ELSEVIER

European Journal of Pharmacology 392 (2000) 51–59

ejp

www.elsevier.nl/locate/ejphar

Role of endothelium in thapsigargin-induced arterial responses in rat aorta

Yu Huang [a,*], Xiaoqiang Yao [a], Chi-Wai Lau [a], Franky Leung Chan [b], Nickie Wai Kei Chan [a], Yee-Man Cheng [a], Zhen-Yu Chen [c]

[a] *Department of Physiology, Faculty of Medicine, Chinese University of Hong Kong, Shatin, Hong Kong, People's Republic of China*
[b] *Department of Anatomy, Chinese University of Hong Kong, Hong Kong, People's Republic of China*
[c] *Department of Biochemistry, Chinese University of Hong Kong, Hong Kong, People's Republic of China*

Received 28 December 1999; accepted 21 January 2000

Abstract

We assessed the role of endothelium in the arterial response to thapsigargin, the Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum, in rat isolated aortic rings. Thapsigargin induced an endothelium-dependent relaxation of phenylephrine-contracted aortic rings with an EC_{50} of 2.6 ± 0.4 nM and a 75% maximum relaxation, while it was less effective against 30 mM K^+-induced contraction. Pretreatment of aortic rings with N^G-nitro-L-arginine methyl ester (30 μM) or methylene blue (1 μM) reduced thapsigargin-induced relaxation by approximately 85%. Thapsigargin failed to relax the endothelium-denuded rings. L-Arginine (3 mM) partially, but significantly, antagonized the effect of 30 μM N^G-nitro-L-arginine methyl ester. Pretreatment with indomethacin (3 μM), glibenclamide (1 μM) or iberiotoxin (100 nM) did not alter the thapsigargin-induced relaxation. In contrast, pretreatment with tetrapentylammonium ions (TPA^+, 1–3 μM) or with 300 μM Ba^{2+} suppressed the relaxant response to thapsigargin. TPA^+ (3 μM) also attenuated acetylcholine-induced relaxation. Thapsigargin-induced endothelium-dependent relaxation was primarily dependent on the presence of extracellular Ca^{2+}. Interestingly, when the tissues were exposed to very low concentrations of thapsigargin (1–3 nM) the nitric oxide-dependent relaxation induced by acetylcholine or A23187 was markedly reduced. While thapsigargin (3 nM) did not influence the relaxation induced by endothelium-independent dilators, sodium nitroprusside and verapamil. These results indicate that thapsigargin produced complex vascular effects primarily by acting on the endothelial cells. Thapsigargin causes an endothelial nitric oxide-dependent relaxation; on the other hand, it inhibits nitric oxide-mediated relaxation at the similar concentrations. Activation of TPA^+- and Ba^{2+}-sensitive but not Ca^{2+}-activated or ATP-sensitive K^+ channels may be also involved in thapsigargin-induced relaxation of rat isolated aortic rings.

Keywords: Thapsigargin; Nitric oxide (NO); Endothelium; K^+ channel; Relaxation; Aorta; (Rat)

1. Introduction

Thapsigargin is a tumor-promoting agent (Thastrup et al., 1987) extracted from the root of the umbelliferous plant *Thapsia garganica* (Rasmussen et al., 1978), which has been demonstrated to be the most specific and potent inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA pump) (Thastrup et al., 1990, Sagara and Inesi, 1991). There are at least five isoforms of the SERCA pump encoded by three genes, termed SERCA1, SERCA2, and SERCA3 (Guerini and Carafoli, 1999). SERCA1 pump isoform is highly expressed in fast-twitch skeletal muscles, from which it was originally cloned (Brandl et al., 1986). Transcripts of SERCA2 pump have been found in cardiac and smooth muscle cells while SERCA3 was cloned from a rat kidney library (Guerini and Carafoli, 1999). Intracellular Ca^{2+} stores play an essential role in the regulation of the cytoplasmic free Ca^{2+} concentration by mobilization and refilling of stored Ca^{2+}. Emptying of intracellular Ca^{2+} stores results in the activation of store-operated entry channels mediating influx of extracellular Ca^{2+} (Parekh and Penner, 1997; Putney and McKay, 1999) and this process is suggested to be mediated by a physical ''secre-

* Corresponding author. Tel.: +852-2609-6787; fax: +852-2603-5022.
E-mail address: yu-huang@cuhk.edu.hk (Y. Huang).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00074-1

tion-like'' mechanism coupling interactions between the endoplasmic reticulum and the plasma membrane (Patterson et al., 1999).

In response to hormonal stimuli which trigger Ca^{2+} entry (Himmel et al., 1993), the endothelial cells in intact arteries modulate the tone of the underlying arterial smooth muscle by liberating various vasoactive factors, some of which cause relaxation via different mechanisms. Thapsigargin elevates cytosolic Ca^{2+} in a diversity of cells including endothelium and vascular smooth muscle (Thastrup et al., 1987; Gericke et al., 1993; Sekiguchi et al., 1996) probably through depleting intracellular Ca^{2+} stores (Jackson et al., 1988) but not through inhibition of the plasma membrane Ca^{2+}-ATPase (Lytton et al., 1991). Thapsigargin induces endothelium-dependent relaxation in rat and guinea pig aorta (Matsuyama et al., 1993; Zheng et al., 1994) and endothelium-dependent N^G-nitro-L-arginine-insensitive hyperpolarization in rat mesenteric artery (Fukao et al., 1995). These vascular response may be associated with the stimulatory effect of thapsigargin on intracellular Ca^{2+} levels in endothelium (Schilling et al., 1992; Gericke et al., 1993) since the release of endothelium-derived vasoactive factors depends upon increased cytosolic free Ca^{2+} concentration (Chen and Suzuki, 1990; Himmel et al., 1993). Interestingly, thapsigargin in a micromolar range inhibited the calcium mobilization induced by bradykinin in venular endothelium (Ziche et al., 1993) and relaxation induced by substance P and acetylcholine in rabbit aorta (Amerini et al., 1996). Thapsigargin at 1 μM was also shown to inhibit nitric oxide and prostacyclin release stimulated by bradykinin and substance P in cultured rabbit endothelial cells (Macarthur et al., 1993). However, the mechanisms underlying the complex vascular response to thapsigargin are not very clear. Besides, thapsigargin at 1 μM had a constricting effect on isolated aortic rings (Amerini et al., 1996), indicating that thapsigargin at micromolar concentrations may have multiple sites of action in endothelium-intact arteries. This would complicate explanations for inhibitory effect of thapsigargin at higher concentrations on endothelium-dependent relaxation. This study was intended to examine what endothelium-derived factors may be involved in the vascular response to thapsigargin and whether thapsigargin at nanomolar concentrations could inhibit endothelium-dependent relaxation through inhibition of nitric oxide formation and/or release in rat isolated thoracic aorta.

