BIBLID: ISSN 0253-9756 中国药理学报 *Acta Pharmacologica Sinica* 1993 Jan; **14** (1) : 21-26

Cyclopiazonic acid causes endothelium-dependent relaxation in rat aorta[1]

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ABSTRACT The effects of cyclopiazonic acid (CPA), a selective inhibitor of Ca^{2+}-pump ATPase for endoplasmic reticulum (ER), on the contractility of rat aorta with and without intact endothelium were studied to investigate the possible involvement of endothelial ER Ca^{2+}-pump in the release of endothelium-derived relaxing factor (EDRF), which is known to cause vascular relaxation or inhibition of phenylephrine (PE)-precontracted aorta. When added to the organ bath cumulatively, CPA concentration-dependently caused gradual development of contraction, which was much less in aortic rings with intact endothelium than in endothelium-denuded aortic rings. But CPA at low concentrations (1-3 $\mu mol \cdot L^{-1}$) induced vascular relaxation when added to PE (3 $\mu mol \cdot L^{-1}$)-precontracted aortic rings with intact endothelium, but not in denuded aortic rings. This relaxant effect of CPA is very similar to the effect of acetylcholine (ACh), which is well recognized to be mediated by the release of EDRF from the endothelium. N^G-nitro-*L*-arginine methyl ester (*L*-NAME), a nitric oxide synthase inhibitor, completely prevented the vascular relaxation induced by CPA or ACh and the inhibitory effect of *L*-NAME was partially reversed by *L*-arginine (*L*-Arg). Treatment of the aortic rings with nifedipine (Nif) 0.3 $\mu mol \cdot L^{-1}$ did not affect the relaxant effect of ACh or CPA on PE-induced contraction indicating that the Ca^{2+}-entry to the endothelial cells as a result of receptor activation by ACh or ER Ca^{2+}-pump inhibition by CPA was via channels other than *L*-type Ca^{2+} channels. We conclude that CPA causes vascular relaxation by releasing EDRF (most likely nitric oxide) from the endothelium, via its inhibitory action on endothelial ER Ca^{2+}-pump causing elevation of cytosolic Ca^{2+} concentration.

KEY WORDS cyclopiazonic acid; endothelium; endothelium-derived relaxing factor; thoracic aorta; vascular smooth muscle; calcium; phenylephrine; acetylcholine; nifedipine

Cyclopiazonic acid (CPA) has been shown to be a potent and selective Ca^{2+}-pump inhibitor for the sarcoplasmic reticulum (SR) of skeletal muscle[1]. Recently, we have also presented functional evidence that CPA also selectively and reversibly inhibited SR Ca^{2+}-pump in vascular smooth muscle of rat aorta[2] and dog mesenteric artery[3]. The intense interests for the past decade on the role of endothelium in modulating vascular reactivity have led to the identification of nitric oxide (NO) as one major endothelium-derived relaxing factor (EDRF) released from the endothelial cells[4]. Calcium ions (Ca^{2+}) apparently play an important role in the release of NO from the endothelial cells[5]. In an earlier study of the contractility of rat thoracic aorta[6], the herbal drug, thapsigargin, which selectively inhibits ER Ca^{2+}-pump ATPase in non-excitable cells[7] elicited a dual effect, eg, contraction in unstimulated tissue and relaxation in precontracted tissue and the relaxant effect was endothelium-dependent. In view of the above findings, we hypothesize that CPA, being a selective and

Received 1992-07-05 Accepted 1992-08-26

[1] Supported by a grant-in-aid and a Career Investigator Award (to CYK) from the Heart and Stroke Foundation of Ontario. We appreciate the constructive criticisms and suggestions from Professor E E Daniel.

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reversible SR Ca^{2+}–pump inhibitor structually different from thapsigargin, may also elicit such a dual effect on rat aortic contraction and causes endothelium–dependent relaxation via the release of EDRF or NO.

