中国药理学报 *Acta Pharmacologica Sinica* 1991 Jan; **12** (1) : 53-58

Cyclopiazonic acid is a sarcoplasmic reticulum Ca^{2+}-pump inhibitor of rat aortic muscle[1]

DENG Han-Wu[2](邓汉武), KWAN Chiu-Yin[3](关超然), (*Smooth Muscle Research Program, Division of Physiology and Pharmacology, Department of Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada L8N 3Z5*)

ABSTRACT The effects of a mycotoxin, cyclopiazonic acid (CPA), on the contractile function of rat aortic smooth muscle rings were investigated to test the hypothesis that CPA selectively inhibits the sarcoplasmic reticulum Ca^{2+}-pump. This hypothesis was tested by two types of experimental approaches. First, we compared the ability of the vascular muscle rings to relax upon washout of the agonists following the contraction induced by high concentration of depolarizing KCl (60 mmol · L^{-1}) and supramaximal concentration of phenylephrine (Phe; 10^{-5} mol · L^{-1}). In this case, we found that CPA treatment resulted in slowing the rate of relaxation to both stimuli. In the second approach, the status of Phe-sensitive intracellular Ca^{2+} pool was assessed by the ability of Phe to elicit sequential responses in Ca^{2+}-free medium with loading of Ca^{2+} by reintroduction of extracellular Ca^{2+} in the presence and absence of CPA following the first response to Phe 10^{-5} mol · L^{-1} and the washout of the agonist. We found that effective Ca^{2+} loading resulted in total restoration of the second response to Phe, while CPA treatment caused ineffective loading leading to ultimate prevention of the second response to Phe. Our finding are consistent with the hypothesis that CPA acts by inhibiting the SR Ca^{2+}-pump, thus interfering an effective refilling of the agonist-sensitve intracellular Ca^{2+} stores.

KEY WORDS cyclopiazonic acid; mycotoxins; sarcoplasmic reticulum; vascular smooth muscle; aorta; calcium

Received 1990 Oct 19 Accepted 1990 Nov 14

[1] Supported by the Heart and Stroke Foundation of Ontario.

[2] Now in *Department of Pharmacology, Hunan Medical University, Changsha 410078, China.*

[3] Career Investigator of the Heart and Stroke Foundation of Ontario.

Cytosolic concentration of calcium ions ($[Ca^{2+}]_i$) play an absolutely essential role in the normal physiological function of smooth muscle under rigorous cellular regulation[1]. In vascular smooth muscle, extensive derangement of the Ca^{2+} regulatory mechanisms will inevitably result in contractile dysfunction leading to cardiovascular diseases[2]. It is now generally accepted that both plasma membrane (PM) and sarcoplasmic reticulum (SR) contributed to the control of the level of $[Ca^{2+}]_i$ in vascular smooth muscle under physiological conditions. The exact roles of and the functional relationship between SR and PM have been a matter of continuing debate[1,3,4]. The intrinsic difficulty associated with the studies of SR function in vascular smooth muscle is its meager quantity on one hand and the lack of selective pharmacological tool on the other hand[1,4]. Recently, a novel selective internal membrane Ca^{2+}-pump inhibitor, thapsigargin, originally characterized in several nonexcitable cells[5], has been studied in vascular smooth muscle[6,7]. The action of thapsigargin was consistent with its role as a SR Ca^{2+}-pump inhibitor. However, the action of thapsigargin was slow onset, practically irreversible and tissue selective[5,7]. Recently, a mycotoxin, cyclopiazonic acid (CPA), has been identified to be a highly selective and potent inhibitor of the SR Ca^{2+}-pump in skeletal muscle[8], which interacts directly with the Ca^{2+} transport ATPase molecules[9]. We have undertaken the investigation on the functional effects of CPA on the vascular muscle

contraction in order to determine whether CPA also serves as a SR Ca^{2+}–pump inhibitor in vascular smooth muslce cells. Our results are consistent with the mechanism of action of CPA in vascular muscle as a SR Ca^{2+}–pump inhibitor. The reasonably fast and readily reversible effects would make CPA an attractive pharmacological tool in probing the role of SR function at cellular as well as subcellular membrane level.

