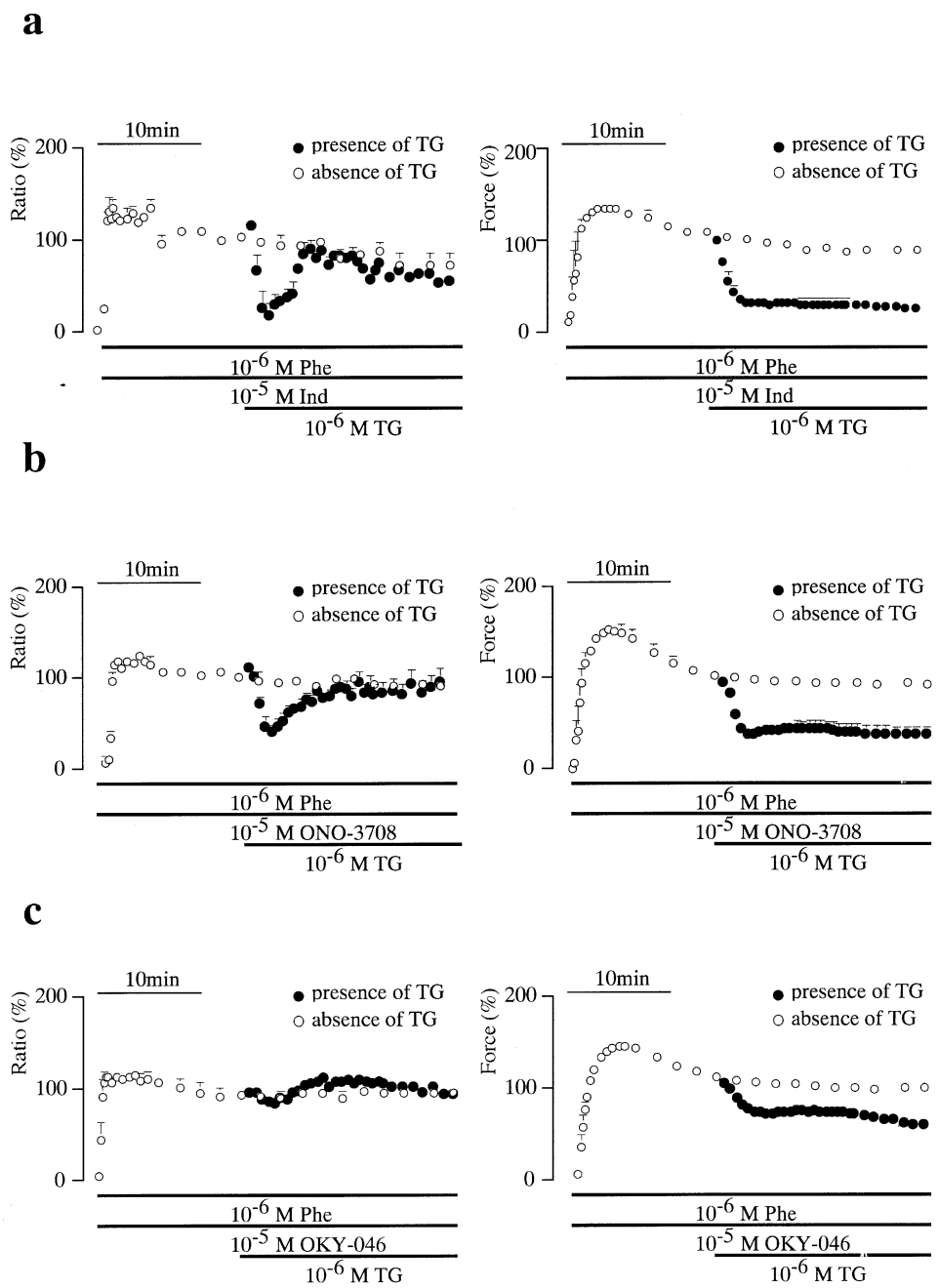

Figure 3 Effects of indomethacin (Ind: a cyclo-oxygenase inhibitor), ONO-3708 (a thromboxane A_2/prostaglandin H_2 receptor antagonist), and OKY-046 (a thromboxane A_2 synthase inhibitor) on thapsigargin (TG)-induced endothelium-dependent contraction. Time courses of TG-induced changes in $[Ca^{2+}]_i$ (left panels) and force (right panels) in the presence of (a) 10^{-5} M Ind, (b) 10^{-5} M ONO-3708, and (c) 10^{-5} M OKY-046 in porcine renal artery. After recording the reference response to 10^{-6} M phenylephrine (Phe), each inhibitor was applied just prior to the initiation of contraction by Phe. TG was applied at 15 min after the initiation of Phe-precontraction. The levels of $[Ca^{2+}]_i$ and force at rest and during Phe-induced sustained contraction were designated as 0 and 100%, respectively. All data are the mean ± s.e.mean ($n=5-6$).

