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Electrical activity in rat tail artery during asynchronous activation of postganglionic nerve terminals by ciguatoxin-1

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1 The effects of ciguatoxin-1 (CTX-1) on the membrane potential of smooth muscle cells have been examined in rat proximal tail arteries isolated *in vitro*.

2 CTX-1 ($\geqslant$10 pM) increased the frequency of spontaneous excitatory junction potentials (s.e.j.ps). At 100-400 pM, there was also a marked and maintained depolarization (19.7 ± 1.4 mV, $n=14$, at 400 pM).

3 In 20-400 pM CTX-1, perivascular stimuli evoked excitatory junction potentials (e.j.ps) which were prolonged in time course relative to control.

4 Although threshold and latency of the e.j.p. were not affected by CTX-1 ($\leqslant$400 pM), propagated impulses were blocked at $\geqslant$100 pM.

5 The spontaneous activity and the depolarization produced by CTX-1 were reduced in the presence of Ca^{2+} (0.1 mM)/Mg^{2+} (25 mM), ω-conotoxin (0.1 μM) or Cd^{2+} (50-100 μM).

6 All effects of CTX-1 were abolished by tetrodotoxin (0.3 μM).

7 Raised Ca^{2+} (6 mM) reduced the depolarization and spontaneous activity produced by CTX-1.

8 In 400 pM CTX-1, the membrane repolarized (17 ± 3.2 mV, $n=4$) following the addition of phentolamine (1 μM). S.e.j.ps and e.j.ps were selectively abolished by suramin (1 mM), and the membrane repolarized by 1.3 ± 1.6 mV ($n=4$).

9 We conclude that CTX-1 releases noradrenaline and ATP by initiating asynchronous discharge of postganglionic perivascular axons. In 100-400 pM CTX-1, the smooth muscle was depolarized to levels resembling those recorded in this artery during ongoing vasoconstrictor discharge *in vivo*.

Keywords: Ciguatoxin; postjunctional activity; electrophysiological; rat tail artery; noradrenaline; ATP; sympathetic nerve terminals; postganglionic terminals; neuroeffector transmission; excitability

Introduction

Noradrenaline (NA) initiates contraction of arterial smooth muscle cells both by releasing Ca^{2+} from intracellular stores and by depolarization of the muscle leading to voltage-dependent calcium entry (Itoh *et al.*, 1992; Nilsson *et al.*, 1994). However, the mechanisms by which activity of sympathetic postganglionic axons affects arterial smooth muscle *in vivo* remain unclear. There is evidence that neurally evoked arterial vasoconstriction is mediated by α-adrenoceptor and non-α-adrenoceptor-mediated mechanisms *in vivo* (see Hirst & Edwards, 1989). In arterial vessels isolated and stimulated electrically *in vitro*, the relative contributions of α- and non-α adrenoceptor-mediated mechanisms are dependent on the frequency of stimulation (e.g. Sjöblom-Widfeldt *et al.*, 1990), with the non-α-adrenoceptor-mediated component increasing in importance in smaller diameter arteries and in arterioles (Bao *et al.*, 1989; Evans & Surprenant, 1992). As the non-α-adrenoceptor-mediated component is blocked by P_2-purinoceptor antagonists, e.g. suramin, it has been taken to result from ATP released together with NA from the sympathetic nerve endings (Evans & Surprenant, 1992; Bao & Stjärne, 1993; Morris, 1994).

When arterial vessels are studied electrophysiologically *in vitro*, synchronous activation of perivascular nerve terminals evokes an excitatory junction potential (e.j.p.) (Hirst, 1977; Bolton & Large, 1986; Hirst & Edwards, 1989). The e.j.p. is resistant to α-adrenoceptor antagonists but sensitive to suramin (Evans & Surprenant, 1992; Jobling *et al.*, 1992; Brock & Cunnane, 1993; Jobling, 1994). In the rat tail artery, perivascular stimuli in addition evoke an α_2 -adrenoceptor-mediated slow depolarization that follows the e.j.ps (Cheung, 1982; Itoh *et al.*, 1983; Cassell *et al.*, 1988; Jobling & McLachlan, 1992b), so that the release of NA and ATP can be examined concomitantly. Single e.j.ps in most arteries are not associated with arterial constriction but, during repetitive stimulation, summation of e.j.ps can open voltage-activated Ca^{2+} channels, thereby triggering contraction (Hirst & Edwards, 1989).

Vasoconstrictor axons supplying arterial vessels discharge asynchronously *in vivo* (Jänig, 1988) and the form of the postjunctional electrical activity will be different from that recorded in *in vitro* experiments where many axons innervating a length of vessel are activated synchronously. It has proved difficult to record electrical activity from arterial smooth muscle in arteries perfused with blood *in vivo* (e.g. Bryant *et al.*, 1985; Neild & Keef, 1985), but in general membrane potentials are depolarized relative to those recorded *in vitro*. The relative contributions of NA and ATP to the generation of constriction by asynchronous nerve activity are unknown.

