

Dual effect of cobra cardiotoxin on vascular smooth muscle and endothelium¹

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KEY WORDS cobra venoms; toxins; thoracic aorta; vascular smooth muscle; vascular endothelium; phenylephrine; potassium chloride; *N*^G-nitroarginine methyl ester; calcium channel blockers

AIM: To assess the cytotoxic effects of cobra cardiotoxin (CTX) on rat aorta. **METHODS:** Measure of contractility of aortic rings with or without endothelium. **RESULTS:** In endothelium-intact rings, CTX 10 $\mu\text{mol}\cdot\text{L}^{-1}$ induced a transient relaxation followed by a sustained contraction. Removal of the endothelium or pre-incubation of the rings with NO synthase inhibitor *N*^G-nitro-*L*-arginine methyl ester (*L*-NAME) abolished the transient relaxation but did not affect the magnitude of the contractile response induced by CTX. CTX itself induced contraction of vascular smooth muscle but also reduced contractions induced by phenylephrine (PhE) or KCl stimulation in a concentration-dependent manner. Contraction induced by CTX was dependent on the external Ca^{2+} concentration. Maximal contractile response to CTX was obtained in medium containing Ca^{2+} 1 $\text{mmol}\cdot\text{L}^{-1}$. This response decreased with higher Ca^{2+} concentration and disappeared when Ca^{2+} 7 $\text{mmol}\cdot\text{L}^{-1}$, organic and inorganic calcium channel blockers were present in the external solution before CTX addition. In preparations with the endothelium intact and incubated with CTX, relaxation by acetylcholine (ACh) stimulation of the tension induced by PhE was decreased. Endothelium-dependent relaxation to ACh was preserved when Ca^{2+} 5 $\text{mmol}\cdot\text{L}^{-1}$ was added to the medium prior to CTX. **CONCLUSION:** CTX first triggers the release of

NO from the endothelium which results in muscle relaxation, and then causes smooth muscle contraction. Ca^{2+} and Ca^{2+} channel blockers prevented the effect of CTX.

Cobra venom from *Naja naja atra* containing a cardiotoxin (CTX) is also named cytotoxin, cytolyisin, direct lytic factor, membrane-active polypeptide, and membrane-disruptive polypeptide. CTX causes systolic heart arrest by depolarizing myocardial cells, induces contractures and myonecrosis in skeletal muscle, hemolyzes erythrocytes, and induces platelet aggregation^[1-3]. The mechanisms responsible for these cytotoxic effects are unclear. Either CTX interacts with specific molecules of the cell membrane to affect Ca^{2+} regulation as proposed for myocardium and skeletal muscle^[4,5] or CTX disrupts the membrane through various non-specific interactions^[6].

The efficiency of excitation-contraction coupling in vascular smooth muscle is controlled by cytosolic Ca^{2+} concentration^[7] and is also modulated by activation of muscle guanylate cyclase by nitric oxide (NO) released from the endothelium^[8]. Therefore, a substance may affect vascular reactivity by acting at distinct sites in the vessel; directly on the smooth muscle, or on endothelial cells, or on both. This study was to look at the effects of CTX *in vitro* on vascular smooth muscle and endothelial cells.

MATERIALS AND METHODS

Drugs CTX was purified from the venom of *Naja naja atra* by successive chromatography on DEAE Sephadex A-50 column, CM Sephadex C-25 column and Sephadex G-50 column^[9]. Its *iv* LD₅₀ in mice was determined to be 2.0 $\text{mg}\cdot\text{kg}^{-1}$. No phospholipase A₂ activity was detectable with a standard pH-sat assay^[10] in this CTX fraction. Tetrandrine (Aldrich) was dissolved in HCl 0.1 $\text{mol}\cdot\text{L}^{-1}$ and diluted with distilled water before use. SK&F 96365

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(Biomol Res Lab) was prepared in absolute ethanol as $30 \text{ mmol} \cdot \text{L}^{-1}$ stock solution; nifedipine (Sigma) was prepared in absolute ethanol as $1 \text{ mmol} \cdot \text{L}^{-1}$ stock solution. These drugs were added to the baths in which the final concentration of ethanol was $< 0.1 \%$. Phenylephrine (PhE) and acetylcholine (ACh, Sigma) were prepared in double-distilled water. All other chemicals were of laboratory standard.

