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# Relaxation of rat thoracic aorta induced by the $Ca^{2+}$-ATPase inhibitor, cyclopiazonic acid, possibly through nitric oxide formation

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**1** The effect of the $Ca^{2+}$-ATPase inhibitor, cyclopiazonic acid (CPA), was studied on rat thoracic aortic ring preparations.

**2** At concentrations above 0.3 μM, CPA induced relaxation in the arteries precontracted with phenylephrine. Removal of the endothelium abolished CPA-induced relaxation.

**3** The nitric oxide (NO) synthase inhibitor $N^G$-nitro L-arginine (3–300 μM), the free radical scavenger haemoglobin (0.1–3 μM), the soluble guanylate cyclase inhibitor, LY83583 (0.1–10 μM), each inhibited the endothelium-dependent relaxation to CPA. The potassium channel blocker, glibenclamide (10 μM) and cyclo-oxygenase inhibitor, indomethacin (100 μM for 60 min and then washed out) did not alter the action of CPA.

**4** The calmodulin inhibitors calmidazolium (3–10 μM) and W-7 (100 μM) also abolished CPA-induced relaxation.

**5** CPA (10 μM) increased guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in arteries with an intact endothelium, without affecting adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels.

**6** The inhibitors of NO synthesis and actions, the calmodulin inhibitor and removal of the endothelium abolished the CPA-stimulated increase in the levels of cyclic GMP.

**7** In $Ca^{2+}$-free solution, CPA failed to induce relaxation or to stimulate cyclic GMP production. Relaxation to nitroprusside was not affected under these conditions.

**8** These results suggest that CPA can stimulate NO synthesis, possibly by inhibiting a $Ca^{2+}$-ATPase, which replenishes $Ca^{2+}$ in the intracellular storage sites in endothelial cells. Depletion of the $Ca^{2+}$ store in the endothelium may then trigger influx of extracellular $Ca^{2+}$, contributing to an increase in free $Ca^{2+}$ in the endothelial cells, which activates NO synthase and NO formation.

**Keywords:** Cyclopiazonic acid; rat aorta; relaxation; endothelium; nitric oxide; cyclic GMP; calcium; calmodulin; $Ca^{2+}$-ATPase inhibition

## Introduction

Biosynthesis of nitric oxide (NO) is correlated to the free $Ca^{2+}$ concentration in the cytoplasm of endothelial cells (Johns *et al.*, 1987; Mülsch *et al.*, 1989; Lückhoff *et al.*, 1988; Buchan & Martin, 1991). In the endothelial cells, an agonist-induced increase in $Ca^{2+}$ concentration follows an influx of extracellular $Ca^{2+}$ via $Ca^{2+}$ channels and the release of intracellularly stored $Ca^{2+}$ (Schilling *et al.*, 1988; 1989; 1992; Hallam *et al.*, 1989; Jacob, 1990; Dolor *et al.*, 1992).

Cyclopiazonic acid (CPA) is a specific inhibitor of $Ca^{2+}$-dependent ATPase in sarcoplasmic/endoplasmic reticulum vesicles (Goeger *et al.*, 1988), and inhibits $Ca^{2+}$ uptake by the sarcoplasmic reticulum (Goeger *et al.*, 1988; Goeger & Riley, 1989; Seidler *et al.*, 1989). It has been documented that CPA can deplete inositol trisphosphate-sensitive $Ca^{2+}$ stores, and subsequently activate $Ca^{2+}$ influx in several cell types (Mason *et al.*, 1991; Foskett & Wong, 1992; Demaurex *et al.*, 1992). In vascular smooth muscle, CPA, besides raising resting tension (Shima & Blaustein, 1992) and inducing $Ca^{2+}$-dependent contraction (Deng & Kwan, 1991), has been shown to inhibit contractions induced by phenylephrine (Deng & Kwan, 1991) in $Ca^{2+}$-free solution, possibly by inhibiting ATP-dependent $Ca^{2+}$ uptake into sarcoplasmic reticulum and subsequently depleting stored $Ca^{2+}$. However, an effect of CPA on the vascular endothelium has not been reported.

The present study was carried out to determine whether CPA could induce relaxation through the activation of NO synthase in the rat aorta, which may reflect an effect of $Ca^{2+}$ entry.

A preliminary account of some of these data was presented at the 66th Annual Meeting of the Japanese Pharmacological Society, Yokohama, Japan, 1993 (Kondoh *et al.*, 1993).

## Methods

### *Organ bath experiments*

Male Wistar rats (9–11 weeks old) were killed by a blow to the head, cervical dislocation, and rapid exsanguination. The thoracic aortae were rapidly removed, freed of adjacent connective tissue under a dissecting microscope, and cut into ring segments of 3 mm length. The ring segments were placed in a 10 ml organ bath at 34°C containing oxygenated (95% $O_2$ and 5% $CO_2$) Krebs solution of the following composition (mM): NaCl 115.3, KCl 4.9, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.1. $Ca^{2+}$-free solution was made by omitting $CaCl_2$ from the Krebs solution and adding 2 mM EGTA.

