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Thapsigargin- and cyclopiazonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery

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1 The present study was designed to determine whether putative, selective inhibitors of the Ca^{2+}-pump ATPase of endoplasmic reticulum, thapsigargin (TSG) and cyclopiazonic acid (CPA), induce endothelium-dependent hyperpolarization in the rat isolated mesenteric artery. The membrane potentials of smooth muscle cells of main superior mesenteric arteries were measured by the microelectrode technique.

2 In tissues with endothelium, TSG ($10^{-8}-10^{-5}$ M) caused sustained hyperpolarization in a concentration-dependent manner. In tissues without endothelium, TSG did not cause any change in membrane potential. CPA (10^{-5} M) also hyperpolarized the smooth muscle membrane, an effect that was endothelium-dependent and long-lasting.

3 The hyperpolarizing responses to these agents were not affected by indomethacin or N^G-nitro-L-arginine (L-NOARG).

4 In Ca^{2+}-free medium, neither TSG nor CPA elicited hyperpolarization, in contrast to acetylcholine which generated a transient hyperpolarizing response.

5 In rings of mesenteric artery precontracted with phenylephrine, TSG and CPA produced endothelium-dependent relaxations. L-NOARG significantly inhibited the relaxations to these agents, but about 40-60% of the total relaxation was resistant to L-NOARG. The L-NOARG-resistant relaxations were abolished by potassium depolarization.

6 These results indicate that TSG and CPA can cause endothelium-dependent hyperpolarization in rat mesenteric artery possibly by releasing endothelium-derived hyperpolarizing factor and that membrane hyperpolarization can contribute to the endothelium-dependent relaxations to these agents. The mechanism of hyperpolarization may be related to increased Ca^{2+} influx into endothelial cells triggered by depletion of intracellular Ca^{2+} stores due to inhibition of endoplasmic reticulum Ca^{2+}-pump ATPase activity.

Keywords: Thapsigargin; cyclopiazonic acid; acetylcholine; endothelium; hyperpolarization; vasorelaxation; vascular smooth muscle

Introduction

Endothelial cells modulate the tone of underlying vascular smooth muscle cells by releasing prostacyclin (Moncada *et al.*, 1977), endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) and contracting factors (Lüscher *et al.*, 1992). EDRF is now considered to be identical to nitric oxide (NO) (Palmer *et al.*, 1987) or to a related nitroso compound (Myers *et al.*, 1990). Endothelial cells also mediate hyperpolarization of smooth muscle cells in response to agents such as acetylcholine (Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). Some electrophysiological studies have demonstrated that endothelium-dependent hyperpolarization does not appear to be mediated by NO (Bény & Brunet, 1988; Garland & McPherson, 1992), suggesting the existence of another biologically-active, endothelium-derived factor. The yet undefined factor causing membrane hyperpolarization has been named endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Furchgott & Vanhoutte, 1989; Suzuki *et al.*, 1992).

Thapsigargin (TSG) is a tumour-promoting agent extracted from the umbelliferous plant *Thapsia garcanica* (Christensen *et al.*, 1981) which has been shown to inhibit selectively the Ca^{2+}-pump ATPase of endoplasmic reticulum in a variety of tissues (Thastrup *et al.*, 1990). TSG causes endothelium-dependent relaxation in rat aorta (Mikkelsen *et al.*, 1988; Zheng *et al.*, 1994) and in guinea-pig aorta (Matsuyama *et al.*, 1993). TSG elevates cytosolic Ca^{2+} concentration in endothelial cells (Gericke *et al.*, 1993) and the release of EDRF is dependent on intracellular Ca^{2+} (Singer & Peach, 1982). These findings suggest that the increase in cytosolic Ca^{2+} concentration in endothelial cells may be responsible for the TSG-induced endothelium-dependent relaxation.

Increased cytosolic Ca^{2+} concentration in endothelial cells has also been proposed as an obligatory step in the production and/or release of EDHF (Chen & Suzuki, 1990). Thus, TSG might induce endothelium-dependent hyperpolarization by releasing EDHF. To assess this possibility, the present study was designed to investigate the effect of TSG on the membrane potential of smooth muscle cells in rat isolated mesenteric arteries. We also examined the effect of cyclopiazonic acid (CPA), another selective inhibitor of endoplasmic reticulum Ca^{2+} pump ATPase (Seidler *et al.*, 1989; Georger *et al.*, 1988) in order to determine whether the observed electrical effect of TSG, if any, is attributed to the selective inhibition of the enzyme, and compared the effects of TSG and CPA with those of acetylcholine (Chen & Suzuki, 1990). We found that both TSG and CPA elicited endothelium-dependent hyperpolarization. Further experiments were carried out to assess the contribution of membrane hyperpolarization to endothelium-dependent relaxations induced by these agents.

