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Inhibition by SK&F96365 of NO-mediated relaxation induced by Ca^{2+}-ATPase inhibitors in rat thoracic aorta

[1]Hideki Moritoki, Tetsuhiro Hisayama, Shougo Takeuchi, Wataru Kondoh, Shinnichi Inoue & Kan Kida

Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Tokushima, Shomachi, Tokushima 770, Japan

1 We investigated the effect of SK&F96365, a putative inhibitor of receptor-operated Ca^{2+} entry, on the endothelium-dependent, NO-mediated relaxation and cyclic GMP formation induced by Ca^{2+}-ATPase inhibitors in rat thoracic aorta.

2 SK&F96365 inhibited cyclopiazonic acid or thapsigargin-induced relaxation and cyclic GMP formation mediated by a constitutive NO synthase, which is known to be activated by the Ca^{2+} that enters into the endothelial cells via plasma membrane Ca^{2+} channels subsequent to depletion of stored Ca^{2+} by Ca^{2+}-ATPase inhibitors.

3 SK&F96365 also inhibited relaxation and cyclic GMP formation induced by acetylcholine, without affecting those induced by nitroprusside and A23187.

4 Ni^{2+} attenuated relaxation and cyclic GMP formation induced by cyclopiazonic acid and acetylcholine.

5 In contrast, the voltage-dependent Ca^{2+} channel blocker, nifedipine, did not affect the relaxation caused by Ca^{2+}-ATPase inhibitors.

6 These results suggest that endothelium-dependent, NO-mediated relaxation of the arteries induced by Ca^{2+}-ATPase inhibitors is triggered by the Ca^{2+} that enters into endothelial cells via receptor-operated channels (SK&F96365-sensitive channels) subsequent to depletion of stored Ca^{2+} as a result of inhibition of the Ca^{2+}-ATPase (Ca^{2+} pump) of the stores.

Keywords: SK&F96365; cyclopiazonic acid; thapsigargin; aorta; endothelium; Ca^{2+} influx; relaxation; NO formation

Introduction

Ca^{2+}-ATPase inhibitors, such as cyclopiazonic acid (CPA) and thapsigargin (TG), have been shown to cause discharge of intracellular Ca^{2+} by inhibiting the Ca^{2+}-ATPase of inositol trisphosphate-sensitive Ca^{2+} stores (Seidler *et al.*, 1989; Takemura *et al.*, 1989; Thastrup, 1990; Thastrup *et al.*, 1990). The effect of TG on Ca^{2+} movement in cultured endothelial cells has been examined by use of the fluorescent indicator fura-2 as a marker (Dolor *et al.*, 1992; Schilling *et al.*, 1992). However, little is known about the pharmacological effects of Ca^{2+}-ATPase inhibitors on isolated vascular preparations. It has been demonstrated that CPA and TG induced endothelium-dependent relaxations and cyclic GMP production in rat thoracic aorta, and that these effects were inhibited by nitric oxide (NO) pathway inhibitors, calmodulin inhibitors and removal of Ca^{2+}, suggesting that NO is involved in the relaxations induced by Ca^{2+}-ATPase inhibitors (Moritoki *et al.*, 1994a,b; Zheng *et al.*, 1994). Therefore, we concluded that Ca^{2+}-ATPase inhibitors may deplete Ca^{2+} stores in the endothelial cells by inhibiting Ca^{2+}-ATPase, a Ca^{2+} pump, which in turn triggers influx of extracellular Ca^{2+}, leading to activation of constitutive NO synthase and resultant NO generation. The NO thus formed may activate soluble guanylate cyclase to produce guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in the vascular smooth muscle (Kondoh *et al.*, 1993; Moritoki *et al.*, 1994a, b).