MATERIALS AND METHODS

Male adult Wistar rats (300–350 g) were killed by stunning and decapitation. The thoracic aortas were promptly removed and placed in Krebs' physiological solution at pH 7.4 containing (mmol · L^{-1}) NaCl 119, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1 and glucose 11. Fat and connective tissues were removed under a dissecting microscope and cut into 4–5 mm rings. Soon after the endothelial cells were removed by rubbing against the teeth of a pair of forceps, the aortic rings were mounted on a 3 ml organ bath connected to a force transducer (Grass FT03C) and a pen recorder. The organ baths and Krebs' solution were bubbled continuously with 95% O_2 + 5% CO_2 and warmed to 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 . For Ca^{2+}–free Krebs, Ca^{2+} was omitted and EGTA 50 μmol · L^{-1} was added. Aortic rings were allowed to be incubated in this Ca^{2+}–free solution for not more than 5 min. Such a protocol ensures total elimination of extracellular Ca^{2+} without deleterious effects on intracellular Ca^{2+} [10].

To assess the status of agonist–sensitive intracellular Ca^{2+}–stores, contractile response to phenylephrine (Phe, Sigma) 10 μmol · L^{-1} was obtained and maintained until the contraction had returned to or near its original baseline. After washout of the agonist, the aortic rings were allowed to be exposed to Ca^{2+} 2.5 mmol · L^{-1} in the presence of KCl 60 mmol · L^{-1} in order to recharge the intracellular Ca^{2+} stores. The effectiveness of the Ca^{2+} repletion was evaluated by the magnitude of the subsequent response to Phe 10 μmol · L^{-1} in Ca^{2+}–free medium.

All organic chemicals including cyclopiazonic acid were purchased from Sigma. All inoganic chemicals were obtained from Fischer and Biorad. All drugs were dissolved in deionized and distilled water.

RESULTS

Effects of CPA on the basal tension of aortic muscle Cumulative doses of CPA were applied to the vascular muscle strips under applied resting tension in Krebs' solution containing Ca^{2+} 2.5 mmol · L^{-1} (normal Krebs' solution). Fig 1 shows that force development occurred variably with increasing concentration of CPA. There was no indication of plateau response even at CPA 100 μmol · L^{-1}. Studies of CPA at concentrations higher than 100 μmol · L^{-1} were not possible due to interference resulting from excessive foam formation upon aeration in the tissue bath chamber. On the other hand, similar treatment of the aortic strips with CPA in Ca^{2+}–free medium containing EGTA 50 μmol · L^{-1} (hereafter Ca^{2+}–free medium) did not cause force development up to 30 μmol · L^{-1}, but CPA 100 μmol · L^{-1} caused an abrupt, sustained and irreversible elevation of tension. In view of these findings, we carried out most of our studies using 10 μM CPA.

Effects of CPA on agonist–induced contraction and subsequent relaxation after washout Fig 2A shows the effects of CPA 10 μmol · L^{-1} on the contractile responses to

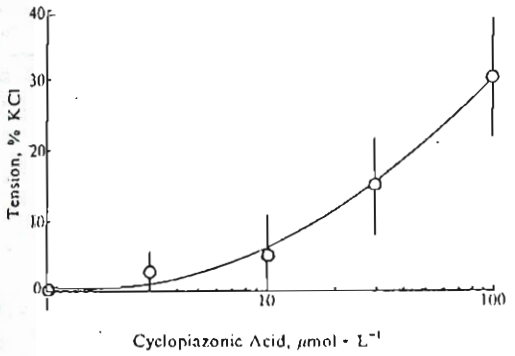

Fig 1. Effects of CPA on basal tension of rat aortic rings. After equilibration of rat aortic rings devoided of endothelium in normal Krebs' solution at a passive tension of approximately 2 g, CPA were added cumulatively to the aortic preparation. The force development was expressed as % of the contraction induced by KCl 60 mmol · L^{-1}. $n=6$. $\bar{x} \pm$SD.