an initial relaxation and a subsequent transient contraction, followed by a sustained relaxation. The initial relaxation and the transient contraction were associated with a decrease and a subsequent increase in $[Ca^{2+}]_i$, respectively, while the sustained relaxation was accompanied by little change in $[Ca^{2+}]_i$. Figure 1c shows a summary of the endothelium-dependent responses induced by the application of various concentrations of TG (10^{-8}–10^{-5} M) during the second 10^{-6} phenylephrine-induced contraction. At 10^{-8} M TG induced an early transient decrease in $[Ca^{2+}]_i$ and only a small but sustained relaxation. At 10^{-7} M, TG caused an initial relaxation followed by a transient contraction, accompanied by an initial decrease and a following transient increase in $[Ca^{2+}]_i$, respectively. No apparent sustained relaxation was observed over the following 30 min of observation. TG (10^{-5} M) caused similar changes in $[Ca^{2+}]_i$ and force to those observed with 10^{-6} M TG. However, the extent of the transient contraction was smaller than that obtained with 10^{-6} M ($P<0.05$). The time to reach the lowest point of the first relaxation and the peak of the transient contraction became progressively shorter in a concentration-dependent manner. The first relaxation reached its lowest point at 4.4 ± 0.8 min (10^{-8} M), 3.1 ± 0.3 min (10^{-7} M), 1.8 ± 0.1 min (10^{-6} M), and 1.47 ± 0.33 min (10^{-5} M) ($n=6$). The transient contraction reached its peak at 9.9 ± 0.5 min (10^{-7} M), 6.4 ± 0.3 min (10^{-6} M), and 5.0 ± 0.2 min (10^{-5} M) ($n=6$).

Figure 2 summarizes the concentration-dependent effect of TG on the initial relaxation, transient contraction and sustained relaxation. The initial relaxation and transient contraction were evaluated at the time of the lowest point and peak, respectively. The sustained relaxation was evaluated at 30 min after the application of TG. A significant initial relaxation was observed with 10^{-7} M and higher concentrations. The maximum relaxation ($55.2\pm6.8\%$, $n=6$) was obtained with 10^{-6} M TG. The decreases in force were accompanied by decreases in $[Ca^{2+}]_i$. The maximal decrease in $[Ca^{2+}]_i$ ($63.1\pm7.8\%$, $n=6$) during the initial relaxation was also obtained with 10^{-6} M. The level of $[Ca^{2+}]_i$ ($76.1\pm5.8\%$, $n=6$) and force ($64.5\pm6.5\%$, $n=6$) obtained with 10^{-5} M TG