MATERIALS AND METHODS

Male adult Wistar rats (300–350 g) were killed by stunning and decapitation. The thoracic aorta was placed in Krebs' solution at pH 7.4 containing NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1, and glucose 11 mmol· L^{-1}. Fat and connective tissues were removed under a dissecting microscope and cut into 4–5 mm rings. The endothelium was either left intact or removed by gently rubbing against the teeth of a pair of forceps. The effectiveness of removal of endothelium was functionally tested with the ability of ACh 1 μmol· L^{-1} to induce relaxation of rat aorta precontracted with PE 3 μmol· L^{-1}.

The aortic rings were mounted on a 3–ml organ bath connected to a force transducer (Grass FT03C) and a pen recorder. The baths containing Krebs' solution were bubbled continuously with 95% O_2 +5% CO_2 at 37℃. The solutions in the baths were changed every 20–30 min. The rings were equilibrated for 20 min before stretching the arteries to approximately 2 g and were allowed to further equilibrate for at least 90 min. Before data collection, stimulation of the arteries with K^+ 60 mmol· L^{-1} was repeated every 15–20 min until a reproducible contractile response was obtained.

All organic chemicals including CPA were purchased from Sigma. Inorganic chemicals were obtained from Fischer and Biorad. All drugs were dissolved in deionized and distilled water except that CPA was dissolved in dimethyl sulfoxide (Me_2SO).

RESULTS

Effects of CPA on resting tone of aorta with (+E) and without (−E) endothelium Fig 1 shows the contractile effects of CPA, in a concentration– dependent manner, on aortic rings +E and −E. As we have demonstrated previously, CPA caused a gradual develo ment of contraction in −E rings[2]. Howev the magnitude of tension development due CPA was substantially attenuated in the pr ence of intact endothelium. This is consiste with the notion that CPA induced contracti in aortic smooth muscle, whereas CPA m also cause the release of EDRF from t endothelial cells leading to depression CPA–evoked contraction.

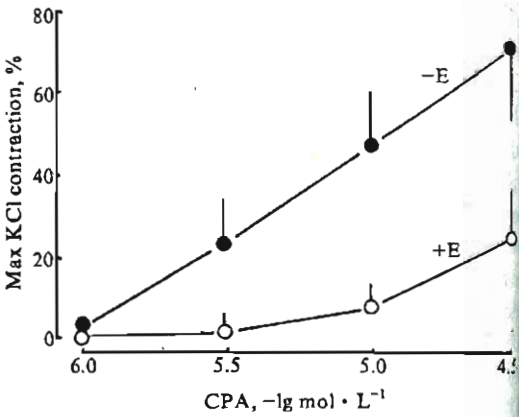

Fig 1. Effect of CPA concentration on tension deve opment in unstimulated rat aortic rings in the presen (+E) and absence (− E) of endothelium. Data were contraction of maximal response obtained with KCl mmol· L^{-1}. n= 6–8 pairs of aortic rings. $\bar{x}\pm s$.

Endothelium–dependent relaxation induce by CPA and ACh ACh– and CPA–induce relaxation occurred in aortic rings +E and − precontracted with PE 3 μmol· L^{-1} (Fig 2). It is noted that at low CPA concentration (1– μmol· L^{-1}) only relaxation was seen, but a higher CPA concentrations (>10 μmol· L^{-1}), biphasic response was frequently observed. This was characterized by a rapid relaxatio followed by a gradual reversal to contraction. Such a biphasic response was not as common ly seen in ACh–induced responses unles extremely high concentrations of ACh (>10 μmol· L^{-1}) was used. Fig 3 summarizes th results on the concentration–dependent effect of CPA on aortic rings precontracted with PE