KCl 60 mmol · L^{-1} in normal Krebs' solution. The most prominent change was in the rate of relaxation. Fig 2B shows a more quantitative analysis of the time course of relaxation to KCl–induced contraction in the presence of CPA. CPA markedly reduced the rate of relaxation within 1 min of washout. The fact that a subsequent KCl stimulation following washout of the CPA–treated aortic preparations resulted in contractile responses indistinquishable from those of the control aortic rings not treated with CPA indicated that the effect of CPA at 10 μmol · L^{-1} was readily reversible.

We have obtained similar findings in the vascular responses to Phe 10 μmol · L^{-1} (not shown). Tab 1 summarized the analysis of these data. In both cases, CPA 10 μmol · L^{-1} had no significant effects on the magnitude of the contraction. However, the effects of CPA resided in the kinetics of the force development. For example, the time required to reach half–maximal contraction was substantially shortened by CPA in KCl–induced contraction, but not in the Phe–induced contraction. Furthermore, CPA significantly prolonged the rate of relaxation after washout,

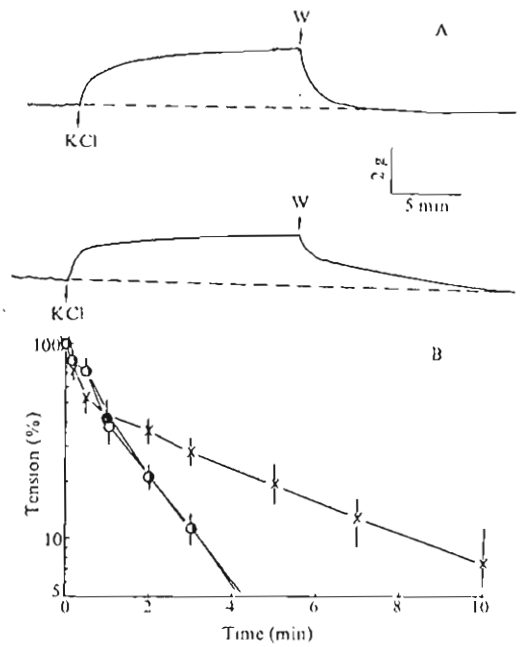

Fig 2. Effects of CPA (10 μmol · L^{-1}) on KCl (60 mmol · L^{-1}) contracture. (A) Representative tracings in the presence (lower tracing) and absence (upper tracing) of CPA, which was added 15 min before the addition of KCl. (B) Temporal changes of force generation upon washout of KCl by normal Krebs' solution with (×) and without (○) CPA. △: relaxation of KCl–precontracted aortic rings after prior washout of CPA as for the same aortic rings represented by (×). $n=6$. $\bar{x} \pm$SD.

the effect being more potent in aortic rings precontracted with KCl 60 mmol · L^{-1}.

Effects of CPA on Phe–induced response in Ca^{2+}–free medium It is well recognized that Phe induced contraction via α_1–adrenoceptor activation utilizing both extra– and intra–cellular Ca^{2+} in Ca^{2+}–containing normal Krebs' solution[11]. However, in Ca^{2+}–free medium, the source of Ca^{2+} for activation of contraction is primarily intracellular. The contraction is transient and the intracellular Ca^{2+} store is readily depleted upon repeated application of Phe[12]. However, upon washout of Phe, reintroduction of extracellular Ca^{2+} is known to result in Ca^{2+}–influx and thus refilling of the empty store[11,13]. If the empty

Tab 1. Effects of CPA 10 μmol · L^{-1} on the contractile responses of rat aortic rings induced by KCl 60 mmol · L^{-1} and Phe 10 μmol · L^{-1} in normal Krebs' solution. The magnitude of the contraction was expressed as % of the initial contraction to KCl 60 mmol · L^{-1} prior to the addition of CPA $T_{\frac{1}{2}}$ the time (minutes) required to reach half-maximal response in contraction or in relaxation following washout of stimuli. $n=6$, $\bar{x} \pm$ SD. $^{*}P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Group	Magnitude (% KCl)	$T_{\frac{1}{2}}$ Contraction	$T_{\frac{1}{2}}$ Relaxation
KCl-induced contraction			
Control	95.58 ± 3.28	1.05 ± 0.20	2.06 ± 0.38
CPA	97.72 ± 6.12	0.25 ± 0.12***	5.64 ± 1.68***
Phenylephrine-induced contraction			
Control	111.91 ± 6.97	0.84 ± 0.06	4.80 ± 0.82
CPA	111.08 ± 12.19	0.92 ± 0.16	6.73 ± 1.73**