Methods

Electrophysiological experiments

Male Wistar rats between 10 and 15 weeks old and weighing 250-340 g, were anaesthetized with diethyl ether. The main branch of the superior mesenteric arteries was excised carefully

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and placed on a plate containing oxygenated physiological salt solution (PSS) at room temperature. The arteries were dissected free from surrounding tissues, cut into rings 3-mm in length, and opened longitudinally. Care was taken to ensure that the endothelial cell layer was not damaged during tissue preparation. Where indicated, the endothelial cells were removed by gently rubbing the intimal surface of the vessel with a moistened cotton ball. The tissue was pinned down, intimal side upward, on the bottom of an organ chamber (capacity 3 ml), and superfused at a constant flow rate of 7 ml min^{-1} with normal PSS aerated with 95% O_2 and 5% CO_2. The temperature of the perfusate was kept constant at 37°C. Normal PSS contained the following (mM); NaCl 118.2, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 10.0. For Ca^{2+}-free PSS, Ca^{2+} was omitted and 0.2 mM EGTA was added. After the preprations were allowed to equilibrate for 60 min, glass microelectrodes filled with 3 M KCl (tip resistance 40–80 MΩ) were inserted into the smooth muscle cells from the intimal surface. Electrical signals were monitored continuously on an oscilloscope (Nihon Kohden, VC-10, Tokyo, Japan) and recorded on a chart recorder (Watanabe Sokki, WR3101, Tokyo, Japan). For data analysis we used only the experiments in which a single impalement was maintained.

Mechanical experiments

Rat mesenteric arterial rings were prepared as described above. Each ring was suspended by a pair of stainless steel hooks in a water-jacketed bath filled with 25 ml of normal PSS. The solution in the bath was gassed with 95% O_2 and 5% CO_2 and its temperature was maintained at 37°C. The rings were stretched until a resting tension of 1 g was reached and then allowed to equilibrate for 60 min. Force generation was monitored using an isometric transducer (Sanei-Sokki, 45196, Tokyo, Japan) and a carrier amplifier (Sanei-Sokki, 1236). The output of the force transducer was registered on a pen recorder (TOA Electronics, ERP-241A, Tokyo, Japan) through a polygraph recorder (Sanei-Sokki, 142-8).

After the equilibration period, the rings were exposed several times to high K^+ PSS (40 mM $[K^+]_0$) until reproducible contractile responses were obtained. High K^+ PSS was made by substituting NaCl with equimolar KCl. The vessels were then precontracted with 10^{-6} M phenylephrine. After the contraction had reached a plateau level, 10^{-6} M TSG or 10^{-5} M CPA was applied. When 3×10^{-5} M N^G-nitro-L-arginine (L-NOARG) was used in order to exclude the involvement of EDRF-NO in the responses to TSG and CPA, it was added 15 min before the addition of phenylephrine. Because pretreatment with L-NOARG markedly enhanced the phenylephrine-induced contractions, care was taken to match the contractions induced by phenylephrine in control and pretreated rings. Thus, in the experiments with L-NOARG, the tissues were precontracted with 10^{-7} M phenylephrine. Relaxations were expressed as a percentage of the height of the contraction induced by phenylephrine.

Drugs

The compounds used were as follows: TSG (Calbiochem-Novabiochem Corp., San Diego, CA, U.S.A.); CPA, L-NOARG, indomethacin and (−)-phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); and acetylcholine chloride (Wako Pure Chemical Industries, Osaka, Japan). TSG was prepared as a stock solution (2×10^{-2} M) in dimethyl sulphoxide and diluted in ethanol. CPA was dissolved in dimethyl sulphoxide. L-NOARG and indomethacin were prepared in 0.2 N HCl and 50 mM Tris, respectively. Other chemicals were dissolved in distilled water. Further dilutions were made with PSS.

Statistical analysis

All values are presented in terms of the means ± s.e.mean. Analysis by Student's t test was performed for paired and unpaired observations. P values less than 0.05 were considered significant.