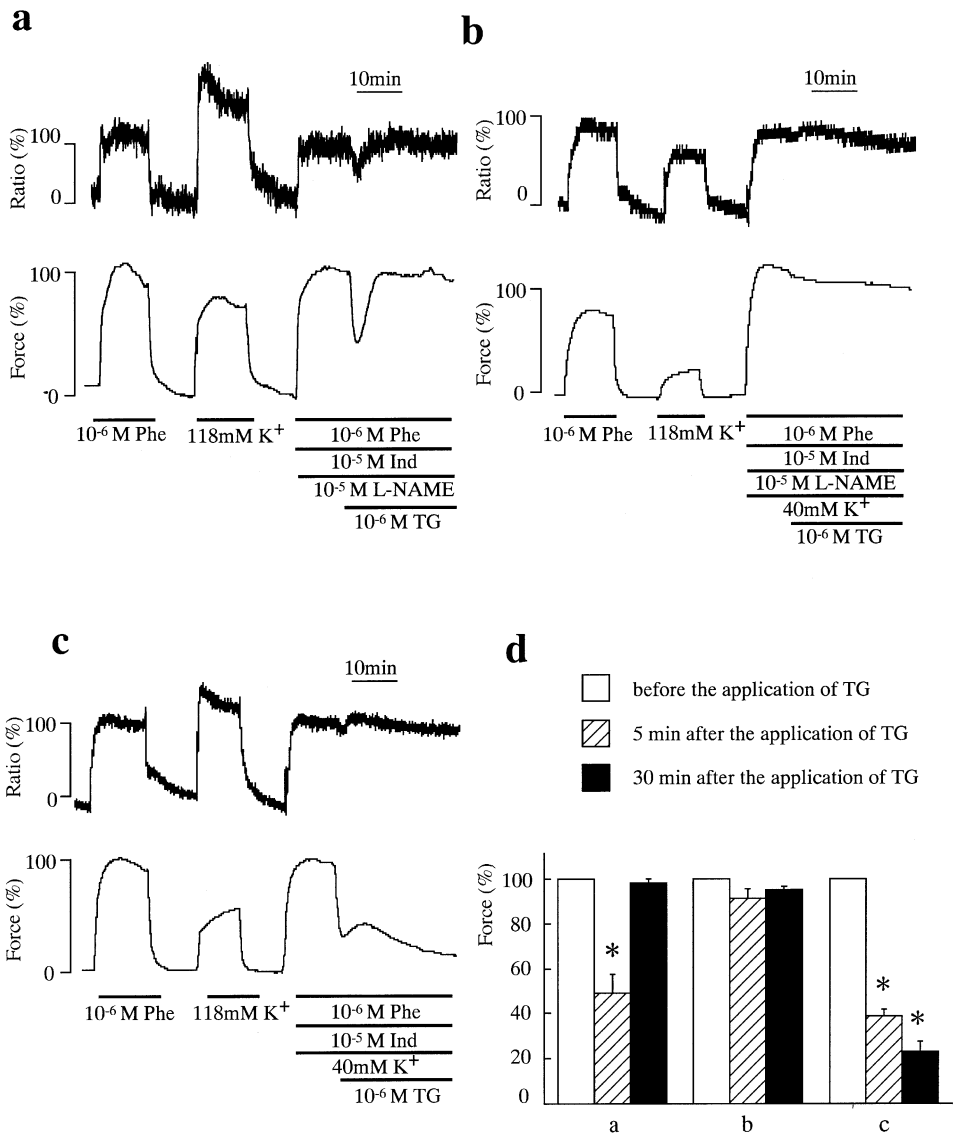

Figure 4 Involvement of nitric oxide and endothelium-derived hyperpolarizing factor in thapsigargin-induced relaxation in the porcine renal artery. Representative traces of changes in $[Ca^{2+}]_i$ and force induced by thapsigargin (TG) during the contraction induced by phenylephrine (phe) (a) or phenylephrine plus 40 mM K^+ (b) in the presence of 10^{-5} M indomethacin (Ind) and 3×10^{-5} M N^{ω}-nitro-L-arginine methylester (L-NAME), and during the contraction induced by phenylephrine plus 40 mM K^+ in the presence of 10^{-5} M Ind (c). (d) Summary on the level of forces just before, and 5 min and 30 min after the application of thapsigargin in protocol a, b, and c. The level of force at rest and just prior to the application of thapsigargin during the sustained contraction under each protocol were designated as 0 and 100%. All data are the mean±s.e.mean ($n=4$).

was not significantly different from that obtained with 10^{-6} M. The transient contraction was observed at 10^{-7} M and higher concentrations. The maximum increases in $[Ca^{2+}]_i$ ($142.1 \pm 7.0\%$, $n=6$) and force ($225.8 \pm 18.0\%$, $n=6$) were obtained with 10^{-6} M TG, while 10^{-5} M TG induced smaller increases in $[Ca^{2+}]_i$ ($118.4 \pm 6.0\%$, $n=6$) ($P<0.01$) and force ($132.1 \pm 9.4\%$, $n=6$) ($P<0.01$). A sustained relaxation was observed with 10^{-8} M, 10^{-6} M and 10^{-5} M TG. The levels of $[Ca^{2+}]_i$ and force obtained at 30 min after the application of 10^{-7} M TG did not differ from those for the control contraction induced by phenylephrine. The maximum relaxation ($50.7 \pm 3.8\%$, $n=6$) was obtained with 10^{-6} M. The relaxation induced by 10^{-5} M TG was similar to that obtained with 10^{-6} M TG. No significant decreases were observed in $[Ca^{2+}]_i$ level during the sustained relaxation at all concentrations examined. In the following experiments, we thus used 10^{-6} M TG to investigate the mechanisms of the TG-induced endothelium-dependent contractions and relaxations.

Cyclopiazonic acid (CPA), another inhibitor of endoplasmic Ca^{2+} ATPase (Demaurex *et al*., 1992; Schilling *et al*., 1992), induced similar changes in the $[Ca^{2+}]_i$ level and force to those seen with TG. When applied during phenylephrine-induced contraction, 3×10^{-5} M CPA produced an initial relaxation ($53.5 \pm 10.4\%$, $n=4$) followed by a transient contraction ($299.8 \pm 40.2\%$, $n=4$) and subsequently by a sustained relaxation ($42.9 \pm 11.6\%$, $n=4$).