The ring preparations were maintained at 1.0 g resting tension, and equilibrated for 2 h before the start of an experiment. For the measurement of relaxation, the arteries were precontracted with phenylephrine (PHE) at a concentration corresponding to the $EC_{80}$ (0.3 μM for the preparations with

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endothelium and 0.1 μM for those without endothelium). In $Ca^{2+}$-free solution, the contraction induced by PHE was transient and greatly attenuated. However, under these conditions, as has been reported (Bradley & Morgan, 1987), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induced an attenuated, but sustained tonic contraction. In addition, a larger maximum contraction was produced by $PGF_{2\alpha}$ than by PHE. Therefore, in the experiments using $Ca^{2+}$-free conditions, an $EC_{80}$ concentration of $PGF_{2\alpha}$ (around 10 μM) was used to produce tone.

Responses were recorded isometrically with a force displacement transducer (Nihon Kohden SB 1TH). Concentration-response curves were constructed by adding CPA cumulatively to the 10 ml organ bath in a volume of 10–20 μl, and relaxations were plotted as percentages of the contraction induced by the $EC_{80}$ concentrations of PHE.

The endothelium was removed by rubbing the lumen of the artery with a cotton thread, and was assessed by a loss of relaxation to acetylcholine (ACh) at the start and end of each experiment.

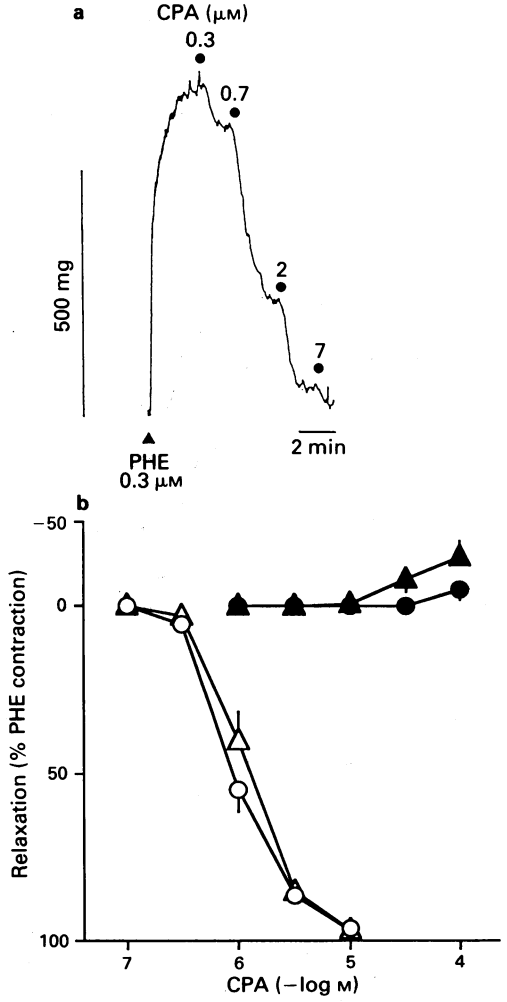


**Figure 1** (a) Representative trace showing relaxation in the rat thoracic aorta induced by cyclopiazonic acid (CPA). The arteries were first contracted with an $EC_{80}$ concentration of phenylephrine (PHE, 0.3 μM). (b) Effect of CPA on rat thoracic aorta with (○) and without endothelium (●). Initial tension, 576 ± 62 mg with endothelium and 994 ± 58 mg without endothelium. The ordinate scale shows the relaxation of the arteries expressed as a percentage of the contraction induced by 0.3 μM PHE. Preparations with matched levels of tension (386 ± 54 mg with (△) vs. 327 ± 47 mg without (▲) endothelium, $n = 6$) by lowering the concentration of PHE to 0.03–0.1 μM. Values are means with s.e.mean of preparations from 10 rats.

### *Assay of cyclic nucleotides*

Segments of aorta were equilibrated in Krebs solution bubbled with $O_2$ containing 5% $CO_2$ for 2 h before the start of experiments. Arteries were first incubated with 0.3 μM PHE for 5 min, and then challenged with 3 or 10 μM CPA for 90 s. To study the effect of removal of $Ca^{2+}$, 2 min after replacing normal Krebs solution with $Ca^{2+}$-free EGTA-containing medium, the arteries were incubated with 10 μM $PGF_{2\alpha}$ for 5 min, and then challenged with 10 μM CPA. NO inhibitors, when used, were applied before and throughout the incubation with 10 μM CPA. After incubation with CPA for 90 s, the preparations were quickly frozen in liquid nitrogen and then homogenized in ice-cold 6% trichloroacetic acid in a Potter glass-glass homogenizer. The homogenates were centrifuged at 1700 *g* for 15 min at 4°C, and the supernatants were extracted with three volumes of water-saturated ether. Cyclic GMP and cyclic AMP were measured by radioimmunoassay. Briefly, the cyclic nucleotides in the supernatant were succinylated and incubated with [$^{125}$I]-succinyl cyclic GMP tyrosine methyl ester or [$^{125}$I]-succinyl cyclic AMP tyrosine methyl ester, and antisera for 18 h at 4°C. Then dextran-coated charcoal was added to the reaction medium to terminate the reaction. The radioactivity in the supernatant was counted in a gamma spectrophotometer. Amounts of cyclic nucleotides were expressed as pmol $mg^{-1}$ protein.