Ca^{2+} is known to play a central role in production of NO in the vascular endothelium (Mülsch *et al.*, 1989; Schmidt *et al.*, 1989). In the endothelial cells, agonist-induced increase in Ca^{2+} concentration has been attributed to an influx of extracellular Ca^{2+} via Ca^{2+} channels and in part to the release of intracellularly stored Ca^{2+} (Schilling *et al.*, 1988; 1989; 1992; Jacob, 1990; Dolor *et al.*, 1992). Therefore, we carried out studies to characterize the Ca^{2+} entry channels through which Ca^{2+} enters endothelial cells in response to depletion of the internal stores by Ca^{2+}-ATPase inhibitors.

A preliminary account of some of these data was presented at the 67th Annual Meeting of the Japanese Pharmacological Society (Kondoh *et al.*, 1994a) and 12th International Congress of Pharmacology (Kondoh *et al.*, 1994b).

Methods

The thoracic aorta from male Wistar rats of 8 to 9 weeks old was dissected free and cut into ring segments, 3 mm in length. Each ring was suspended under 1 g tension between two parallel wires in a 2-ml organ bath filled with oxygenated (95% O_2 and 5% CO_2) Krebs solution of the following composition (in mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 1.46, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1, EDTA disodium salt 0.03 and ascorbic acid 0.11. Ca^{2+}-free solution contained 2 mM EGTA and the same salts as Krebs solution but without Ca^{2+}. The ring preparations were equilibrated for 2 h before the start of experiments. For measurement of relaxation, the arteries were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at a concentration corresponding to the EC_{80} (10 or 30 μM). Responses were recorded isometrically with a force-displacement transducer (Nihon Kohden SB 1TH). Concentration-response curves were constructed by adding CPA cumulatively to the 2 ml organ bath in a volume of 10~20 μl, and relaxations were plotted as percentages of the contractions induced by the EC_{80} concentrations of $PGF_{2\alpha}$.

The endothelium was removed from the arterial segments by rubbing the lumen of the artery with cotton thread, and was confirmed by the loss of a relaxant response to acetylcholine (ACh) at the start and end of the experiments.

[1] Author for correspondence.

Assay of cyclic GMP

Helical strips were cut into small pieces, and equilibrated for 120 min at 34°C in Krebs solution bubbled with 95% O_2 and 5% CO_2. The preparations were first incubated with 30 μM $PGF_{2\alpha}$ for 5 min, then with 10 μM CPA or 1 μM TG for 90 s. SK&F96365 and nickel, when used, were added to the medium 40 and 10 min, respectively, before addition of the Ca^{2+}-ATPase inhibitors. Then the preparations were quickly frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid in ice. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C, and the supernatants were extracted with 3 volumes of water-saturated ether before lyophilization. Cyclic GMP was measured by radioimmunoassay with a cyclic GMP assay kit.

Statistical analysis

Values are expressed as means ± s.e.mean. The statistical significance of differences between means was analyzed by Student's unpaired *t* test, and *P* values of less than 0.05 were considered as significant.

Materials

The following were used: 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxy-phenyl}-1H-imidazole hydrochloride (SK&F96365, Calbiochem), cyclopiazonic acid, thapsigargin, nickel chloride, A23187, sodium nitroprusside, acetylcholine chloride and prostaglandin $F_{2\alpha}$ (all from Sigma Chemical Co., St Rouis, MO, U.S.A.), nifedipine (Carbiochem, San Diago, CA, U.S.A.) and a kit for radioimmunoassay of cyclic GMP (Yamasa Shoyu Co., Japan).

Results

Effect of SK&F96365 on the relaxation induced by Ca^{2+}-ATPase inhibitors

Because SK&F96365 considerably decreased noradrenaline induced tone, the aorta was contracted with 30 μM $PGF_{2\alpha}$. In the arteries with intact endothelium, treatment with the receptor-operated Ca^{2+} channel blocker, SK&F96365, at concentrations of 10, 30 and 50 μM for 40 min (while decreasing the $PGF_{2\alpha}$-induced tension from the control level of 943.5 ± 99.1 to 990.4 ± 98.4 mg, 668.8 ± 164.8 mg and 782.2 ± 217.0 mg, respectively) inhibited the relaxation induced by CPA (Figure 1). The relaxations induced by TG and another endothelium-dependent relaxant, ACh, were also inhibited by 50 μM SK&F96365 (Figure 2). In contrast, 50 μM SK&F96365 scarcely affected relaxation induced by A23187 and nitroprusside used for comparison (Figure 2).

