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Calcium-dependent contractile response of arterial smooth muscle to a jellyfish toxin (pCrTX: *Carybdea rastonii*)

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1 The purpose of the present experiments was to investigate the pharmacological mechanisms of the vasoconstriction caused by the toxin (pCrTX) which had been partially purified from the tentacles of the jellyfish *Carybdea rastonii* ('Andonkurage').

2 pCrTX (0.1 to 10 $\mu g\,ml^{-1}$) produced a tonic contraction of rabbit aortic strips, which was nearly abolished in Ca^{2+}-free medium and was significantly reduced by verapamil or diltiazem.

3 pCrTX stimulated $^{45}Ca^{2+}$-influx and this effect was markedly attenuated by verapamil.

4 pCrTX-induced vasoconstriction was significantly attenuated by phentolamine, 6-hydroxydopamine (6-OHDA) and in low Na^+-medium, but not by bretylium, guanethidine, reserpinization or tetrodotoxin (TTX).

5 pCrTX continuously and significantly increased the ^3H-efflux from [^3H]-noradrenaline preloaded aortic strips and this effect was completely inhibited by pretreatment with 6-OHDA and in Ca^{2+}-free medium, but not by phentolamine, bretylium, guanethidine or TTX.

6 A single exposure to pCrTX for 30 min greatly reduced the contractile responses to tyramine, nicotine and transmural electrical stimulation, but not those to noradrenaline or KCl. In addition, incorporation of [^3H]-noradrenaline was reduced.

7 Pretreatments with chlorphenylamine or indomethacin failed to modify the contractile response to pCrTX.

8 These results suggest that the pCrTX-induced vasoconstriction is caused by a presynaptic action, releasing noradrenaline from the intramural adrenergic nerve terminals, and by a postsynaptic action, which consists at least in part of stimulation of the transmembrane calcium influx. Both pre- and postsynaptic actions depend on the external calcium concentration. The data further suggest that pCrTX damages the noradrenaline uptake and/or storage mechanisms without damaging postsynaptic contractile systems.

Introduction

During the summer months, one of the species of box jellyfish, *Carybdea rastonii*, 'Andonkurage', is commonly present in sea waters along the coast of Japan. The nematocyst of this jellyfish is capable of producing severe cutaneous pain, erythema, wheeling and haemorrhagic skin lesions in humans who accidentally come into contact with its tentacles.

The physiological and toxicological effects such as lethal toxicity on mammals, haemolytic activity, dermatonecrotic activity and cardiotoxic effect of various coelenterate toxins have been well documented (Larsen & Lane, 1966; Hastings *et al.*, 1967; Lane, 1968; Burnett & Goldner, 1969; Endeen & Henderson, 1969; Freeman, 1974), particularly those derived from the Portuguese Man-of-War (*Physalia physalis*), the sea wasp (*Chironex fleckeri*) and the sea nettle (*Chrysaora quinque cirrha*). The toxin (pCrTX) partially purified from tentacles of *Carybdea rastonii* jellyfish also possesses physiological and toxicological effects similar to those described above (Satoh *et al.*, 1983;

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1984). However, the detailed mechanisms of its action are unknown.

Recently, we have found that pCrTX causes a marked and sustained contraction of vascular smooth muscle. Thus, the present experiments were undertaken to investigate the pharmacological mechanisms of the pCrTX-induced vasoconstriction in comparison with other vasoactive substances.

Methods

Preparation of pCrTX

Specimens of *Carybdea rastonii* jellyfish ('Andonkurage') were captured at Abuzuri located on Sagami Bay and identified by Dr M. Imajima, Department of Zoology, National Science Museum, Tokyo. Tentacles were cut from living specimens immediately after capture, packed in dry ice, lyophilized and stored at −80°C until required.

Aliquots of the lyophilized tentacles were sonicated in 50 mM sodium acetate (pH 6.0). The supernatant was separated and treated with 40% ammonium sulphate for 60 min. Centrifugation at 6000 *g* for 30 min was carried out to obtain supernatant, which was treated with 60% ammonium sulphate for 60 min. The precipitate was separated by decantation of the supernatant after centrifugation at 6000 *g* for 30 min and dissolved in 50 mM sodium acetate (pH 6.0). Finally, the solution was dialyzed by means of an Amicon YM-2 to obtain pCrTX. All the procedures described above were carried out at 4°C. The concentration of pCrTX was expressed as μg protein ml^{-1} in *in vitro* experiments. Protein was assayed by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as a standard. The pCrTX prepared in this manner had an i.v. LD_{50} of 0.127 μg g^{-1} body weight in mice, which had been determined by the up-and-down method (Brownlee *et al.*, 1953). Periodic checks of lethality conducted on aliquots of the toxin used in this investigation established that the lethality did not decrease for at least 5 weeks when stored at 4°C. The pCrTX was diluted to appropriate concentrations in physiological saline just before use.

Measurements of contractile responses of aortic strips

Albino rabbits of either sex weighing 2.2 to 2.8 kg were killed by a blow on the head and exsanguination, and the thoracic aorta was rapidly excised. After removal of the adventitial connective tissue, helical strips 3 mm wide and 25 mm long were prepared. These preparations were mounted vertically in an organ bath containing 20 ml of modified Krebs solution continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. One end of each strip was secured to the bottom of the organ bath and the other was attached to a force-displacement transducer (SB-1T, Nihon Kohden Kogyo Co. Tokyo). Isometric changes in tension were recorded on a pen-writing oscillograph (Rectigraph RJG-3004, Nihon Kohden Kogyo Co. Tokyo). The length of the strips was adjusted several times until a stable basal tension of 2 g was attained. Before beginning the experiments, strips were allowed to equilibrate for at least 60 min in the bathing solution and during this period, the bathing solution was replaced every 20 min with fresh solution. The composition of the modified Krebs solution (in mM) was as follows: NaCl 115.0, KCl 4.7, $MgSO_4 7H_2O$ 1.2, $CaCl_2 2H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and glucose 10.0. The Ca^{2+}-free medium was prepared by omitting $CaCl_2$ from the solution. For the low Na^+-solution, 60 mM NaCl was replaced by 60 mM choline chloride. Each point on the dose-response curves was obtained by applying of a single concentration of pCrTX to one preparation. In order to examine the effects of various blocking agents on the contractile responses, aortic strips were pretreated with those agents for 30 min before the addition of the contractile agonists.