2. Methods and materials

2.1. Preparation of rat aorta

After approval from the Animal Ethical Committee of the Chinese University of Hong Kong was obtained, male Sprague–Dawley rats weighing about 300 g were killed by cervical dislocation. The thoracic cavity was exposed and the descending thoracic aorta was dissected out and placed in cold physiological saline solution. After surrounding connective tissues were cleaned off, aortic rings of ~3 mm in length were prepared and suspended between two stainless steel hooks in 10-ml organ bath filled with the Krebs-Henseleit solution of the following composition (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 11.1. The bath solution was constantly gassed with carbogen (95% O_2 and 5% CO_2) and kept at 37°C. One of the hooks was mounted at the bottom of the bath while the other was connected to a Grass FT03 force-displacement transducers (Grass Instruments) and the isometric contraction was measured. One and a half gram basal tension was applied in all experiments. Thirty minutes after being set up in the organ baths, the tissues were first contracted with 0.3 μM phenylephrine to test their contractility after which time they were rinsed three times in Krebs solution. The basal tone was always monitored and adjusted to 1.5 g. The tissues were allowed to equilibrate further for 60 min before experiments were started. Three aortic rings were normally prepared from one rat and each experiment was performed on the arteries from different rats. A total of 56 rats were used in this study.

2.2. Effect of thapsigargin on agonist-induced contraction

In endothelium-intact arteries, the sustained contraction was induced by 0.3 μM phenylephrine or 30 mM high K^+, thapsigargin (1–100 nM) was then applied cumulatively to the bath solution. Since the relaxant effect of thapsigargin was difficult to wash out, the effects of nitric oxide activity inhibitors or putative K^+ channel blockers were examined on another set of arteries. The tissues were pretreated with each inhibitor for 30 min prior to application of phenylephrine. Since the nitric oxide activity inhibitors enhanced phenylephrine-induced contraction, the concentration of phenylephrine was lowered to 0.1 μM in order to induce a comparable level of the evoked tension. In some experiments, the endothelium was denuded mechanically by inserting plastic tubing into the lumen of an artery and rolling the preparation gently. Successful removal of the functional endothelium was verified by the abolition of the relaxant response to 3 μM acetylcholine at the start of each experiment. The removal of endothelium was also evaluated by light microscopy of the histological section of the artery. In experiments using high K^+ concentrations, the equimolar concentration of Na^+ was replaced by K^+ to maintain a constant ionic strength.

In some experiments using Ca^{2+}-free Krebs solution, the tissues were exposed to Ca^{2+}-free solution containing 0.3 mM Na_2-EGTA, washed with this solution twice and left for 15 min before application of 1 μM phenylephrine to induce the first contraction (C_1). The tissues were thereafter washed twice with normal Krebs solution (30 min contact time for refilling of the intracellular stores)

and twice with Ca^{2+}-free Krebs solution (15 min contact time). The second contraction (C_2) was then induced by 1 μM phenylephrine in control or in the presence of 10 nM thapsigargin (10 min contact time). A ratio (%) of the second contraction over the first contraction (C_2/C_1) was calculated.

2.3. Effect of thapsigargin on endothelium-dependent relaxation

The aortic rings with endothelium were first exposed to thapsigargin (1–3 nM) or to nitric oxide activity inhibitors for 30 min and then constricted with phenylephrine. Once the contraction became steady, acetylcholine (0.01–3 μM) or A23187 (0.003–1 μM) was applied cumulatively to induce a concentration-dependent response.

2.4. Effect of thapsigargin on endothelium-independent relaxation

The endothelium-denuded tissues were first exposed to thapsigargin for 30 min and then contracted with phenylephrine (0.1 μM), sodium nitroprusside (1–100 nM) or verapamil (0.1–300 μM) was applied cumulatively to the bath.

2.5. Chemicals and drugs

Drugs used in this study were phenylephrine hydrochloride, acetylcholine hydrochloride, indomethacin, methylene blue, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, L-arginine, calcium ionophore A23187, sodium nitroprusside, verapamil, $BaCl_2$ (Sigma, St. Louis, MO, USA). Thapsigargin, glibenclamide, iberiotoxin (Research Biochemicals, Natick, MA, USA). Tetrapentylammonium chloride (Aldrich Chem. St. Louis, MO, USA). All drugs were dissolved in Krebs solution except for thapsigargin, A23187 and glibenclamide which were dissolved in dimethyl sulfoxide. Dimethyl sulfoxide at 0.2% (v/v) did not affect the phenylephrine-induced tension.

2.6. Statistical analysis

The relaxant effect was expressed as percentage relaxation from the agonist-induced contractile response of the aortic ring. Cumulative concentration-relaxation relationship was analyzed with a non-linear curve fitting by the following equation:

$$\%\,\text{Relaxation} = \frac{E_{\max}}{1 + \left([\text{dilator}]/\text{IC}_{50}\right)^{n}}$$

Where % relaxation is the percentage relaxation of the contractile response at a given dilator concentration ([dilator]), $E_{\max}$ is the maximal relaxation, IC_{50} is the drug concentration that induces a half-maximum inhibition and n is the Hill coefficient. Data were presented as means ± S.E.M. of n experiments on the rings prepared from separate rats. Student's two-tailed t-test was used and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Thapsigargin-induced endothelium-dependent relaxation

In the endothelium-intact rat aorta, phenylephrine (0.3 μM) caused a steady increase of muscle tension (12.1 ± 1.4 mN, $n = 14$). Thapsigargin relaxed phenylephrine-contracted arteries in a concentration-dependent manner with an IC_{50} of 2.57 ± 0.35 nM ($n = 7$) and a 75.4 ± 6.5% maximal relaxation ($n = 7$). Traces in Fig. 1 show that the