Sprague-Dawley rats (200 – 250 g) were decapitated. Thoracic aortae were placed in Krebs' solution containing NaCl 120, NaHCO_3 25, glucose 11.1, KH_2PO_4 1.2, MgSO_4 1.2, KCl 4.5, and CaCl_2 $1.25 \text{ mmol} \cdot \text{L}^{-1}$ (pH 7.4). Rings (3 – 4 mm) were cut, and in some rings, endothelium was denuded with forceps. The absence of functional endothelium was evidenced by the inability of ACh $3 \mu\text{mol} \cdot \text{L}^{-1}$ to relax preparations precontracted with PhE ($1 \mu\text{mol} \cdot \text{L}^{-1}$). Rings were suspended in a 5-mL organ bath connected to a force transducer (Grass FT03) for isometric measurement. Krebs' solution was aerated with 95 % O_2 + 5 % CO_2 at 37 °C. Rings were equilibrated in the medium for > 1 h during which the solution was changed every 15 min. Resting tension was maintained at 2 g, which was found to be optimal for the generation of active tension by these rings in response to KCl $60 \text{ mmol} \cdot \text{L}^{-1}$ stimulation.

Rings were exposed to a depolarizing solution to contract by adding KCl $60 \text{ mmol} \cdot \text{L}^{-1}$ hypertonically to the baths. When a steady-state tonic contraction was reached, preparations were washed 3 times with fresh Krebs' solution and allowed to relax back to resting tension. Stimulation with KCl $60 \text{ mmol} \cdot \text{L}^{-1}$ was repeated every 15 – 20 min until a reproducible contractile response (usually after 3 applications of KCl) was obtained.

CTX treatment Contractions obtained in response to CTX addition to the bath were expressed as % of the contractile response to KCl $60 \text{ mmol} \cdot \text{L}^{-1}$ prior to CTX treatment in that ring. CTX was added to the baths for 10 min only, unless otherwise stated.

CTX effect on endothelium-dependent relaxation Tone in rings was generated by stimulating with PhE $1 \mu\text{mol} \cdot \text{L}^{-1}$. When the contraction reached a plateau, a control endothelium-dependent relaxation to ACh $3 \mu\text{mol}$

$\cdot \text{L}^{-1}$ was recorded. Rings were then washed and brought back to resting tension. Various concentrations of CTX were then added to the different organ baths (one concentration per ring), and after 10 min thoroughly washed. The ability of ACh to induce relaxation of PhE-precontracted rings was tested again and compared to a time control.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with *t* test.

RESULTS

CTX induced vascular smooth muscle contraction and endothelium-dependent relaxation: CTX caused slowly developing contractions in aortic rings in a concentration-dependent manner. These contractions were usually preceded by a small, variable, and transient relaxation in endothelium-intact rings. This transient relaxation was never seen in endothelium-denuded preparation (Fig 1A & 2B), but was seen more distinctly when the tone was raised (Fig 2A), or the presence of *L*-NAME $100 \mu\text{mol} \cdot \text{L}^{-1}$ (Fig 1A & 2C).

Contractions developing following 10-min incubation with CTX were transient, reaching a peak within 10 – 15 min and lasting 0.5 h at all CTX concentrations. Similar peak contractions were observed in aortic rings with or without intact endothelium (Fig 1B). After CTX, the response to stimulation with KCl or PhE were decreased. This effect of CTX was concentration- (Fig 3B) and time-dependent (Fig 3C).

Effect of extracellular Ca^{2+} concentration on contractions induced by CTX The largest contractions induced by CTX were obtained when extracellular Ca^{2+} concentrations were within the physiologic ranges. Increasing Ca^{2+} concentrations, prior to CTX incubation, resulted in a decrease in CTX-induced contraction (Fig 4).

