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# Possible Involvement of $Ca^{2+}$ Entry and its Pharmacological Characteristics Responsible for Endothelium-dependent, NO-mediated Relaxation Induced by Thapsigargin in Guinea-pig Aorta

HARUYORI TANIGUCHI, HARUKO HIRANO, YOSHIO TANAKA, HIKARU TANAKA AND KOKI SHIGENOBU

*Department of Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1 Miyama, Funabashi-City, Chiba 274-8510, Japan*

**Abstract**

Thapsigargin, a specific inhibitor of $Ca^{2+}$-pump $Ca^{2+}$-ATPase in the sarcoplasmic/endoplasmic reticulum (SR/ER), produces an endothelium-dependent vascular relaxation. In the present study, pharmacological features of thapsigargin-induced endothelium-dependent relaxation were functionally characterized in the isolated guinea-pig aorta especially focusing on the $Ca^{2+}$ mobilization mechanisms in endothelial cells.

Thapsigargin-induced endothelium-dependent vascular relaxation was markedly suppressed by $N^G$-nitro-L-arginine (L-NNA) and calmidazolium, suggesting that the vascular relaxation to thapsigargin is largely attributable to endothelium-derived nitric oxide (NO) produced as a result of the activation of $Ca^{2+}$, calmodulin-dependent NO synthase (NOS). Removal of $Ca^{2+}$ from the external solution abolished the endothelium-dependent relaxation of guinea-pig aorta in response to thapsigargin. Thapsigargin-induced endothelium-dependent relaxation was inhibited more strongly compared with the endothelium-independent relaxation to an NO donor, SIN-1 (3-(4-morpholinyl)-sydnonimine), when the artery preparation was preconstricted with a high concentration (80 mM) of KCl instead of agonistic stimulation. Endothelium-dependent relaxation induced by thapsigargin was not affected by diltiazem, a blocker of L-type voltage-gated $Ca^{2+}$ channels. SK&F96365 (1-[$\beta$-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1 *H*-imidazole) and $Ni^{2+}$, both of which block capacitative $Ca^{2+}$ entry, did not show any appreciable inhibitory effects on the endothelium-dependent relaxation to thapsigargin.

These findings suggest that in guinea-pig aorta, endothelium-dependent NO-mediated relaxation induced by thapsigargin is preceded by the increase in the cytosolic free $Ca^{2+}$ concentrations ($[Ca^{2+}]_{cyt}$) following the depletion of stored $Ca^{2+}$ in thapsigargin-sensitive store sites in endothelial cells. Although the increase in $[Ca^{2+}]_{cyt}$ responsible for the activation of endothelium NOS leading to thapsigargin-induced vascular relaxation may be ascribed to the capacitative $Ca^{2+}$ entry from extracellular space, the $Ca^{2+}$ entry mechanism stimulated with thapsigargin is deficient in sensitivity to SK&F96365 and $Ni^{2+}$ in the endothelium of guinea-pig aorta.

Thapsigargin, a nonphorbol ester tumour promoter, is a sesquiterpene lactone extracted from the roots of the umbelliferous Mediterranean plant *Thapsia garganica* L. (Linnaeus) (Christensen et al 1981; Treiman et al 1998). It belongs to a group of related, naturally occurring 6,12-guaianolides with a 1$\beta$-hydrogen and a 7$\beta$-hydroxy group (Treiman et al 1998). Thapsigargin depletes $Ca^{2+}$ in the sarcoplasmic/endoplasmic reticulum (SR/ER) by specifically inhibiting SR/ER $Ca^{2+}$-pump $Ca^{2+}$-ATPase activity in a variety of excitable and non-excitable cells (Thastrup et al 1990), which leads to

Correspondence: Y. Tanaka, Department of Pharmacology, Toho University School of Pharmaceutical Sciences, 2-2-1 Miyama, Funabashi-City, Chiba 274-8510, Japan. E-Mail: yotanaka@phar.toho-u.ac.jp

an increase in cytosolic free $Ca^{2+}$ concentrations ($[Ca^{2+}]_{cyt}$) and subsequent alterations in cellular and tissue functions. In blood vessels, thapsigargin produces an endothelium-dependent relaxation in the aortae isolated from the rat (Mikkelsen et al 1988; Zheng et al 1994; Moritoki et al 1996) and the guinea-pig (Matsuyama et al 1993; Tanaka et al 1997), and in the mesenteric artery from the rat (Fukao et al 1995). Since thapsigargin-induced relaxations in the aortae from the rat and the guinea-pig are inhibited by compounds which affect the nitric oxide (NO)-cGMP pathway, thapsigargin-induced endothelium-dependent vascular relaxation seems to be attributable mainly to the release of endothelium-derived NO as a result of the increased activation of $Ca^{2+}$, calmodulin-dependent constitutive NO synthase (NOS) in the aorta (Busse & Mülsch 1990; Förstermann et al 1991; Matsuyama et al 1993; Moritoki et al 1994a; Moritoki et al 1996; Tanaka et al 1997).

As to the $Ca^{2+}$ source and $Ca^{2+}$ handling mechanisms responsible for thapsigargin-induced endothelium-dependent NO-mediated vascular relaxation, an important role of capacitative $Ca^{2+}$ entry into the endothelial cells through store-operated $Ca^{2+}$ channels (SOCCs) (Gibson et al 1998; Treiman et al 1998), which are opened following the depletion of stored $Ca^{2+}$ within endothelial cells and sensitive to SK&F96365 and $Ni^{2+}$, has been implicated in rat aorta (Moritoki et al 1994a, 1996). By comparison, in guinea-pig aorta, intracellular $Ca^{2+}$ mobilization from endothelial $Ca^{2+}$ store sites, following the inhibition of store sites $Ca^{2+}$-pump $Ca^{2+}$-ATPase by thapsigargin, is thought to be sufficient for the stimulation of NOS activity leading to endothelium-dependent relaxation to thapsigargin (Matsuyama et al 1993). Thus, it has not been clarified whether endothelial capacitative $Ca^{2+}$ entry contributes to endothelium-dependent NO-mediated functional vascular relaxation to thapsigargin in guinea-pig aorta. The present study was carried out to elucidate pharmacological characteristics of endothelium-dependent relaxation of guinea-pig aorta to thapsigargin, focusing on the possible contribution of capacitative $Ca^{2+}$ entry to the vascular relaxation.