Ciguatoxin-1 (CTX-1), a lipid soluble toxin arising from the benthic dinoflagellate, *Gambierdiscus toxicus*, is responsible for the disease *ciguatera*, a form of food poisoning with prolonged symptoms involving the peripheral nervous system (see Gillespie *et al.*, 1986). CTX-1 has been shown to increase spontaneous and evoked release of transmitter at skeletal (Molgó *et al.*, 1990) and autonomic (Lewis & Endean, 1984; 1986) neuromuscular junctions and in sympathetic ganglia (Hamblin *et al.*, 1995). The toxin produces membrane depolarization, apparently through a selective action on tetrodotoxin (TTX)-

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sensitive Na^+ channels (Bidard *et al.*, 1984; see Hamblin *et al.*, 1995). Its effect is to shift the voltage-dependence of the channels so that they open at or close to the resting membrane potential leading to the initiation of action potentials in both myelinated and unmyelinated axons.

The present experiments have examined the effects of low concentrations of CTX-1 on smooth muscle membrane potential in segments of rat proximal tail arteries. Our results show that the tail artery is sensitive to picomolar concentrations of CTX-1 and that asynchronous nerve activity induced by CTX-1 results in maintained depolarization of arterial smooth muscle produced primarily by activation of postjunctional α-adrenoceptors by released NA. It seems possible that the postjunctional electrical activity induced by CTX-1 is similar to that produced by asynchronous activity of perivascular axons *in vivo*.

Methods

All experiments were performed on tissue isolated from female rats (70–160 g) anaesthetized with pentobarbitone (80 mg kg^{-1} i.p.) and decapitated. A segment of the main ventral caudal artery was dissected from 5–25 mm distal to the base of the tail and a 10 mm length transferred to a small organ bath (volume < 0.8 ml) on the stage of an inverted microscope. The preparation was superfused with physiological saline ('control solution') of the following composition (mM): Na^+ 151, K^+ 4.7, Ca^{2+} 2, Mg^{2+} 1.2, Cl^- 144, H_2PO_4- 1.3, HCO_3- 16.3, glucose 7.8, gassed with 95% O_2 /5% CO_2 (pH 7.2), and warmed to 35°C. Usually two pieces of artery from the same animal were used in an experiment, with the second being held in oxygenated physiological saline at room temperature until required. No differences could be detected in the properties or responses of the two arterial segments.

Intracellular recordings were made with microelectrodes filled with 0.5 M KCl (resistance 80–160 MΩ) in smooth muscle cells located either superficially or deep within the vascular smooth muscle. Records were collected on a PC based data acquisition system using sampling frequencies of 0.1–1 kHz as described previously (Cassell *et al.*, 1986); criteria for accepting impalements in control solution were the same as in Cassell *et al.* (1988). When the membrane depolarized in CTX-1, reimpalements were accepted if the electrode noise was the same as that in the bathing solution. Perivascular nerves were stimulated either through a suction electrode, into which approximately 5 mm of the proximal end of the arterial segment was introduced, or through a pair of platinum wires (125 μm diameter) placed vertically in the Sylgard coated base of the organ bath 100–150 μm from either side of the artery and 1–2 mm from the mouth of the suction electrode. Recordings were made at sites between the transmural electrodes where the amplitudes and time course of responses evoked by stimulation through the suction electrode were generally similar between preparations (Sittiracha *et al.*, 1987). Stimuli of pulse width 1 ms and voltage between 1 and 30 V were presented; larger voltages were avoided so that slow depolarizing potentials arising from non-neural sources did not distort the decay phase of the excitatory junction potential (Jobling & McLachlan, 1992b).

A recirculation system of 20 ml volume was used to minimize the amount of toxin used (see Hamblin *et al.*, 1995). In order to maintain the Cl^- -concentration when $MgCl_2$ concentration was raised, the NaCl concentration was reduced accordingly. Between experiments or preparations, the set up including the organ bath and perfusion tubing, etc., was rinsed in ethanol (to remove CTX-1) followed by thorough washing in distilled water.

Membrane potentials were always confirmed on withdrawal of the microelectrode and the values given are the most negative recorded just prior to withdrawal. Amplitude and time course of e.j.ps and slow potentials were measured from digitized recordings after the experiment.

Ciguatoxin-1 (CTX-1)

CTX-1 was extracted from the moray eel (*Lycodontis javanicus*) and purified as described previously (Lewis *et al.*, 1991). Stock CTX-1 was dissolved in 50% aqueous methanol and stored at −20°C. It was applied within a concentration range of 10–400 pM.

Statistical analysis

Data are reported as mean ± 1 standard error of the mean, unless otherwise stated. Where appropriate data were compared using a paired *t*-test. P values < 0.05 were considered significant.

Drugs

The following drugs were used as indicated in the Results: tetrodotoxin (TTX, MMA International, Kingston, ACT, Australia); ω-conotoxin GVIA (Peninsula Laboratories, Belmont, CA, U.S.A.); phentolamine (Ciba); suramin (a kind gift from Bayer Australia).