Effect of SK&F96365 on the cyclic GMP formation

CPA (10 μM) and TG (0.1 μM) elevated cyclic GMP levels in 90 s. Treatment with 10, 30 and 50 μM SK&F96365 for 40 min dose-dependently attenuated the cyclic GMP formation stimulated by 10 μM CPA, without affecting the basal level of cyclic GMP (Figure 3). Similarly, 50 μM SK&F96365 suppressed cyclic GMP formation stimulated by 0.1 μM TG to the basal level (Figure 4). On the other hand, this concentration of SK&F96365 (50 μM) abolished cyclic GMP formation stimulated by 0.1 μM ACh, but decreased that stimulated by 1 μM ACh by 50%. In contrast, 50 μM SK&F96365 did not affect cyclic GMP formation stimulated by 0.1 μM A23187 or 0.1 μM nitroprusside (Figure 4).

Effect of Ni^{2+} on the CPA-induced responses

Pretreatment with 300 μM Ni^{2+} for 10 min shifted the concentration-response curves for CPA and ACh to the right with reduction of maximum relaxations (Figure 5a). This concentration of Ni^{2+} did not affect nitroprusside-induced relaxation (data not shown).

Ni^{2+} (300 μM) also reduced cyclic GMP formation of the artery stimulated by 10 μM CPA or 0.3 μM ACh to 50% (Figure 5b).

Effect of nifedipine on the CPA-induced relaxation

Treatment with the voltage-dependent Ca^{2+} channel blocker, nifedipine (1 μM), did not affect either endothelium-dependent relaxations induced by CPA and TG, or endothelium-independent relaxation caused by nitroprusside (data not shown), although nifedipine to some extent attenuated the initial tone induced by 30 μM $PGF_{2\alpha}$.

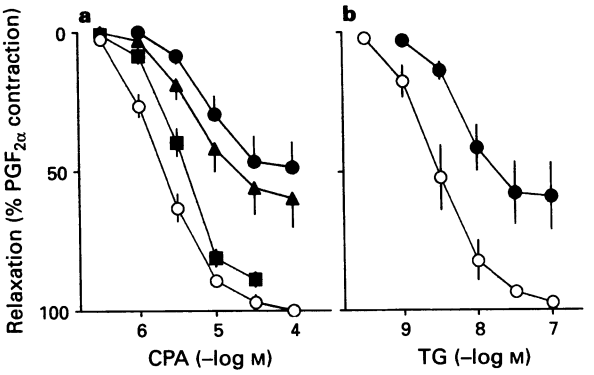

Figure 1 Inhibition by SK&F96365 of the relaxations of the rat thoracic aorta induced by (a) cyclopiazonic acid (CPA) and (b) thapsigargin (TG). The arteries were contracted with the EC_{80} concentration of $PGF_{2\alpha}$ (30 μM) to study relaxation. Tissues were exposed to 10, 30 and 50 μM SK&F96365 for 40 min before application of the Ca^{2+}-ATPase inhibitors. Because of the irreversible nature of TG, the effect of TG in the presence and absence of SK&F96365 was tested in separate preparations: (○) control; in the presence of (■) 10, (▲) 30 and (●) 50 μM SK&F96365. The ordinates show the relaxations of the arteries as percentages of the contraction induced by 30 μM $PGF_{2\alpha}$. Values are means with s.e.mean of preparations from 6 rats.