## Materials and Methods

*Animals*

Hartley guinea-pigs of either sex were housed under controlled conditions (21–22°C, relative humidity 50 ± 5%). Food and water were freely available to all animals. This study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Toho University School of Pharmaceutical Sciences (which is accredited by the Ministry of Education, Science, Sports and Culture, Japan).

*Preparation of thoracic aortic rings*

Guinea-pigs of either sex were killed by cervical dislocation, and bled by section of the carotid arteries. A section of the thoracic aorta between aortic arch and diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS) of the following composition (in mM): NaCl, 118·4; KCl, 4·7; $NaHCO_3$, 24·9; $CaCl_2$, 2·5; $KH_2PO_4$, 1·2; $MgSO_4$, 1·2; glucose, 11·0 (pH = 7·4). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring segments about 2 mm in length. The tissue was then mounted using stainless steel hooks under a resting tension of 1·0 g in a 5-mL organ bath (UC-5, UFER Medical Instrument, Kyoto, Japan) containing KHS. Tension changes in the muscle preparation were isometrically recorded with a force-displacement transducer (TB-611T, Nihon Kohden, Tokyo, Japan) connected to a minipolygraph (RM-6100, Nihon Kohden, Tokyo, Japan). After arterial preparations were incubated for 90 min to be equilibrated, the rings were preconstricted with noradrenaline ($3 \times 10^{-6}$ M) and then challenged with carbachol ($10^{-5}$ M) or acetylcholine ($10^{-5}$ M) to verify the presence of functional endothelium. In some experiments, aortic-ring preparations were preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $10^{-5}$ M). For the study with vascular preparations without functional endothelium, the intimal surface of ring preparations was rubbed gently with a cotton thread moistened with KHS solution to remove endothelial cells. Removal of functional endothelium was confirmed by the loss of relaxant responses to carbachol ($10^{-5}$ M) or acetylcholine ($10^{-5}$ M). After confirming the presence or absence of endothelium, the bath solution was exchanged with fresh one, and ring preparations were left to be equilibrated for 30 min before starting subsequent experiments. Concentration–response relationships for the relaxations in response to test compounds (thapsigargin, SIN-1) were constructed by cumulative application of the compounds to the 5-mL organ bath, and plotted as percentage inhibitions against the contraction induced by noradrenaline ($3 \times 10^{-6}$ M) or $PGF_{2\alpha}$ ($10^{-5}$ M). The vasorelaxant effects of thapsigargin were tested in separate ring preparations since the effects were irreversible in nature.

A $Ca^{2+}$-free solution was prepared by omitting $CaCl_2$ and adding 2 mM *O*, *O*′-bis(2-aminoethyl) ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). A $Ca^{2+}$-free solution without EGTA was also used. The KHS solution was continuously bubbled with 95% $O_2$–5% $CO_2$, and kept at 36·5 ± 0·5°C (pH = 7·35).

*Drugs*

The following drugs were used in the present study: prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Cayman Chemical, Ann Arbor, MI); L-adrenaline bitartrate, diltiazem hydrochloride (Wako Pure Chemical, Osaka, Japan); acetylcholine chloride (Daiichi, Tokyo, Japan); carbachol (carbamylcholine chloride), thapsigargin, $N^G$-nitro-L-arginine (L-NNA), papaverine hydrochloride (Sigma, St Louis, MO); calmidazolium chloride (R24571), SK&F96365 (1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole) (Biomol Research Laboratories Inc., Plymouth Meeting, PA); SIN-1 (3-(4-morpholinyl)-sydnonimine hydrochloride) (Dojindo, Kumamoto, Japan).

Thapsigargin and $PGF_{2\alpha}$ were dissolved in pure ethanol as a stock solution of $10^{-3}$ M and $10^{-2}$ M, and diluted with distilled water to the desired concentrations. Final ethanol concentration in the bath medium did not exceed more than 1%, which did not affect the vascular responses.

*Statistics*

Results were expressed as mean values ± s.e.m. The data obtained were analysed for statistical significance using unpaired Student's *t*-test, unpaired *t*-test with Welch's correction, or one-way analysis of variance followed by Tukey's multiple comparison test. *P* values less than 0·05 were considered statistically significant.