Results

General observations

The effects of CTX-1 were studied in 46 proximal tail artery preparations from 30 rats. The strength of perivascular stimulation through a suction electrode positioned 1-2 mm proximal to the recording site was adjusted so as to evoke an EJP of 5 to 15 mV amplitude. Transmural stimulation at the same voltage strength usually produced an EJP of larger amplitude and with a decay having a faster initial time course than that evoked by propagated impulses; the transmural stimulus strength was adjusted such that it also evoked an EJP in the range 5 to 15 mV amplitude. Following 5 stimuli at 1 or 10 Hz, the EJPs evoked by either stimulus were followed by a slow depolarization lasting nearly 1 min. Resting membrane potentials (RMPs) ranged from -52 to -75 mV, with a mean value of -63.4 ± 0.4 mV (154 impalements in 46 preparations). These data are similar to those described elsewhere in similar preparations (Cassell *et al.*, 1988; Jobling & McLachlan, 1992b).

Spontaneous activity

Spontaneous excitatory junction potentials (s.e.j.ps) in the absence of nerve stimulation occurred at very low frequency in control solution (< 10 per min). In superficial cells (i.e. those impaled immediately after contacting the outermost smooth muscle cells which bear neuromuscular junctions; Luff & McLachlan, 1989), amplitudes varied from about 15 mV down to the noise level (~1 mV), rise times varied from 10 to 50 ms, and half-widths ranged from 50 to 200 ms as previously described (Cassell *et al.*, 1988; Jobling & McLachlan, 1992b). In cells deeper in the media (i.e. further from the contacting nerve terminals), s.e.j.ps recorded in control solution were not readily distinguished from the noise. In some preparations spontaneous transient depolarizations (s.t.ds, Van Helden, 1990) could also be distinguished by their time course (Jobling & McLachlan, 1992b).

Addition of CTX-1 (10 to 400 pM) to the solution superfusing the artery produced an increase in the frequency of s.e.j.ps within 5 min of introducing the toxin into the perfusion system. In superficial cells, the lowest concentrations used (10 pM) produced single or short bursts of s.e.j.ps (Figure 1a(ii))) with amplitudes similar to those recorded in control solution, together with many larger amplitude s.e.j.ps. The frequency of s.e.j.ps was often high and summation of individual events made it difficult to determine their amplitudes.

In deep cells, this low concentration of CTX-1 sometimes induced transient depolarizing waves lasting 0.5 to 1 s (Figure 1a(iii).

As the concentration of CTX-1 was increased ($\geqslant$20 pM), bursts of spontaneous potentials became more common. The amplitudes of s.e.j.ps in each burst recorded in superficial cells were characteristically similar (Figure 1b). However, successive s.e.j.ps during the bursts tended to increase slightly in amplitude (Figure 1b), i.e. they facilitated (Jobling & McLachlan, 1992b). The frequency of occurrence of s.e.j.ps within the bursts differed between preparations, values ranging between 8 and 25 Hz in concentrations of CTX-1 between 20 and 50 pM. In deep cells, the frequency of the depolarizing waves increased and the membrane potential varied up and down in an unstable manner (see Figure 2b). At the highest concentrations used in this study (100–400 pM), membrane activity in both superficial cells and deeper cells became more or less continuous (Figures 2, 4, 5 and 6).