### *Statistical analysis*

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

### *Materials*

The drugs used were cyclopiazonic acid, acetylcholine chloride, sodium nitroprusside, methylene blue, haemoglobin, calmidazolium, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalene-sulphonamide), glibenclamide, indomethacin, phenylephrine hydrochloride (all from Sigma Chemical Co., St Louis, MO, U.S.A.), LY83583 (6-anilino-5,8-quinolinedione, Calbiochem, San Diego, CA, U.S.A.), $N^G$-nitro L-arginine and $N^G$-nitro D-arginine (Peptide Institute, Osaka, Japan) and prostaglandin $F_{2\alpha}$ (Ono Pharmaceutical Co. Ltd., Osaka, Japan). Kits for radioimmunoassay of cyclic GMP and cyclic AMP were obtained from Yamasa Shoyu Co. Ltd. (Choshi, Japan).

## Results

### *Relaxation induced by cyclopiazonic acid*

As shown in Figure 1a, the cumulative application of CPA at concentrations above 0.3 μM caused relaxation of the rat thoracic aorta with an $EC_{50}$ value of 0.86 ± 0.16 μM ($n = 10$). The maximal relaxation caused by 10 μM CPA was sustained until CPA was washed out. CPA-induced relaxation was reproducible, but more than 60 min between exposures were necessary for a complete recovery of responses to either CPA or PHE.

In the arteries without endothelium, CPA did not induce relaxation, even at a concentration as high as 100 μM (Figure 1b). Although removal of the endothelium augmented the PHE-induced tone (576 ± 62 mg with and 994 ± 58 mg without endothelium, $n = 10$), nitroprusside-induced relaxation was not attenuated but slightly potentiated ($EC_{50}$ values: 3.28 ± 0.49 nM with endothelium vs. 1.52 ± 0.25 nM without endothelium, $n = 6$, $P < 0.05$). Moreover, even when the initial tension was normalized by lowering the concentration of PHE, CPA did not induce relaxation.

Treatment with 100 μM indomethacin for 60 min (and then washed for 60 min) or 10 μM glibenclamide for 30 min did not affect the CPA-induced relaxation (data not shown).

In unstimulated, endothelium-denuded arteries, CPA at higher concentrations than those inducing relaxation (above 10 μM) evoked contraction. At a concentration of 100 μM, the contraction reached a plateau, and the maximal response was 11.0 ± 5.9% ($n = 4$) of that induced by 0.3 μM PHE. However, the contraction was not observed in endothelium-intact arteries

### *Effect of inhibitors of NO synthesis and action on CPA-induced relaxation*

In arteries with a functional endothelium, $N^G$-nitro L-arginine (L-NOARG) inhibited CPA-induced relaxation (Figure 2). In the presence of 300 μM L-NOARG, CPA did not induce any relaxation. In contrast to L-NOARG, its enantiomer $N^G$-nitro D-arginine did not alter the CPA-induced relaxation. Haemoglobin (Hb, for 5 min) and LY83583 (for 20 min) had essentially similar inhibitory actions on the relaxation to CPA (Figure 2a).

Because inhibitors of the NO pathway augmented the initial tension caused by the $EC_{80}$ concentration of PHE (Table 1), we examined whether the increased tone by the inhibitors of NO pathway attenuated the relaxant effect of CPA. However, even when the initial tensions were elevated by 100 μM L-NOARG, relaxations to nitroprusside and cromakalim were not weakened (the $EC_{50}$ values of nitroprusside being 1.79 ± 0.30 nM in control and 2.01 ± 0.37 nM ($n = 5$) in treated rings, and those of cromakalim being 203.3 ± 40.1 nM in control and 236.4 ± 46.5 nM ($n = 5$) in treated rings).

Furthermore, even after the initial tension of the arteries treated with 100–300 μM L-NOARG, 1–3 μM LY83583 or

**Table 1** Changes in the initial tensions in the presence of inhibitors of NO pathway

| *Inhibitor* | μM | *Tension* (mg) |
|---|---|---|
| L-NOARG | 0 | 608 ± 47 |
| | 10 | 1101 ± 136 |
| | 100 | 1070 ± 63 |
| | 300 | 1146 ± 92 |
| LY 83583 | 0 | 707 ± 88 |
| | 0.3 | 1009 ± 114 |
| | 3 | 948 ± 83 |
| Haemoglobin | 0 | 582 ± 69 |
| | 0.3 | 698 ± 39 |
| | 3 | 803 ± 58 |
| Calmidazolium | 0 | 623 ± 97 |
| | 3 | 402 ± 69 |
| | 10 | 717 ± 61 |
| W-7 | 0 | 675 ± 62 |
| | 10 | 238 ± 38 |

Tensions induced by 0.3 μM phenylephrine are expressed as mg. Values are mean ± s.e.mean ($n = 5$–8). L-NOARG: $N^G$-nitro-L-arginine.