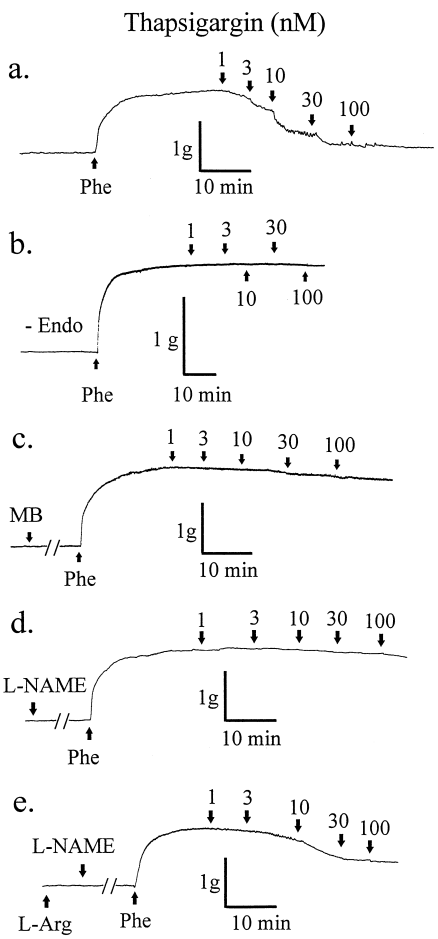

Fig. 1. Traces showing the representative records for thapsigargin-induced concentration-dependent aortic relaxation in the presence (a) and absence (b) of the functional endothelium, in the presence of 1 μM methylene blue in endothelium-intact arteries (c). The inhibitory effect of L-NAME (30 μM) on thapsigargin-induced relaxation (d) and partial reversal (e) of the L-NAME effect by L-arginine (3 mM). L-NAME or methylene blue were incubated for 30 min prior to application of 0.1 μM phenylephrine and L-arginine was added 10 min before application of L-NAME.

removal of the functional endothelium (Fig. 1b) or pretreatment of the endothelium-intact ring with 1 μM methylene blue (Fig. 1c) or with 30 μM L-NAME markedly reduced thapsigargin-induced relaxation (Fig. 1d). While L-arginine at 3 mM partially antagonized the inhibitory effect of L-NAME (Fig. 1e). The concentration–response curves for the relaxant response to thapsigargin under various treatment are summarized in Fig. 2 (IC_{50} with E_{max}: 2.57 ± 0.35 nM with 75.4 ± 6.5%, $n = 7$ for control; 9.94 ± 0.68 nM with 66.9 ± 7.2%, $n = 5$ for L-arginine plus L-NAME. E_{max}: 16.2 ± 1.6%, $n = 5$ for 30 μM L-NAME; 15.7 ± 5.0%, $n = 5$ for 1 μM methylene blue, $P < 0.05$ compared with control). Pretreatment with tetrapentylammonium ion (TPA^+) significantly attenuated thapsigargin-induced maximum relaxation (Fig. 3a, Table 1). TPA^+ at 3 μM also inhibited acetylcholine-induced relaxation (IC_{50} with E_{max}: 39.2 ± 4.3 nM with 74.1 ± 9.0% ($n = 7$) and 182.4 ± 6.1 nM with 82.1 ± 8.9% ($n = 3$) in the absence and presence of TPA^+, respectively, $P < 0.05$). Pretreatment with 300 μM Ba^{2+} attenuated thapsigargin-induced relaxation (Fig. 3b, Table 1). In contrast, pretreatment with either glibenclamide (1 μM) or iberiotoxin (100 nM) did not affect thapsigargin-induced relaxation (Fig. 3c, Table 1). In addition, indomethacin (10 μM) had no effect (IC_{50}: 3.01 ± 0.22 nM, $n = 4$, $P > 0.05$ compared with control). Thapsigargin also relaxed 30 mM K^+ contracted aortic rings with an IC_{50} of 1.52 ± 0.09 nM and a 56.6 ± 1.5% maximum response ($n = 3$).

In another series of experiments on endothelium-intact aortic rings, pretreatment for 10 min with 10 nM thapsigargin markedly reduced the phenylephrine (0.3 μM)-induced sustained contraction in normal Krebs solution (C_2/C_1: 111.4 ± 4.4%, $n = 4$ and 10.3 ± 2.8%, $n = 4$ in the absence and presence of thapsigargin, respectively, $P < 0.05$). In Ca^{2+}-free Krebs solution, 1 μM phenylephrine induced transient contractile responses (C_1: 3.46 ± 0.3 mN and C_2: 3.61 ± 0.3 mN, $n = 4$). Thapsigargin also inhibited the amplitude of the second contraction induced by phenylephrine (C_2/C_1: 104.5 ± 3.6% for control and 54..8 ± 10.9% for thapsigargin, $n = 4$ in each case, $P < 0.05$).

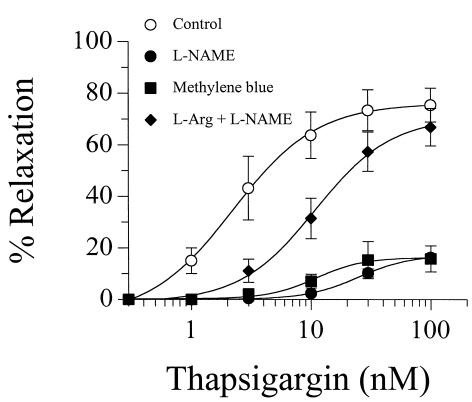

Fig. 2. The log concentration–response curves for the relaxant effect of thapsigargin under different treatments (○, $n = 7$ in the presence of endothelium; ●, $n = 5$ in 30 μM L-NAME; ■, $n = 5$ in 1 μM methylene blue; ◆, $n = 5$ in 3 mM L-arginine plus 30 μM L-NAME). Tissues were incubated with L-NAME for 30 min prior to application of phenylephrine and L-arginine was added 10 min before application of L-NAME. Data are means ± S.E.M. of n experiments.