Effect of calcium channel blockers on CTX-induced contraction Ni^{2+} , like high Ca^{2+} , prevented CTX-induced contraction. However, after washout of high Ca^{2+} or Ni^{2+} , contraction developed. Organic calcium channel blockers, tetrandrine, SK&F 96365, and nifedipine, also prevented the development of contractions induced by CTX. However, contrary to inorganic calcium channel blocker (Ni^{2+}), contraction did not develop following

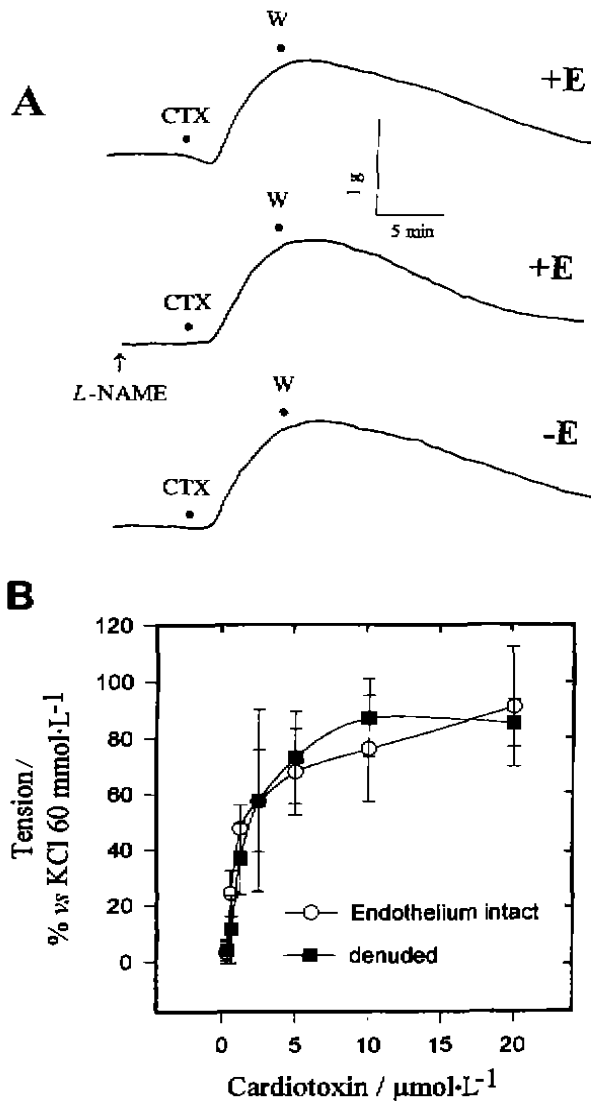


Fig 1. Effects of CTX on isolated rat aortic rings: A) Typical examples of contractions caused by CTX $10 \mu\text{mol}\cdot\text{L}^{-1}$ in isolated rat aortic rings. (+E) with, (-E) without endothelium. (W) washout. B) Summary of CTX-induced contraction in endothelium-intact and -denuded preparation ($n = 6$ rats, $\bar{x} \pm s$).

repeated wash of these compounds (Fig 5).

Inhibition of ACh-induced endothelium-dependent relaxation by CTX The relaxation induced by ACh in endothelium-intact rings ($55 \% \pm s 11 \%$, $n = 8$ rats) was decreased by CTX in a concentration-dependent manner (Fig 6).

When rings were treated with CTX $10 \mu\text{mol}\cdot\text{L}^{-1}$ for 10 min, ACh-induced relaxation was lost. But, when Ca^{2+} $5 \text{ mmol}\cdot\text{L}^{-1}$ was added before CTX, ACh-induced relaxation was