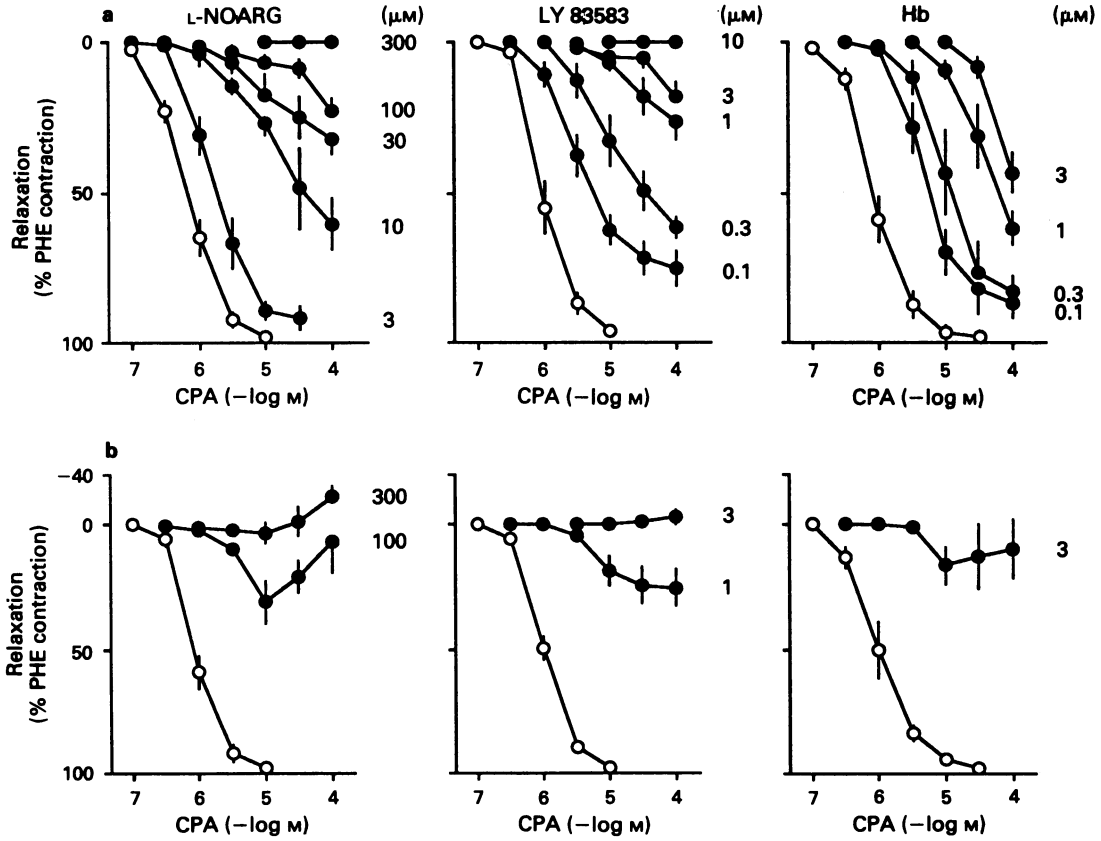


**Figure 2** (a) Inhibitory effects of $N^G$-nitro L-arginine (L-NOARG), haemoglobin (Hb) and LY83583 on the cyclopiazonic acid (CPA)-induced relaxation in the rat thoracic aorta. Tissues were exposed to Hb for 5 min and to the other inhibitors for 20 min before the application of CPA. The ordinate scale shows the relaxation of the arteries expressed as a percentage of the contraction induced by 0.3 μM phenylephrine (PHE). (○), control; (●), in the presence of the inhibitors. (b) Effects of the inhibitors in preparations in which the initial tensions were matched by lowering the concentration of PHE to 0.03–0.1 μM. The ordinate scale shows the relaxation of the arteries expressed as a percentage of the respective initial tension. (○), control; (●), in the presence of the inhibitors. The initial tensions were as follows: L-NOARG (573 ± 48 mg in control vs. 546 ± 74 mg in treated rings), LY85383 (563 ± 52 mg in control vs. 642 ± 70 mg in treated rings), Hb (597 ± 43 mg in control vs. 620 ± 57 mg in treated rings). Experimental conditions were as for Figure 1. Values are means ± s.e.means of preparations from 6 rats.

3 μM Hb was adjusted to an equivalent level by lowering the concentration of PHE to 0.03 to 0.1 μM, the inhibitory effects of these inhibitors on the CPA-induced relaxation were not substantially different from those observed in un-normalized preparations (Figure 2b).

### *Effect of CPA on cyclic GMP formation*

The time course of cyclic GMP formation in the rat aorta stimulated with 10 μM CPA is shown in Figure 3a. In preparations with a functional endothelium, CPA at a concentration of 10 μM increased the level of cyclic GMP from a basal level of 1.28 ± 0.32 pmol $mg^{-1}$ protein, to 28.04 ± 2.40 pmol $mg^{-1}$ protein within 90 s ($n = 5-6$). The level then decreased, but remained elevated (10.05 ± 1.70 pmol $mg^{-1}$ protein) above basal, even after 20 min (Figure 3a). On the basis of the time course of cyclic GMP formation, amounts of the cyclic GMP were measured after incubation with CPA for 90 s. CPA-stimulated cyclic GMP formation was concentration-dependent (Figure 4).

In the endothelium-denuded preparations, 10 μM CPA did not increase the cyclic GMP level.

In contrast to the marked elevation of the cyclic GMP level, the cyclic AMP level was not significantly increased by 10 μM CPA (Figure 3b).

### *Effect of inhibitors of NO pathway on the CPA-stimulated cyclic GMP production*

Treatment with 10 μM L-NOARG for 20 min, besides reducing the basal level of cyclic GMP to 0.12 ± 0.03 pmol $mg^{-1}$ protein, suppressed the cyclic GMP production stimulated by 10 μM CPA from the control value of 28.04 ± 2.40 pmol $mg^{-1}$ protein to 0.69 ± 0.07 pmol $mg^{-1}$ protein, $n = 5-6$ (Figure 4).

Similarly, treatment with 3 μM Hb for 5 min or 1 μM LY83583 for 20 min suppressed the CPA-stimulated increase in cyclic GMP production. Removal of the endothelium also abolished any significant stimulation of cyclic GMP synthesis by CPA.