Inhibition of TG-induced endothelium-dependent contraction by a cyclo-oxygenase inhibitor, a TXA_2/PGH_2 receptor antagonist, and a TXA_2 synthase inhibitor

To determine whether thromboxanes or other cyclo-oxygenase metabolites of arachidonic acid were involved in the TG-induced endothelium-dependent contractions, the effects of indomethacin (a cyclo-oxygenase inhibitor), ONO-3708 (a TXA_2/PGH_2 receptor antagonist), and OKY-046 (a TXA_2 synthase inhibitor) were examined (Figure 3). After recording the first control response to phenylephrine, the strips were treated with these agents just prior to the second phenylephrine-induced contractions. Treatment with 10^{-5} M indomethacin had no significant effect on the phenylephrine-induced $[Ca^{2+}]_i$ elevation and force (Figure 3a). The levels of $[Ca^{2+}]_i$ and force induced by 10^{-6} M phenylephrine in the presence of indomethacin were $89.7 \pm 4.4\%$ and $106.8 \pm 3.1\%$ ($n=4$) at 15 min after the stimulation by phenylephrine, respectively. In the presence of indomethacin, an addition of 10^{-6} M TG during phenylephrine-induced sustained contraction caused a rapid relaxation ($20.0 \pm 5.3\%$, $n=5$) with a transient decrease in $[Ca^{2+}]_i$ ($26.0 \pm 8.9\%$, $n=5$), followed by a sustained relaxation ($21.5 \pm 7.7\%$, $n=5$) with little decrease in $[Ca^{2+}]_i$ (Figure 3a). No apparent contraction was observed in the presence of indomethacin. The extents of the transient decrease in $[Ca^{2+}]_i$ and the decrease in force were significantly greater than those observed without pretreatment with indomethacin ($P<0.05$). Indomethacin apparently augmented the TG-induced relaxation while it completely abolished the TG-induced contraction.

Figure 3b shows the time courses of the endothelium-dependent responses induced by 10^{-6} M TG in the presence of 10^{-5} M ONO-3708. Treatment with 10^{-5} M ONO-3708, *per se*, had no effect on the phenylephrine-induced contraction (Figure 3b). ONO-3708 completely inhibited the TG-induced transient contraction as observed with indomethacin. The TG-induced changes in the $[Ca^{2+}]_i$ level and force during phenylephrine-induced contraction in the presence of ONO-3708 were similar to those observed in the presence of indomethacin. In the presence of ONO-3708, the addition of 10^{-6} M TG during phenylephrine-induced sustained contraction caused a rapid relaxation ($39.8 \pm 6.4\%$, $n=5$) with a transient decrease in $[Ca^{2+}]_i$ ($31.4 \pm 8.4\%$, $n=5$), followed by a sustained relaxation ($19.5 \pm 3.8\%$, $n=5$) with little decrease in $[Ca^{2+}]_i$ (Figure 3b). When the precontraction was caused by U46619, a TXA_2 mimetics, TG induced a transient relaxation but no contraction (data not shown). Collectively, these observations indicate that TXA_2 and/or PGH_2 are therefore involved in the TG-induced contraction.

To determine the relative contribution of TXA_2 and PGH_2 in TG-induced contraction, the effects of a TXA2 synthase inhibitor (OKY-046) were examined (Figure 3c). Treatment with 10^{-5} M OKY-046 had no direct effect on the phenylephrine-induced contraction (Figure 3c). In the presence of OKY-046, TG induced only a slowly developing sustained relaxation with no significant changes in $[Ca^{2+}]_i$. No apparent contraction was observed in the presence of OKY-046. However, in contrast to data using indomethacin and ONO-3708, the extent of the relaxation was not augmented by treatment with OKY-046. The level of $[Ca^{2+}]_i$ and force at 2 min after the stimulation with TG were $83.4 \pm 1.0\%$ and $66.9 \pm 2.8\%$ ($n=6$), respectively. These values were signifi-