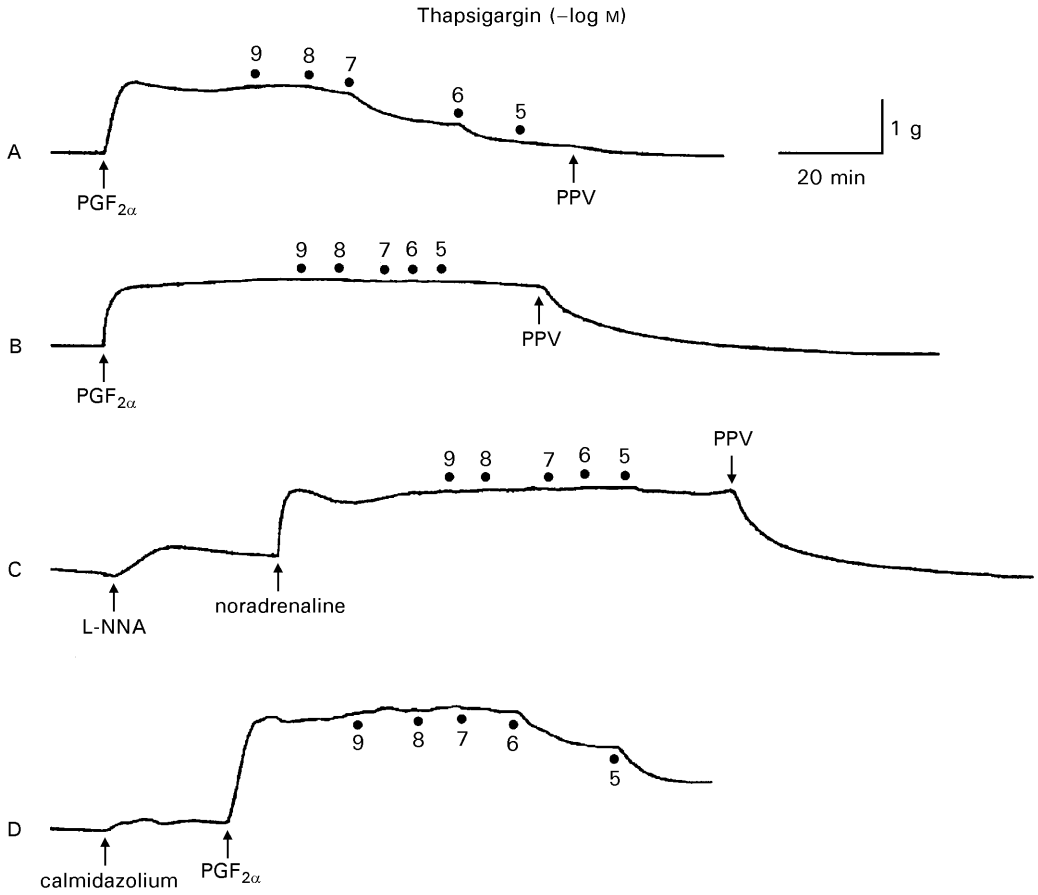


Figure 1. Typical traces showing the vasorelaxant actions of thapsigargin on isolated guinea-pig aorta. Guinea-pig aortic preparations with (A, C, D) or without (B) functional endothelium were preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $10^{-5}$ M) (A, B, D) or with noradrenaline ($3 \times 10^{-6}$ M) (C). Thapsigargin was added cumulatively ($10^{-9}$–$10^{-5}$ M) to the bath solution. $N^G$-Nitro-L-arginine (L-NNA, $3 \times 10^{-4}$ M) (C) and calmidazolium ($3 \times 10^{-5}$ M) (D) were added to the bath solution 30 min before application of noradrenaline (C) or $PGF_{2\alpha}$ (D). Numbers appearing in the traces show the negative logarithm of the concentration of thapsigargin. PPV = papaverine at $10^{-4}$ M.

## Results

*Endothelium-dependent vasorelaxant effects of thapsigargin on guinea-pig thoracic aorta*

In ring preparations of the isolated guinea-pig thoracic aorta, application of noradrenaline ($3 \times 10^{-6}$ M) or $PGF_{2\alpha}$ ($10^{-5}$ M) induced sustained contraction which lasted for at least 3 h without any prominent decline. Thapsigargin, when applied cumulatively to the bath solution, relaxed endothelium-intact vascular preparations that had been preconstricted with $PGF_{2\alpha}$, in a concentration-dependent manner at a concentration range $10^{-8}$–$10^{-5}$ M (Figure 1A). Thapsigargin-induced relaxation developed slowly and reached a maximal level within 10–25 min after application of the compound. The maximum vasorelaxant response to thapsigargin was obtained at $10^{-5}$ M. By comparison, in endothelium-denuded preparations, thapsigargin did not produce vascular relaxation at concentrations up to $10^{-5}$ M (Figure 1B). These findings indicate that vascular relaxation of guinea-pig aorta in response to thapsigargin requires the presence of intact endothelium. Figure 2A shows the concentration–response relationships for the thapsigargin-induced relaxation in the vascular preparations with or without intact endothelium. The pIC50 value (minus logarithm of the concentration of thapsigargin required to induce 50% inhibition of the $PGF_{2\alpha}$-induced contraction) was calculated to be $6{\cdot}57 \pm 0{\cdot}20$ (n = 4) in the preparations with intact endothelium. The maximum relaxation induced by $10^{-5}$ M thapsigargin was $81{\cdot}6 \pm 2{\cdot}7\%$ (n = 4) of the contraction induced by $PGF_{2\alpha}$. By comparison, in endothelium-denuded preparations, thapsigargin failed to produce vascular relaxations at concentrations up to $10^{-5}$ M (Figure 2A).

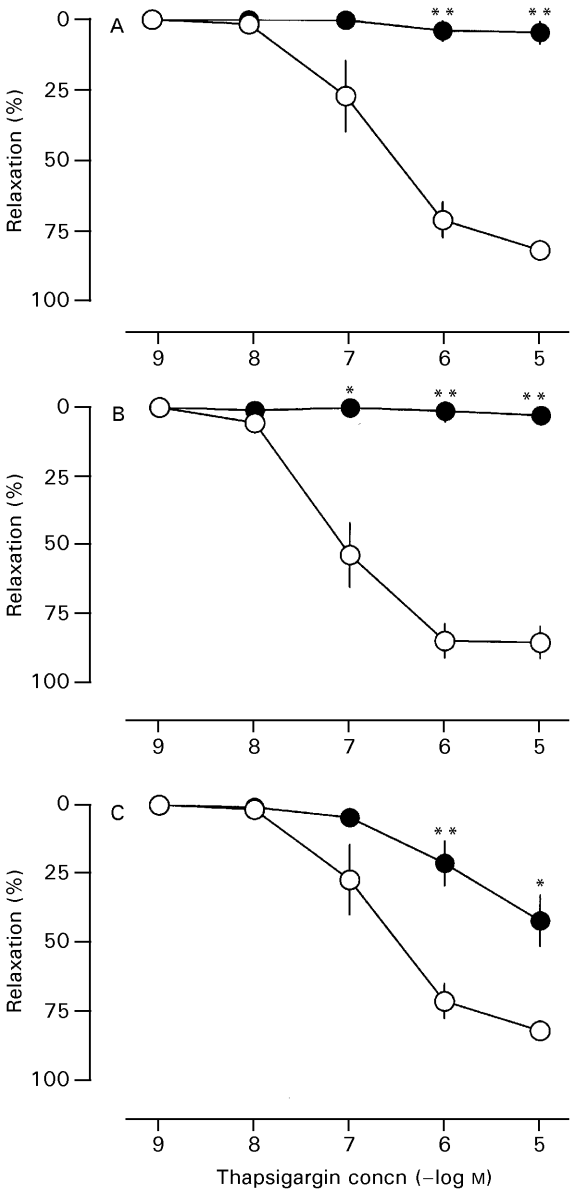


Figure 2. Concentration–response relationships for the vasorelaxant effects of thapsigargin on guinea-pig aorta. Guinea-pig aortic preparations with or without functional endothelium were preconstricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $10^{-5}$ M) (A, C) or with noradrenaline ($3 \times 10^{-6}$ M) (B). Thapsigargin was added cumulatively ($10^{-9}$–$10^{-5}$ M) to the bath solution. Vascular relaxation is expressed on the ordinate as a percentage inhibition against the contractions induced by $PGF_{2\alpha}$ or noradrenaline. Effects with (○) and without (●) functional endothelium (A), in the absence (○) and in the presence (●) of $N^G$-nitro-L-arginine (B), with functional endothelium in the absence (○) and in the presence (●) of calmidazolium (C). Data are mean values ± s.e.m. of four experiments. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$ compared with the corresponding controls.