### *Effect of calmodulin inhibitors on CPA-induced relaxation and cyclic GMP formation*

Pretreatment of artery segments with the calmodulin inhibitor, calmidazolium at a concentration of 3 μM for 20 min attenuated CPA-induced relaxation (Figure 5a). Increasing the concentration of calmidazolium to 10 μM completely suppressed CPA-induced relaxation and CPA-stimulated cyclic GMP production (Figure 5b). Under these conditions, ACh-induced relaxation and cyclic GMP production were similarly blocked by calmidazolium, whereas those induced by nitroprusside were not affected by the inhibitor (Figure 5).

W-7 at a concentration of 100 μM, which attenuated the contraction induced by the $EC_{80}$ concentration of PHE (0.3 μM) to 38% (Table 1), completely suppressed CPA-induced relaxation (Figure 6) and CPA-stimulated cyclic GMP production (29 pmol $mg^{-1}$ protein in control and 3.5 pmol $mg^{-1}$ protein in treated preparation, $n = 5$). This concentration of W-7 nearly suppressed ACh-induced relaxation without affecting that induced by nitroprusside.

### *Effect of removal of $Ca^{2+}$*

PHE-induced contraction was transient and greatly attenuated in the absence of $Ca^{2+}$; $PGF_{2\alpha}$ was therefore used to induce tone. Two minutes after replacing normal Krebs solution with $Ca^{2+}$-free EGTA-containing medium, the arteries were incubated with $PGF_{2\alpha}$ at a concentration corresponding to the $EC_{80}$ (10 μM) to produce background tone. When the contraction induced by 10 μM $PGF_{2\alpha}$ reached a plateau, a single dose of CPA was applied. In $Ca^{2+}$-free conditions, although the $PGF_{2\alpha}$-induced contraction was reduced to 34% (528 ± 63 mg in control and 181 ± 21 in $Ca^{2+}$-free solution, $n = 6$), CPA at concentrations of up to 100 μM did not relax the arteries (Figure 7a). In addition, 10 μM CPA failed to stimulate cyclic GMP production (Figure 7b). Under these conditions, the relaxation and cyclic GMP production induced by 0.1 μM ACh were attenuated, whereas similar responses to 0.1 μM nitroprusside were not altered (Figure 7).

## Discussion

CPA is a selective inhibitor of the $Ca^{2+}$-ATPase which mediates uptake of $Ca^{2+}$ into endoplasmic/sarcoplasmic reticulum, and is known to deplete $Ca^{2+}$ stores (Goeger & Riley, 1989; Seidler *et al.*, 1989).

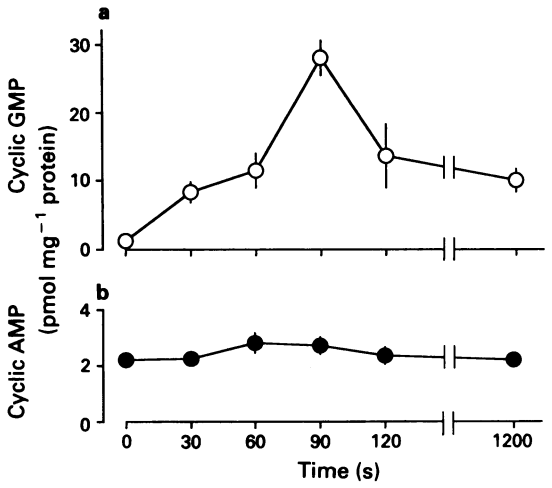


**Figure 3** (a) Time course of cyclopiazonic acid (CPA)-stimulated formation of cyclic GMP in rat aortic preparations with a functional endothelium. The arteries were incubated with an $EC_{80}$ concentration of phenylephrine for 5 min before exposure to 10 μM CPA. (b) Time course of cyclic AMP levels in the presence of 10 μM CPA. Each point represents mean ± s.e.mean of values ($n = 5-6$) in preparations from 8 to 12 rats.

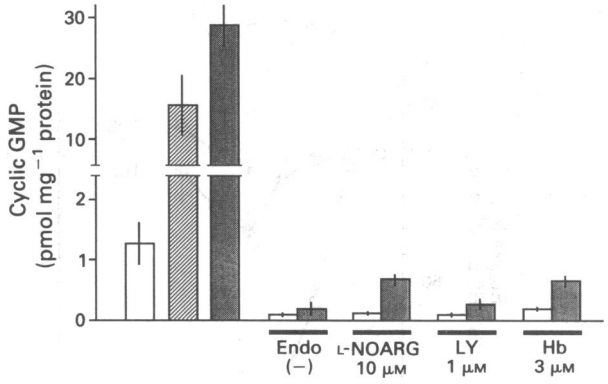


**Figure 4** Effects of NO pathway inhibitors and removal of the endothelium on the cyclopiazonic acid (CPA)-induced formation of cyclic GMP in rat thoracic aorta. Amounts of cyclic GMP were measured after incubation with the $EC_{80}$ concentration of phenylephrine (0.3 μM) for 5 min and then with CPA for 90 s. Open columns represent corresponding control values in the absence of CPA. Hatched and stippled columns show values in the presence of 3 μM and 10 μM CPA, respectively. $N^G$-nitro-L-arginine (L-NOARG) and LY 83583 (LY) were applied 20 min before and during incubation with CPA. Haemoglobin (Hb) was applied 5 min before and during application of CPA. Endo(−), removal of the endothelium. Each column represents the mean ± s.e.mean of values ($n = 5-6$) in preparations from 10 to 12 rats.