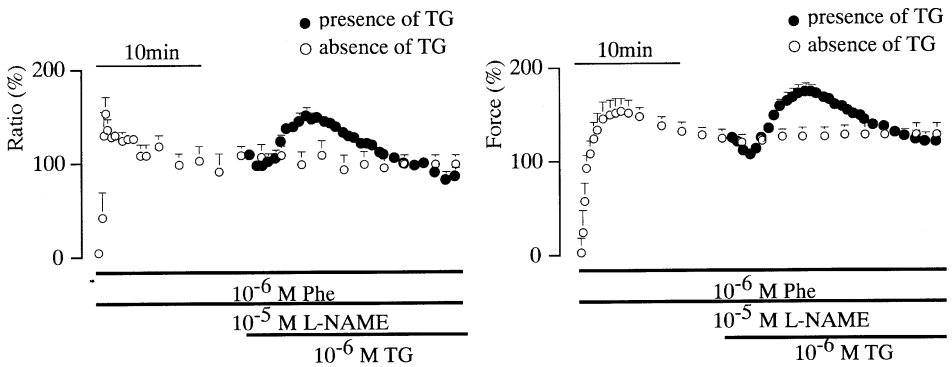

Figure 5 Effect of N^{ω}-nitro-L-arginine methylester on the endothelium-dependent relaxation induced by thapsigargin. After recording the reference response to 10^{-6} M phenylephrine (Phe), N^{ω}-nitro-L-arginine methylester (L-NAME) were added just prior to the initiation of precontraction by Phe. Phe-induced changes in $[Ca^{2+}]_i$ (left panel) and force (right panel) in the presence of L-NAME were shown. Thapsigargin (TG) was applied at 15 min after the initiation of the precontraction. The levels of $[Ca^{2+}]_i$ and force at rest and during Phe-induced sustained contraction were designated as 0 and 100%, respectively. All data are the mean ± s.e.mean ($n=6$).

cantly ($P<0.05$) higher than those obtained in the presence of indomethacin (Figure 3a) and ONO-3708 (Figure 3b), and did not significantly ($P>0.05$) differ from those obtained in the absence OKY-046 (Figure 2a). The extent of the decrease in force was $61.3\pm6.1\%$ ($n=6$) at 30 min after the application of TG. This value did not significantly ($P>0.05$) differ from that obtained in the absence of OKY-046 (Figure 2c).

Relative contribution of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) to the TG-induced endothelium-dependent relaxation

To examine the contribution of nitric oxide and endothelium-derived hyperpolarizing factor to the TG-induced relaxation, the effects of L-NAME and of elevation of external K^+ concentration on the relaxation were examined under conditions in which the TG-induced contraction was inhibited by 10^{-5} M indomethacin (Figure 4). In the presence of 10^{-5} M L-NAME, TG induced only a transient, initial relaxation during the phenylephrine-induced contraction (Figure 4a). Compared with Figure 3a, L-NAME markedly inhibited the sustained phase of the TG-induced relaxation. However, this transient relaxation was completely abolished when the external K^+ concentration was elevated from 5.9 to 40 mM (Figure 4b). Elevation of external K^+ concentration to 30 mM partially inhibited the TG-induced transient relaxation observed in the presence of L-NAME and indomethacin (data not shown). Removing L-NAME caused a re-appearance of the sustained phase of the TG-induced relaxation during the contraction initiated by phenylephrine plus 40 mM K^+ in the presence of indomethacin (Figure 4c). Treating tissues with L-NAME augmented the phenylephrine-induced force development ($163.4\pm12.2\%$, $n=4$) in the presence of 10^{-5} M indomethacin, while it had little effect on the $[Ca^{2+}]_i$ elevation ($98.8\pm1.9\%$, $n=4$). In the presence of indomethacin, the force development induced by phenylephrine was also augmented by 40 mM K^+ ($124.4\pm13.7\%$, $n=4$), while the $[Ca^{2+}]_i$ elevation obtained in the presence of 40 mM K^+ was similar to that obtained in its absence. Therefore, the effects of L-NAME and high K^+ were evaluated by assigning the level of force obtained just before the application of TG as 100% in each experimental condition (Figure 4d). In the presence of L-NAME and indomethacin, the force decreased to $49.1\pm8.2\%$ ($n=4$) at 5 min after the application of TG during the phenylephrine-induced contraction, while it returned to the level obtained before the application of TG at 30 min ($97.8\pm2.2\%$, $n=4$). In the presence of L-NAME and 40 mM K^+, no significant decrease in force was obtained either at 5 min ($91.0\pm4.7\%$, $n=4$) or at 30 min ($94.9\pm1.5\%$, $n=4$) after the application of TG.

Figure 5 shows the effects of L-NAME alone on the TG-induced response without inhibiting the contractile component. Treatment with 10^{-5} M L-NAME augmented phenylephrine-induced force development ($136.5\pm8.6\%$, $n=4$) with little effect on the $[Ca^{2+}]_i$ elevation ($105.7\pm9.1\%$, $n=4$). L-NAME markedly inhibited the decrease in $[Ca^{2+}]_i$ and force of the first relaxation induced by TG. The level of $[Ca^{2+}]_i$ was $90.7\pm7.6\%$ ($n=6$) and the level of force was $118.0\pm5.1\%$ ($n=6$). A transient contraction was observed, however, no sustained relaxation was seen. The levels of $[Ca^{2+}]_i$ and force of the contraction ($139.5\pm6.0\%$ and $185.6\pm7.5\%$, $n=6$, respectively) was not significantly different from those obtained without L-NAME ($[Ca^{2+}]_i$, $142.1\pm7.0\%$; force, $225.8\pm18.0\%$, Figure 2). The duration of the contraction was thus apparently extended.