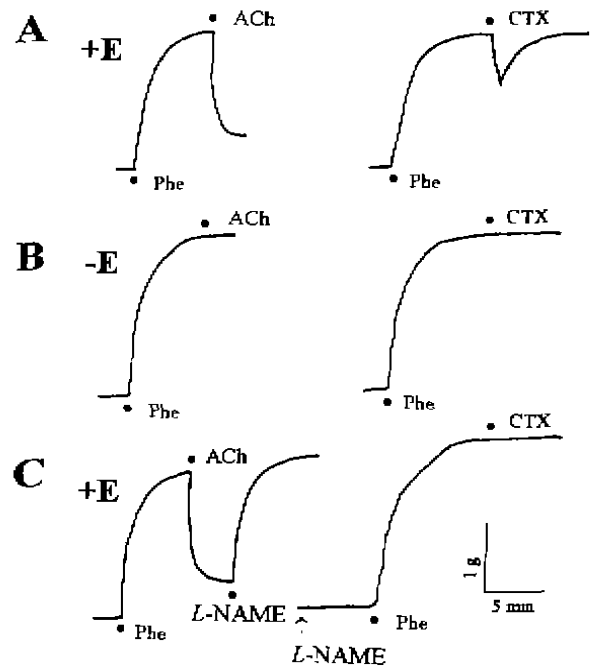


Fig 2. Typical examples of the effect of ACh and CTX on phenylephrine-precontracted rings. In the presence (A) and absence (B) of endothelium (E), and (C) in the presence of endothelium (E), and following pre-incubation for 10 min with L-NAME $0.1 \text{ mmol}\cdot\text{L}^{-1}$.

preserved ($39 \% \pm s 3 \%$, $n = 4$) (Fig 7).

DISCUSSION

Our data indicated that CTX interacted with endothelial cells to release NO. This effect most probably initially contributes to the drop in blood pressure observed *in vivo*. Production of endothelium-derived NO is Ca^{2+} -dependent, and any event which elevates the cytosolic Ca^{2+} concentration in endothelial cells should cause NO biosynthesis and increase NO levels. For example, drugs other than agonists such as the Ca^{2+} -ionophore calcimycin, or the endoplasmic reticulum Ca^{2+} -ATPase pump inhibitor, cyclopiiazonic acid, which both increase intracellular Ca^{2+} concentration, can induce endothelium-dependent relaxation^[11, 12]. We speculate that CTX increased the membrane permeability of endothelial cells to Ca^{2+} which in turn activated the production of NO and was responsible for the initial vasorelaxation. This relaxation was transient because direct contractile effect of CTX on smooth muscle eventually offsetted the relaxant effect of NO released from the endothelium.