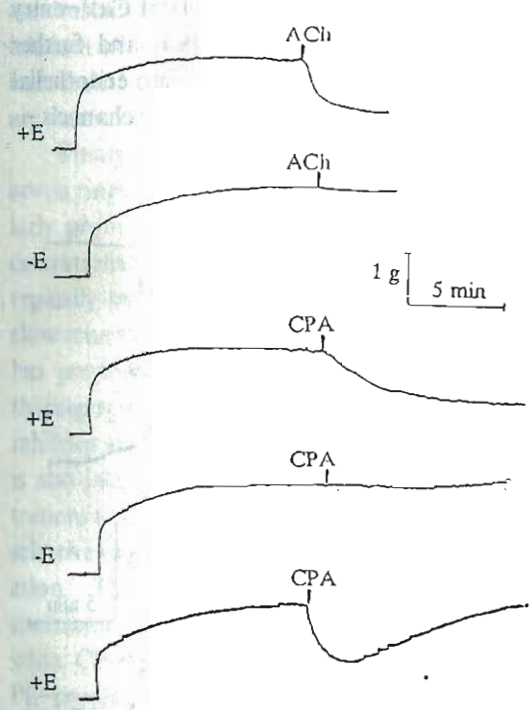

Fig 2. Effects of ACh (0.1 μmol· L^{-1}) and CPA (1 and 10 μmol· L^{-1}) on PE (3 μmol· L^{-1})-precontracted rat aorta with endothelium intact (+E) or denuded (−E). Note the biphasic response of +E aortic ring preparation after addition of CPA 10 μmol· L^{-1} (bottom tracing).

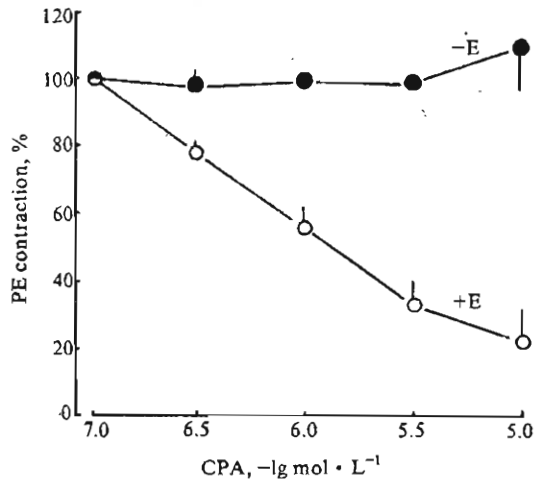

Fig 3. Effect of CPA on tension development in rat aorta with endothelium intact (+E) or denuded (−E) precontracted with PE 3 μmol· L^{-1}. Not that relaxation occurred at CPA concentrations below the threshold level to cause contraction (see Fig 1). n= 5 pairs of aortic rings. $\bar{x}\pm s$.

Effects of *L*-NAME on CPA-induced relaxation of aortic rings precontracted with PE Since NO is likely the EDRF mediating the endothelium-dependent relaxation, we tested the effects of *L*-NAME, a NO synthase inhibitor[8], on the relaxation induced by CPA 3 μmol· L^{-1} in PE (3 μmol· L^{-1})-precontracted aortic rings. Fig 4 shows that *L*-NAME (100 μmol· L^{-1}) almost completely inhibited CPA-induced relaxation and the inhibitory effect of *L*-NAME lasted at least 80 min after extensive washout. The relaxation of *L*-NAME-treated aortic rings failed to respond to the addition of ACh 0.1 μmol· L^{-1}, which elicited prominent relaxation in control rings not treated with *L*-NAME. However, a complete relaxation of *L*-NAME-treated aortic rings was effectively achieved by endothelium-independent vasodilatory drug sodium nitroprusside (SNP) 10 nmol· L^{-1} indicating that the fundamental relaxation mechanism in smooth muscle remains intact in spite of the long effect of *L*-NAME on the endothelium. Since *L*-arginine (*L*-Arg) is the precursor for NO generation in endothelial cells, incubation of the aortic rings with *L*-Arg 100 μmol· L^{-1} was apparently effective in partially restoring the endothelium-dependent relaxation induced by CPA. *D*-Arg (100 μmol· L^{-1}), on the other hand, was without effect.