Results

Endothelium-dependent hyperpolarization by acetylcholine

The resting membrane potentials of vascular smooth muscle cells in preparations with endothelium averaged −55.5 ± 0.4 mV ($n=68$). Removal of the endothelium produced no significant change in the resting membrane potential (−56.0 ± 1.5 mV, $n=5$, $P>0.5$). When the preparations were exposed to Ca^{2+}-free PSS, the resting membrane potentials were gradually depolarized (see Figures 1b, 4b and 5b). The membrane potentials were depolarized by 8 ± 1 and 16 ± 2 mV ($n=10$, $P<0.001$) at 5 and 10 min after exposure to Ca^{2+}-free PSS, respectively.

In tissues with intact endothelium, application of 10^{-6} M acetylcholine promptly hyperpolarized the membrane potential (Figure 1a). The hyperpolarizing response to acetylcholine was composed of two components as previously reported (Chen & Suzuki, 1990); i.e., an initial transient and following sustained phase. The time required to reach the peak amplitude of the initial transient component was within 10 s. The sustained component reached maximum amplitudue at 20–60 s after the addition of acetylcholine, and decayed with a very slow time course. In Ca^{2+}-free PSS, acetylcholine produced only a transient hyperpolarizing effect (Figure 1b). After the endothelium was removed, acetylcholine no longer caused hyperpolarization (data not shown).

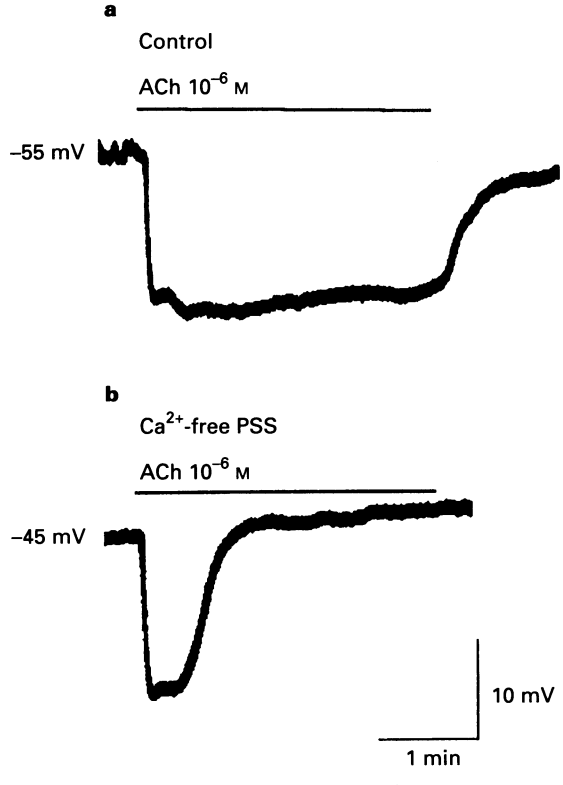

Figure 1 Effect of acetylcholine (ACh, 10^{-6} M) on cell membrane potential of rat mesenteric artery with endothelium in normal PSS (a) and in Ca^{2+}-free PSS (b). ACh was applied 5 min after exposure to Ca^{2+}-free PSS. Traces were recorded in different tissues.

Endothelium-dependent hyperpolarization of TSG and CPA

Figure 2 shows typical membrane potential changes elicited by TSG at different concentrations in the presence of endothelial cells. Application of TSG in concentrations less than 3×10^{-9} M caused no detectable change in the membrane potentials. At concentrations of 10^{-8} M or higher, TSG produced a gradual, progressive hyperpolarization of the membrane potential in a concentration-dependent manner. The time required to reach a peak effect after the addition of TSG was much longer than that after the addition of acetylcholine. Figure 3 summarizes the concentration-dependent hyperpolarizing effect of TSG on the membrane potential of smooth muscle cells of rat mesenteric arteries. TSG hyperpolarized by -18.3 ± 0.9 mV ($n=4$, $P<0.001$) at a concentration of 10^{-6} M, which was required to reach the maximum amplitude. In tissues without an endothelium, 10^{-6} M TSG produced no significant change in the membrane potential ($n=4$, Figure 4a). The hyperpolarizing effect of 10^{-6} M TSG was not observed in Ca^{2+}-free PSS even in endothelium-intact tissues ($n=4$, Figure 4b).