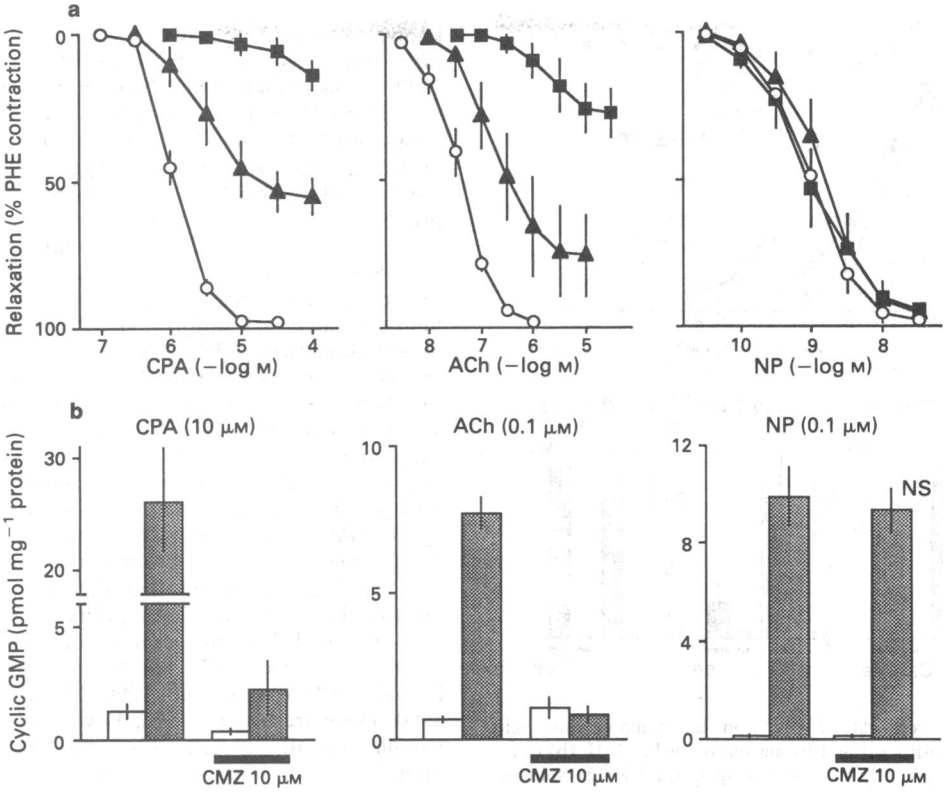


**Figure 5** Effect of calmidazolium on cyclopiazonic acid (CPA)-induced relaxation (a) and cyclic GMP formation (b) in rat thoracic aorta with a functional endothelium. Calmidazolium, when used, was applied 20 min before and during incubation with CPA. Acetylcholine (ACh) and nitroprusside (NP) were used as reference relaxants. (○) Control; (▲) in the presence of 3 μM calmidazolium; (■) in the presence of 10 μM calmidazolium. Open columns, basal level of cyclic GMP; stippled columns, cyclic GMP level in the presence of 10 μM CPA, 0.1 μM ACh or 0.1 μM NP; underlined columns, cyclic GMP formation in the presence of 10 μM calmidazolium (CMZ). Other experimental conditions were as for Figures 1 and 4. Each point and column represent the mean ± s.e.mean of values ($n = 5$) in preparations from 6 to 8 rats. NS, not significantly different from the control preparations (unpaired $t$ test).

It has been suggested that in aortic smooth muscle, CPA induced either contraction by increasing free $Ca^{2+}$ as a result of inhibition of ATP-dependent $Ca^{2+}$ uptake into storage sites (Deng & Kwan, 1991; Shima & Blaustein, 1992), or inhibition of PHE-induced contraction in $Ca^{2+}$ free conditions (Deng & Kwan, 1991). However, the effect of CPA on the vascular endothelial cells has not been documented.

We demonstrated that in rat thoracic aorta, CPA induced relaxation, which was mediated by an endothelium-dependent mechanism. CPA-induced relaxation is not due to hyperpolarization via activation of glibenclamide-sensitive $K^+$-channels or production of prostanoids, because neither the $K^+$-channel blocker, glibenclamide nor the cyclo-oxygenase inhibitor, indomethacin affected the response.

The relaxant effect of CPA was found to be closely associated with an elevation of cyclic GMP level. It is known that cyclic GMP production is involved in endothelium-dependent vasorelaxation, and that NO stimulates cyclic GMP production by activating soluble guanylate cyclase. Therefore it is suggested that NO is involved in the CPA-induced relaxation of rat arteries. Further evidence supporting this idea is that the NO pathway inhibitors, such as the NO synthase inhibitor, L-NOARG (Moore *et al.*, 1990), the NO scavenger, haemoglobin (Gruetter *et al.*, 1981) and the soluble guanylate cyclase inhibitor with cyclic GMP-lowering activity, LY83583 (Malta *et al.*, 1988; Mülsch *et al.*, 1988) each suppressed both the CPA-induced relaxation and cyclic GMP formation.