Transmural electrical stimulation

Transmural electrical stimulation was applied through a pair of platinum wire electrodes. In this case, the preparation was placed in parallel between the electrodes. The distance between the electrodes was approximately 2 mm, this being sufficiently narrow not to disturb tension change. The stimulus had a duration of 0.3 ms and supramaximum intensity (20 V) at a frequency of 20 Hz for 10 s.

6-Hydroxydopamine (6-OHDA) treatment

The strips were incubated with 10^{-4}M 6-OHDA (including 100 mg l^{-1} ascorbic acid) for 30 min at 37°C. After washing, the contractile responses to various stimuli were recorded and compared with those responses obtained with no 6-OHDA treatment. Although 6-OHDA is a very labile and easily oxidized compound, there are reports indicating that 6-OHDA used *in vitro* is not destroyed to any great extent (Jonsson & Sacks, 1970; 1971). Functional denervation was confirmed by the lack of any responses to tyramine or transmural electrical stimulation in the thoracic aorta after treatment with 6-OHDA.

Reserpinization

Reserpine at a dose of 0.5 mg kg^{-1} was given i.v. once daily for 2 days. Twenty four hours after the last injection, the rabbits were killed and the thoracic aorta isolated. Functional denervation was confirmed by the

lack of a response to tyramine or to transmural electrical stimulation in the thoracic aorta isolated from these rabbits.

$^{45}Ca^{2+}$-influx in aortic strips

Calcium influx was estimated by measuring changes in the specific activity of the calcium fraction resistant to displacement by lanthanum (Godfraind, 1976; Godfraind & Dieu, 1981). In brief, helical strips of thoracic aorta, the same size as those used in measurements of mechanical response, were mounted in a 20 ml organ bath under a 2 g load. An equilibration period of 60 min was allowed in a physiological solution (composition, mM: NaCl 112.0, KCl 5.0, $NaHCO_3$ 25.0, $MgSO_4 7H_2O$ 1.2, $CaCl_2 2H_2O$ 1.25 and glucose 11.5) bubbled with 95% O_2 and 5% CO_2, maintained at 37°C (during this period, the bathing solution was replaced every 20 min). Then $^{45}CaCl_2$ (1 μCi ml^{-1}) was added to the medium and 5 min later either the contractile agonists or the vehicle were also added. Five, 15 or 30 min after the addition of the contractile agonists, aortic strips were exposed for an additional 5 min to a lanthanum solution (composition, mM: NaCl 122.0, KCl 5.9, $MgCl_2 7H_2O$ 1.25, glucose 11.0, $LaCl_3$ 50.0 and Tris maleate 15.0, pH 6.8) bubbled with 100% O_2. Thereafter, the strips were blotted with filter paper and weighed. Each strip was dissolved in 1 ml of Soluene-350 (United Technologies Packard) by heating for 60 min at 60°C. After cooling, 4 ml of Aquazol-2 (New England Nuclear) was added and the radioactivity of the sample counted in a liquid scintillation counter (Packard 460-C). For determination of the effect of verapamil, the strips were treated with the agent for 30 min prior to the contractile agonists. The results of each determination have been converted to the apparent tissue content (μmol kg^{-1}) of $^{45}Ca^{2+}$ according to the following formula (Godfraind, 1976): $^{45}Ca^{2+}$ (μmol kg^{-1}) = (d.p.m. kg^{-1}) × ((μmol Ca^{2+} in litre medium) × (d.p.m. in litre medium)$^{-1}$).

Release and uptake of [^{3}H]-noradrenaline

Aortic strips were preincubated with [^{3}H]-noradrenaline (3×10^{-7}M) in modified Krebs solution containing ascorbic acid 100 mg l^{-1} and corticosterone 10^{-5}M for 90 min at 37°C. Then the strips were mounted in a superfusion apparatus. The superfusion apparatus and the procedure were essentially the same as described by Su & Bevan (1970). Briefly, the strip was mounted vertically between a stationary, supporting Lucite rod and a force-displacement transducer, under an initial load of 2 g. The modified Krebs solution containing ascorbic acid 100 mg l^{-1}, was bubbled with 95% O_2 and 5% CO_2 and constantly superfused using a peristaltic pump (Varioperpex II pump, LKB, Sweden) at a flow rate of 1 ml min^{-1}. The agents to be tested were dissolved in oxygenated and warmed Krebs solution immediately before use. The strips were then superfused with test agents for a given period. The strips were equilibrated for 90 min before starting the experiments. The superfusate was collected at 1 min intervals and the radioactivity determined by counting in a liquid scintillation counter. Six millilitres of Instagel (United Technologies Packard) was used as a scintillant. After termination of the superfusion experiments, the strip was digested in Soluene-2 and total residual radioactivities were measured. After the spontaneous efflux of ^{3}H had decreased exponentially and reached a plateau at 60 min to 90 min, the change in ^{3}H-efflux with pCrTX was determined. In these experiments, although a part of the [^{3}H]-noradrenaline taken up by the vascular tissue is metabolized, it has been found that the total ^{3}H-efflux correlates well with the release of unchanged [^{3}H]-noradrenaline from vascular tissue (Pinto & Trifaro, 1976; Vanhoutte *et al.*, 1981; Karaki *et al.*, 1984). We did not make an analysis of the composition of the ^{3}H-effluent in the present experiments.