agonist-sensitive store is effectively refilled, a subsequent application of Phe in Ca^{2+}-free medium will again elicit a full magnitude of transient response. If the refilling process is inhibited, no Ca^{2+} will be available in the store for the subsequent release. Fig 3 shows this was exactly the case in rat aortic rings. Note two remarkable differences between the responses of control rings and CPA-treated rings. First, reintroduction of Ca^{2+} resulted in sustained contraction in CPA-treated but not the control rings. Second, the second application of Phe completely restored (to better than 95 % in 12 aortic rings from 6 rats) the transient contraction in Ca^{2+}-free medium in the control rings, but the same responses were completely eliminated in the presence of CPA.

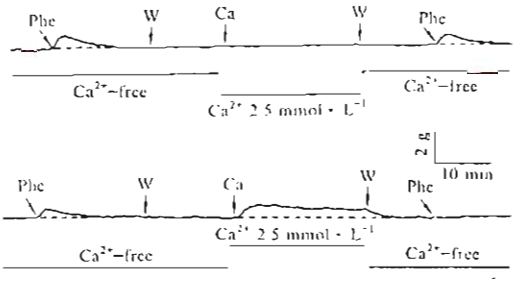

Fig 3. Representative tracings illustrating the effects of CPA (10 μmol · L^{-1}) on Phe (10 μmol · L^{-1}) -induced contraction in Ca^{2+}-free medium, reintroduction of extracellular Ca^{2+}(2.5 mmol · L^{-1}) and a subsequent response to Phe 10 μmol · L^{-1} in Ca^{2+}-free medium. The initial double applications of Phe ensured a complete depletion of agonist-sensitive Ca^{2+} store. Washout (W), addition of CPA or Ca^{2+}, and changes to Ca^{2+}-free medium were as indicated. Note in the control aortic rings (upper tracing) the absence of contraction upon reintroduction of Ca^{2+} and an effective repetition of the transient response to Phe 10 μmmol · L^{-1} in Ca^{2+}-free medium. The reverse was true in CPA-treated aortic rings (lower tracing), in which CPA was present throughout the experiment (CPA was also present in the Ca^{2+}-containing or Ca^{2+}-free wash solutions).

DISCUSSION

In this work we have investigated for the first time in smooth muslce the effects of CPA on vascular contractile function. The premise of this study comes from the observation that CPA, a fungal toxin commonly found as a common contaminant in agricultural products, produces toxic effects in skeletal muscle by inhibiting the SR Ca^{2+}-pump[8]. The effect of CPA on SR Ca^{2+}-ATPase has been shown to be highly selective, since it failed to inhibit the erythrocyte cell membrane Ca^{2+}-transport ATPase or the basal Mg^{2+}-ATPase or the Na^{+}, K^{+}-ATPase[9]. Our results on functional studies in rat aorta are consistent with the contention that CPA is also an inhibitor of SR Ca^{2+}-pump in smooth muscle. We arrived at such a contention from two separate pieces of functional evidence; namely, the effects of CPA on the kinetics of contractile responses to KCl and Phe and the status of agonist-sensitive intracellular Ca^{2+} store as assessed by the ability of Phe to induce contractile responses in Ca^{2+}-free medium following repletion of the agonist-sensitive Ca^{2+} store previously emptied by agonist-induced Ca^{2+}-release.