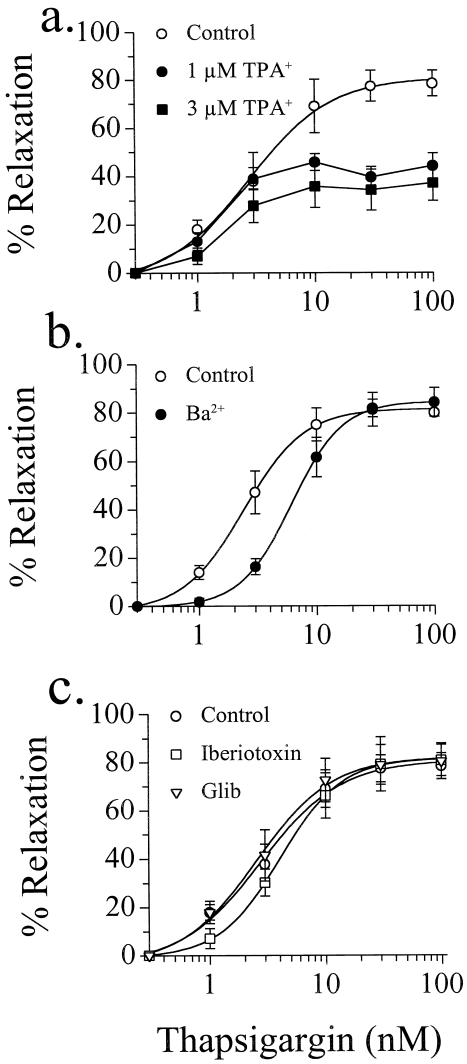

Fig. 3. (a) The log concentration–response curves for the relaxant effect of thapsigargin in the presence of tetrapentylammonium ions (○, $n = 5$, 0 μM TPA^+, ●, $n = 5$, 1 μM TPA^+, ■, $n = 5$, 3 μM TPA^+). (b) The log concentration–response curves for the relaxant effect of thapsigargin in the absence (○, $n = 6$) and presence of 300 μM $BaCl_2$ (●, $n = 4$). (c) The log concentration–response curves for the relaxant effects of thapsigargin in the absence (○, $n = 5$) and presence of 1 μM glibenclamide (▽, $n = 5$) or of 100 nM iberiotoxin (□, $n = 5$). Data are means ± S.E.M. of n experiments.

3.2. Inhibitory effect of thapsigargin on endothelium-dependent relaxation

The traces in Fig. 4 show that after the endothelium-intact aortic ring was exposed for 30 min to 3 nM thapsigargin (Fig. 4b), the relaxant effects induced by acetylcholine were significantly attenuated (compared to control in Fig. 4a). The maximum relaxation induced by acetylcholine

Table 1
Effects of putative K^+ channel blockers on thapsigargin-induced relaxation

Drug	IC_{50} (nM)	E_{max} (%)	n
Control	2.43 ± 0.38	75.4 ± 6.5	6
1 μM TPA^+	1.39 ± 0.13*	44.2 ± 5.3*	5
3 μM TPA^+	1.87 ± 0.12*	37.2 ± 7.8*	5
Control	2.63 ± 0.29	81.6 ± 3.6	6
300 μM Ba^{2+}	6.07 ± 0.15*	84.2 ± 7.0	4
Control	3.27 ± 0.43	78.5 ± 5.4	7
1 μM Glibenclamide	2.64 ± 0.28	80.5 ± 7.5	5
100 nM Iberiotoxin	3.98 ± 0.37	80.9 ± 6.6	5

The IC_{50} values and the maximum relaxation (E_{max}) induced by thapsigargin in the absence and presence of various putative K^+ channel blockers in the endothelim-intact rat isolated aortic rings. TPA^+, tetrapentylammonium ions. Significance between the control and treatment groups (*$P < 0.05$, Student's two-tailed t-test) is indicated. Data are mean ± S.E.M. of n experiments.

was 74.1 ± 9% ($n = 7$), 62.3 ± 4.5% ($n = 5$) and 28.5 ± 12% ($n = 5$), respectively, in the presence of 0, 1 and 3 nM thapsigargin (Fig. 4c). Pretreatment with 100 μM N^G-nitro-L-arginine (L-NNA) or removal of the endothelium eliminated the acetylcholine response (Fig. 4d). Similarly, A23187-induced relaxation was significantly reduced by pretreatment with 3 nM thapsigargin (Fig. 5a and b). Calcium ionophore A23187 relaxed the precontracted tissues with an IC_{50} of 49.4 ± 4 nM ($n = 7$) and a maximum relaxation of 75.2 ± 4.7% ($n = 6$, Fig. 5c) Thapsigargin at 1 and 3 nM decreased A23187-induced maximum response by 70% and 60%, respectively ($n = 5$ in each case, Fig. 5c). Pretreatment of 100 μM L-NNA (Fig. 5d) or removal of the endothelium abolished relaxation induced by A23187 (Fig. 5d).

3.3. Lack of effect of thapsigargin on endothelium-independent relaxation

In the endothelium-denuded rings contracted by 0.1 μM phenylephrine, verapamil and sodium nitroprusside caused a concentration-dependent relaxation with respective IC_{50} values of 4.49 ± 0.65 μM ($n = 5$) and 0.63 ± 0.05 nM ($n = 5$) (Fig. 6a and b). Both dilators induced almost a full relaxation. Pretreatment with thapsigargin (3 nM) did not affect relaxation induced by these two agents. The IC_{50} values were 7.67 ± 1.27 μM ($n = 5$) for verapamil in the presence of 3 nM thapsigargin ($P > 0.05$ compared with control, Fig. 6a), and 0.81 ± 0.07 nM ($n = 5$) for sodium nitroprusside in the presence of 3 nM thapsigargin ($P >$