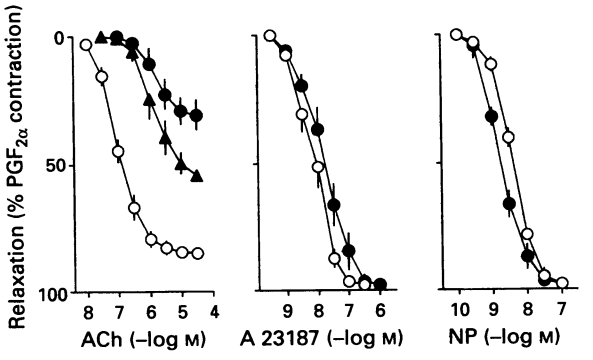

Figure 2 Inhibition by SK&F96365 of the relaxations of the rat thoracic aorta induced by acetylcholine (ACh), A23187 and nitroprusside (NP), used for comparison. Experimental conditions were as for Figure 1: (○) control; in the presence of (▲) 30 and (●) 50 μM SK&F96365. Values are means with s.e.mean of preparations from 6 rats.

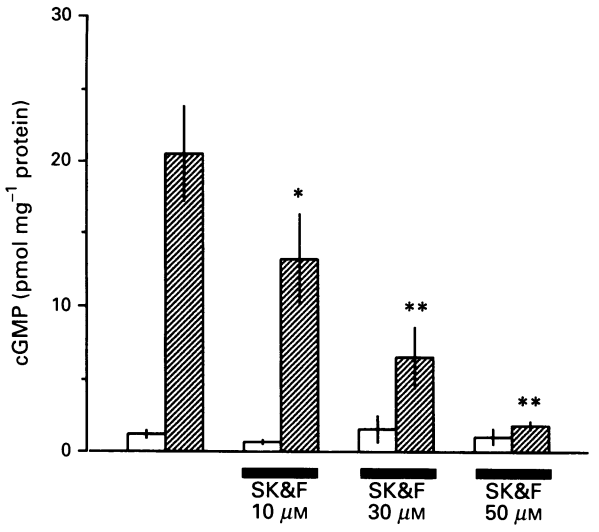

Figure 3 Effect of SK&F96365 on the formation of cyclic GMP (cGMP) stimulated by 10 μM cyclopiazonic acid (CPA) in the rat thoracic aorta. Amounts of cyclic GMP were measured after incubation with the EC_{80} concentration of $PGF_{2\alpha}$ (30 μM) for 5 min and then with CPA for 90 s. SK&F96365, when used, was applied 40 min before and during incubation with CPA. Open columns represent control levels of cyclic GMP; hatched columns show values in the presence of 10 μM CPA. Each column represents the mean ± s.e.mean of values ($n=5$) for preparations from pooled segments of 9 rats. $^*P<0.05$; $^{**}P<0.01$, significantly different from the respective control value (unpaired t test).

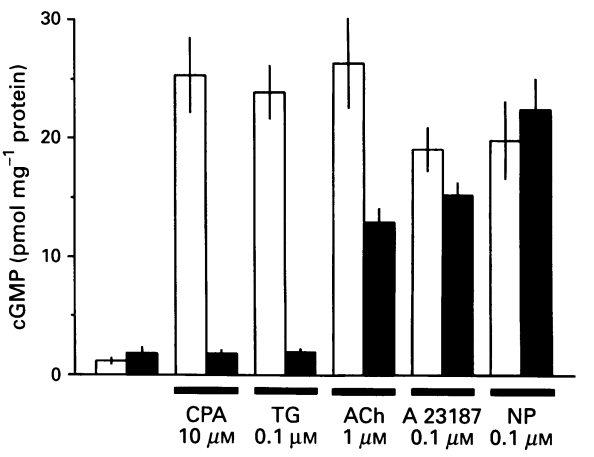

Figure 4 Effects of SK&F96365 on the formation of cyclic GMP (cGMP) stimulated by thapsigargin (TG), and acetylcholine (ACh), A23187 and nitroprusside (NP) used for comparison. Amounts of cyclic GMP were measured after incubation with the EC_{80} concentration of $PGF_{2\alpha}$ (30 μM) for 5 min and then with relaxants for 90 s (for 15 s in case of ACh). Open columns represent control levels of cyclic GMP; solid columns show values in the presence of 50 μM SK&F96365. Horizontal bars below columns show values in the presence of 10 μM CPA, 0.1 μM TG, 1 μM ACh, 0.1 μM A23187 and 0.1 μM nitroprusside, respectively. Other experimental conditions were as for Figure 3. Each column represents the mean ± s.e.mean of values ($n=5\sim6$) in preparations from pooled segments of 12 rats.