In order to test the effects of pCrTX and cocaine on the [^{3}H]-noradrenaline uptake, aortic strips were preincubated with or without 1 μg ml^{-1} pCrTX or 3×10^{-6}M cocaine at 37°C for 30 min and then equilibrated for 120 min in the modified Krebs solution without any agent. During this period, the bathing solution was replaced every 20 min. Thereafter, the strips were incubated with 3×10^{-7}M [^{3}H]-noradrenaline in the Krebs solution containing ascorbic acid 100 mg l^{-1} and corticosterone 10^{-5}M for 90 min. After termination of the incubation, strips were rinsed in the Krebs solution containing ascorbic acid for 90 min. Then, the tissue level of ^{3}H was determined as stated above.

Chemicals

The following chemicals were used in these experiments: $^{45}CaCl_2$ (specific activity 22.2 and 19.8 mCi mg^{-1}, Amersham), (±)-[17-^{3}H(N)]-noradrenaline hydrochloride (specific activity 11.8 mCi mmol^{-1}, New England Nuclear), (−)-noradrenaline bitartrate, tetrodotoxin (TTX), procaine hydrochloride, 6-hydroxydopamine hydrochloride (6-OHDA), corticosterone acetate, tyramine hydrochloride, histamine dihydrochloride and arachidonic acid (all from Sigma), verapamil hydrochloride (Knoll), diltiazem (Tanabe), chlorphenylamine maleate (Kongo Chemicals Co.), indomethacin (Merck), phentolamine mesylate (Regitine) and guanethidine sulphate (Ciba), bretylium tosylate (Burroughs Wellcome Co.), nicotine (Nakarai Chemicals Co.), reserpine (Apoplon, Daiichi Pharmaceutical Co.), cocaine hydrochloride (Takeda) and sheep seminal vesicle microsomes (SSVM, Funakoshi

Chemicals Co.).

Indomethacin was dissolved in dimethylsulphoxide (DMSO) and then diluted with deionized and distilled water to make a solution. The final concentration of DMSO did not exceed 0.1% v/v.

Arachidonic acid was dissolved in ethanol and diluted with an aqueous solution of Na_2CO_3 (2 mol of Na_2CO_3 correspond to 1 mol of arachidonic acid) with stirring on ice and kept at −20°C until use.

Statistical analysis

Values are expressed or plotted as the mean ± s.e. and Student's *t* test was used to evaluate the results in all of the experiments.

Results

Contractile effect of pCrTX

pCrTX at concentrations of 0.1 to 10 μg ml^{-1} produced a long-lasting, tonic contraction of aortic strips in a concentration-dependent manner. Figure 1a shows a representative trace of the contractile response to 1 μg ml^{-1} pCrTX. After washout (6 to 7 times at 20 min intervals) of the preparation with Krebs solution, the tension was restored to the pre-pCrTX level. At this time, if pCrTX was again applied to the preparation, a smaller contraction which had a longer onset time was produced (Figure 1b). Figure 2 shows the concentration-response curve for the contractions to pCrTX. The correlation coefficient between the developed tension and the log concentration of pCrTX (0.1 to 10 μg ml^{-1}) was calculated to be 0.7382 ($P < 0.05$). The developed tension produced by 10 μg ml^{-1} pCrTX was 2.5 ± 0.1 g ($n = 6$).

Effects of various treatments on the pCrTX-induced vasoconstriction

The effects of various treatments employed to analyse the mechanisms of the pCrTX-induced vasoconstriction are summarized in Table 1. The concentrations of noradrenaline (3×10^{-8}M, approximate ED_{60}) and KCl (20 mM, approximate ED_{40}) used, produced the same magnitude of contraction (approx. 2 g) as did pCrTX.

Since it is well known that stimulation of Ca^{2+}-influx triggers a variety of physiological functions including smooth muscle contraction and neurotransmitter release, we first examined the role of Ca^{2+} in the medium in the pCrTX-induced vasoconstriction. After incubation of aortic strips in Ca^{2+}-free Krebs solution for 60 min, the contractile response to 1 μg ml^{-1} pCrTX was markedly reduced or abolished, but was nearly completely restored by the addition of 2.5 mM Ca^{2+}. In this case, contraction was induced immediately after addition of calcium (Figure 3). Under the same conditions, KCl-induced vasoconstriction was also markedly reduced or abolished, but restored by calcium, while the contraction to noradrenaline was significantly attenuated but never abolished in Ca^{2+}-free medium.

Pretreatment with verapamil (or diltiazem) at a concentration (10^{-6}M each) sufficient to inhibit the

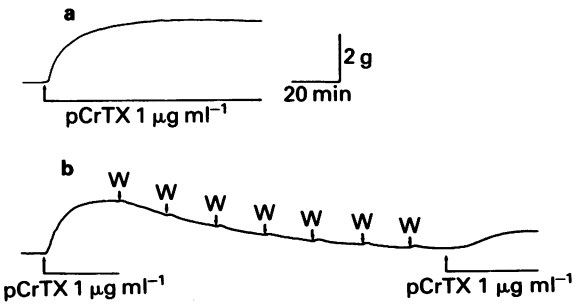

Figure 1 Representative tracings of the contractile response of the aortic strip to pCrTX. (a) pCrTX at a concentration of 1 μg ml^{-1} produced a long-lasting, tonic contraction. (b) When pCrTX was again applied at an interval of 120 min during which the bathing solution was replaced every 20 min (W), the amplitude of the second contraction was significantly reduced and the time to onset of the contraction markedly prolonged.