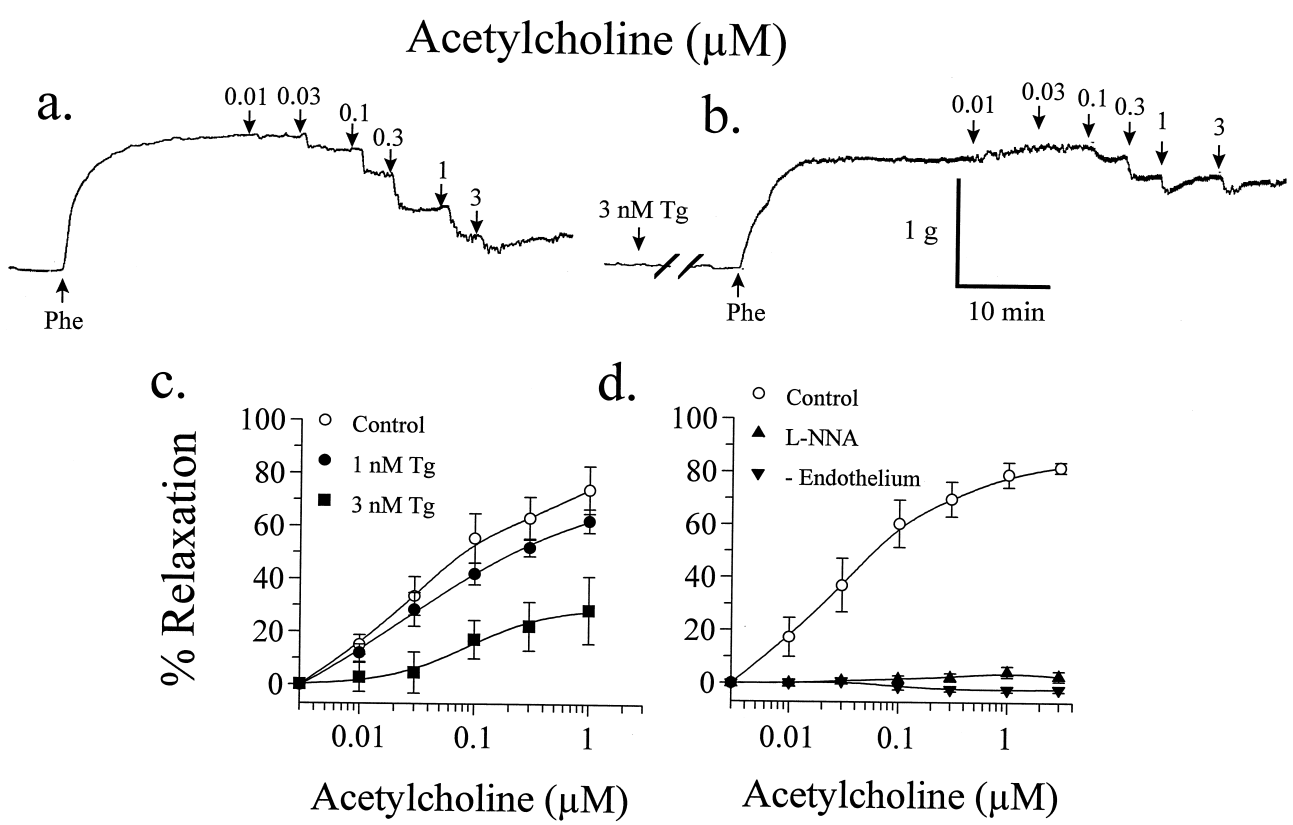

Fig. 4. The traces showing acetylcholine-induced aortic relaxation in the absence (a) and presence of 3 nM thapsigargin (b, 30-min contact time). (c) The log concentration–response curves for the relaxant effect of acetylcholine in the absence (○, $n = 7$) and presence of thapsigargin (●, $n = 5$ for 1 nM and ■, $n = 5$ for 3 nM thapsigargin). (d) The log concentration–response curve for the relaxant effect of acetylcholine in the absence (○, $n = 7$) and presence of 100 μM L-NNA (▲, $n = 5$) in endothelium-intact rings, and in endothelium-denuded rings (▼, $n = 5$). Tissues were exposed to thapsigargin or L-NNA for 30 min prior to application of phenylephrine. Data are means ± S.E.M. of n experiments.