Figure 5 shows the typical membrane potential changes elicited by CPA. CPA also produced sustained membrane hyperpolarization of smooth muscle cells (Figure 5a), an effect not observed in tissues from which the endothelium had been removed ($n=4$). CPA at a concentration of 10^{-5} M hyperpolarized the membrane potential by -16.2 ± 1.2 mV ($n=5$, $P<0.001$). However, CPA caused no hyperpolarization in Ca^{2+}-free PSS even if the endothelium was intact ($n=4$, Figure 5b).

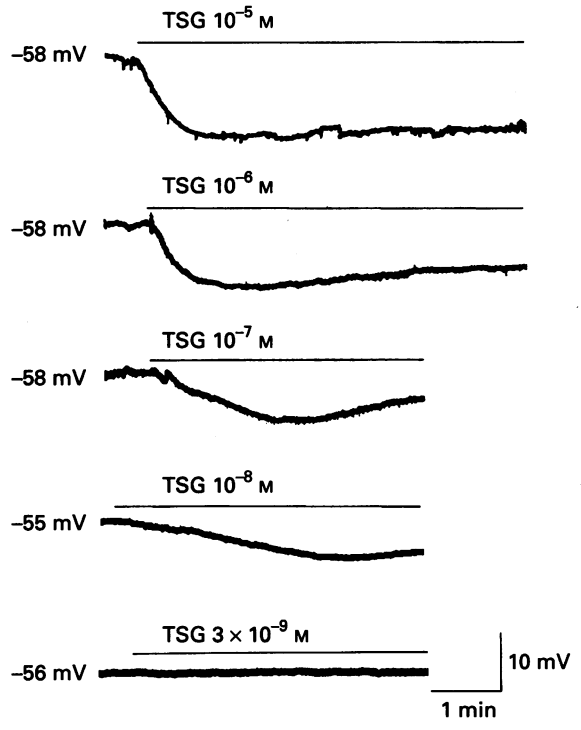

Figure 2 Effect of thapsigargin (TSG) at different concentrations on cell membrane potential of rat mesenteric artery with endothelium. Each trace is typical of the effect of that concentration of TSG.

Influence of L-NOARG and indomethacin on endothelium-dependent hyperpolarization

In order to assess the possible involvement of endogenous vasoactive prostanoids and EDRF-NO in the endothelium-dependent hyperpolarization, the influence of indomethacin and L-NOARG on the responses to acetylcholine, TSG and CPA was examined. Neither indomethacin nor L-NOARG affected the resting membrane potential. The maximal amplitude of the hyperpolarizing effect elicited by 10^{-6} M TSG was not affected by pretreatment of the tissues with either 10^{-5} M indomethacin or 3×10^{-5} M L-NOARG (Figure 6). Similarly, pretreatment with indomethacin or L-NOARG had no influence on the hyperpolarizing responses to 10^{-6} M acetylcholine and 10^{-5} M CPA ($n=4$, for each group).

Endothelium-dependent relaxation by TSG and CPA

As shown in Figure 7, 10^{-6} M TSG induced marked relaxation in the endothelium-intact arterial ring preparations which had been precontracted with phenylephrine. In the presence of 3×10^{-5} M L-NOARG, the relaxant response to TSG was significantly reduced (control $93 \pm 5\%$, and L-NOARG treated $53 \pm 4\%$; $P<0.001$). The intriguing finding was that even in the presence of L-NOARG, TSG produced transient relaxations (Figures 7 and 8a). In the arterial rings precontracted with phenylephrine after both high K^+ PSS (25 mM) and L-NOARG were applied, TSG no longer caused relaxations (Figure 8b). Removal of the endothelium virtually abolished the relaxant responses to TSG regardless of whether L-NOARG was present (Figure 8c). CPA at 10^{-5} M also caused endothelium-dependent relaxations of the phenylephrine-precontracted arterial rings (Figure 7). Pretreatment with L-NOARG attenuated the relaxant effect induced by CPA. However, a small but substantial relaxant response to CPA was still observed even in the presence of L-NOARG.