Since the rate of the force development or relaxation of vascular muscle is dependent

upon the equilibrium among various integrated dynamic processes of Ca^{2+} movement, such as Ca^{2+} entry via voltage–operated and/or receptor–operated Ca^{2+}–channels, Ca^{2+}–release, Ca^{2+}–sequestration (by SR Ca^{2+}–pump) and Ca^{2+}–extrusion (by PM Ca^{2+}–pump and/or Na^{+}–Ca^{2+} exchange)[1,13], a precise assignment of the change in the rate of tensoin development to any one of the above processes would be formidable. However, the lack of effect of short–term treatment of CPA on the magnitude of the initial responses to either KCl (via membrane depolarization) or Phe (via alpha$_1$–adrenoceptor activation) would be expected when the SR Ca^{2+} is full (e.g., in the absence of prior depletion by Phe in Ca^{2+}–free medium; as in Fig 2) and particularly when the Ca^{2+}–influx mechanisms are not altered. Thus, the enhancement in the rate of force development and the retardation in the relaxation rate of KCl–induced contraction in the presence of CPA at a concentration causing very little change in basal tensoin, is suggestive of an imbalance between the SR Ca^{2+}–sequestration and PM Ca^{2+}–extrusion activities. Our results are inconsistent with the hypothesis that the PM Ca^{2+} extrusion (presumably mainly due to the PM Ca^{2+}–pump ATPase) is the one that is inhibited by CPA. If it were the case, the rate of elevation of $[Ca^{2+}]_i$, and thus force development, would be expected to increase in the presence of CPA since the Ca^{2+} would neither be extruded (due to assumed inhibition of PM Ca^{2+} extrusion by CPA) nor be sequestered further into the SR (which had not been depleted). Similarly, if the SR Ca^{2+}–pump remained intact while the PM Ca^{2+}–pump were inhibited by CPA, one would also expect to observe an effective recycling of the SR upon repeated Phe stimulation in Ca^{2+}–free medium. It is very clear that none of these expectations were met according to our results. On the contrary, these results strongly suggest that the SR sequestration mechanism is selectively inhibited by CPA such that the rate of force development to KCl depolarization was enhanced (as reflected by reduced time to reach half–maximal contraction) in the face of a weak Ca^{2+}–extrusion. The more convincing evidence is derived from the fact that the responses to Phe 10 μmol · L^{-1} in Ca^{2+}–free medium with Ca^{2+} repletion between two sequential applications of Phe was completely eliminated (Fig 3). It can occur only if the refilling of SR Ca^{2+} store becomes ineffective. It is also interesting to note that although the rate of relaxation to KCl– and Phe– induced contraction was different in the control rings, in the presence of CPA the difference was primarily eliminated suggesting that the CPA–insensitive component of the rate of relaxation may be of the same entity, most likely the PM Ca^{2+}–pump. In sum, these results from functional studies strongly indicated that SR Ca^{2+}–pump in vascular smooth muscle is selectively inhibited by CPA. A more definitive answer shall require a direct demonstration of the inhibitory action of CPA on the ATP–driven Ca^{2+}–transport as well as the Ca^{2+}–transport ATPase activity in SR–enriched membrane fractions of vascular smooth muscle. Further studies using such a subcellular membrane approach are currently in progress in this laboratory.

REFERENCES

1 Daniel EE, Grover AK, Kwan CY. Calcium. In: Stephens NL, ed. *Biochemistry of smooth muscle*; vol 3. Boca Raton : CRC Press, 1983 : 1

2 Kwan CY. Dysfunction of calcium handling by smooth muscle in hypertension. *Can J Physiol Pharmacol 1985; 63* : 366

3 Grover AK. Ca–pumps in smooth muslce: one in plasma membrane and another in endoplasmic reticulum. *Cell Calcium* 1984; **6** : 227

4 Kwan CY, Grover AK, Daniel EE. Cell membrane and regulation of cytoplasmic level of Ca in vascular muscle. In: Vanhoutte PM , ed. *Vaso–dilatation: vascular smooth muscle, autonomic nerves and endothelium*. NY: Raven Press; 1988 : 21

5 Thastrup O, Cullen PJ, Drobak BK, Hanley MR,

Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specificinhibition of the endoplasmic reticulum Ca^{2+}–ATPase. *Proc Natl Acad Sci* 1990; **87** : 2466