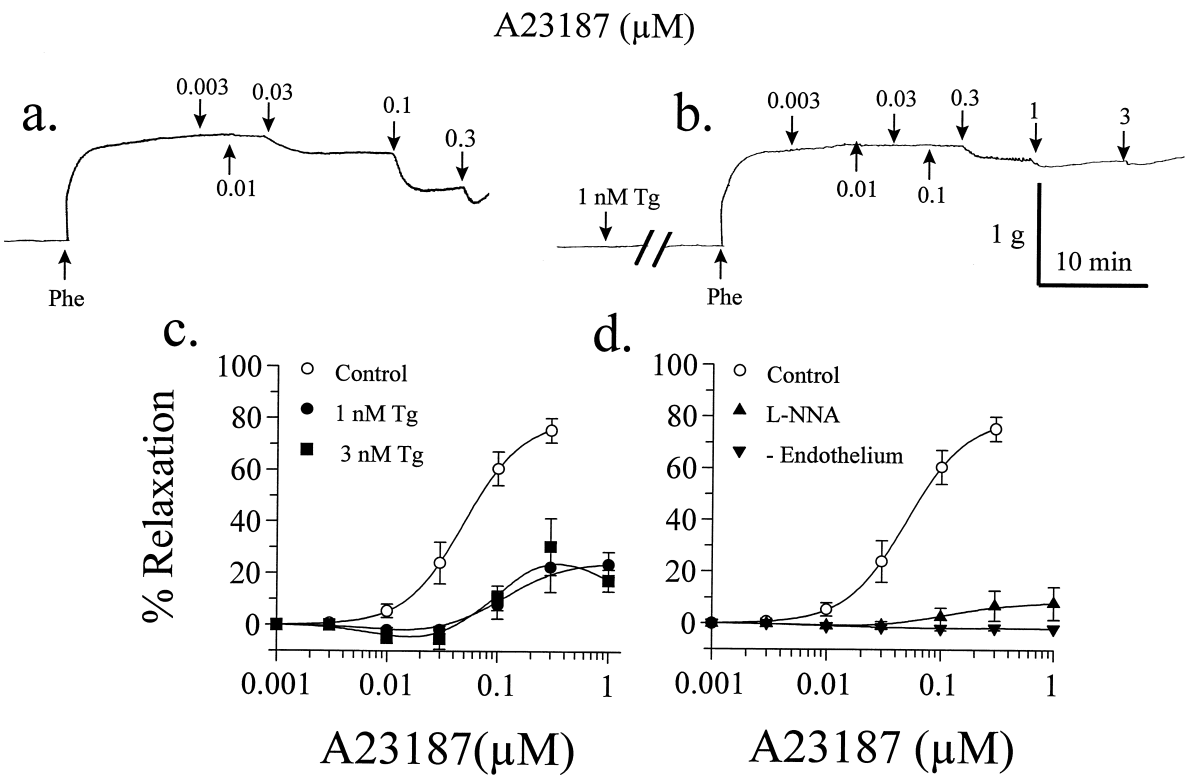

Fig. 5. The traces showing A23187-induced aortic relaxation in the absence (a) and presence of 3 nM thapsigargin (b, 30-min contact time). (c) The log concentration–response curves for the relaxant effect of A23187 in the absence (○, $n = 7$) and presence of thapsigargin (●, $n = 5$ for 1 nM and ■, $n = 6$ for 3 nM thapsigargin). (d) The log concentration–response curve for the relaxant effect of A23187 in the absence (○, $n = 7$) and presence of 100 μM L-NNA (▲, $n = 5$) in endothelium-intact rings, and in endothelium-denuded rings (▼, $n = 5$). Tissues were exposed to thapsigargin or L-NNA for 30 min prior to application of phenylephrine. Data are means ± S.E.M. of n experiments.

0.05 compared with control, Fig. 6b). In addition, the relaxation induced by sodium nitroprusside was unaltered by 100 μM L-NNA (IC_{50}: 0.75 ± 0.06 nM, $n = 5$), but this relaxation was inhibited by 3 μM methylene blue (38.5 ± 3.8 nM, $n = 7$, $P < 0.05$ compared with control).

4. Discussion

In this report, we show a complex vascular response to the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin in rat isolated thoracic artery. Thapsigargin concentration dependently caused endothelium-dependent relaxation. The effect of thapsigargin is similar to those produced by acetylcholine and A23187, the known endothelium-dependent dilators. L-NAME, the L-arginine reversible inhibitor of endothelial nitric oxide synthase, and methylene blue, an inhibitor of soluble gunaylyl cyclase, markedly reduced the relaxant effect of thapsigargin. L-Arginine significantly antagonized the effect of L-NAME on thapsigargin-induced relaxation. Inhibitors of nitric oxide activity almost abolished relaxation induced by acetylcholine and A23187. These results are in agreement with thapsigargin-mediated endothelium-dependent relaxation reported previously in guinea pig aorta (Matsuyama et al., 1993), rat aorta (Zheng et al., 1994) and rat mesenteric artery (Fukao et al., 1995) except that the concentration of thapsigargin used to relax the arteries in our study is much lower than that used in aforementioned studies. It is possible that thapsigargin and other intracellular Ca^{2+}-ATPase inhibitors prevent refilling of Ca^{2+} of internal stores in the endothelium, which in turn stimulates influx of extracellular Ca^{2+} and, thus, nitric oxide synthesis since thapsigargin was found to increase the intracellular Ca^{2+} concentration in human umbilical vein endothelial cells (Gericke et al., 1993) and also in porcine renal artery endothelial cells

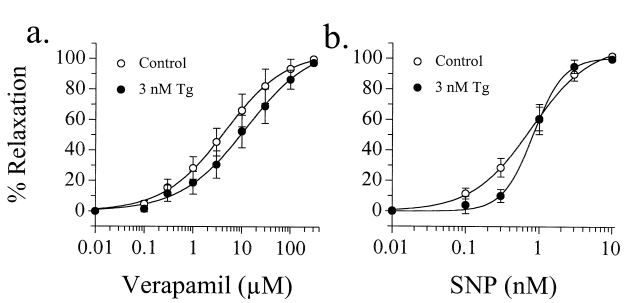

Fig. 6. The log concentration–response curves for the relaxation effects of verapamil (a) and sodium nitroprusside (SNP, b) in the absence (○, $n = 5$ in each case) and presence of 3 nM thapsigargin (●, $n = 5$ in each case). Thapsigargin was applied 30 min before the addition of phenylephrine. Data are means ± S.E.M. of mean from five experiments.

(Ihara et al., 1999). Thus, the increased cytosolic free Ca^{2+} level in endothelial cells with thapsigargin might be responsible for nitric oxide-dependent relaxation. Furthermore, pretreatment with thapsigargin markedly reduced phenylephrine-induced sustained contraction in normal Ca^{2+}-containing Krebs solution as well as the transient contractile response to phenylephrine in Ca^{2+}-free bath solution but to a lesser extent. These new data show that extracellular Ca^{2+} influx is required for thapsigargin-induced endothelium-dependent relaxation and thapsigargin may also inhibit phenylephrine-sensitive intracellular Ca^{2+} pool. The thapsigargin-induced relaxation was unaffected by indomethacin, an inhibitor of cyclooxygenase which is responsible for biosynthesis of prostacyclin. This indicates that endothelial prostanoids are not involved.

Activation of Ca^{2+}-activated K^+ channels may partly mediate relaxation induced by endothelium-derived relaxing factor (EDRF)/nitric oxide or by nitric oxide donors (Huang 1998; Mistry and Garland, 1998). Present results show that thapsigargin-induced relaxation was unchanged by the Ca^{2+}-activated K^+ channel blocker iberiotoxin or the ATP-sensitive K^+ channel blocker glibenclamide (Standen et al., 1989), but this relaxation was significantly inhibited by TPA^+, a non-selective K^+ channel blocker, or by Ba^{2+}, a blocker of inwardly rectifying K^+ channels in rat arterial smooth muscle cells (Bradley et al., 1999). In addition, thapsigargin induced a significantly less relaxation in arteries contracted by 30 mM K^+ than by phenylephrine, suggesting that thapsigargin-induced relaxation is, at least in part, mediated through activation of K^+ channels which were insensitive to iberiotoxin or glibenclamide. It appears that the TPA^+-sensitive component of thapsigargin-induced relaxation might be caused by EDRF/nitric oxide because TPA^+ at 3 μM also inhibited the relaxation induced by acetylcholine in this study and by other nitric oxide donors such as hydroxylamine and sodium nitroprusside in the same preparations (Huang, 1998). There has been no available evidence showing the stimulatory effect of nitric oxide on inward rectifier K^+ channels in vascular smooth muscle even though Ba^{2+} significantly inhibited thapsigargin-induced endothelium-dependent relaxation in the present study. It, therefore, remains to be known whether thapsigargin can activate inward rectifier K^+ channels in endothelium since Ba^{2+} inhibited hypercapnia-induced endothelium-dependent relaxation by blocking endothelial inward rectifier K^+ channels (Okazaki et al., 1998). Pretreatment with nitric oxide synthase inhibitors almost abolished relaxation induced by thapsigargin in rat aorta in our study or in guinea-pig aorta (Matsuyama et al., 1993), while thapsigargin induced endothelium-dependent hyperpolarization is resistant to nitric oxide synthase inhibitors in rat mesenteric artery (Fukao et al., 1995). This discrepancy may be due to a regional difference in the contribution of nitric oxide and endothelium-derived hyperpolarizing factor between the two arteries.