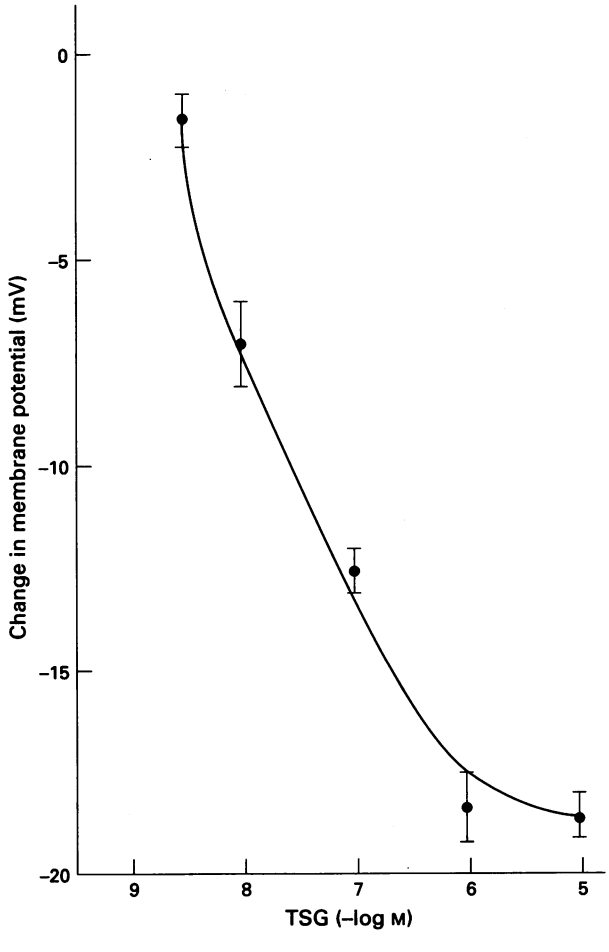

Figure 3 Concentration-response curve for the hyperpolarizing effect of thapsigargin (TSG) in rat mesenteric artery with endothelium. The points are the means ± s.e.mean of four to five experiments.

Discussion

The present study demonstrated that TSG and CPA each caused sustained endothelium-dependent hyperpolarization in rat mesenteric arteries. Recently, it has been shown that TSG releases NO from endothelial cells and thereby induces relaxation of guinea-pig aorta precontracted with phenylephrine (Matsuyama *et al.*, 1993). Exogenous NO produces membrane hyperpolarization of smooth muscle cells of rat small mesenteric artery (Garland & McPherson, 1992) and of guinea-pig uterine artery (Tare *et al.*, 1990), although the hyperpolarizing effect of NO could be blocked by prior depolarization (Garland & McPherson, 1992). In the present experiments, however, NO released by TSG and CPA seems an unlikely candidate to mediate endothelium-dependent hyperpolarization, because the hyperpolarizing responses to both agents were insensitive to L-NOARG, an inhibitor of NO synthase (Moore *et al.*, 1990). The production of vasoactive prostanoids is not likely to contribute to the endothelium-dependent hyperpolarizing responses. Although prostacyclin has been reported to produce hyperpolarization of smooth muscle cells in canine carotid artery (Siegel *et al.*, 1989), the magnitude and time course of hyperpolarization induced by TSG and CPA remained unchanged by pretreatment with indomethacin. Hence, another substance released from the endothelial cells, presumably EDHF, is likely to account for endothelium-dependent hyperpolarization elicited by TSG and CPA.

In rat mesenteric arterial rings precontracted with phenylephrine, TSG and CPA caused endothelium-dependent relaxations. The relaxant responses to these agents, which were observed in the presence of indomethacin, were significantly reduced by pretreatment with L-NOARG. It thus appears that EDRF-NO partly contributes to the endothelium-dependent relaxant responses to TSG and CPA. However, the major part of the vasorelaxant responses was resistant to L-NOARG. The possibility that the relaxation in the presence of L-NOARG was caused by residual NO, which was not blocked by L-NOARG at the concentration used in this study, is unlikely,