It has been reported that the level of the initial tone is an important factor affecting relaxation (Furchgott, 1983; Dainty *et al.*, 1990). Indeed, blockers of the NO pathway and removal of the endothelium enhanced the PHE-induced tone of the aorta. Then the question arises of whether an increased initial tone due to blockade of the NO pathway or removal of the endothelium, in part contributes to abolition of CPA-induced relaxation. Therefore, the inhibitory effects of these blockers on the CPA-induced relaxation were examined at the equivalent level of the initial tension by lowering the concentration of PHE. However, the inhibitory effects of these blockers were not substantially altered, irrespective of whether the tone was normalized or not. In addition, the

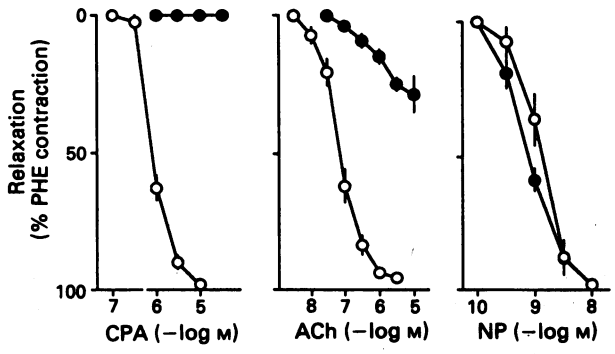


**Figure 6** Effect of W-7 on cyclopiazonic acid (CPA)-induced relaxation in rat thoracic aorta with a functional endothelium. Arteries were stimulated with 10 μM prostaglandin $F_{2\alpha}$ for 5 min to produce tone. (○) Control; (●) in the presence of 100 μM W-7. W-7, when used, was applied 20 min before and during incubation with CPA. Other experimental conditions were as for Figures 1 and 4. Each point represents the mean ± s.e.mean of values ($n = 4$) in preparations from 4 rats.

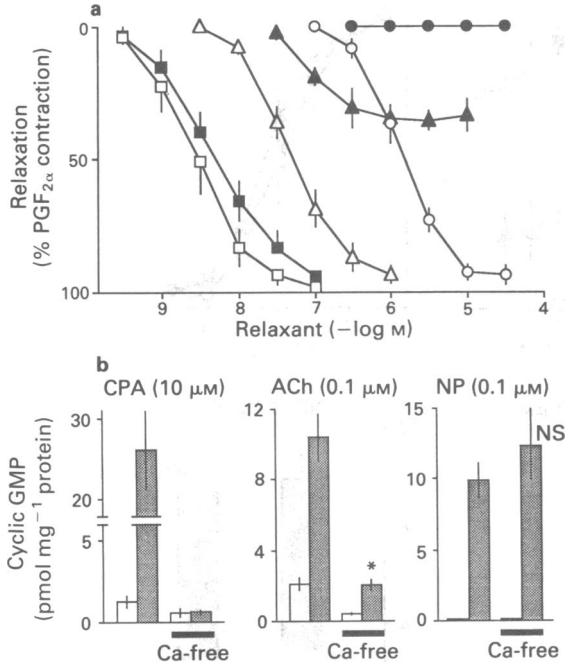


**Figure 7** Effect of removal of $Ca^{2+}$ on the cylcopiazonic acid (CPA)-induced relaxation (a) and formation of cyclic GMP (b) in rat thoracic aorta. Two minutes after replacing normal Krebs solution with $Ca^{2+}$-free Krebs solution, the arteries were incubated with 10 μM $PGF_{2\alpha}$ for 5 min to produce background tone, and then a single dose of CPA was applied. Acetylcholine (ACh) and nitroprusside (NP) were used as reference relaxants. (○ ●) CPA; (△ ▲) ACh; (□ ■) NP. Open symbols represent responses obtained in normal Krebs solution with 2.5 mM $Ca^{2+}$ (initial tension, 528 ± 63 mg), and closed symbols represent those obtained in $Ca^{2+}$-free medium (initial tension, 181 ± 21 mg). Open columns, basal level of cyclic GMP; stippled columns, cyclic GMP level in the presence of 10 μM CPA, 0.1 μM ACh or 0.1 μM NP; underlined columns, cyclic GMP formation in $Ca^{2+}$-free medium. Other experimental conditions were as for Figures 1 and 4. Each point and column represent the means of values ($n = 5$) in preparations from 6 to 8 rats; vertical lines indicate s.e.mean. $^*P < 0.05$, compared with the value obtained in normal Krebs solution containing 2.5 mM $Ca^{2+}$ (unpaired $t$ test). NS, not significantly different from corresponding control value.

relaxant effects of cromakalim and nitroprusside were not substantially altered irrespective of the increased tone. Furthermore, L-NOARG, LY83583 and Hb also suppressed CPA-stimulated increases in the cyclic GMP level. Therefore, abolition of the CPA-induced relaxation does not seem to be secondary to this enhanced tone.

It is known that endothelium-derived hyperpolarizing factor (EDHF), distinct from NO, in part contributes to endothelium-dependent relaxation induced by A23187 or bradykinin (Nagao & Vanhoutte, 1991; 1992), and that the EDHF-mediated component of the relaxations is dependent on $Ca^{2+}$/calmodulin and resistant to L-NOARG (Illiano *et al.*, 1992). From these considerations, it is conceivable that CPA-induced relaxation is in part mediated by EDHF. However, we found that the relaxant effect of CPA was almost abolished by the inhibitors of the NO pathway, L-NOARG, Hb and LY83583. Therefore, it is suggested that the contribution of EDHF in CPA-induced relaxation is negligible, if any, and NO plays a major role in the CPA-induced relaxation.