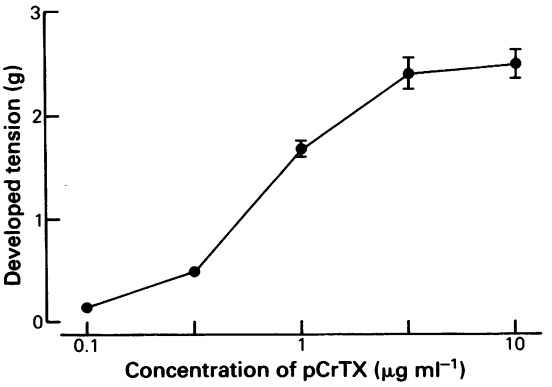

Figure 2 Log concentration-response curve for the contractile response of the aortic strip to pCrTX. Each point represents the mean of 6 observations and vertical lines show s.e.

Table 1 Effects of various treatments on the vasoconstriction induced by pCrTX, noradrenaline and KCl

Treatment	*pCrTX* ($1\ \mu g\ ml^{-1}$)	*Noradrenaline* (3×10^{-8} M)	*KCl* (20 mM)
Control	2.1 ± 0.1	2.2 ± 0.2	2.3 ± 0.2
Ca^{2+}-free medium	0.2 ± 0.1** (6)	1.2 ± 0.1* (5)	0.2 ± 0.1**(5)
Verapamil 10^{-6}M	1.0 ± 0.1** (6)	1.4 ± 0.1**(5)	0.2 ± 0.1**(5)
Diltiazem 10^{-6}M	1.0 ± 0.2** (6)	–	–
Phentolamine 10^{-6}M	1.4 ± 0.4**(12)	0**(5)	2.3 ± 0.3 (5)
6-OHDA 10^{-4}M	1.2 ± 0.2** (6)	2.6 ± 0.4* (5)	2.4 ± 0.3 (5)
Guanethidine 10^{-5}M	2.5 ± 0.2** (7)	2.6 ± 0.2**(7)	2.4 ± 0.2 (7)
Bretylium 10^{-5}M	2.1 ± 0.2 (5)	–	–
Reserpinization	2.5 ± 0.1** (8)	2.8 ± 0.1**(8)	2.8 ± 0.2**(8)
Phentolamine + verapamil	0.2 ± 0.0** (5)	–	–
6-OHDA + verapamil	0.3 ± 0.0** (7)	–	–
TTX 3×10^{-6}M	2.1 ± 0.2 (6)	2.3 ± 0.1 (5)	2.4 ± 0.1 (5)
Low-Na^+ medium	1.3 ± 0.1** (5)	2.1 ± 0.2 (5)	1.8 ± 0.2**(7)
Chlorphenylamine 10^{-5}M	2.0 ± 0.1 (5)	–	–
Indomethacin 10^{-5}M	1.9 ± 0.1 (6)	–	–

Results are given as mean developed tension (g) ± s.e. Figures in parentheses indicate the number of experiments. *$P<0.05$, **$P<0.01$ vs. each control, (–): not determined 6-OHDA = 6-hydroxydopamine and TTX = tetrodotoxin.

KCl-induced contraction, markedly attenuated but did not abolish the contractile response to $1\ \mu g\ ml^{-1}$ pCrTX. Noradrenaline-induced vasoconstriction was inhibited by about 40% by verapamil.

These observations suggest that pCrTX causes vasoconstriction via a mechanism dependent on external calcium which is mediated, at least in part, through the stimulation of calcium channels that are blocked by verapamil and diltiazem.

The contractile response to $1\ \mu g\ ml^{-1}$ pCrTX was significantly attenuated but not abolished by pretreatment with 10^{-6}M phentolamine or 10^{-4}M 6-OHDA (Table 1). Mechanical responses to exogenous noradrenaline and to KCl were not reduced after 6-OHDA treatment, although the contractile responses to tyramine and transmural electrical stimulation were abolished, confirming the presynaptic action of 6-OHDA. The pCrTX-induced residual contraction after chemical sympathectomy with 6-OHDA was unaffected by phentolamine (10^{-6}M) (data not shown). On the other hand, pCrTX-induced vasoconstriction was almost abolished after combined pretreatment with phentolamine + verapamil or 6-OHDA + verapamil. In contrast, 10^{-5}M guanethidine and 10^{-5}M bretylium did not inhibit, and guanethidine actually potentiated, the vasoconstriction (Table 1). Furthermore, TTX at a relatively high concentration of 3×10^{-6}M had no effect on the contractile response to pCrTX (Table 1). These agents, except verapamil, abolished the contractile response of the aortic strip to

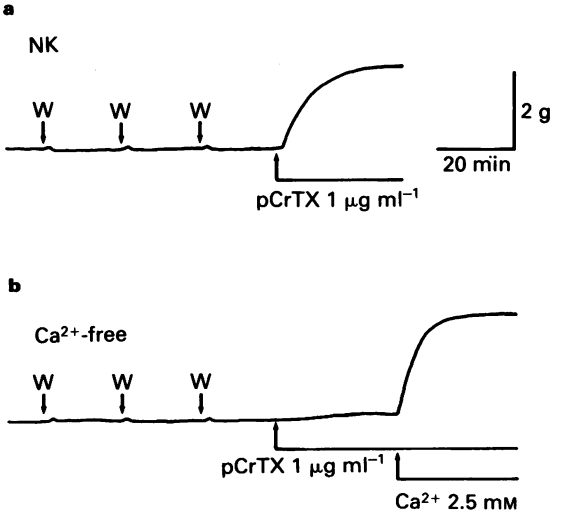

Figure 3 Abolition of the contractile response to pCrTX in Ca^{2+}-free medium and its restoration on addition of Ca^{2+}. (a) pCrTX-induced vasoconstriction in normal Krebs solution (NK). (b) The contractile response to pCrTX was nearly abolished after incubation in Ca^{2+}-free medium for 60 min, but was restored by addition of 2.5 mM $CaCl_2$. W: the bathing solution was replaced every 20 min.