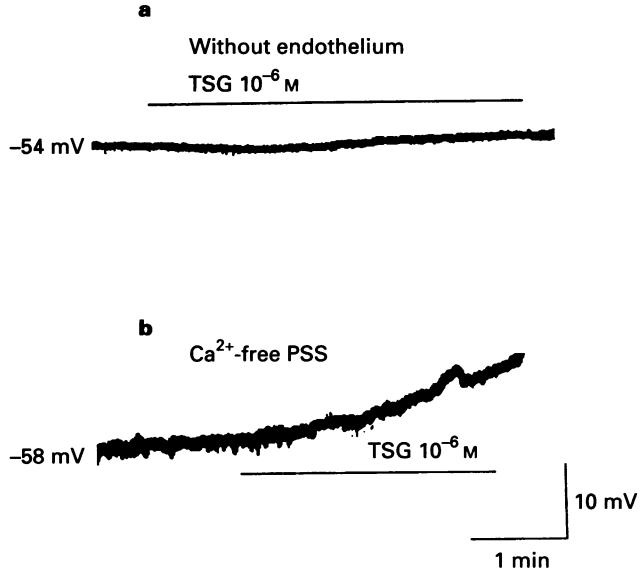

Figure 4 Influence of removal of endothelium (a) and exposure to Ca^{2+}-free PSS (b) on the effect of 10^{-6} M thapsigargin (TSG) on cell membrane potential of rat mesenteric artery. The experiment with Ca^{2+}-free PSS was performed using a tissue with endothelium intact. TSG was applied 5 min exposure to Ca^{2+}-free PSS.

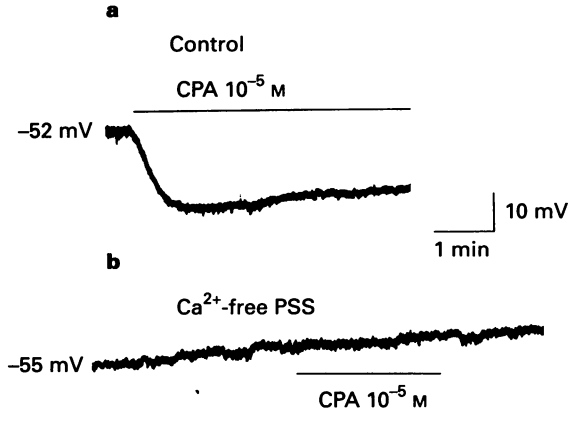

Figure 5 Effect of cyclopiazonic acid (CPA, 10^{-5} M) on cell membrane potential of rat mesenteric artery with endothelium in normal PSS (a) and in Ca^{2+}-free PSS (b). CPA was applied 5 min after exposure to Ca^{2+}-free PSS. Traces were recorded in two different tissues.

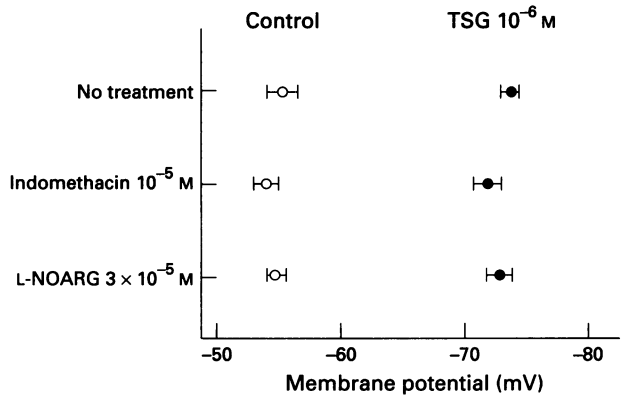

Figure 6 Influences of pretreatment with 10^{-5} M indomethacin or 3×10^{-5} M N^G-nitro-L-arginine (L-NOARG) on membrane hyperpolarization produced by 10^{-6} M thapsigargin (TSG) in rat mesenteric artery. TSG was applied 15–30 min after the addition of indomethacin or L-NOARG. Membrane potentials and peak amplitude of hyperpolarization are expressed as the mean ± s.e.mean of four experiments.