This study demonstrated another interesting effect of thapsigargin in endothelium-intact tissues. Exposure of aortic rings to very low concentrations (1–3 nM) of thapsigargin significantly impaired nitric oxide-mediated relaxation induced by acetylcholine or A23187. It appears that thapsigargin might inhibit the agonist-induced nitric oxide release as indicated previously in cultured bovine aortic endothelial cells (Macarthur et al., 1993) and, thus, attenuated arterial smooth muscle relaxation. It is unlikely that thapsigargin counteracts with acetylcholine at the plasma membrane receptor levels since thapsigargin also inhibited relaxation induced by A23187, a receptor-independent agonist. In contrast, thapsigargin had no effect on the endothelium-independent relaxation induced by sodium nitroprusside, an exogenous nitric oxide donor or by verapamil, the putative voltage-sensitive Ca^{2+} channels blocker. These results contrast with a previous report in which thapsigargin was shown to noncompetitively inhibit the vasodilator effects of nitroglycerin and atrial natriuretic factor on the rabbit thoracic aorta (Luo et al., 1993). It is at present unknown what had caused this discrepancy between the results of Luo et al. (1993) and ours. Luo et al. (1993) used reserpinized rabbit while we used untreated Sprague–Dawley rats in our study.

Impaired endothelium-dependent relaxation by thapsigargin might be caused by the following mechanisms: (1) decreased Ca^{2+} influx or internal Ca^{2+} release; (2) reduced activity of nitric oxide synthase; (3) a decreased activity of guanylate cyclase; and (4) decreased diffusion of nitric oxide into the underlying vascular smooth muscle. Firstly, thapsigargin may deplete the $InsP_3$-sensitive Ca^{2+} stores and, thus, markedly attenuates the subsequent release of intracellular Ca^{2+} from the same pools in response to agonists as proposed by Macarthur et al. (1993). In cultured endothelial cells, a transient increase in $[Ca^{2+}]_i$ was seen with agonists such as bradykinin and substance P while thapsigargin induced relatively slower but much more sustained rise in $[Ca^{2+}]_i$ (Macarthur et al., 1993). Nevertheless, it cannot be ruled out that thapsigargin may first increase the cytosolic Ca^{2+} concentration that then inhibits Ca^{2+} influx through the endothelial non-selective cation channels which may be activated by some agonists or that thapsigargin may inhibit Ca^{2+}-release-activated Ca^{2+} influx. In most studies, a higher concentration (1 μM) of thapsigargin was used and a threshold concentration of 1–10 nM was found to inhibit agonist-induced nitric oxide release in the endothelium (Dolor et al., 1992; Macarthur et al., 1993), but thapsigargin at 1–3 nM significantly reduced by over 60% the endothelium-dependent relaxation seen in this study. These concentrations of thapsigargin induced approximately 30% relaxation of phenylephrine-contracted arteries. It is, therefore, possible that thapsigargin acts at additional sites on the endothelium apart from elevating $[Ca^{2+}]_i$. Thapsigargin at low concentrations may have a direct inhibitory effect on nitric oxide synthase. This possible mechanism could explain the in-

hibitory effect of thapsigargin on nitric oxide release stimulated by bradykinin and substance P in cultured endothelial cells (Macarthur et al., 1993) even though this possibility needs to be further substantiated by biochemical assay of the enzyme activity. The present results discount the possible inhibitory effect on guanylate cyclase since thapsigargin had no effect on nitroprusside-induced relaxation. In contrast, methylene blue significantly antagonized the relaxant response to thapsigargin. However, it is currently unknown whether thapsigargin could inhibit nitric oxide diffusion from the endothelium to smooth muscle. Finally, endothelial cells may contain a Ca^{2+}/calmodulin-dependent constitutive nitric oxide synthase and thapsigargin may inhibit calmodulin activity despite the fact that a sustained level of cytosolic Ca^{2+} is raised by thapsigargin. However, in human neuroblastoma cells treated with thapsigargin, cytosolic calmodulin was elevated while particulate calmodulin was reduced (McGinnis et al., 1998).

Taken together, the present results show a significant role of endothelium in a complex aortic response to thapsigargin. Thapsigargin caused an endothelium-dependent relaxation primarily through stimulation of EDRF/nitric oxide release from the endothelium and, at least in part, through the activation of K^+ channels which are sensitive to inhibition by TPA^+ or Ba^{2+} but not by iberiotoxin or glibenclamide. Thapsigargin within a low concentration range (1–3 nM) significantly attenuated the endothelium-dependent relaxation induced by acetylcholine and A23187 probably through inhibition of EDRF/nitric oxide synthesis in the endothelium while it had no effect on the endothelium-independent relaxation induced by nitroprusside or verapamil. These complex effects suggest that thapsigargin may have multiple sites of action in endothelium-intact arterial rings.

Acknowledgements

This work was supported by the Hong Kong Research Grants Committee. YMC was supported by CUHK Summer Work Scheme and NWKC was supported by CUHK Postgraduate Studentship.

References

Amerini, S., Filippi, S., Parenti, A., Ziche, M., Ledda, F., 1996. Thapsigargin inhibits the response to acetylcholine and substance P but not interfere with the responses to endothelium-independent agents. J. Cardiovasc. Pharmacol. 28, 82–88.

Bradley, K.K., Jaggar, J.H., Bonev, A.D., Heppner, T.J., Flynn, E.R., Nelson, M.T., Horowitz, B., 1999. Kir2.1 encodes the inward rectifier potassium channel in rat arterial smooth muscle cells. J. Physiol. 515, 639–651.

Brandl, C.J., Green, N.M., Korczak, B., MacLennan, D.H., 1986. Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. Cell 44, 597–607.