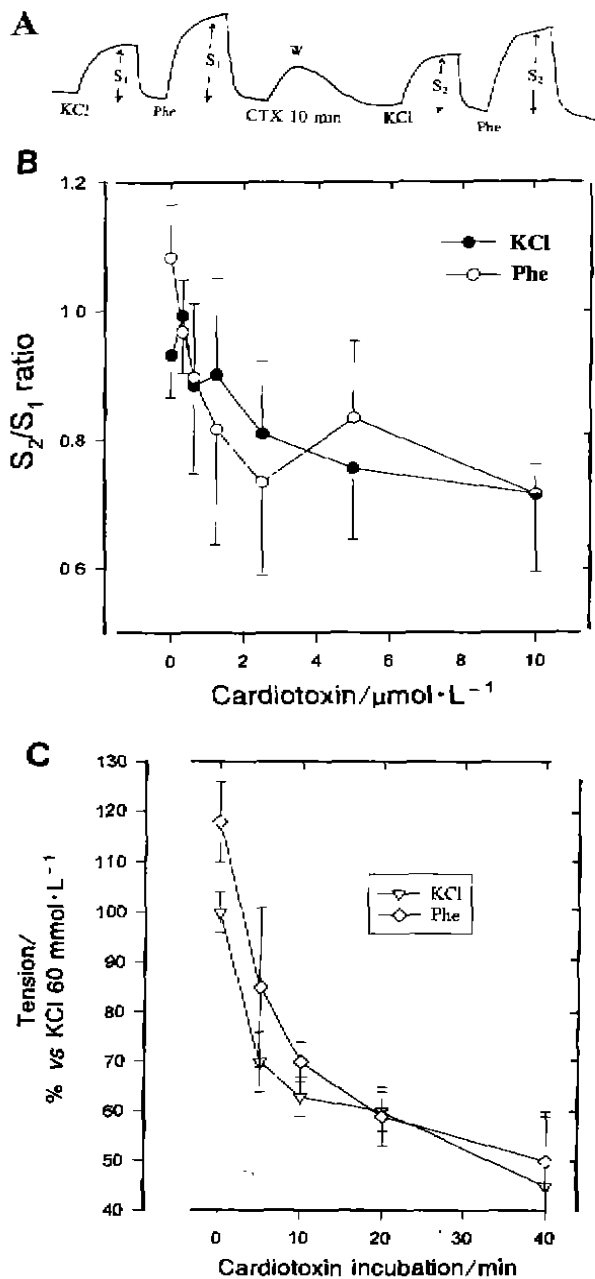


Fig 3. Effect of CTX on contractions induced by PhE $1 \mu\text{mol} \cdot \text{L}^{-1}$ and KCl $60 \text{mmol} \cdot \text{L}^{-1}$. A) Experimental protocol. The amplitude of control was denoted as S_1 . Rings were then challenged with CTX $10 \mu\text{mol} \cdot \text{L}^{-1}$ for a 10-min period and then repeatedly washed (W) for about 30-min after which contractions to KCl and PhE were assessed again (S_2). Concentration-dependent (B, $n = 7$ rats, $\bar{x} \pm s$;) and time-dependent (C, $n = 6$ rats, $\bar{x} \pm s$) effect of CTX on KCl and PhE-induced contractions.

CTX, although acting on both endothelial and smooth muscle cells induced contraction, and maximal contractile responses obtained were

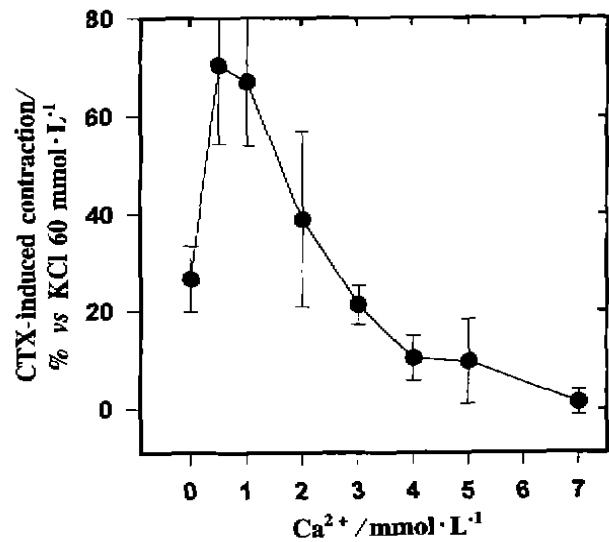


Fig 4. Relationship between external Ca^{2+} concentration and CTX-induced contractions. $n = 6$, $\bar{x} \pm s$.