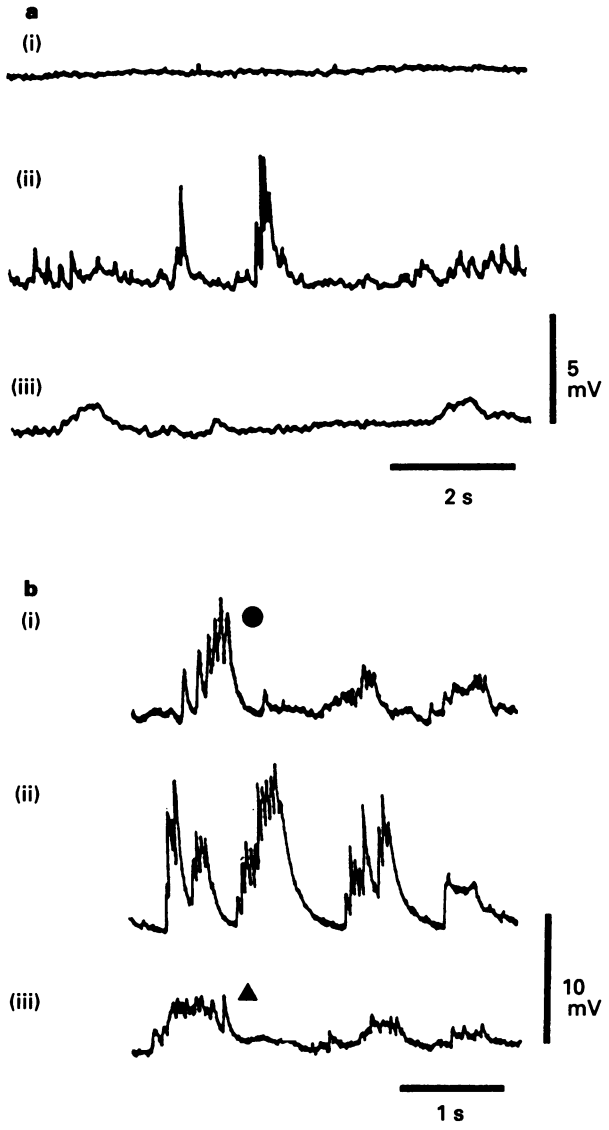

Figure 1 Effects of low concentrations of CTX-1 on spontaneous electrical activity. (a) (i) Control record from a surface cell (resting membrane potential (RMP) – 63 mV). (ii) Record from another surface cell in the same preparation in the presence of 10 pM CTX-1 (RMP –58 mV). (iii) Record from a cell deeper in the media in the same preparation in the presence of 10 pM CTX-1 (RMP –60 mV). (b) Activity in a superficial cell in the presence of 50 pM CTX-1. (i-iii) Records showing bursts of brief e.j.ps of two configurations (indicated by ● in (i) and ▲ in (iii)) produce transient depolarizations lasting about 1 s. Most negative membrane potential –69 mV. At these concentrations, CTX-1 increased the rate of spontaneous synaptic activity without significant change in membrane potential.

When the artery was exposed to > 10 pM CTX-1, the bursts of s.e.j.ps were superimposed on an overall membrane depolarization which developed gradually over a few minutes. The magnitude of this depolarization was concentration dependent (Figure 2b) and, in superficial cells, the amplitudes of s.e.j.ps decreased as the muscle depolarized. Following the addition of 200–400 pM CTX-1, the membrane depolarized rapidly sometimes triggering muscle action potentials (Figure 2a), but subsequently the membrane continued to depolarize and additional spontaneous action potentials were not observed. During the onset of the depolarization induced by $\leqslant$100 pM CTX-1, the microelectrode was frequently dislodged from the muscle, implying that contraction had occurred. Direct observation of the artery through the microscope revealed a marked and maintained constriction. However, it was relatively easy to impale another cell within 1–2 min, and these impalements could be maintained for long periods (up to hours).

During continuous exposure to 200–400 pM CTX-1 (n=44), the initial increase in membrane activity partially subsided over the first 10–20 min. The membrane potential typically reached about -35 mV following the addition of 400 pM CTX-1 and then declined so that the most negative level was between –40 and –56 mV (–46.5$\pm$1.3 mV, n=14) about 15–20 min after applying CTX-1. At this time the membrane was depolarized by 19.7$\pm$1.4 mV (n=14) relative to pretreatment control values. This depolarization was maintained in the presence of these concentrations of CTX-1 for periods of 40 min and longer (n=5). Similarly the contraction of the artery observed shortly after addition of 200–400 pM CTX-1 tended to subside in the continued presence of this agent.

In 5 experiments, the preparation was exposed to CTX-1 (50–200 pM) for only 5–15 min, and then washed continuously in control solution. In all cases, depolarization and the increased spontaneous activity decreased progressively during the washing period. Although the membrane potential was close to pretreatment values within 10–20 min, s.e.j.ps were still present after 30–60 min at frequencies higher (>0.3 Hz) than in experiments on the tail artery in which CTX-1 had not been used (Cassell *et al.*, 1988; Jobling & McLachlan, 1992b). Further, despite rigorous washing between experiments, occasional bursts of s.e.j.ps in control solution suggested that traces of CTX-1 remained in the perfusion system. This indicates that, despite the ready reversal of membrane depolarization when CTX-1 was washed out, the artery preparation was much more sensitive to CTX-1 than the sympathetic ganglia studied in the same laboratory (Hamblin *et al.*, 1995).

Responses to nerve stimulation

The amplitude and time course of e.j.ps evoked by single stimuli were little affected by the addition of very low concentrations of CTX-1 (<20 pM), although in superficial cells individual s.e.j.ps were often observed overlying the decay phase of the e.j.p. Following exposure to 20–400 pM CTX-1, the e.j.ps had an initial rate of rise similar to that in control solution but their decay phase was prolonged and it could no longer be described by a single exponential (Figures 3a, b, d). In some records, a burst of similar amplitude s.e.j.ps was observed which prolonged the decay phase (Figure 3d(ii)). In other instances, the shape of the e.j.ps was distorted so that, following the initial rising phase, the amplitude continued to increase more slowly before decaying over a prolonged and irregular time course (Figure 3b(ii)). The simplest explanation for these altered configurations is that individual perivascular axons discharged repetitively following a single stimulus in the presence of CTX-1, as occurs in preganglionic axons (Hamblin *et al.*, 1995). In addition, again as for preganglionic axons,