Chen, G., Suzuki, H., 1990. Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. J. Physiol. 421, 521–534.

Dolor, R.J., Hurwitz, L.M., Mirza, Z., Strauss, H.C., Whorton, A.R., 1992. Regulation of intracellular calcium entry in endothelial cells: role of intracellular calcium pool. Am. J. Physiol. 262, C171–C181.

Fukao, M., Hattori, Y., Kanno, M., Sakuma, I., Kitabatake, A., 1995. Thapsigargin- and cyclopiazonic acid-induced endothelium-dependent hyperpolarization in rat mesenteric artery. Br. J. Pharmacol. 115, 987–992.

Gericke, M., Droogmans, G., Nilius, B., 1993. Thapsigargin discharges intracellular calcium stores and induces transmembrane currents in human endothelial cells. Pflugers. Arch. 422, 552–557.

Guerini, D., Carafoli, E., 1999. The calcium pumps. In: Carafoli, E., Klee, C. (Eds.), Calcium as a Cellular Regulator. Oxford Univ. Press, Oxford, pp. 249–278.

Himmel, H.M., Whorton, A.R., Strauss, H.C., 1993. Intracellular calcium, currents, and stimulus-response coupling in endothelial cells. Hypertension 21, 112–127.

Huang, Y., 1998. Hydroxylamine-induced relaxation inhibited by K^+ channel blockers in rat aortic rings. Eur. J. Pharmacol. 349, 53–60.

Ihara, E., Hirano, K., Nishimura, J., Nawata, H., Kanaide, H., 1999. Thapsigargin-induced endothelium-dependent triphasic regulation of vascular tone in the porcine renal artery. Br. J. Pharmacol. 128, 689–699.

Jackson, T.R., Patterson, S.I., Thastrup, O., Hanley, M.R., 1988. A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free Ca^{2+} without generation of inositol phosphates in NG 115-401L neuronal cells. Biochem. J. 253, 81–86.

Luo, D.L., Nakazawa, M., Ishibashi, T., Kato, K., Imai, S., 1993. Putative, selective inhibitors of sarcoplasmic reticulum Ca^{++}-pump ATPase inhibit relaxation by nitroglycerin and atrial natriuretic factor of the rabbit aorta contracted by phenylephrine. J. Pharmacol. Exp. Ther. 265, 1187–1192.

Lytton, J., Westlin, M., Hanley, M.R., 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase gamily of calcium pumps. J. Biol. Chem. 266, 17067–17071.

Macarthur, H., Hecker, M., Busse, R., Vane, J.R., 1993. Selective inhibition of agonist-induced but not shear stress-dependent release of endothelial autacoids by thapsigargin. Br. J. Pharmacol. 108, 100–105.

Matsuyama, S., Shuntoh, H., Katayama, S., Tanaka, C., 1993. Thapsigargin induces an endothelium-dependent, intracellular calcium ion-dependent vasodilation in intro. Life Sci. 53, 618–688.

McGinnis, K.M., Shariat-Madar, Z., Gnegy, M.E., 1998. Cytosolic calmodulin is increased in SK-N-SH human neuroblastoma cells due to release of calcium from intracellular stores. J. Neurochem. 70, 139–146.

Mistry, D.K., Garland, C.J., 1998. Nitric oxide (NO)-induced activation of large conductance Ca^{2+}-dependent K^+ channels (BK(Ca)) in smooth muscle cells isolated from the rat mesenteric artery. Br. J. Pharmacol. 124, 1131–1140.

Okazaki, K., Endou, M., Okumura, F., 1998. Involvement of barium-sensitive K^+ channels in endothelium-dependent vasodilation produced by hypercapnia in rat mesenteric vascular beds. Br. J. Pharmacol. 125, 168–174.

Parekh, A.B., Penner, R., 1997. Store depletion and calcium influx. Physiol. Rev. 77, 901–930.

Patterson, R.L., van Rossum, D.B., Gill, D.L., 1999. Store-operated Ca^{2+} entry: evidence for a secretion-like coupling model. Cell 98, 487–499.

Putney, J.W., McKay, R.R., 1999. Capacitative calcium entry channels. Bioessays 21, 38–46.

Rasmussen, U., Christensen, S.B., Snadberg, F., 1978. Thapsigargin and thapsigargicin, two new histamine bilerators from *Thapsia-garganica*. Acta Pharmaceut. Suec. 15, 133–140.

Sagara, Y., Inesi, G., 1991. Inhibition of the sarcoplasmic reticulum Ca^{2+} transport ATPase by thapsigargin at subnanomolar concentrations. J. Biol. Chem. 266, 13503–13506.

Schilling, W.P., Cabello, O.A., Rajan, L., 1992. Depletion of the inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store in vascular endothelial cells activated the agonist-sensitive Ca^{2+}-influx pathway. Biochem. J. 284, 521–530.

Sekiguchi, F., Shimamura, K., Akashi, M., Sunano, S., 1996. Effects of cyclopiazonic acid and thapsigargin on electromechanical activities and intracellular Ca^{2+} in smooth muscle of carotid artery of hypertensive rats. Br. J. Pharmacol. 118, 857–864.

Standen, N.B., Quayle, J.M., Davies, N.W., Brayden, J.E., Huang, Y., Nelson, M.T., 1989. Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. Science 245, 177–180.

Thastrup, O., Cullen, P.J., Drobrak, B.K., Hanley, M.R., Dawson, A.P., 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Proc. Natl. Acad. Sci. USA 87, 2466–2470.

Thastrup, O., Foder, B., Scharff, O., 1987. The calcium mobilizing and tumour promoting agent, thapsigargin, elevates the platelet cytoplasmic free calcium concentration to a higher steady state level. A possible mechanism of action for the tumour promotion. Biochem. Biophys. Res. Commun. 142, 654–660.

Zheng, X.F., Kwan, C.Y., Daniel, E., 1994. Role of intracellular Ca^{2+} in EDRF release in rat aorta. J. Vasc. Res. 31, 18–24.

Ziche, M., Zawieja, D., Hester, R.K., Granger, H., 1993. Calcium entry, mobilization, and extrusion in postcapillary venular endothelium exposed to bradykinin. Am. J. Physiol. 265, H569–H580.