To determine whether NO is involved in thapsigargin-induced endothelium-dependent vascular relaxation of guinea-pig aorta, the effect of $N^G$-nitro-L-arginine (L-NNA), an inhibitor of NO synthase (Moore et al 1990), was examined on thapsigargin-induced relaxation. L-NNA ($3 \times 10^{-4}$ M) was applied to the bath solution 30 min before application of noradrenaline ($3 \times 10^{-6}$ M). L-NNA was found to abolish carbachol-induced endothelium-dependent relaxation in guinea-pig aorta at a concentration less than $3 \times 10^{-4}$ M ($10^{-5}$ M) (Agata et al 1997). L-NNA itself induced a small contraction in the preparation with functional endothelium (Figure 1C), which corresponded to $23{\cdot}2 \pm 9{\cdot}1\%$ (n = 4) of the noradrenaline ($3 \times 10^{-6}$ M)-induced contraction. As shown in Figure 1C, thapsigargin-induced endothelium-dependent relaxation was largely suppressed by treatment with L-NNA. Inhibitory effects of L-NNA on thapsigargin-induced relaxation are summarized in Figure 2B. L-NNA ($3 \times 10^{-4}$ M) significantly inhibited the relaxation to $10^{-6}$ M thapsigargin by 98·7% from $84{\cdot}8 \pm 6{\cdot}1\%$ (n = 4) to $1{\cdot}1 \pm 3{\cdot}9\%$ (n = 4) ($P < 0{\cdot}01$) and that to $10^{-5}$ M

thapsigargin by 97·1% from 85·3 ± 5·8% (n = 4) to 2·5 ± 3·5% (n = 4) ($P < 0·01$). L-NNA ($3 \times 10^{-4}$ M) did not affect endothelium-independent relaxation by papaverine ($10^{-4}$ M) (Figures 1A and 1C); papaverine ($10^{-4}$ M)-induced relaxations were 125·0 ± 20·7% (n = 4) and 120·8 ± 7·6% (n = 4) in the absence and presence of L-NNA, and these values were not significantly different from each other ($P > 0·05$).

Activation of NO synthase (NOS) in vascular endothelial cells is dependent on $Ca^{2+}$ and calmodulin (Busse & Mülsch 1990; Förstermann et al 1991). To find out whether calmodulin is involved in endothelium-dependent NO-mediated relaxation of guinea-pig aorta in response to thapsigargin, the effect of calmidazolium, a putative calmodulin inhibitor, was examined on the thapsigargin-induced relaxation. Calmidazolium ($3 \times 10^{-5}$ M), when applied 30 min before $PGF_{2\alpha}$, significantly suppressed thapsigargin-induced relaxation (Figures 1D and 2C). This concentration of calmidazolium did not suppress endothelium-independent vascular relaxation induced by SIN-1; the pIC50 value of SIN-1 in the absence of calmidazolium was 6·23 ± 0·32 (n = 4) and that in the presence of calmidazolium ($3 \times 10^{-5}$ M) was 6·54 ± 0·14 (n = 4). These pIC50 values were not significantly different each other ($P > 0·05$). Calmidazolium itself induced a small contraction in the aortic preparation with functional endothelium (Figure 1D), which corresponded to 7·4 ± 8·8% (n = 4) of the contraction induced by $PGF_{2\alpha}$ ($10^{-5}$ M).

Taken together, these findings indicate that thapsigargin-induced endothelium-dependent vascular relaxation of guinea-pig aorta is mainly attributable to the synthesis of NO in endothelial cells, release of NO from endothelial cells, or both, following the activation of $Ca^{2+}$, calmodulin-dependent NOS due to the possible increase in cytosolic free $Ca^{2+}$ concentrations ($[Ca^{2+}]_{cyt}$) in endothelial cells.

### *Extracellular $Ca^{2+}$ dependence of thapsigargin-induced endothelium-dependent NO-mediated relaxation*

To determine whether thapsigargin-induced endothelium-dependent NO-mediated vascular relaxation of guinea-pig aorta is dependent on extracellular $Ca^{2+}$, the effect of removal of $Ca^{2+}$ from the bathing solution was examined on the thapsigargin-induced vasorelaxant response. For this purpose, aortic preparations were incubated in $Ca^{2+}$-free solution with 2 mM EGTA for 30 min before vascular contraction was produced by $PGF_{2\alpha}$ ($10^{-5}$ M). Under this condition, thapsigargin-induced relaxation was abolished (Figure 3A). Thapsigargin at $10^{-5}$ M induced a small contraction instead of relaxation in $Ca^{2+}$-free + 2 mM EGTA solution, which corresponded to 21·2 ± 15·1% (n = 4) of the contraction induced by $10^{-5}$ M $PGF_{2\alpha}$. When the incubating condition in $Ca^{2+}$-free solution was changed (incubation time, 10 min; EGTA was not added to the solution), the thapsigargin-induced relaxation was not observed (n = 4).