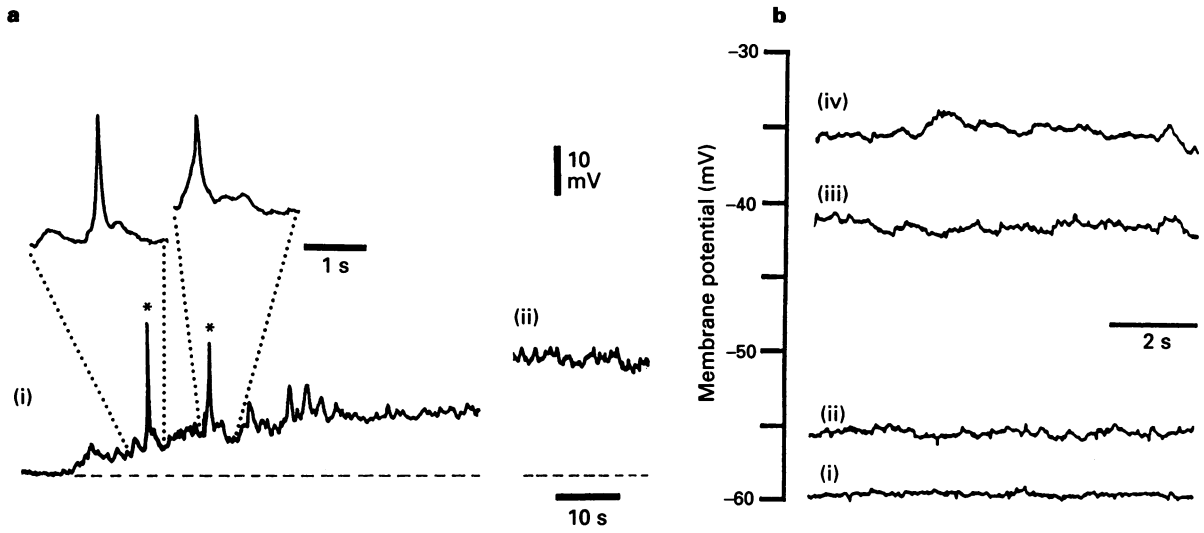

Figure 2 The effects of higher concentrations of CTX-1. (a) Initial effects of 200 pM CTX-1 on membrane potential in a cell deep in the media: (i) shows the muscle beginning to depolarize as the toxin reached the tissue. When the membrane potential reached about 10 mV positive of its initial resting value, transient depolarizations triggered two muscle action potentials (*, displayed on a faster time base above). The membrane then continued to depolarize without further active responses until the electrode was dislodged by contraction of the muscle (end of (i)). (ii) When another cell was impaled 2 min later, the membrane was unstable at a potential about 20 mV positive to control RMP (−55 mV). (b) Records from a cell deep in the media in (i) control solution and following the sequential addition of (ii) 20, (iii) 100 and (iv) 200 pM CTX-1. The effects of CTX-1 in depolarizing the membrane were concentration-dependent. Individual s.e.j.ps were not detected in this cell.