Two types of NO synthase have been identified in the vasculatures; one is constitutive type present in the endothelial cells and $Ca^{2+}$.calmodulin-dependent (Busse & Mülsch, 1990), and the other is an inducible type found in macrophages and independent of $Ca^{2+}$ and calmodulin (Hauschildt *et al.*, 1990). NO formation induced by ACh or bradykinin is known to be mediated by $Ca^{2+}$/calmodulin-dependent constitutive NO synthase in the endothelium (Busse & Mülsch, 1990; Förstermann *et al.*, 1991) and endothelium-dependent relaxations have been shown to be inhibited by calmodulin antagonists such as calmidazolium and W-7 (Weinheimer & Ossward, 1986; Adeagbo & Triggle, 1991; Schini & Vanhoutte, 1992). Therefore, calmodulin antagonists have been proposed as useful tools for identifying the type of NO synthase involved in relaxation (Schini & Vanhoutte, 1992).

In this respect, in the present experiments, it was found that the calmodulin inhibitors selectively abolished endothelium-dependent relaxation and increase in cyclic GMP formation induced by CPA as well as by ACh, without affecting the endothelium-independent nitroprusside-induced responses. These results provide evidence for the idea that the NO synthase involved in the CPA-induced relaxation is of the calmodulin-dependent constitutive type. In addition, the present results in the absence of $Ca^{2+}$, showing that CPA but not nitroprusside, failed to relax the arteries and to stimulate cyclic GMP formation, also suggests that $Ca^{2+}$ plays a crucial role in the CPA-induced relaxation.

It has been suggested that the amount of $Ca^{2+}$ in the intracellular stores regulates influx of $Ca^{2+}$; the influx of $Ca^{2+}$ is coupled to the level of $Ca^{2+}$ in the internal store (Merritt & Rink, 1987; Pandol *et al.*, 1987; Takemura & Putney, 1989; Kwan *et al.*, 1990; Mertz *et al.*, 1990; Putney, 1990; Demaurex *et al.*, 1992; Low *et al.*, 1992). In cultured vascular endothelial cells, discharge of $Ca^{2+}$ from intracellular $Ca^{2+}$ stores has been reported to trigger $Ca^{2+}$ influx from the extracellular space via $Ca^{2+}$ channels in the plasma membrane (Hallam *et al.*, 1989; Jacob, 1990; Dolor *et al.*, 1992; Schilling *et al.*, 1992). It has been shown that, in HL-60 cells, parotid acinar cells and thymic lymphocytes, CPA can induce a rapid release of $Ca^{2+}$ from intracellular storage sites by inhibiting $Ca^{2+}$-ATPase and subsequently increasing the rate of influx of extracellular $Ca^{2+}$ via the plasma membrane (Mason *et al.*, 1991; Demaurex *et al.*, 1992; Foskett & Wong, 1992), and that in vascular endothelial cells, $Ca^{2+}$-ATPase inhibitors increased the cytosolic $Ca^{2+}$ (Dolor *et al.*, 1992; Schilling *et al.*, 1992). On the basis of these considerations, it is hypothesized that in rat thoracic aorta, the $Ca^{2+}$-ATPase inhibitor CPA prevents the $Ca^{2+}$ uptake into intracellular stores in the endothelium and thereby depletes stored $Ca^{2+}$, which in turn triggers an influx of extracellular $Ca^{2+}$ and stimulates NO synthase. Whether the $Ca^{2+}$ influx pathway activated by CPA-induced depletion of the internal stores is the same as that activated by receptor agonists was not elucidated in the present study.

In endothelium-denuded arteries, in contrast to endothelium-intact preparations, high concentrations of CPA evoked contractions, suggesting that CPA elevates the cytosolic free $Ca^{2+}$ level to trigger contraction. When the endothelium is present, the contractile effect is masked by a predominant endothelium-dependent relaxing effect.

Intracellular free $Ca^{2+}$ is known to be sequestered into the sarcoplasmic reticulum via a $Ca^{2+}$-pump. In this respect, it is also possible that in the aortic endothelial cells, CPA prevents $Ca^{2+}$ uptake into intracellular stores by inhibiting $Ca^{2+}$-ATPase ($Ca^{2+}$ pump) and the $Ca^{2+}$ thus accumulated stimulates constitutive NO synthase to trigger production of NO. If this were the case, CPA would induce relaxation and cyclic GMP production even in the absence of extracellular $Ca^{2+}$. However, this was not the case. In $Ca^{2+}$-free medium, CPA failed to induce relaxation of the arteries previously contracted with $PGF_{2\alpha}$, and did not stimulate cyclic GMP production. Under these conditions, ACh, although greatly attenuated, still induced relaxation and cyclic GMP production, which may be mediated in part by the $Ca^{2+}$ released from the internal stores. From these results, it appears 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. Therefore, the $Ca^{2+}$- that enters into the endothelial cells via plasma mem-

brane $Ca^{2+}$- channels subsequent to depletion by CPA of stored $Ca^{2+}$ may activate NO synthase.

In summary, we have demonstrated that CPA induced relaxation of smooth muscle in the rat thoracic aorta by a mechanism involving NO synthesis. It seems likely that CPA blocks the $Ca^{2+}$-ATPase ($Ca^{2+}$-pump) in the endothelium. Depletion of the stored $Ca^{2+}$ may then trigger an influx of extracellular $Ca^{2+}$ by some as yet unknown mechanisms, which in turn activates $Ca^{2+}$/calmodulin-dependent constitutive NO synthase and produces NO.

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