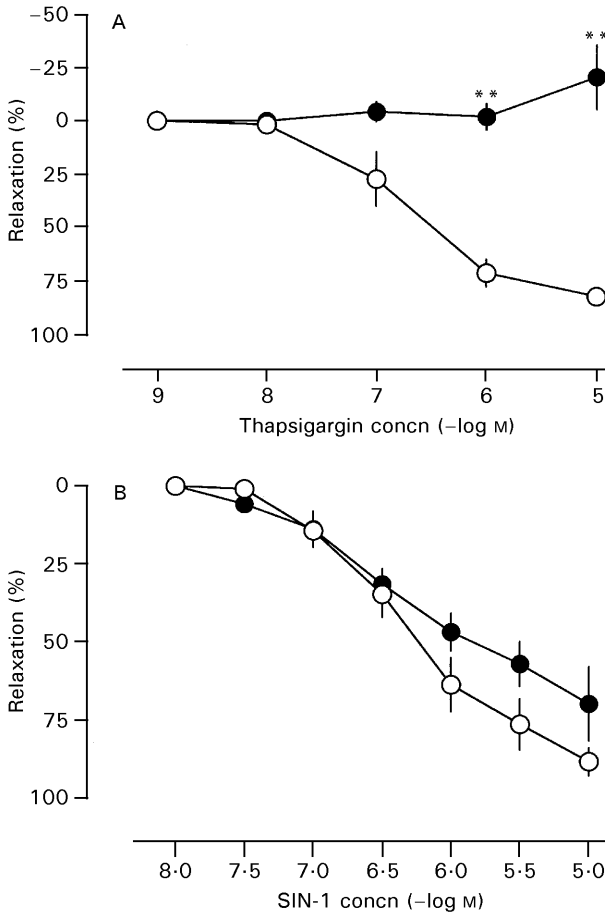


Figure 3. Effects of removal of $Ca^{2+}$ from the bath solution on the concentration–response relationships for the vasorelaxation by thapsigargin (A) and SIN-1 (B) of guinea-pig aorta with (A) and without (B) functional endothelium. ○ in the presence of $Ca^{2+}$, ● in $Ca^{2+}$-free solution containing 2 mM EGTA. Data are mean values ± s.e.m. of four experiments. ** $P < 0·01$, compared with the corresponding controls.

By comparison, endothelium-independent relaxation to SIN-1 was not significantly affected by the removal of extracellular $Ca^{2+}$ (Figure 3B). This suggests that $Ca^{2+}$ removal from the bathing solution does not affect the vascular smooth muscle response to NO. It seems possible that elimination of thapsigargin-induced relaxation in $Ca^{2+}$-free solution is ascribed to the inhibited activation of endothelium NOS as a result of the suppressed

increase in $[Ca^{2+}]_{cyt}$ following the inhibition of the $Ca^{2+}$ store sites' $Ca^{2+}$-ATPase by thapsigargin.

*Effects of increasing $K^+$ in the bathing solution on thapsigargin-induced endothelium-dependent relaxation*

Effects of increasing $K^+$ in the bathing solution were examined on thapsigargin-induced endothelium-dependent NO-mediated relaxation of guinea-pig aorta. For this purpose, thapsigargin-induced relaxation was examined in an endothelium-intact aortic ring preparation preconstricted with a high concentration (80 mM) of KCl rather than with $PGF_{2\alpha}$ ($10^{-5}$ M) (Figure 4A). As compared with the relaxation against the contraction produced by $PGF_{2\alpha}$, thapsigargin-induced relaxation was profoundly attenuated against the contraction produced by 80 mM KCl.

In contrast, endothelium-independent relaxation to SIN-1 against 80 mM KCl-induced contraction of guinea-pig aorta was not suppressed so profoundly as compared with that against $PGF_{2\alpha}$-induced contraction over the concentration range $10^{-8}$–$10^{-5}$ M (Figure 4B). These findings imply that suppression of thapsigargin-induced relaxation in depolarizing high KCl solution is possibly related to the inhibition of synthesis or release of NO. Therefore, it is likely that inhibition of thapsigargin-induced relaxation in high KCl solution is mostly due to the suppression of thapsigargin-stimulated endothelial capacitative $Ca^{2+}$ entry. Slight inhibition of SIN-1-induced relaxation in high KCl solution implies that activation of plasma membrane $K^+$ channels is partly involved in thapsigargin-induced endothelium-dependent NO-mediated relaxation of guinea-pig aorta.

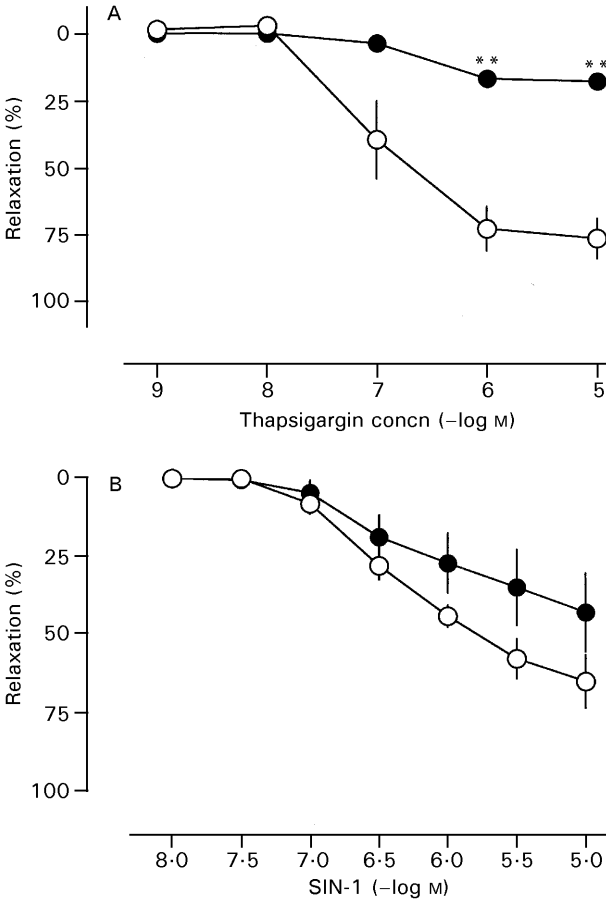


Figure 4. Concentration–response relationships for the vasorelaxation by thapsigargin (A) or SIN-1 (B) of guinea-pig aorta with (A) or without (B) functional endothelium. Guinea-pig aortic preparations with (A) or without (B) functional endothelium were constricted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, $10^{-5}$ M, (○) or with high (80 mM) KCl (●). Thapsigargin or SIN-1 was added cumulatively to the bath solution. Vascular relaxations are expressed as percentage inhibition of the contractions induced by $PGF_{2\alpha}$, or by 80 mM KCl. Data are mean values ± s.e.m. of four or five experiments. ** $P < 0.01$, compared with data for high KCl.