transmural electrical stimulation (data not shown). It was of interest to note that pCrTX-induced vasoconstriction was not reduced but significantly augmented in reserpinized preparations as compared with that in normal preparations (Table 1). This potentiated contraction in the reserpinized preparations was markedly reduced by verapamil but unaffected by phentolamine (Figure 5).

Since pCrTX-induced vasoconstriction was significantly attenuated by pretreatment with the α-adrenoceptor blocking agent, phentolamine, or after chemical sympathectomy with 6-OHDA, it appears that an indirect sympathomimetic action is involved in the contractile response to pCrTX.

The contractile responses to pCrTX and KCl were significantly decreased in low Na^+ medium. However, the noradrenaline-induced response was unaffected in the same medium (Table 1).

Pretreatment with chlorphenylamine (10^{-5}M) or indomethacin (10^{-5}M) failed to modify the pCrTX-induced vasoconstriction (Table 1). These agents completely inhibited the contractile responses of aortic strips to histamine (10^{-6}M) or to a mixture of microsomes (SSVM, 1 mg protein ml^{-1}) and arachidonic acid (100 μM) (data not shown).

pCrTX-induced increase in $^{45}Ca^{2+}$-influx and its inhibition by verapamil

Figure 4 shows the time course of $^{45}Ca^{2+}$-influx induced by some contractile agonists. The $^{45}Ca^{2+}$-influx was significantly increased 5 and 15 min after addition of 1 μg ml^{-1} pCrTX as compared with each control, but not 30 min after. Similar results were obtained with KCl and noradrenaline. As shown in Table 2, pCrTX, KCl and noradrenaline significantly increased the $^{45}Ca^{2+}$-influx 15 min after addition of these agonists. The increases in $^{45}Ca^{2+}$-influx caused by these contractile agonists were markedly inhibited in the presence of 10^{-6}M verapamil.

pCrTX-induced increase in ^{3}H-efflux and effects of various treatments on the efflux

pCrTX, 1 μg ml^{-1}, produced a significant, maintained increase in the ^{3}H-efflux from [^{3}H]-noradrenaline preloaded aortic strips. The tension of the strips increased with increasing ^{3}H-efflux (Figure 6). This increase in ^{3}H-efflux did not occur after pretreatment with 6-OHDA, and was significantly attenuated (approx. 80%) after a 60 min exposure to low Na^+-

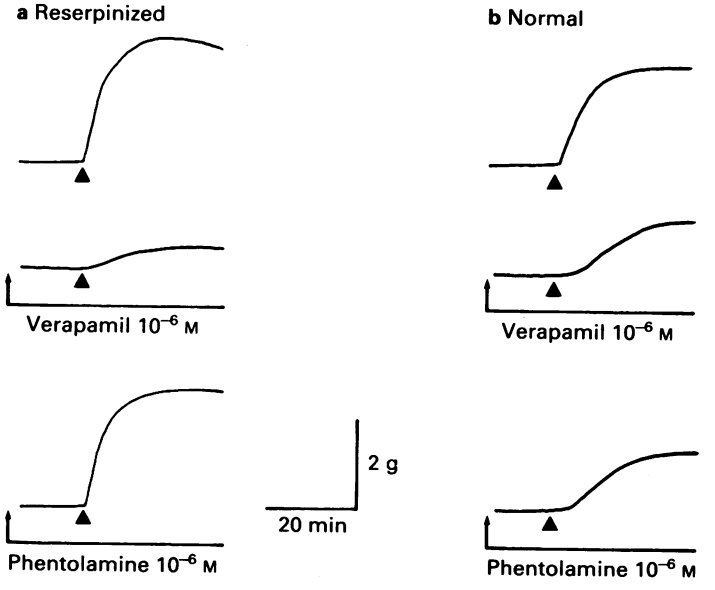

Figure 5 Comparison of contractile responses to pCrTX, and effects of verapamil and phentolamine, in reserpinized (a) and normal (b) preparations. pCrTX-induced vasoconstriction was significantly greater in the reserpinized preparation than that in the normal one. The potentiated contraction in the reserpinized preparation was markedly attenuated by 10^{-6}M verapamil, but was unaffected by 10^{-6}M phentolamine. Similar results were obtained with 4 other preparations. Verapamil and phentolamine were added 30 min before pCrTX. ▲: 1 μg ml^{-1} pCrTX.