The effect of TG on the $[Ca^{2+}]_i$ level of endothelial cells in primary culture

Cultured endothelial cells obtained from porcine renal artery were stimulated with 10^{-5} M ATP every 15 min until the similar steady response was obtained (Miyagi *et al*., 1996). In the presence of extracellular Ca^{2+}, 10^{-6} M TG caused a rapid increase in $[Ca^{2+}]_i$, reaching its first peak ($388.9\pm75.1\%$, $n=3$) at 4.5 ± 0.10 min (Figure 6a). After a slight dip, the $[Ca^{2+}]_i$ reached a second peak ($403.3\pm70.7\%$, $n=3$) at 15.5 ± 4.5 min, followed by gradual decline. The $[Ca^{2+}]_i$ level at 30 min was $277.0\pm84.0\%$. Applying indomethacin, ONO-3708, OKY-046 or L-NAME during the sustained phase of the TG-induced $[Ca^{2+}]_i$ elevation had no effect on the changes in $[Ca^{2+}]_i$ (data not shown). CPA also caused a large sustained increase in $[Ca^{2+}]_i$. The levels of $[Ca^{2+}]_i$ at 5 min, the peak (11.0 ± 1.2 min, $n=3$) and 30 min were $160\pm7.5\%$, $294\pm51.3\%$, and $177.7\pm44.7\%$ ($n=3$), respectively. Figure 6b summarizes the time course of $[Ca^{2+}]_i$ elevation induced by various concentrations of TG (10^{-8}–10^{-5} M). A slight increase in $[Ca^{2+}]_i$ was observed at 10^{-8} M. At 10^{-7} M, TG induced $[Ca^{2+}]_i$ elevation

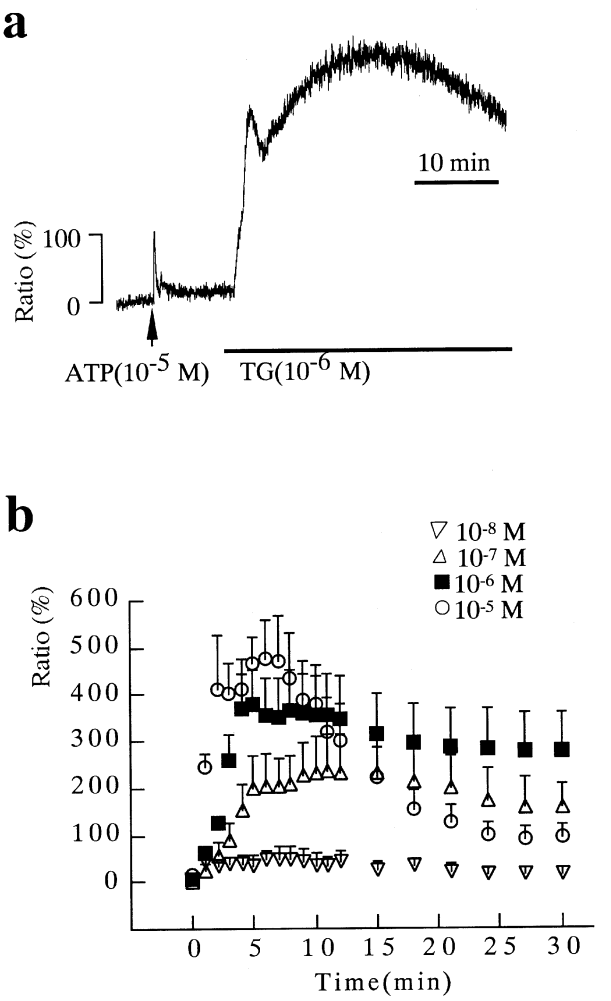

Figure 6 Ca^{2+} transients induced by thapsigargin (TG) in endothelial cells of renal artery in primary culture. (a) The representative recording of changes in $[Ca^{2+}]_i$ induced by 10^{-6} M TG in endothelial cells of renal artery in primary culture. The responses to 10^{-5} M ATP were recorded as control reference (100%) and TG was applied 15 min later. (b) The time course of changes in $[Ca^{2+}]_i$ in endothelial cells in primary culture induced by 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M TG. $[Ca^{2+}]_i$ levels at rest and at peak response to 10^{-5} M ATP were designated as 0 and 100%, respectively. All data are the mean±s.e.mean ($n=3$).

with a single peak (232.2±80.0%, $n=3$) at 9.7±1.1 min, followed by the slightly declining sustained $[Ca^{2+}]_i$ elevation. The $[Ca^{2+}]_i$ level at 30 min was 158.1±51.3%. At 10^{-5} M, $[Ca^{2+}]_i$ rapidly reached the first peak (472.7±103.4%, $n=3$) at 2.3±0.33 min and then the second peak (519.9±77.4%, $n=3$) at 6.1±0.56 min. However, the $[Ca^{2+}]_i$ elevation induced by 10^{-5} M TG thereafter rapidly declined, and the $[Ca^{2+}]_i$ level at 30 min was 94.3±27.8%.