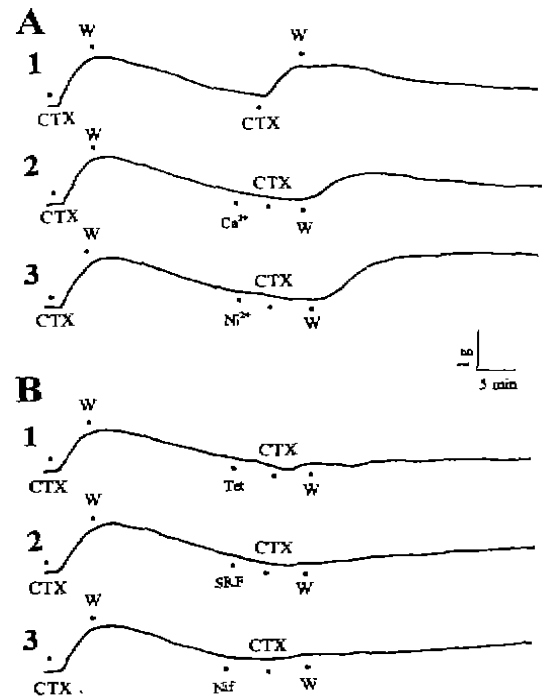


Fig 5. Typical examples of the effect of inorganic and organic calcium blocker on CTX-induced contraction. A) CTX $10 \mu\text{mol} \cdot \text{L}^{-1}$ stimulation for 10-min induced contraction (A_1) can be repeated and $\text{Ca}^{2+} 5$ (A_2) and $\text{Ni}^{2+} 3 \text{mmol} \cdot \text{L}^{-1}$ when added before CTX prevented the contraction induced by CTX. Contraction developed when cations were washed (W) away. B) Incubation with tetrandrine ($30 \mu\text{mol} \cdot \text{L}^{-1}$), SK&F96365 ($6 \mu\text{mol} \cdot \text{L}^{-1}$) and nifedipine ($1 \mu\text{mol} \cdot \text{L}^{-1}$) before CTX challenge, prevented development of contraction (B1, B2, and B3). Tracings are typical of 4 separate experiments.

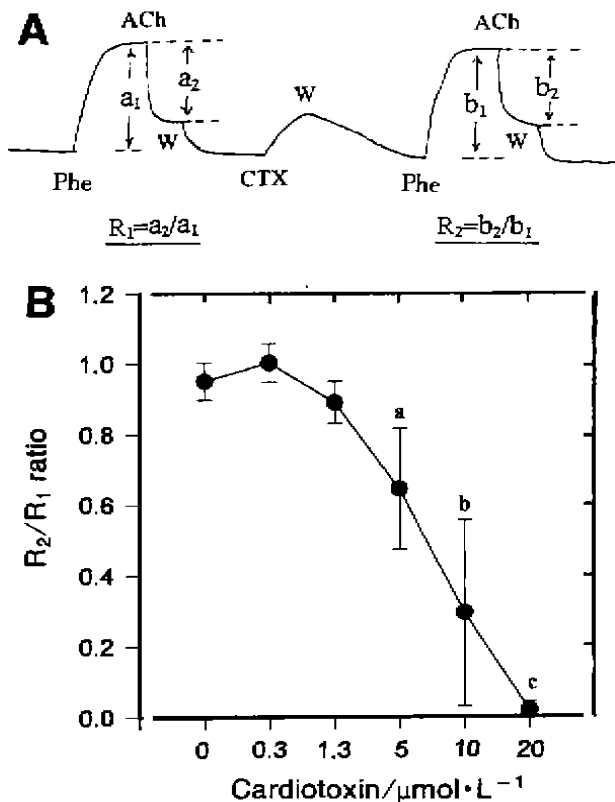


Fig 6. Effect of CTX on ACh-induced relaxation. A) Experimental protocol. B) After assessing control endothelium-dependent relaxation (R_1), rings were challenged with CTX $10 \mu\text{mol} \cdot \text{L}^{-1}$ for 10-min only. Rings were then repeatedly washed (W) for 30-min and ACh-induced relaxation was assessed again (R_2). B) Summary of the effect of CTX on ACh-induced relaxation. $n = 6$ rats, $\bar{x} \pm s$. ^a $P > 0.05$, ^b $P < 0.05$, ^c $P < 0.01$.

identical in endothelium-intact and endothelium-denuded preparations (see Fig 1B) which suggests that CTX has impaired the function of endothelial cells (no more NO release). This is supported by the data in Fig 4.

Maximal contractions induced by CTX were obtained in solution containing physiological concentration of Ca^{2+} . When Ca^{2+} concentration was increased beyond the physiologic range, CTX-induced contraction was progressively decreased. The inhibitory effect of high Ca^{2+} could have not been the consequence of a direct interaction between CTX and Ca^{2+} as CTX is positively charged. High concentration of several divalent cations, including Ca^{2+} , can reduce by 50% the binding of ^{125}I -CTX to skeletal muscle cell's plasma membrane^[5], but other mechanisms must have been operative in