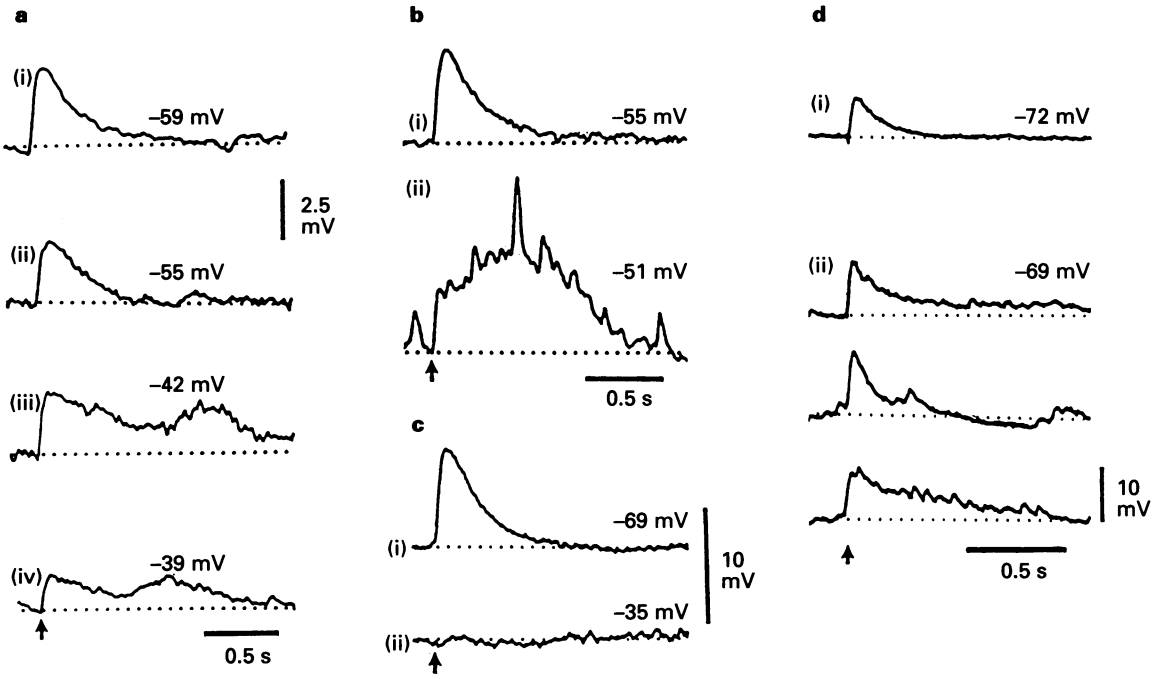

Figure 3 Effects of CTX-1 on e.j.ps evoked by submaximal perivascular electrical stimuli (at the times indicated by arrows) in different preparations (a-d). (a) E.j.ps evoked by transmural stimuli in a cell deep in the media (i) in control solution and following the addition of (ii) 20, (iii) 100 and (iv) 200 pM CTX-1. (b) E.j.ps evoked from the suction electrode in a superficial cell in (i) control solution and (ii) in the presence of 60 pM CTX-1. (c) (i) E.j.p. evoked from the suction electrode in a deep cell in control solution and (ii) no response in the presence of 400 pM CTX-1 (cf. a(iv)). (d) (i) E.j.p. evoked by transmural stimuli in a superficial cell in control solution and (ii) consecutive responses at 60 s intervals in the presence of 50 pM CTX-1 showing variable configuration and repetitive activity resembling the spontaneous bursts shown in Figure 1b. Membrane potentials indicated above each record. In the presence of CTX-1, the decay phase of the e.j.p. was distorted (a.b.d) and, in high concentrations, the propagated response was blocked (c(ii)). Voltage calibration bar for (a) also applies to (b). Time calibration bar for (b) also applies to (c).

stimulation also markedly accelerated the rate of occurrence of s.e.j.ps in superficial cells for several seconds after each stimulus.

The peak amplitudes of e.j.ps evoked by single stimuli sometimes increased slightly in relatively low concentrations (<100 pM) of CTX-1 but, at higher concentrations (100–400 pM), they always became smaller as the membrane depolarized (Figure 3a, c). Ten to 15 min after application of 200 or 400 pM CTX-1, single stimuli applied through the suction electrode commonly failed to evoke a detactable e.j.p. (Figure 3c(ii)), even when the stimulus intensity was increased, although e.j.ps could always be evoked by transmural stimuli

(Figure 3a(iv). This suggests that action potential propagation along axons within the preparation was blocked by CTX-1 (see Lewis & Endean, 1984; Molgó *et al.*, 1990). However, prior to the failure to evoke e.j.ps, there was no evidence of any change in latency or voltage threshold for activation from those determined in control solution.

The following sections describe experiments designed to clarify the mechanism of action of CTX-1. Most of these experiments used relatively high concentrations of CTX-1 (200 or 400 pM).

Effects of low Ca^{2+}/high Mg^{2+} concentrations and calcium antagonists

In order to determine whether the effects of CTX-1 on vascular smooth muscle were due to calcium-dependent transmitter release, the effects of reducing the Ca^{2+} /Mg^{2+} ratio in the bathing solution were examined. When the concentrations of Ca^{2+} and Mg^{2+} ions were changed to 0.1 mM and 25 mM respectively ($n=2$), addition of CTX-1 (200 pM) did not affect the membrane potential or increase the spontaneous activity. Subsequent perfusion of these tissues with control solution (i.e. with 2 mM Ca^{2+} and 1.2 mM Mg^{2+} respectively) containing 200 pM CTX-1 induced levels of spontaneous activity and membrane depolarization similar to those observed in other cells exposed to this solution initially. These observations imply that the activity induced by CTX-1 resulted from calcium-dependent transmitter release from postganglionic nerve terminals.