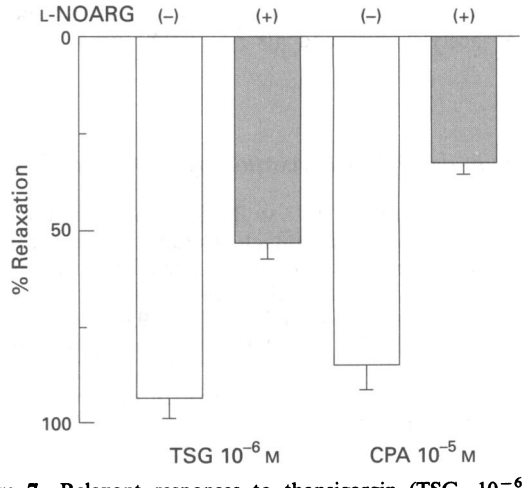

Figure 7 Relaxant responses to thapsigargin (TSG, 10^{-6} M) and cyclopiazonic acid (CPA, 10^{-5} M) in rat mesenteric artery with endothelium in the absence and presence of N^G-nitro-L-arginine (L-NOARG 3×10^{-5} M). The arterial rings were precontracted with phenylephrine at 10^{-7} M (with L-NOARG) or 10^{-6} M (without L-NOARG). L-NOARG was added to the bath 15 min before a precontraction was elicited. The values are shown as the means ± s.e.mean of four to six experiments. All experiments were performed in the presence of 10^{-5} M indomethacin.

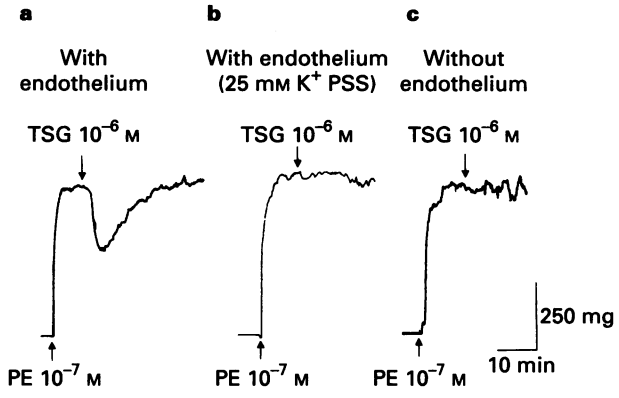

Figure 8 The vasorelaxant effect of thapsigargin (TSG, 10^{-6} M) in rat arterial rings precontracted with phenylephrine (PE, 10^{-7} M) in the presence of L-NOARG (3×10^{-5} M) and indomethacin (10^{-5} M). (a) Control; (b) rings depolarized with 25 mM K^+ PSS: note the disappearance of the vasorelaxant effect of TSG; (c) rings without endothelium.

because at this concentration L-NOARG has been reported to eliminate the production of EDRF-NO (Nagao & Vanhoutte, 1992; Fujii *et al.*, 1992). Furthermore, the relaxant response to TSG was abolished by the combined application of L-NOARG and high K^+ solution (25 mM). Endothelium-dependent hyperpolarization and the associated relaxation have been reported to be impaired by elevating the extracellular K^+ concentration (Chen & Suzuki, 1990; Nagao & Vanhoutte, 1992). Thus, it may be concluded that the relaxation of rat mesenteric artery evoked by TSG and CPA was caused in part by endothelium-dependent hyperpolarization of the smooth muscle cells in addition to the production of EDRF-NO. Interestingly, a recent report has shown that endothelium-dependent hyperpolarization can account for approximately 50% of the relaxation elicited by acetylcholine in the rat mesenteric artery (Waldron & Garland, 1994).

Contrary to the present findings in rat mesenteric artery, previous studies have demonstrated that pretreatment with the NO synthase inhibitors caused an almost complete inhibition of the relaxant responses to TSG and CPA in aortae from guinea-pigs and rats (Matsuyama *et al.*, 1993; Zheng *et al.*, 1994). Possible reasons for this discrepancy may be related to a difference in contribution of EDHF to relaxation between the two vessels. Nagao *et al.* (1992) have shown that in the presence of L-NOARG (3×10^{-5} M), acetylcholine caused about 70% relaxation in rat mesenteric artery but no longer caused relaxation in rat aorta.