Discussion

We have previously demonstrated that the Ca^{2+}-ATPase inhibitors, CPA and TG, induced relaxation of rat thoracic aorta, which was mediated by an endothelium-dependent, NO-mediated mechanism. Removal of Ca^{2+} from the medium abolished the effects of the Ca^{2+}-ATPase inhibitors, suggesting that the Ca^{2+} that enters into the endothelial cells plays a crucial role in the relaxations induced by Ca^{2+}-ATPase inhibitors (Moritoki *et al.*, 1994a, b). In this connection, it has been reported that in cultured vascular endothelial cells, discharge of Ca^{2+} from intracullular stores triggered influx of Ca^{2+} from the extracellular space via Ca^{2+} channels of the plasma membrane (Hallam *et al.*, 1989; Jacob, 1990; Dolor *et al.*, 1992; Schilling *et al.*, 1992), and that Ca^{2+}-ATPase inhibitors increased cytosolic Ca^{2+} (Dolor *et al.*, 1992; Schilling *et al.*, 1992). In human umbilical vein, increase in the intracellular Ca^{2+} concentration by Ca^{2+}-ATPase inhibitors is dependent on the extracellular Ca^{2+} concentration (Hallam *et al.*, 1989; Gericke *et al.*, 1993). On the basis of these considerations, it is hypothesized that in rat thoracic aorta, the Ca^{2+}-ATPase inhibitors CPA and TG prevent Ca^{2+} uptake into intracellular stores in the endothelium and thereby deplete stored Ca^{2+}, which in turn triggers an influx of extracellular Ca^{2+} and stimulates NO synthase. Alternatively, as has been proposed (Pasyk *et al.*, 1995), transient elevation of cytosolic Ca^{2+} near the cell membrane as a consequence of inhibition of the Ca^{2+} pump of Ca^{2+} stores by the Ca^{2+}-ATPase inhibitors, could modulate plasma membrane Ca^{2+} channels and stimulate influx of external Ca^{2+}.

We characterized further the Ca^{2+} entry channels responsible for Ca^{2+} influx triggered by the Ca^{2+}-ATPase inhibitors. Increase in the intracellular Ca^{2+} necessary for NO release is mediated by an opening of receptor-operated ion channels (Johns *et al.*, 1987). It has been reported that SK&F96365 inhibited Ca^{2+} entry mediated by receptor-operated or non-selective cation channels in human platelets, neutrophils (Merritt *et al.*, 1990) and endothelial cells (Merritt *et al.*, 1990; Demirel *et al.*, 1993; Weber *et al.*, 1993) and that SK&F96365 reduced CPA-activated non-specific cation currents in cultured endothelial cells (Inazu *et al.*, 1995). In the present study, we found that in rat thoracic aorta, SK&F96365 concentration-dependently inhibited relaxation and cyclic GMP formation induced by the Ca^{2+}-ATPase inhibitors. In addition, receptor-mediated, endothelium-dependent relaxation and cyclic GMP formation induced by ACh were also inhibited by SK&F96365. In contrast, the responses induced by the Ca^{2+} ionophore, A23187, were not affected. These results suggest that the channels mediating Ca^{2+}-influx triggered by the Ca^{2+}-ATPase inhibitors as well as by ACh are receptor-operated channels (Johns *et al.*, 1987) or SK&F96365-sensitive, non-selective cation channels as observed in HL-60 cells (Krautwurst *et al.*, 1993).