Effects of nifedipine (Nif) on CPA-induced relaxation We have previously demonstrated that inhibition of SR Ca^{2+}-pump by CPA resulted in vasoconstriction which was dependent on extracellular Ca^{2+} [2,3] and we have

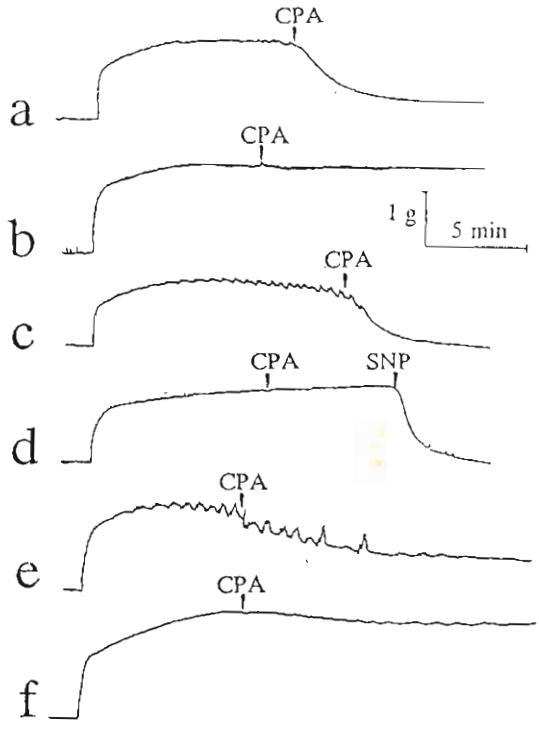

Fig 4. Rat aortic ring with endothelium. (a) Relaxation by CPA 3 μmol· L^{-1} in aorta precontracted with PE 3 μmol· L^{-1}. (b) After treatment of the same ring with *L*-NAME 10 μmol· L^{-1}, CPA 3 μmol· L^{-1} no longer relaxed. (c) In the same ring treated as in (a) after repeated washes for 1 h, CPA still relaxed the ring. (d) *L*- NAME-treated ring as in (b) was repeatedly washed for 30 min, CPA caused no relaxation, but SNP 10 nmol· L^{-1} totally relaxed PE-induced contraction. In separate rings under conditions of treatment as in (d) except that *L*-Arg 100 μmol· L^{-1} (e) or *D*-Arg (f) was added 15 min before PE to cause contraction. Note that CPA restored relaxation in *L*-Arg but not in *D*-Arg rings.

recently shown that the pathway of Ca^{2+}-entry was insensitive to L-type Ca^{2+} channel antagonist, Nif[9]. Therefore, we also investigated the effect of Nif on the endothelium-dependent relaxation induced by ACh and CPA. Fig 5 shows that the relaxation evoked by ACh or CPA was not inhibited by Nif 30 nmol· L^{-1}, a concentration very effectively inhibiting KCl-induced response. These results indicate the important role of Ca^{2+}-entry mediating the release of EDRF and further suggest that the entry of Ca^{2+} into endothelial cells does not utilize L- type Ca^{2+} channels.

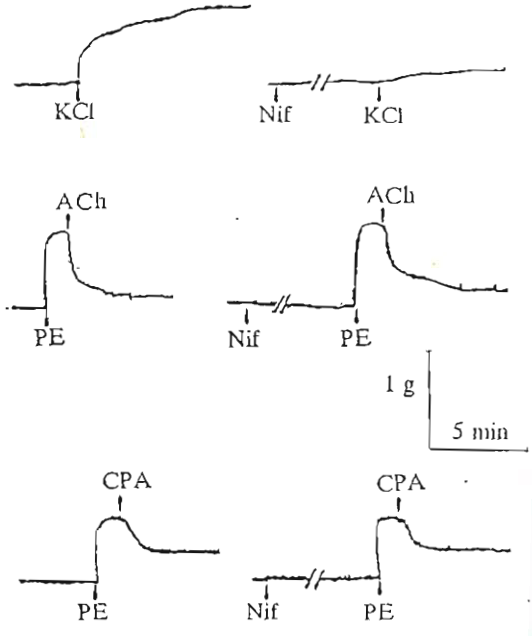

Fig 5. Effect of nifedipine (Nif, 30 nmol· L^{-1}) on endothelium-dependent relaxation of PE-precontracted aortic rings induced by ACh 0.2 μmol· L^{-1} or CPA 3 μmol· L^{-1}. The effectiveness of Nif as an effective L-type Ca^{2+} channel antagonist was demonstrated in KCl-induced contraction. Nif was not effective in inhibiting the relaxation caused by either CPA or ACh.