Discussion

Vascular endothelial cells play an important role in the regulation of vascular tone by producing both relaxing and contracting factors (Rubanyi, 1991; Vanhoutte & Eber, 1991). Endothelium-dependent relaxation has been well documented in many species and types of blood vessel (Shepherd & Katusic, 1991). In contrast, reports on endothelium-dependent contraction are limited to specific combinations of agonist and types of blood vessels (Katusic & Shepherd, 1991; Luscher & Vanhoutte, 1986). There are few reports showing that both relaxation and contraction may be induced by the same stimulus in one tissue. In the present study, we demonstrated that TG caused both an endothelium-dependent relaxation and contraction in the porcine renal artery. We also demonstrated characteristic changes in $[Ca^{2+}]_i$ accompanied by this endothelium-dependent alteration of contractile state of smooth muscle. Both TG and CPA induced similar endothelium-dependent triphasic responses composed of an initial relaxation, a subsequent transient contraction and a sustained relaxation. Both TG and CPA also induced large increases in $[Ca^{2+}]_i$ in cultured endothelial cells of the porcine renal artery, at the same concentrations as those required to produce endothelium-dependent relaxation and contraction. These findings suggest that the thapsigargin-induced $[Ca^{2+}]_i$ elevation of endothelial cells was associated with the triphasic changes in force in the porcine renal artery.

TXA_2, PGH_2, endothelin and histamine are possible mediators of endothelium-dependent contraction (Dai *et al*., 1992; Gruetter *et al*., 1994; Katusic & Shepherd, 1991). Endothelium-dependent contractions have been reported with nicotine in the canine cerebral artery (Shirahase *et al*., 1998a), angiotensin I and angiotensin II in the canine cerebral artery (Manabe *et al*., 1989), arachidonic acid and methacholine in the rabbit pulmonary artery (Buzzard *et al*., 1993), and somatostatin in the canine cerebral artery (Shirahase *et al*., 1993). These studies suggested that TXA_2 mediated the endothelium-dependent contractions. On the other hand, it was suggested that PGH_2 might mediate endothelium-dependent contraction induced by acetylcholine in rat aorta because a TXA_2/PGH_2 receptor antagonist (ONO-3708), but not a TXA_2 synthase inhibitor (OKY-046), inhibited the contraction (Kato *et al*., 1990). In the present study, a cyclooxygenase inhibitor (indomethacin), a TXA_2/PGH_2 receptor antagonist (ONO-3708) and a TXA_2 synthase inhibitor (OKY-046) all inhibited TG-induced contraction. These findings suggest that TXA_2 is a major mediator of TG-induced endothelium-dependent contraction in the porcine renal artery. This is consistent with the finding that TG failed to produce any contraction when precontraction was initiated with U46619. However, the TG-induced relaxation was apparently augmented by indomethacin and ONO-3708, but not by OKY-046. This observation suggests that PGH_2 also contributed to TG-induced contraction, since OKY-046 is expected to eliminate TXA_2 but indomethacin and ONO-3708 could eliminate contributions by both TXA_2 and PGH_2. In the porcine renal artery, PGH_2, *per se*, caused as large a contraction as U46619 (data not shown). It is thus concluded that PGH_2 production plays a minor role in mediating TG-induced contraction. Endothelin is also known to be a potent endothelium-derived vasoconstrictor, and endothelin-1 has been shown to induce contraction mediated by ET_A receptor in the renal artery (Brooks *et al*., 1994; Clark & Pierre, 1995). In porcine renal artery, endothelin-1 caused a contraction, which was abolished by a selective ET_A receptor antagonist BQ-123 (RBI, Natick, MA, U.S.A.). However, BQ-123 had no effect on the TG-induced contraction (data not shown), thus ruling out the involvement of endothelin.