At high concentrations SK&F96365 has been shown also to block dihydropyridine-sensitive, voltage-dependent Ca^{2+} channels in arterial smooth muscle cells (Merritt *et al.*, 1990). However, we found that the dihydropyridine Ca^{2+} channel blocker, nifedipine, did not affect CPA-induced relaxation. In addition, it has been reported that vascular endothelium and endothelial cells are devoid of voltage-dependent Ca^{2+} channels (Colden-Stanfield *et al.*, 1987; Jayakody *et al.*, 1987) and that Ca^{2+} channel blockers had no significant effect on the endothelium-dependent relaxation (Jayakody *et al.*, 1987; Adeagbo & Triggle, 1991). Therefore, it is unlikely that the inhibitory effect of SK&F96365 observed in the present experiments is due to inhibition of Ca^{2+} influx via voltage-dependent channels.

SK&F96365 did not inhibit NO-mediated relaxation and cyclic GMP formation induced by the Ca^{2+} ionophore, A23187, indicating that SK&F96365 does not directly inhibit constitutive NO synthase.

SK&F96365 has been shown to interfere with release of Ca^{2+} from internal stores (Merritt *et al.*, 1990), but this effect is not the major cause of inhibition of relaxation and cyclic GMP formation induced by the Ca^{2+}-ATPase inhibitors for the following reasons: (1) it has been reported that SK&F96365 at 50 μM had little or no effect on agonist-induced mobilization of stored Ca^{2+} (Krautwurst *et al.*, 1993); (2) concentrations of SK&F96365 necessary to inhibit release of Ca^{2+} are 10 fold higher than those affecting receptor-operated Ca^{2+} entry

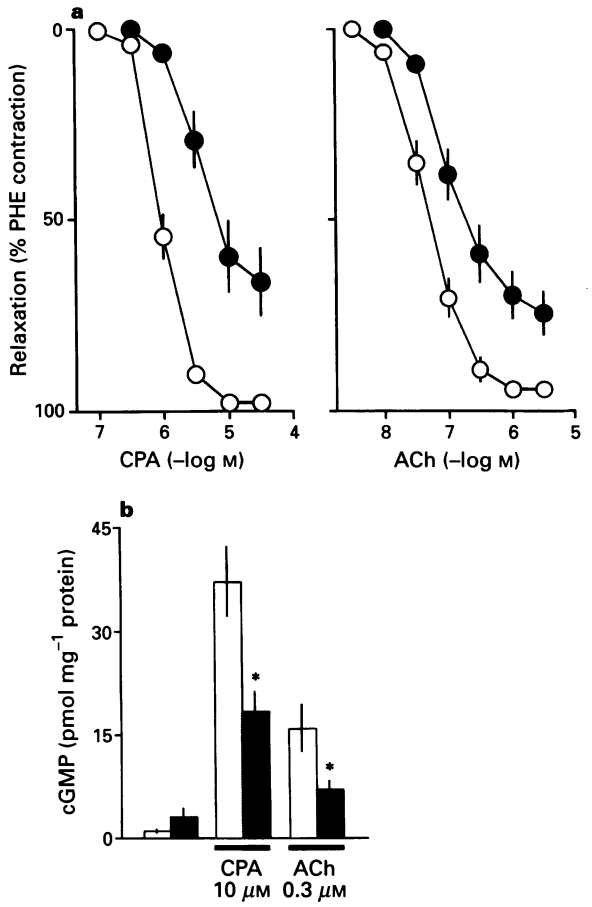

Figure 5 Inhibitory effect of Ni^{2+} on the relaxation [a] and cyclic GMP formation [b] induced by cyclopiazonic acid (CPA) or acetylcholine (ACh) in the rat thoracic aorta. (a) To study relaxation, the arteries were contracted with the EC_{80} concentration of phenylephrine (PHE, 1 μM): (○) control; (●) in the presence of 300 μM Ni^{2+}. Values are means ± s.e.mean of preparations from 6 rats. Other experimental conditions for relaxation experiments were as for Figure 1. (b) Amounts of cyclic GMP (cGMP) were measured after incubation with the EC_{80} concentration of phenylephrine (1 μM) for 5 min and then with CPA and ACh. Other experimental conditions for cyclic GMP measurement were as for Figure 3. Open columns represent control levels of cyclic GMP; solid columns show value in the presence of 300 μM Ni^{2+}; horizontal bars below columns show values in the presence of 10 μM CPA or 0.3 μM ACh. Columns represent mean ± s.e.mean of values for 5~6 preparations from pooled segments of 6 rats. $^*P<0.01$, significantly different from the respective control value (unpaired t test).