The importance of the elevation of cytosolic Ca^{2+} concentration in the endothelial cells in the release of EDHF has been proposed (Chen & Suzuki, 1990). This hypothesis has been supported by the findings that A-23187 is capable of inducing endothelium-dependent hyperpolarization in several kinds of arteries including rat mesenteric artery (Chen & Suzuki, 1990; Nakashima & Vanhoutte, 1993). TSG has been shown to elevate the cytosolic Ca^{2+} concentration in a variety of cells including human umbilical vein endothelial cells (Takemura *et al.*, 1989; Thastrup *et al.*, 1990; Thastrup, 1990; Gericke *et al.*, 1993). CPA has been also reported to result in an increase in intracellular Ca^{2+} concentration in thyroid FRTL-5 cells (Tornquist, 1993) and in Friend erythroleukaemia cells (Schaefer *et al.*, 1994). Thus, the increased cytosolic Ca^{2+} concentration in endothelial cells with TSG and CPA may be responsible for the endothelium-dependent hyperpolarizing effects demonstrated in this study. The hyperpolarizing responses to TSG and CPA were not generated in Ca^{2+}-free solution, indicating that the responses are not due to release of Ca^{2+} from intracellular stores. In contrast, acetylcholine produced a transient hyperpolarizing effect in Ca^{2+}-free solution. In endothelial cells, the agonist-stimulated hydrolysis of membrane phosphatidylinositol results in a production of inositol polyphosphates (Derian & Moskowitz, 1986; Lambert *et al.*, 1986). Thus the acetylcholine-induced transient hyperpolarization is likely to be related to a mobilization of Ca^{2+} from endothelial intracellular stores by inositol 1,4,5-trisphosphate (Chen & Suzuki, 1990). Although TSG activates the release of Ca^{2+} from intracellular stores without generation of inositol polyphosphates in NG115-401L cells (Jackson *et al.*, 1988) and in parotid acinar cells (Takemura *et al.*, 1989), it has been shown in human umbilical vein endothelial cells that the increase in intracellular Ca^{2+} concentration induced by TSG depends on the extracellular Ca^{2+} concentration (Gericke *et al.*, 1993).

TSG and CPA can deplete the rapidly exchanging intracellular Ca^{2+} stores by blocking the refilling of Ca^{2+} stores, possibly due to inhibition of activity of the Ca^{2+}-pump ATPase located on the endoplasmic reticulum (Goerger *et al.*, 1988; Seidler *et al.*, 1989; Thastrup *et al.*, 1990). The depletion of intracellular Ca^{2+} stores has been proposed as the signal for Ca^{2+} entry (Putney, 1986). It has been recently suggested that a novel messenger named 'Ca^{2+}-influx factor' mediates Ca^{2+} influx triggered by emptying of intracellular Ca^{2+} stores (Randriamampita & Tsien, 1993). The possible pathway for Ca^{2+} entry into endothelial cells is thought to be nonspecific cation channels in human umbilical vein endothelial cells (Nilius, 1990). TSG and CPA activate a nonspecific cation channel in human umbilical vein endothelial cells (Gericke *et al.*, 1993; Zhang *et al.*, 1994). It thus seems likely that both TSG and CPA deplete intracellular Ca^{2+} stores in endothelial cells and the emptying of the Ca^{2+} stores generates an intracellular signal to trigger Ca^{2+} influx through a nonspecific cation channel. Opening of the nonspecific cation channel by these agents could supply sufficient Ca^{2+} into the endothelial cells to promote the production and release of EDHF. This intriguing proposal would best explain the present finding that the endothelium-dependent hyperpolarization elicited by TSG and CPA was exclusively dependent on the presence of extracellular Ca^{2+}.

As previously observed in rabbit carotid artery (Chen & Suzuki, 1990), acetylcholine generated sustained hyperpolarization following an initial transient hyperpolarization in rat mesenteric artery. The sustained hyperpolarizing response was sensitive to the extracellular Ca^{2+} concentration. Chen & Suzuki (1990) have shown that pretreatment with procaine can block preferentially the sustained component of the acetylcholine-induced hyperpolarization. This finding implies that inhibition by procaine of the Ca^{2+} release from intracellular stores (Itoh *et al.*, 1981) prevents the influx of Ca^{2+} from the extracellular medium. This is consistent with the idea that Ca^{2+} entry is initiated by the depletion of the intracellular Ca^{2+} stores. Thus, we assume that the mechanisms for activation of Ca^{2+} entry by acetylcholine and by TSG and CPA may be the same, although the time required to reach the peak amplitude of the sustained hyperpolarization by acetylcholine was somewhat shorter than that by TSG and CPA.

In conclusion, the present findings demonstrate that putative, selective inhibitors of endoplasmic reticulum Ca^{2+}-pump ATPase, TSG and CPA, cause endothelium-dependent hyperpolarization in rat mesenteric artery. The ability of these agents to produce hyperpolarizing effects, possibly *via* release of EDHF, may be mediated by the promotion of Ca^{2+} influx into endothelial cells that can be triggered by the emptying of intracellular Ca^{2+} stores. The results of the present study also indicate that the endothelium-dependent relaxation induced by TSG and CPA is mediated by both EDRF-NO and EDHF.

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