*Effects of diltiazem, SK&F96365 and $Ni^{2+}$ on thapsigargin-induced endothelium-dependent NO-mediated relaxation*

The effect of diltiazem, a blocker of L-type voltage-gated $Ca^{2+}$ channels, was examined on thapsigargin-induced endothelium-dependent relaxation. Diltiazem ($10^{-5}$ M) was applied to the bath solution 30 min before application of $PGF_{2\alpha}$ ($10^{-5}$ M). Diltiazem at $10^{-5}$ M did not affect the basal tone of the vascular preparations and had no effect on endothelium-dependent relaxation to thapsigargin (Figure 5); pIC50 values of thapsigargin were 6·45 ± 0·48 (n = 4) and 6·55 ± 0·18 (n = 4) in the

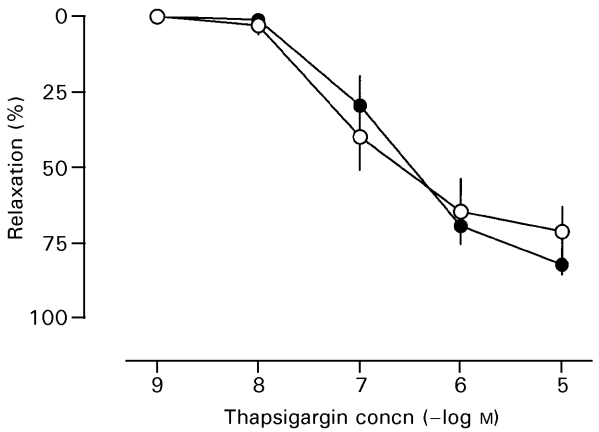


Figure 5. Effects of diltiazem on the concentration–response relationships for the vasorelaxation by thapsigargin of guinea-pig aorta with functional endothelium. Guinea-pig aortic preparations with functional endothelium were preconstricted with prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$, $10^{-5}$ M). ○ Without diltiazem, ● with diltiazem ($10^{-5}$ M). Vascular relaxation is expressed as the percentage inhibition of the contraction induced by $PGF_{2\alpha}$. Data are mean values ± s.e.m. of four experiments.

absence and presence of diltiazem, respectively, and these values were not significantly different from each other ($P > 0.05$).

In a separate series of experiments, the effect of SK&F96365 was examined on thapsigargin-induced endothelium-dependent relaxation. SK&F96365 was applied to the bath solution, at a concentration of $10^{-5}$ or $3 \times 10^{-5}$ M, 30 min before addition of $PGF_{2\alpha}$ ($10^{-5}$ M). The relaxation response to thapsigargin was not affected by either of the concentrations of SK&F96365 used (Figure 6A); pIC50 values of thapsigargin in the absence and presence of SK&F96365 ($10^{-5}$ M and $3 \times 10^{-5}$ M) were $6.38 \pm 0.12$ (n = 4), $5.90 \pm 0.13$ (n = 5) and $6.05 \pm 0.26$ (n = 4), respectively, which were not significantly different ($P > 0.05$). When the concentration of SK&F96365 was increased to $10^{-4}$ M, thapsigargin-induced relaxation remained unaffected (data not shown). $PGF_{2\alpha}$ ($10^{-5}$ M)-induced contraction of guinea-pig aorta was not affected by treatment with SK&F96365 at $3 \times 10^{-5}$ M; the contraction induced by $PGF_{2\alpha}$ ($10^{-5}$ M) in the absence of SK&F96365 was $98.8 \pm 8.8\%$ (n = 3) that of the high (80 mM) KCl-induced contraction, and this was not significantly different from that obtained in the presence of SK&F96365 ($3 \times 10^{-5}$ M) ($101.8 \pm 5.4\%$, n = 4, $P > 0.05$).

The effect of $Ni^{2+}$ was also examined on thapsigargin-induced endothelium-dependent relaxation of guinea-pig aorta. $Ni^{2+}$ at a concentration of $3 \times 10^{-4}$ M was applied to the bath solution 20 min before cumulative application of thapsigargin. Results are shown in Figure 6B. The metal ion did not show any appreciable inhibitory effects on thapsigargin-induced relaxation of guinea-pig aorta; pIC50 values of thapsigargin were $6.50 \pm 0.18$ (n = 4) and $6.16 \pm 0.45$ (n = 3) in the absence and presence of $Ni^{2+}$ ($3 \times 10^{-4}$ M), respectively, and these values were not significantly different from each other ($P > 0.05$). The application of $Ni^{2+}$ ($3 \times 10^{-4}$ M) induced a small contraction, and did not the suppress $PGF_{2\alpha}$ ($10^{-5}$ M)-induced contraction. Augmentation by $Ni^{2+}$ of $PGF_{2\alpha}$-induced contraction was about 20% ($120.4 \pm 2.4\%$ vs control ( = 100%), n = 4). $Cd^{2+}$ ($3 \times 10^{-4}$ M) and $Gd^{3+}$ ($10^{-4}$ M) also did not show any prominent inhibitory effects on thapsigargin-induced relaxation of guinea-pig aorta with functional endothelium (data not shown).