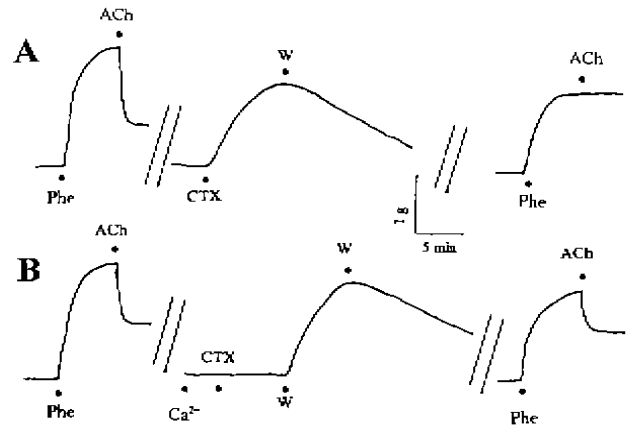


Fig 7. Typical examples of the inhibitory effect of CTX on endothelium-dependent relaxation and its reversal by Ca^{2+} . A) ACh-induced relaxation was tested in rings precontracted with PhE $1 \mu\text{mol} \cdot \text{L}^{-1}$. Rings were then challenged with CTX $10 \mu\text{mol} \cdot \text{L}^{-1}$ for 10-min (middle tracing), after which they were washed (W). Then ACh-induced relaxation was tested again. B) Same as A except that Ca^{2+} $5 \text{ mmol} \cdot \text{L}^{-1}$ was added before CTX. Representative tracings for 5 similar experiments.

our system to account for the almost complete prevention of CTX-induced vascular contraction by Ca^{2+} . Ca^{2+} and CTX may have a common target on the cell membranes, and when binding to this target, Ca^{2+} can prevent or overcome the effect of CTX. When Ca^{2+} in the external solution is lowered, the effect of CTX on membrane resumes. One plausible mechanism may involve inhibition by Ca^{2+} of an increase in the membrane permeability to Ca^{2+} evoked by CTX. It is well known that high concentrations of Ca^{2+} stabilize biological cell membranes and depress the membrane excitability in vascular smooth muscle^[13]. Perhaps this protective effect is at the level of Ca^{2+} channels as Ca^{2+} -channel blockers also prevented CTX cytotoxicity from developing. These organic Ca^{2+} antagonists offer long lasting protection against CTX as they can not be washed away (Fig 5). An effect of CTX at the level of Ca^{2+} channels is consistent with the fact that CTX depolarizes muscle cells and nerves^[14] and is also in agreement with the finding that CTX causes contraction in rabbit aorta associated with nifedipine-sensitive $^{45}\text{Ca}^{2+}$ uptake^[15]. In conclusion, the data presented support the hypothesis that in these experimental conditions CTX exerts its contractile effect primarily via the

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opening of the Ca^{2+} channels.

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眼镜蛇心脏毒素对血管平滑肌和内皮细胞的双向作用

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关键词 眼镜蛇毒; 毒素; 胸主动脉; 血管平滑肌; 血管内皮; 苯福林; 氯化钾; N^G -硝基精氨酸甲酯; 钙通道阻滞剂

目的: 探讨眼镜蛇毒的心脏毒素对大鼠主动脉的毒性反应。 **方法:** 以内皮完整和去内皮后的主动脉环的收缩反应为检测指标。 **结果:** 心脏毒素 (CTX) $10 \mu\text{mol}\cdot\text{L}^{-1}$ 致使去内皮后的血管环产生短暂舒张反应及紧接着的持续收缩反应。去内皮或加入一氧化氮 (NO) 合成酶抑制剂 L-NAME, 虽能防止 CTX 引起的舒张却不影响随后的收缩。CTX 本身虽会引起血管环的收缩, 却又能量效性地抑制苯福林及高钾激素的收缩幅度, CTX 引起的收缩有强烈的外钙依赖性。钙浓度 $1 \text{ mmol}\cdot\text{L}^{-1}$ 产生最高效应然后随钙浓度增加而递减, 外钙浓度增至 $7 \text{ mmol}\cdot\text{L}^{-1}$ 或先加入钙拮抗剂则可完全抑制 CTX 孕育后乙酰胆碱所引起的短暂舒张。虽有显著减少却能受高钙 ($5 \text{ mmol}\cdot\text{L}^{-1}$) 的保护。 **结论:** CTX 明显地影响血管收缩功能。首先产生内皮依赖性的短暂舒张反应, 随后致使血管平滑肌收缩终致细胞坏死, 并使其它激动剂引起的收缩幅度减少。这些现象均与钙协调有密切的关系。