Table 2 pCrTX-, noradrenaline- and KCl-induced $^{45}Ca^{2+}$-influx in the absence or presence of verapamil

	Verapamil 10^{-6} M			
	Absence		*Presence*	
Treatment	*Total*	*Net*	*Total*	*Net*
Control	402.9 ± 55.0 (7)	–	313.3 ± 46.4 (6)	–
pCrTX 1 μg ml^{-1}	548.6 ± 55.1**(7)	145.7 ± 11.5	351.7 ± 45.0**(6)	38.3 ± 4.0
Noradrenaline 3 × 10^{-8} M	505.7 ± 44.4**(7)	102.9 ± 14.9	335.0 ± 47.1* (6)	21.7 ± 7.0
KCl 20 mM	642.9 ± 52.8**(7)	240.0 ± 17.1	315.8 ± 47.2 (6)	2.5 ± 10.1

The results are presented as apparent tissue content of $^{45}Ca^{2+}$ (μmol kg^{-1}). The net increases in $^{45}Ca^{2+}$ were calculated as the mean ± s.e. of the differences between individual tissues in the drug-treated group and corresponding control group. The $^{45}Ca^{2+}$-influx was determined 15 min after the addition of the contractile agonists. Figures in parentheses indicate the number of experiments. *$P<0.05$, **$P<0.01$ vs. each control.

medium, while it was little affected by phentolamine and bretylium (data not shown). 6-OHDA itself and exposure to low Na^+-medium significantly increased the basal ^{3}H-efflux. Guanethidine also increased the efflux and markedly augmented the pCrTX-induced ^{3}H-efflux. TTX, 3 × 10^{-6} M, did not produce any effect on the ^{3}H-efflux (data not shown). When the strips were exposed to Ca^{2+}-free physiological solution for 60 min, pCrTX failed to increase ^{3}H-efflux from the [^{3}H]-noradrenaline preloaded aortic strips or to produce vasoconstriction (Figure 7 and Table 1).

Inhibition of [^{3}H]-noradrenaline uptake by pCrTX

When 1 μg ml^{-1} pCrTX was again applied to the aortic preparation after an interval of 120 min, the amplitude of the second contraction was significantly reduced, and time to onset of the contraction markedly prolonged as compared with that of the first response (Figure 1). Two hours after a 30 min exposure to pCrTX, the contractile responses to 10^{-4} M tyramine, 10^{-4} M nicotine and to transmural electrical stimulation were greatly reduced or abolished, although 10^{-6} M noradrenaline- and 30 mM KCl-induced contractile

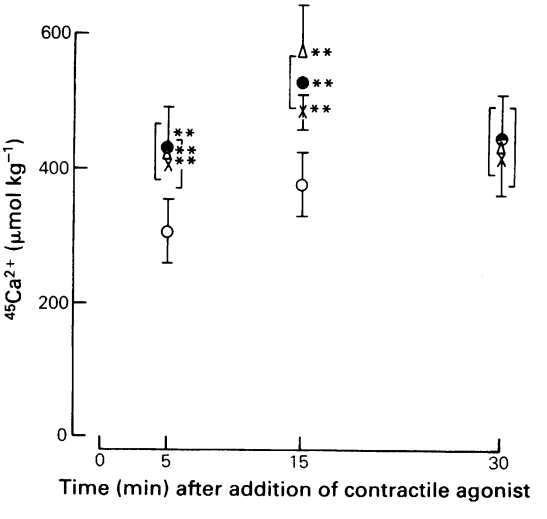

Figure 4 pCrTX-, noradrenaline- and KCl-induced increases in $^{45}Ca^{2+}$-influx in aortic strips. (○) Control; (●) 1 μg ml^{-1} pCrTX; (X) 3 × 10^{-8} M noradrenaline; (△) 20 mM KCl. Each point represents the mean of 5 observations and vertical lines show s.e. The results are presented as apparent tissue content of $^{45}Ca^{2+}$ (μmol kg^{-1}). **$P<0.01$.

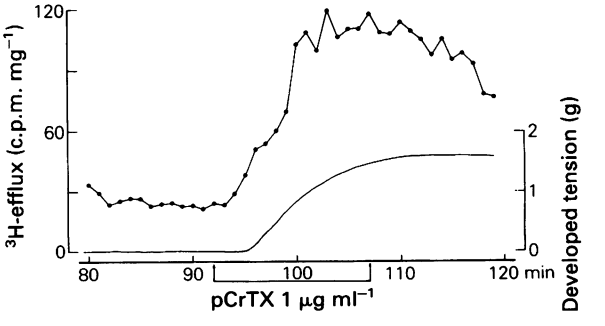

Figure 6 pCrTX-induced vasoconstriction and increase in ^{3}H-efflux in [^{3}H]-noradrenaline preloaded aortic strip. pCrTX 1 μg ml^{-1} was added for 15 min. The residual ^{3}H after termination of the experiments was determined as 4895.3 c.p.m. mg^{-1}, compared with 6182.8 c.p.m. mg^{-1} in the control (no treatment). Similar results were obtained in 3 other preparations.