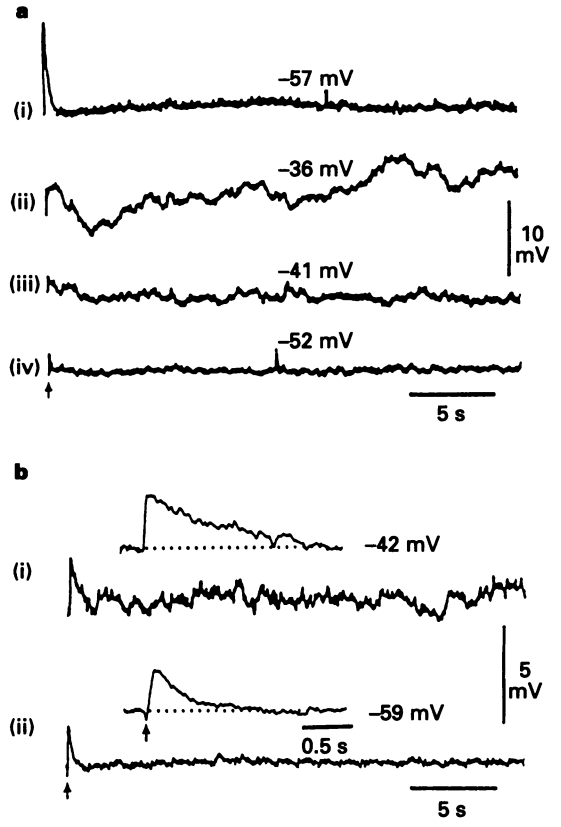

Figure 4 Reversal of the effects of CTX-1 on e.j.ps and membrane depolarization by addition of Cd^{2+} and by raised Ca^{2+} concentration. (a) Records from a surface cell (i) in control solution and following the sequential addition of (ii) 200 pM CTX-1, (iii) 10 μM Cd^{2+} and (iv) 50 μM Cd^{2}. (b) Records from a surface cell in (i) 100 pM CTX-1 and (ii) 100 pM CTX-1 after raising $[Ca^{2+}]$ to 6 mM. Records above show the evoked e.j.ps on a faster time base. These interventions substantially reduced both the depolarization and the spontaneous synaptic activity produced by CTX-1; raised Ca^{2+} also restored the time course of the control e.j.p. Single submaximal stimuli via transmural electrodes (at arrows). Membrane potentials are indicated above each record.

Consistent with this suggestion, addition of Cd^{2+} (0.05–0.1 mM; $n=6$, Figure 4a) totally abolished the activity induced by CTX-1. Application of the N-type Ca^{2+} channel blocker, ω-conotoxin GVIA (0.1 μM; $n=2$) also substantially reduced the effects of CTX-1.

All of these interventions markedly reduced the amplitude of the stimulus-evoked e.j.p.

Effects of raised Ca^{2+} concentration

If the effects of CTX-1 are mediated by action potentials triggered by the opening of Na^{+} channels (Bidard *et al.*, 1984), stabilization of the membrane by raising divalent cation concentration (Frankenhaeuser & Hodgkin, 1957) might be expected to counteract the effects of the toxin, as it did in sympathetic ganglia (Hamblin *et al.*, 1995). Raising the Ca^{2+} content of the bathing solution to 6 mM ($n=6$) substantially reduced the effects of CTX-1 without significantly affecting the amplitude of the initial e.j.ps (Figure 4b, see also Jobling & McLachlan, 1992b). In the presence of 6 mM Ca^{2+}, the effects of CTX-1 on the configuration of the e.j.p. were reversed, so that the decay phase of the e.j.p. in high Ca^{2+} again followed a single exponential time course (Figure 4b). These effects of high Ca^{2+} were reversed within 10 min of returning to a solution containing CTX-1 and 2 mM Ca^{+2}. It seems likely that at least some of the blocking effects of 25 mM Mg^{2+} (see above) may be attributed to the same membrane stabilizing effect.

Effects of tetrodotoxin (TTX)

Addition of TTX (0.3 μM, $n=6$) rapidly and totally reversed the membrane depolarization and abolished the spontaneous synaptic activity induced by CTX-1 (Figure 5). Mean values of membrane potential were -57.3 ± 2.0 mV in control solution, -32.3 ± 1.7 mV after exposure to 200 pM CTX-1 and

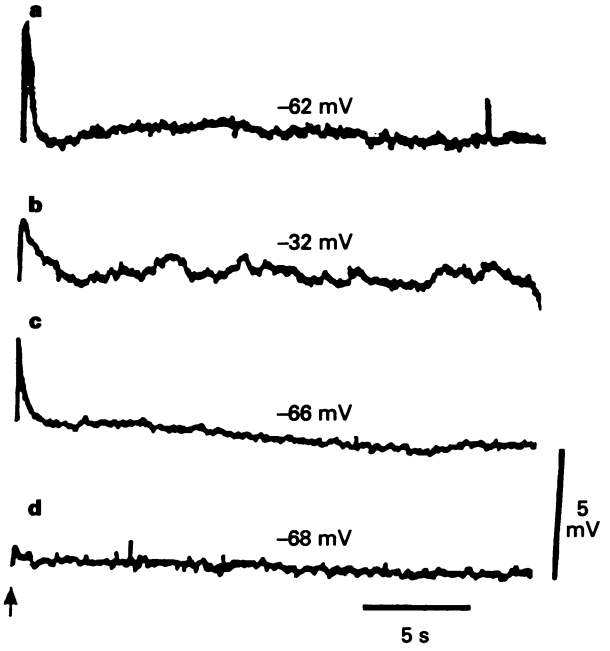

Figure 5 Reversal of the effects of CTX-1 by TTX. Records from a surface cell stimulated with single submaximal stimuli through transmural electrodes (at arrow) (a) in control solution and following the addition of (b) 200 pM CTX-1 and then also 0.3 μM TTX for (c) 5 and (d) 10 min. Membrane potentials are indicated above each record. Note that the effects of CTX-1 on membrane potential and spontaneous activity were blocked (c) before block of the stimulation evoked e.j.p. (d).