(Merritt *et al.*, 1990); (3) we have previously found that CPA-induced relaxation was mainly dependent on the extracellular Ca^{2+} (Moritoki *et al.*, 1994a, b), and (4) in the present study, we found that SK&F96365 at low concentrations (10~50 μM) concentration-dependently inhibited both CPA-induced relaxation and cyclic GMP formation.

On the contrary, SK&F96365 at concentrations higher than 100 μM has been demonstrated to activate Ca^{2+} entry via nonselective cation channels in human cultured endothelial cells (Schwarz *et al.*, 1994). However, this transient increase does not seem to affect CPA- or ACh-induced responses, as the concentrations of SK&F96365 used in the present studies were lower (~50 μM) than those required for activation of Ca^{2+} influx (100 μM~), and SK&F96365-induced transient increase in Ca^{2+}, if any, probably levelled off during pretreatment of the aorta with SK&F96365 for 40 min.

The possibility cannot be ruled out that SK&F96365 blocks Ca^{2+}-activated K^+ channels, resulting in depolarization of the endothelial cells, which in turn reduces the inward driving force for Ca^{2+}, as has been observed in human umbilical endothelial cells (Schwarz *et al.*, 1994).

It has been reported that Ni^{2+} blocked receptor-mediated Ca^{2+} entry found in vascular endothelium (Graier *et al.*, 1992) and platelets (Hallam & Rink, 1985), and that CPA-activated Ca^{2+} influx in HL-60 cells was blocked by Ni^{2+} (Demaurex *et al.*, 1992). In addition, Ni^{2+} markedly inhibited ACh- and histamine-induced relaxations of rat aorta (Adeagbo & Triggle, 1991). The present results, showing that Ni^{2+} attenuated CPA- and ACh-induced relaxations without affecting those induced by nitroprusside, further support the idea that CPA and TG stimulate Ca^{2+} influx through receptor-operated or nonselective cation channels.

In the present study, relaxations induced by the Ca^{2+}-ATPase inhibitors and ACh were not completely suppressed by SK&F96365 in concentrations reported to prevent Ca^{2+} influx selectively (Merritt *et al.*, 1990). In addition, ACh-stimulated cyclic GMP formation was not completely suppressed by SK&F96365 at a concentration sufficient to abolish CPA-induced effects. These results suggest that Ca^{2+} released from internal stores is in part responsible for the SK&F96365-resistant portion of the relaxations, especially in the case of ACh. However, our previous result showing that removal of Ca^{2+} from the medium completely abolished the relaxation induced by Ca^{2+}-ATPase inhibitors (Moritoki *et al.*, 1994a, b) implies that their effects are mainly dependent on extracellular Ca^{2+}, and that the amount of intracellular free Ca^{2+} accumulated as a result of inhibition by CPA of Ca^{2+}-ATPase (Ca^{2+} pump) is not sufficient for stimulation of the NO synthase.

It is therefore concluded that CPA and TG may inhibit Ca^{2+}-ATPase (Ca^{2+} pump) of the Ca^{2+} stores in the endothelium to empty stored Ca^{2+}. Depletion of the stored Ca^{2+} may trigger influx of extracellular Ca^{2+} via receptor-operated channels (SK&F96365-sensitive channels) by some as yet unknown mechanisms involving putative messengers such as calcium influx factor: CIF (Randriamampita & Tsien, 1993). The Ca^{2+} that enters the endothelial cells through SK&F96365-sensitive channels may activate constitutive NO synthase and produce NO, causing relaxation of the arteries.

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