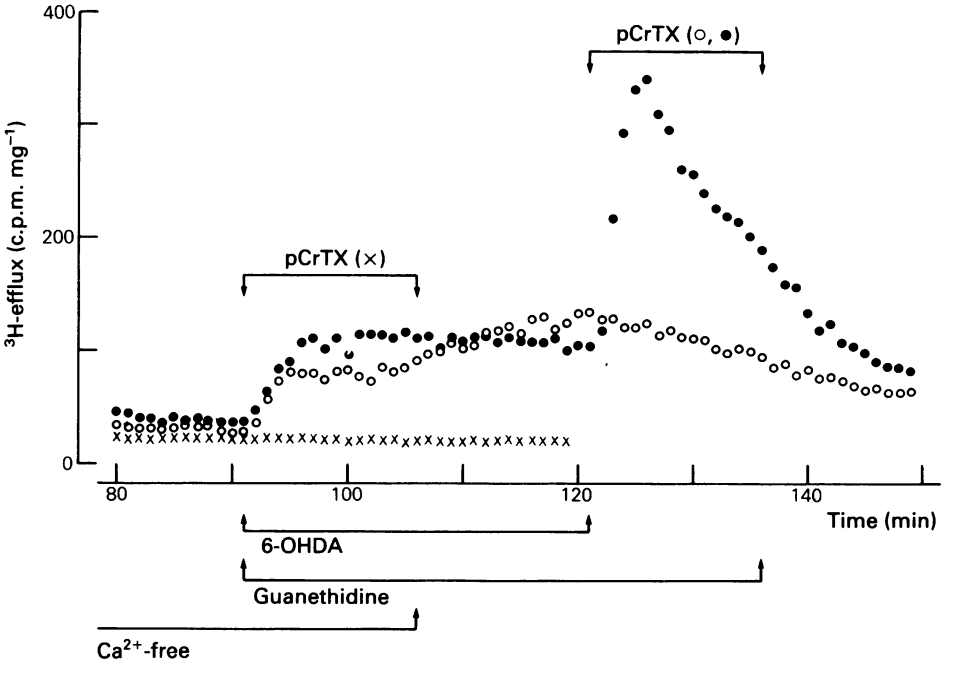

Figure 7 Effects of 6-hydroxydopamine (6-OHDA), guanethidine and Ca^{2+}-free medium on the pCrTX-evoked ^3H-efflux from [^3H]-noradrenaline preloaded aortic strips. 6-OHDA (○): the strip was exposed to 10^{-4}M 6-OHDA for 30 min before pCrTX. Guanethidine (●): the strip was exposed to 10^{-5}M guanethidine 30 min before and during pCrTX. Ca^{2+}-free (X): the strip was exposed to Ca^{2+}-free medium 60 min before and during pCrTX. pCrTX at a concentration of 1 μg ml^{-1} was superfused for 15 min. The residual ^3H after termination of the experiments using 6-OHDA, guanethidine and Ca^{2+}-free medium was determined as 2817.8, 1823.0 and 5567.6 c.p.m. mg^{-1}, respectively compared with 5976.4 c.p.m. mg^{-1} for the control (no treatment). Similar results were obtained in 3 other preparations.

responses remained unchanged (Figure 8a,b). Furthermore, 120 min after a 30 min exposure to 1 μg ml^{-1} pCrTX or 3×10^{-6}M cocaine, [^3H]-noradrenaline uptake by the preparation was inhibited by approximately 74% and 44%, respectively (Figure 9).

Discussion

Extracellular calcium-dependence of the pCrTX-induced vasoconstriction

The pCrTX-induced vasoconstriction was almost abolished in Ca^{2+}-free physiological solution and was significantly attenuated but never abolished by pretreatment with so called calcium antagonists such as verapamil and diltiazem. In addition, the reduced response to pCrTX in Ca^{2+}-free medium was completely restored by the addition of calcium. These results suggest that the pCrTX-induced vasoconstriction depends on the extracellular calcium concentration and at least in part on the stimulation of calcium influx which is blocked by verapamil and diltiazem. This speculation might be supported, in part, by the finding that pCrTX increases $^{45}Ca^{2+}$-influx and this effect of pCrTX is markedly attenuated in the presence of verapamil.

Pre- and postsynaptic component of the pCrTX-induced vasoconstriction

The pCrTX-induced vasoconstriction was partially blocked by an α-adrenoceptor blocking agent, phentolamine, at a concentration sufficient to abolish the contractile response to exogenous noradrenaline, and was more completely inhibited by phentolamine in combination with verapamil. However, a residual contraction to pCrTX after chemical sympathectomy with 6-OHDA remained unaffected by phentolamine (but was almost abolished by verapamil). This appears to eliminate the involvement of direct stimulation of postsynaptic adrenoceptors by pCrTX and suggests

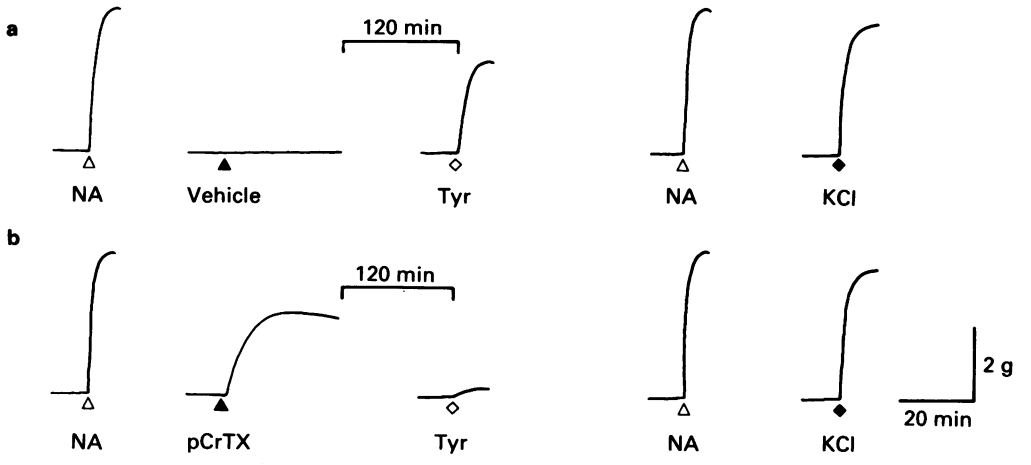

Figure 8 Comparison of the contractile response of aortic strips to tyramine (Tyr, 10^{-4}), noradrenaline (NA, 10^{-6}M) and KCl (30 mM) after a 30 min exposure to pCrTX (1 μg ml^{-1}) (b) or vehicle (a). Two hours after a 30 min exposure to pCrTX (during this period, the bathing solution was replaced every 20 min) the contractile response to tyramine was markedly reduced, although responses to noradrenaline and KCl were unchanged. The preparations were allowed to equilibrate for 60 min before treatments with contractile agonists, except between pCrTX and tyramine.