DISCUSSION

We have previously characterized, for the first time, the contractile effects of CPA, a SR-selective Ca^{2+}-pump inhibitor, on endothelium-denuded rat aorta[2]. Since then, CPA has been proved to be a useful pharmacological tool in studying the role of intracellular Ca^{2+}-stores, namely the ER or SR, in various tissues[3,10,11]. In the present study, we further report a novel observation, ie, endothelium-dependent relaxation, elicited by CPA in rat thoracic aorta,

Therefore, CPA appears to have dual actions on vascular tissues, the contractile and relaxant effects depending on the intactness of endothelial cells. Three lines of novel findings are discussed below:

Firstly, the dual actions of CPA on rat aortic rings with intact endothelium is particularly prominent when CPA was used at concentrations >10 μmol· L^{-1}, characterized typically by a rapid relaxation followed by a slow return of contraction. Such a dual effect has previously been made in rat aorta using thapsigargin[6], also a ER Ca^{2+}-pump inhibitor structurally different from CPA[7]. It is also interesting to note that at low concentrations (<3 μmol· L^{-1}), CPA became more selective on the endothelium-dependent relaxation. This is fully reflected by the lack of contractile effect after the initial relaxation when CPA 1-3 μmol· L^{-1} was added to PE-precontracted rat aortic rings (Fig 2 and 3) and the higher threshold concentration of CPA required for aortic rings with intact endothelium to cause the elevation of tension under unstimulated condition as compared to those devoid of functional endothelium (Fig 1). In the former, the lower responsiveness to CPA is apparently due to the concurrent depression of contractile responses by the release of EDRF from endothelial cells by CPA.

Secondly, we have examined the nature of the EDRF released in the presence of CPA. The relaxing factor released by CPA is very similar to that released by ACh and is likely to be NO, since the NO synthase inhibitor, *L*-NAME, completely inhibited the relaxation responses to CPA and ACh and the inhibitory effect was long-lasting. Furthermore, this inhibitory effect of *L*-NAME can be partially overcome in the presence of *L*-Arg, a precursor for the formation of NO[12]. *D*-Arg, which does not serve as a precursor for NO formation, failed to overcome the long-lasting effect of *L*-NAME. It should also be noted that *L*-NAME itself does not impair endothelium-independent vascular relaxation, since SNP an endothelium-independent vasodilator, still elicited full relaxation.

Thirdly, we have used L-type Ca^{2+} channel antagonist, Nif, to examine the nature of the Ca^{2+} entry resulting from the inhibition of endothelial ER Ca^{2+}-pump by CPA. This experimental protocol was based on our previous findings in vascular smooth muscle that Nif did not inhibit CPA-induced contraction which was dependent on extracellular Ca^{2+} [9]. Indeed, it appears that the lack of sensitivity to Nif of the relaxant effect of CPA on the endothelial cells (Fig 5) seemed to be similar to that of the contractile effect on the smooth muscle cells. It is thus reasonable to propose that Ca^{2+}-entry via channels different from the L-type Ca^{2+} channels may be the final common pathway for the release of NO under different mechanisms, ie, inhibition of ER Ca^{2+}-pump by CPA or activation of surface membrane cholinergic receptors by ACh.

REFERENCES

1 Seidler NW, Jona I, Vegh M, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem* 1989; **264** : 17816-23.

2 Deng HW, Kwan CY. Cyclopiazonic acid is a sarcoplasmic reticulum Ca^{2+}-pump inhibitor of rat aortic muscle. *Acta Pharmacol Sin* 1991; **12** : 53-8.