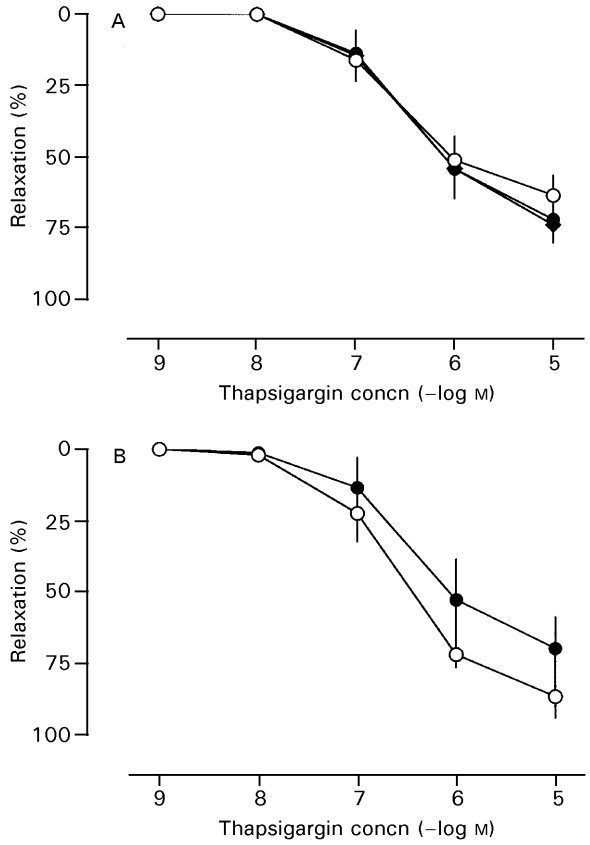


Figure 6. Effects of SK&F96365 and $Ni^{2+}$ on the concentration-response relationships for the vasorelaxation by thapsigargin of guinea-pig aorta with functional endothelium. A. SK&F96365 (○ 0, ● $10^{-5}$ M, ◆ $3 \times 10^{-5}$ M) was added to the bath solution 30 min before application of $PGF_{2\alpha}$. B. $Ni^{2+}$ (○ 0, ● $3 \times 10^{-4}$ M) was added to the bath solution 20 min before application of thapsigargin. Vascular relaxation is expressed as the percentage inhibition of the contraction induced by $PGF_{2\alpha}$. Data are mean values ± s.e.m. of between four and seven experiments.

## Discussion

The present findings indicate that thapsigargin, an inhibitor of $Ca^{2+}$-pump $Ca^{2+}$-ATPase, produces endothelium-dependent NO-mediated relaxation of guinea-pig aorta via activation of $Ca^{2+}$, calmodulin-dependent constitutive NOS. Although endothelial capacitative $Ca^{2+}$ entry from extracellular space is thought to play a key role in thapsigargin-induced endothelium-dependent NO-mediated relaxation of guinea-pig aorta, the $Ca^{2+}$ entry mechanism stimulated with thapsigargin seems to lack sensitivity to SK&F96365 and $Ni^{2+}$ in the endothelium of this artery preparation.

Vascular endothelium releases vasorelaxant substances in response to a wide variety of endogenous vasoactive substances. Endothelium-derived vasorelaxant substances include prostacyclin ($PGI_2$) (Moncada et al 1977), endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki 1980), the chemical identity of which is recognized to be NO (Ignarro et al 1987; Palmer et al 1987), and endothelium-derived hyperpolarizing factor (EDHF) (Garland et al 1995; Mombouli &

Vanhoutte 1997), the chemical identity of which is still unknown. In addition to endogenous vasoactive substances, inhibitors of $Ca^{2+}$-pump $Ca^{2+}$-ATPase such as thapsigargin and cyclopiazonic acid, have been reported to produce endothelium-dependent vascular relaxation in aortae isolated from the rat (Mikkelsen et al 1988; Moritoki et al 1994a, b, 1996; Zheng et al 1994) and the guinea-pig (Matsuyama et al 1993; Tanaka et al 1997), and in mesenteric artery from the rat (Fukao et al 1995). In rat aorta, thapsigargin- and cyclopiazonic acid-induced endothelium-dependent relaxations are mainly attributable to the release of endothelium-derived NO (Mikkelsen et al 1988; Moritoki et al 1994a, b, 1996; Zheng et al 1994). In resistance arteries such as mesenteric artery, not only NO but also EDHF seems to contribute significantly to the thapsigargin-induced endothelium-dependent vascular relaxation (Fukao et al 1995). In the present study, we have shown that thapsigargin-induced endothelium-dependent relaxation of the isolated guinea-pig aorta is mainly caused by endothelium-derived NO and subsequent elevation of smooth muscle cGMP. The reasons for this were as follows: thapsigargin-induced endothelium-dependent relaxation was largely suppressed by L-NNA, an inhibitor of NO synthase (Moore et al 1990); calmidazolium, which is a putative inhibitor of calmodulin, significantly suppressed thapsigargin-induced endothelium-dependent relaxation without affecting endothelium-independent relaxation to SIN-1; and furthermore, we have previously shown that thapsigargin-induced endothelium-dependent relaxation of guinea-pig aorta is largely inhibited by methylene blue, an inhibitor of soluble guanylate cyclase (Tanaka et al 1997). Contribution of $PGI_2$ and EDHF seems to be practically negligible to the endothelium-dependent relaxation of guinea-pig aorta in response to thapsigargin, though the endothelial cells of this arterial preparation are capable of releasing EDHF in response to the stimulation with substance P (Hozumi et al 1997).

Thapsigargin-induced relaxation of guinea-pig aorta was found to be abolished in $Ca^{2+}$-free solution. Since SIN-1-induced endothelium-independent relaxation was not affected by removal of $Ca^{2+}$ from the bathing solution, inhibition of thapsigargin-induced relaxation in $Ca^{2+}$-free solution seems to be associated with suppressed activation of endothelium NOS but not with decreased vascular smooth muscle sensitivity to NO. Elimination of thapsigargin-induced endothelium-dependent relaxation in $Ca^{2+}$-free solution seems to indicate that endothelial capacitative $Ca^{2+}$ entry, which is activated as a result of the depletion of stored $Ca^{2+}$ due to the inhibition of $Ca^{2+}$-pump $Ca^{2+}$-ATPase by thapsigargin, is the crucial step in the vascular relaxation to thapsigargin. In supporting this proposal, increase in endothelium $[Ca^{2+}]_{cyt}$ by $Ca^{2+}$-pump $Ca^{2+}$-ATPase inhibitors was shown to be largely dependent on extracellular $Ca^{2+}$ (Dolor et al 1992; Schilling et al 1992; Gericke et al 1993). Recent studies have suggested that this capacitative $Ca^{2+}$ entry induced by $Ca^{2+}$-pump $Ca^{2+}$-ATPase inhibitors is triggered by an unidentified signal such as calcium influx factor (CIF) (Randriamampita & Tsien 1993) to the plasma membrane SOCCs following the depletion of store sites of $Ca^{2+}$ as a consequence of $Ca^{2+}$-pump $Ca^{2+}$-ATPase inhibition (Moritoki et al 1996). An alternative explanation might be that transient elevation of cytosolic $Ca^{2+}$ concentrations near the cell membrane following inhibition of $Ca^{2+}$-pump $Ca^{2+}$-ATPase by $Ca^{2+}$-pump $Ca^{2+}$-ATP inhibitors, modulates plasma membrane $Ca^{2+}$ permeable channels and stimulates entry of external $Ca^{2+}$ (Pasyk et al 1995).