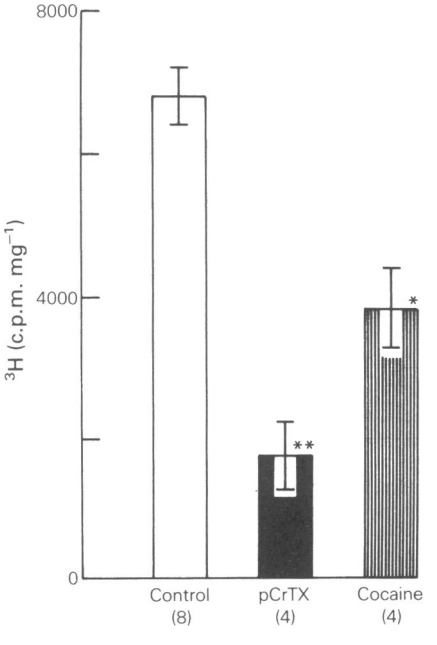

Figure 9 [^{3}H]-noradrenaline uptake by aortic strips pretreated with pCrTX or cocaine. Aortic strips were exposed to pCrTX (1 μg ml^{-1}) or cocaine (3×10^{-6}M) for 30 min, 120 min before incubation with [^{3}H]-noradrenaline (see text). Figures in parentheses indicate the number of observations and vertical bars show s.e. $*P < 0.05$, $**P < 0.01$ vs. control.

that the α-adrenoceptor component is due to the release of noradrenaline from nerve terminals. This is confirmed by the finding that pCrTX increased ^{3}H-efflux from [^{3}H]-noradrenaline preloaded aortic preparations and this was accompanied by an increase in tension. The increase in the ^{3}H-efflux was completely inhibited after chemical sympathectomy with 6-OHDA. However, the mechanical response to pCrTX was only partially attenuated after 6-OHDA, indicating that a postsynaptic action remained. The ^{3}H-efflux and contraction were both abolished in Ca^{2+}-free medium. This shows that the pre- and postsynaptic actions of pCrTX are both dependent on extracellular Ca^{2+}.

All the above findings suggest that the pCrTX-induced vasoconstriction is due to noradrenaline release from intramural adrenergic nerve terminals as well as to direct stimulation of the postsynaptic contractile systems. Neither vasoconstriction nor the increase in ^{3}H-efflux caused by pCrTX were inhibited by adrenergic neurone blocking agents or the nerve conduction blocking agent, TTX (Narahashi, 1974), but both were significantly inhibited in low Na^+ medium, indicating that pCrTX may cause depolarization by increasing the cation permeability indiscriminately and this generalized depolarization, which would not be blocked by TTX, would permit an influx of Ca^{2+} into the terminals. In turn, this would trigger noradrenaline release in such a way as not to be inhibited by bretylium and guanethidine. Since reser-

pine blocks the phentolamine-sensitive response to pCrTX it seems to be effective at blocking noradrenaline release induced by pCrTX.

However, pCrTX-induced vasoconstriction was not inhibited, but was actually potentiated in the reserpinized preparations which had been functionally denervated, i.e. reserpine creates postsynaptic supersensitivity to pCrTX. Hudgins & Harris (1970) have explained supersensitivity after reserpine treatment by showing that the postsynaptic membrane becomes more permeable to extracellular calcium. This suggests that the postsynaptic response (verapamil sensitive) to pCrTX is potentiated as a result of an increase in calcium permeability of the postsynaptic membrane after reserpinization.

Effect of pCrTX on [^{3}H]-noradrenaline uptake

Two hours after the first contraction to pCrTX, the second response was significantly decreased and the contractile responses to tyramine, nicotine and transmural electrical stimulation were greatly reduced or abolished, although noradrenaline- and KCl-induced responses remained unchanged. In addition, only a small amount of [^{3}H]-noradrenaline was incorporated into the preparation. From these findings, it is suggested that pCrTX damages the noradrenaline uptake and/or storage mechanisms without damaging the postsynaptic contractile systems.

Effects of chlorphenylamine and indomethacin on the pCrTX-induced vasoconstriction

The fact that vascular smooth muscle is capable of generating endogenous prostaglandin-like substances that may cause contraction or modulate reactivity (Levy, 1977; Juan, 1979; Berner *et al.*, 1980) led us to postulate that pCrTX may stimulate prostaglandin and thromboxane production. However, pCrTX-induced vasoconstriction remained unchanged in the presence of indomethacin, a cyclo-oxygenase inhibitor, which blocked the contractile response to arachidonic acid. These results rule out the possible involvement of local endogenous prostaglandin generation in the vasoconstriction to pCrTX. Furthermore, the observation that an antihistamine, chlorphenylamine, failed to modify the response to pCrTX would apparently eliminate the possible involvement of local histamine release and direct stimulation of histamine receptors.

In conclusion, we speculate from the present experiments that: (1) presynaptic adrenergic mechanisms are involved in the contractile activity of pCrTX in addition to postsynaptic mechanisms. (2) pCrTX-induced vasoconstriction depends on the extracellular calcium concentration and at least, in part, on the stimulation of calcium influx which is blocked by calcium channel blockers. (3) pCrTX damages the noradrenaline uptake and/or storage mechanisms without damaging postsynaptic contractile systems. (4) Vasoconstriction to pCrTX is not due to local endogenous prostaglandin generation, or local endogenous histamine release and direct stimulation of histamine receptors.

Investigations into how the mechanisms of vasoconstriction to pCrTX relate to the physiological and toxicological effects of the jellyfish toxin, and further purification and characterization of the toxin are in progress.

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