6 Mikkelsen EO, Thastrup O, Christensen SB. Effects of thapsigargin in isolated rat thoracic aorta. *Pharmacol Toxicol* 1988; **62** : 7

7 Low AM, Gaspar V, Kwan CY. Daniel EE. Thastrup O. Thapsigargin inhibits repletion of intracellular calcium store in vascular smooth muscle. *Physiologist* 1990; **33** : A119

8 Goeger DE, Riley RT, Dorner JW, Cole RJ. Cyclopiazonic acid inhibition of the Ca^{2+}–transport ATPase in rat skeletal muscle sarcoplasmic reticulum vesicles. *Biochem Pharmacol* 1988; **37** : 978

9 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

10 Guan YY, Kwan CY, Daniel EE. The effects of EGTA on vascular muscle contractility in Ca–free medium. *Can J Physiol Pharmacol* 1988; **66** : 1053

11 van Breemen C, Saida K. Cellular mechanisms regulating $[Ca^{2+}]_i$ in smooth muscle. *Annu Rev Physiol* 1989; **51** : 315

12 Karaki H, Kubota H, Urakawa N. Mobilization of stored calcium for phasic contraction induced by norepinephrine in rabbit aorta. *Eur J Pharmacol* 1979; **56** : 237

13 Putney JW Jr. A model for receptor–regulated calcium entry. *Cell Calcium* 1986; **7** : 1

中国药理学报 *Acta Pharmacologica Sinica* 1991 Jan; **12** (1) : 58–62

3–(*N*–哌啶甲基偶氮)甲基利福霉素 SV 在大鼠体内的处置[1]

刘 璐、王振钺、李 端、孙时良[2] (上海医科大学药学院药理教研室, 上海 200032, 中国)

Disposition of 3–(*N*–piperidinomethylarino)methyl–rifamycin SV (FCE 22250) in rats[1]

LIU Lu, WANG Zhen–Yue, LI Duan, SUN Shi–Liang[2] (*Department of Pharmacology, School of Pharmacy, Shanghai Medical University, Shanghai 200032, China*)

ABSTRACT After rats being given ig FCE 22250 5, 10 and 25 mg · kg^{-1}, the plasma peak times (T_{max}) were 12–14 h, the max plasma concentrations (C_{max}) were 3.0, 5.6 and 12 $\mu g \cdot ml^{-1}$ respectively, and the half–lives of elimination ($T_{\frac{1}{2}}$) were 24–26 h. The apparent volumes of distribution of the three doses were about 1 L · kg^{-1}, suggesting that FCE 22250 in blood and in tissue was balanced. Total body clearance rate of each of the three doses was 29 ml · kg^{-1} · h^{-1}. The ig absolute bioaviability ranged from 69–84%. Its distribution in rats was follows: the highest in liver, next in lung and then in fat, kidney, intestine, spleen, lymphaticode, heart, muscle, testis, the lowest in brain. It was eliminated mainly via the bile with feces.

The human serum protein binding rate of FCE 22250 was 96.2%. It was shown that the rate was not correlated with drug concentration in serum under our experimental conditions.

KEY WORDS FCE 22250; rifamycins; pharmacokinetics; protein binding

提要 大鼠 ig FCE 22250 5, 10, 25 mg · kg^{-1} 后, T_{max} 12–14 h; C_{max} 分别为 3.0, 5.6, 12 $\mu g \cdot ml^{-1}$ $T_{\frac{1}{2}}$ 24–26h.组织中含量以肝最高,依次为肺、脂肪、肾、肠、脾、淋巴结、心、肌肉、睾丸,脑最低. Ig 绝对生物利用度 69–84%. 本品原形主要经胆汁由粪便排泄,尿中排泄甚少. 血清蛋白结合率为 96.2% 在本实验条件下蛋白结合率与药物浓度无关.

关键词 FCE 22250; 利福霉素; 药物动力学; 蛋白结合

Received 1990 Jan 16 Accepted 1990 Sep 12

[1] Project supported by Sichuan Industrial Institute of Antibiotics

[2] *Department of Instrumental Analysis Center, School of Pharmacy, Shanghai Medical University, Shanghai 200032, China*