3 Low AM, Kwan CY, Daniel EE. Evidence for two types of internal Ca^{2+} stores in canine mesenteric artery with different refilling mechanisms. *Am J Physiol* 1992; **262** : H31-7.

4 Ignarro L, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA* 1987; **84** : 9265-9.

5 Long CJ, Stone TW. The release of endothelium derived relaxant factor is calcium dependent. *Br J Pharmacol* 1985; **84** : 152P.

6 Mikkelsen EO, Thastrup O, Christensen SB. Effects of thapsigargin in isolated rat thoracic aorta. *Pharmacol Toxicol* 1988; **62** : 7–11.
7 Thastrup O. Role of Ca^{2+}–ATPase in regulation of cellular Ca^{2+} signalling, as studied with the selective microsomal Ca^{2+}–ATPase inhibitor, thapsigargin. *Agents Actions* 1990; **29** : 8–15.
8 Rees DD, Palmer RMJ, Schulz R, Hodson HF, Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br J Pharmacol* 1990; **101** : 746–52.
9 Kwan CY, Deng HW, Guan YY. Tetrandrine is not a selective calcium channel blocker in vascular smooth muscle. *Acta Pharmacol Sin* 1992; **13** : 385–90.
10 Foskett JK, Wong D. Calcium oscillations in parotid acinar cells induced by microsomal Ca^{2+}–ATPase inhibition. *Am J Physiol* 1992; **262** : C656–63.
11 Bourreau JP, Abeia AP, Kwan CY, Daniel EE. Acetylcholine Ca^{2+} stores refilling directly involves a dihydropyridine–sensitive channel in dog trachea. *Am J Physiol* 1991; **261** : C497–505.
12 Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L–arginine. *Nature* 1988; **333** : 664–6.

BIBLID: ISSN 0253–9756 中国药理学报 *Acta Pharmacologica Sinica* 1993 Jan; **14** (1) : 26–30

Platelet activating factor production in bovine cerebral microvascular endothelial cells and its drug inhibition

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ABSTRACT The production of platelet activating factor (PAF) in bovine cerebral microvascular endothelial cells (CME cells) and the effects of tetrandrine (Tet) and dauricine (Dau) on the PAF production were investigated. PAF was determined by the aggregation of washed rabbit platelets. The results showed that the CME cells produced PAF 5.93 ng / 8.5×10^5 cells under the calcimycin 2.5 $\mu mol \cdot L^{-1}$ stimulation. Tet and Dau 1, 10, and 100 $\mu mol \cdot L^{-1}$ inhibited the production of PAF by 18.2%, 51.8%, 56.8%, and 26.3%, 63.3%, 65.9%, respectively. Tet concentration–dependently inhibited the PAF 9.1 $nmol \cdot L^{-1}$ induced washed rabbit platelets aggregation with the IC_{50} of 3.05 $\mu mol \cdot L^{-1}$ (95% confidence limits: 0.59–15.86 $\mu mol \cdot L^{-1}$). The binding of [^{3}H]triazolodiazepine to the CME cells was partially displaced by Tet 0.02–33.00 $\mu mol \cdot L^{-1}$. It is suggested that the cerebrovascular system produces PAF at the pathological conditions and the inhibition of Tet and Dau.

KEY WORDS tetrandrine; dauricine; platelet activating factor; vascular endothelium; capillaries; triazoles.

Platelet activating factor (PAF) was originally described as a potent platelet activator derived from antigen–stimulated, IgE– sensitized rabbit basophils[1]. PAF is involved in various disorders, such as asthma, shock, cardiovascular disorders, and renal ischemia[2] PAF is 1000 to 10000 times more potent on a molar basis than histamine in increasing the vascular permeability. It is produced by various cells including basophils, macrophages, monocytes, and mast cells. PAF can be released from endothelial cells of human umbilical veins, bovine aortas, and pulmonary arteries[3,4]. It constricts the pial arteries of newborn pigs[5], and decreases the cerebral blood flow of rats[6]. Our previous studies showed that the PAF specific binding

Received 1991–10–03 Accepted 1992–09–09