EDRF/NO, EDHF and prostacyclin are the three major mediators of endothelium-dependent relaxation (Shepherd & Katusic, 1991). It is unlikely that prostacyclin plays a major role in the TG-induced relaxation in porcine renal artery, because indomethacin not only inhibited TG-induced contraction but also augmented TG-induced relaxation. The observation that the effect of indomethacin was almost identical to that seen with ONO-3708, the TXA_2/PGH_2 receptor antagonist also excludes the involvement of prostacyclin in TG-induced relaxation. Therefore, we examined the mechanism of TG-induced relaxation under conditions in which the contractile component was inhibited by indomethacin. The TG-induced transient relaxation observed during phenylephrine-induced contraction in the presence of L-NAME and indomethacin indicates the involvement of relaxing factors other than NO in the initial relaxation. The absence of this transient initial relaxation during the phenylephrine-induced contraction in the presence of 40 mM K^+ strongly suggests that such NO-independent relaxation was due to EDHF. It has been reported that an elevation of the external K^+ concentration inhibits hyperpolarization due to EDHF in rat artery (Chen & Suzuki, 1989). These authors reported that 30 mM K^+ was sufficient to cause complete inhibition. In the present study, 40 mM K^+ was required to obtain a complete inhibition of the TG-induced transient relaxation. On the other hand, removal of L-NAME from the medium as shown in Figure 4b caused a re-appearance of the sustained relaxation, and L-NAME inhibited the sustained relaxation (Figure 5). These observations suggest that the TG-induced sustained relaxation was mainly due to NO. Furthermore, examining the $[Ca^{2+}]_i$-force relationship during relaxation indicates that the Ca^{2+} sensitivity of the contractile apparatus decreased during sustained relaxation. This observation is 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). Such time-dependent differences in the relative contribution of NO and EDHF in the TG-induced relaxation are similar to those observed in the CPA-induced endothelium-dependent relaxation in the porcine coronary artery (Higuchi *et al*., 1996).

Regarding the effects of OKY-046 (Figure 3c) and L-NAME (Figure 5), there are possibilities other than the involvement of contracting factors such as TXA_2 and PGH_2. The inhibition of the initial fall in $[Ca^{2+}]_i$ by OKY-046 may also be due to the inhibition of cytochrome P450 (Morita *et al*., 1988). It was suggested that epoxyeicosatrienoic acids, metabolites formed by cytochrome P-450 are EDHF (Campbell *et al*., 1996). It is thus possible that OKY-046 inhibited the productions of, not only TXA_2, but also EDHF, thus causing inhibition of the initial fall in $[Ca^{2+}]_i$ and relaxation. Regarding the effect of L-NAME, it is not only due to the inhibition of NO production and the contribution of

contractile component, but also due to the enhancement of production of TXA_2 and PGH_2. It was reported that NO inhibited the activity of cyclo-oxygenase (Kanner *et al*., 1992) and TXA_2 synthase (Wade & Fitzpatrick, 1997). Therefore, it is possible that cyclo-oxygenase activity and the resulting production of TXA_2 and PGH_2 may have been augmented when NO production was inhibited.

The physiological role of endothelium-dependent triphasic regulation of smooth muscle contraction, especially the role of endothelium-dependent contraction is not clear at this moment. Endothelium-dependent contraction has, up to now, generally been reported in relation to pathophysiological conditions. It has been observed in spontaneously hypertensive rats (Boulanger *et al*., 1994; Boulanger & Vanhoutte, 1993; Kato *et al*., 1990; Koga *et al*., 1989; Luscher & Vanhoutte, 1986) but not in normotensive Wistar-Kyoto rats (Diederich *et al*., 1990; Fu-Xiang *et al*., 1992). Hypercholesterolemia (Bank & Aynedjian, 1992; Kaplan *et al*., 1990) and high glucose (Tesfamariam *et al*., 1990) have also been reported to promote endothelium-dependent contraction. These findings suggested that the production of contracting factors may occur in various pathological states, such as hypertension, diabetes and hypercholesterolemia (Katusic & Shepherd, 1991). On the other hand, most of the reports on endothelium-dependent contraction are limited to the cerebral artery and renal artery (Nishimura *et al*., 1995; Shirahase *et al*., 1988a, c; 1993; Usui *et al*., 1993). These arteries are known to show an autoregulation of blood flow (Aukland, 1989; Bugge *et al*., 1991; Wahl & Schilling, 1993). Endothelial cells, perivascular nerves or autacoids may be involved in such autoregulation (Wahl & Schilling, 1993). Therefore, the physiological role of endothelium-dependent contraction may possibly be associated with autoregulation of blood flow.

In conclusion, TG caused endothelium-dependent triphasic responses in the porcine renal artery, comprising an initial transient relaxation, a subsequent contraction and a late sustained relaxation. The initial relaxation and transient contraction were associated with a decrease and an increase in $[Ca^{2+}]_i$, respectively, while the sustained relaxation was not associated with any apparent $[Ca^{2+}]_i$ decrease. The decrease in the Ca^{2+} sensitivity of the contractile apparatus thus plays a major role in sustained relaxation. The endothelium-dependent contraction is mediated mainly by TXA_2 and partly by PGH_2. Both EDHF and NO are involved in the initial relaxation while NO is involved in the sustained relaxation. The thapsigargin-induced $[Ca^{2+}]_i$ elevation in endothelial cells may thus play an important role in the regulation of vascular tone by producing both contracting and relaxing substances.

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