Thapsigargin-induced relaxation of guinea-pig aorta was suppressed more strongly than SIN-1-induced endothelium-independent relaxation when the artery preparation was preconstricted with a high concentration of KCl instead of pharmacological stimulation (Figure 4). This may be interpreted by the explanation that the driving force for $Ca^{2+}$ entry (Busse et al 1988) is decreased by depolarization of endothelium plasma membrane in high KCl solution, which leads to the suppression of $Ca^{2+}$ entry from extracellular spaces and increase in $[Ca^{2+}]_{cyt}$ responsible for the activation of $Ca^{2+}$, calmodulin-dependent NOS. We have also found that the increase in $[Ca^{2+}]_{cyt}$ in response to substance P was greatly reduced in high KCl solution in freshly isolated endothelial cells from pig coronary artery (unpublished observation). Furthermore, SK&F96365-sensitive ATP-induced increase in $[Ca^{2+}]_{cyt}$ in endothelial cells has been reported to be reduced in 80 mM KCl solution (Li & van Breemen 1996). Thus, it is possible that thapsigargin-stimulated capacitative $Ca^{2+}$ entry is suppressed under high KCl-induced contraction. However, it has not yet been confirmed that the $Ca^{2+}$ entry route stimulated by agonists is the same as the capacitative $Ca^{2+}$ entry route that is activated by $Ca^{2+}$-pump $Ca^{2+}$-ATPase inhibitors.

As to the pharmacological characteristics of SOCCs, Moritoki et al (1996) reported that SOCCs responsible for endothelium-dependent NO-mediated relaxation of rat aorta in response to $Ca^{2+}$-ATPase inhibitors (thapsigargin and cyclopiazonic acid) are sensitive to SK&F96365. SK&F96365 has been reported to reduce cyclopiazonic acid-activated non-specific cation currents in cultured

bovine pulmonary artery endothelial cells (Inazu et al 1995) and mouse anococcygeus muscle cells (Wayman et al 1996). In contrast, in the guinea-pig aorta used in the present study, SK&F96365 failed to show any appreciable inhibitory action on endothelium-dependent NO-mediated relaxation induced by thapsigargin (Figure 6A). Since the concentrations of SK&F96365 used in the present study were almost the same as the ones used to inhibit endothelium-dependent relaxation to $Ca^{2+}$-pump $Ca^{2+}$-ATPase inhibitors in rat aorta (Moritoki et al 1996), it is unlikely that SOCCs in the endothelium of guinea-pig aorta possess sensitivity to SK&F96365.

$Ni^{2+}$ has also been reported to inhibit markedly endothelium-dependent NO-mediated relaxation of rat aorta induced by cyclopiazonic acid (Moritoki et al 1996) as well as by acetylcholine or histamine (Adeagbo & Triggle 1991; Moritoki et al 1996), and to block histamine-induced $Ca^{2+}$ entry into human umbilical-vein endothelial cells (Graier et al 1992). However, as in the case of SK&F96365 action, inhibition of thapsigargin-induced relaxation by $Ni^{2+}$ was not so conspicuous in guinea-pig aorta. This finding, together with the observation using SK&F96365, implies that thapsigargin-activated SOCCs in the endothelium of guinea-pig aorta are pharmacologically different from those of rat aorta. It is possible that species differences exist in the sensitivity of SOCCs to SK&F96365 and $Ni^{2+}$. In any event, contribution of L-type voltage-gated $Ca^{2+}$ channels to thapsigargin-induced increase in $[Ca^{2+}]_{cyt}$ can be ruled out because diltiazem failed to inhibit thapsigargin-induced endothelium-dependent relaxation of guinea-pig aorta.

In this study, we have shown that thapsigargin-induced endothelium-dependent relaxation of guinea-pig aorta is strongly dependent on extracellular $Ca^{2+}$. In contrast, Matsuyama et al (1993) reported that thapsigargin-induced relaxation of guinea-pig aorta did not depend on extracellular $Ca^{2+}$, implying that the $Ca^{2+}$ responsible for activation of endothelium NOS can be sufficiently supplied from cytosolic free $Ca^{2+}$ accumulated as a result of the inhibition of $Ca^{2+}$ pump $Ca^{2+}$-ATPase by thapsigargin. However, extracellular $Ca^{2+}$-dependency of thapsigargin-induced action is not restricted only to endothelium-dependent relaxation since endothelium-independent vascular contraction to thapsigargin was also found to show strong dependency on extracellular $Ca^{2+}$ (unpublished observation) whereas Matsuyama et al (1993) observed extracellular $Ca^{2+}$-independent contraction to thapsigargin.

At present, we do not have any reasonable explanation for this discrepancy between our present finding and those of Matsuyama et al (1993). One possible explanation might be that this discrepancy is due to the differences of the vascular preparations employed in the experiments; in our study, we used ring preparations whereas Matsuyama et al used helical strips of the aorta. Suenaga et al (1993) found that the properties of contractile responses to phorbol 12-myristate 13-acetate and $\alpha$-adrenergic stimulation differed between ring preparations and helical strips in the isolated rat aorta. Thus, it may be possible that in guinea-pig aorta, dependency of thapsigargin-induced vascular actions on extracellular $Ca^{2+}$ differs in ring and helical preparations. Furthermore, in the report of Matsuyama et al (1993), the authors compared the thapsigargin-induced endothelium-dependent relaxations in solutions with and without $Ca^{2+}$ only with regard to the vascular sensitivity to thapsigargin. Thus, the maximum relaxation response to thapsigargin might be reduced in the solution without $Ca^{2+}$ compared with that in normal solution. Other possible factors involved would be differences in the strain and age of the guinea-pigs.

In summary, we have shown that thapsigargin-induced endothelium-dependent vascular relaxation of guinea pig aorta can be ascribed to the increased activation of endothelium NOS. Although the $Ca^{2+}$ responsible for the NOS activation due to thapsigargin seems to be supplied mainly via capacitative $Ca^{2+}$ entry from the extracellular space, this $Ca^{2+}$ entry route in the endothelium of guinea-pig aorta lacks sensitivity to SK&F96365 and $Ni^{2+}$.

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