-60.9 ± 3.6 mV after addition of TTX ($n=4$). TTX also blocked e.j.ps evoked by single stimuli, but in all cases the effects produced by CTX-1 were blocked earlier after the addition of TTX than were the nerve-evoked e.j.ps (Figure 5). The effects of TTX were completely reversed by washing in CTX-1 containing control solution for 10 min. The abolition of all effects of CTX-1 by TTX is consistent with the idea that the toxin acts only on TTX-sensitive voltage-dependent Na^+ channels (see Introduction).

When TTX (0.3 μM) was applied 10 min prior to the application of CTX-1 (200 pM, $n=2$), addition of the toxin did not produce either membrane depolarization or an increased rate of spontaneous activity. When the solution containing TTX and CTX-1 was washed out (with control solution), s.e.j.ps increased in frequency and the membrane began to depolarize before a subsequent addition of CTX-1. Later, in the presence of CTX-1 (200 pM), the degree of spontaneous activity and the level of membrane depolarization were similar to those in tissues exposed to this concentration of CTX-1 alone.

Effects of phentolamine

The contribution of α-adrenoceptor activation to the depolarization produced by CTX-1 was tested by adding the α-adrenoceptor antagonist, phentolamine (1 μM), to the preparations. This concentration is without effect on RMP, s.e.j.ps or e.j.ps evoked by single stimuli in untreated control preparations (Cassell *et al.*, 1988). However it abolishes the slow depolarization following the e.j.p. (Cassell *et al.*, 1988; Jobling & McLachlan, 1992b).

In 4 cells, mean values of membrane potential were -63.3 ± 1.0 mV in control solution and -47.5 ± 2.3 mV after exposure to 400 pM CTX-1. Addition of phentolamine (1 μM) repolarized the membrane by 9–24 mV (mean 17.0 ± 3.2 mV), thus returning the membrane potential to its resting value prior to exposure to the toxin (Figure 6c; difference = -1.2 ± 1.4 mV, $P = 0.43$). Phentolamine had no inhibitory effects on the spontaneous activity or on the e.j.ps recorded in the presence of CTX-1. Indeed, when this α-adrenoceptor antagonist was added to the organ bath, both the spontaneous activity and the e.j.ps increased in amplitude as the membrane potential became more negative (Figure 6c).

Effects of suramin

The effects of the P_2 purinoceptor antagonist, suramin (1 mM), on the membrane potential changes induced by CTX-1 were investigated. In untreated preparations, suramin blocks both s.e.j.ps and e.j.ps without affecting the RMP (see Jobling & McLachlan, 1992a). In the presence of CTX-1 (400 pM), application of suramin for periods up to 30 min caused little change in the level of membrane depolarization (mean = -1.3 ± 1.6 mV, $n=4$, $P=0.49$) but abolished the membrane instability. The transmurally activated e.j.p. was also substantially reduced in amplitude or abolished.

When suramin was added following the addition of phentolamine (1 μM), the spontaneous synaptic activity and the electrically evoked e.j.p. were markedly reduced in amplitude (Figure 6d) but effects on the membrane potential were not significant (depolarized by 3 ± 1.4 mV, $n = 4$, $P = 0.13$; Figure 6d).

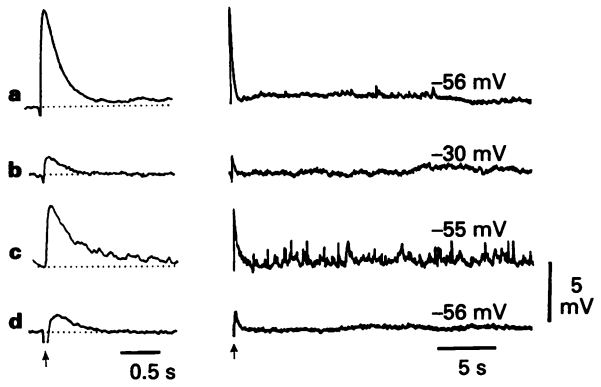

Figure 6 Reversal of the effects of CTX-1 by phentolamine and suramin. Records from a surface cell stimulated through transmural electrodes (at arrow) (a) in control solution and following the sequential addition of (b) 200 pM CTX-1, (c) 1 μM phentolamine and (d) 1 mM suramin. Records on the left show the evoked e.j.ps on a fast time base. Membrane potentials are indicated above each record. Phentolamine reversed the effect of CTX-1 on membrane potential and increased spontaneous synaptic activity and the amplitude of the evoked e.j.p. (c). Subsequent addition of suramin markedly reduced both spontaneous activity and the amplitude of the evoked e.j.p. (d).

Discussion

CTX-1 at sub-nanomolar concentrations induced spontaneous synaptic activity and depolarization in the vascular smooth muscle cells of the rat tail artery. These effects were apparently due solely to activation of postganglionic sympathetic nerve endings as they were totally dependent on Ca^{2+} influx, prevented or reversed by TTX and were completely reversed by antagonists of the transmitter substances released. The toxin therefore had no direct action on vascular smooth muscle cells at the concentrations used in this study. These findings are in accord with the known actions of CTX-1 in causing membrane depolarization and spontaneous action potentials in axons (Bidard *et al.*, 1984; Benoit *et al.*, 1986; Hamblin *et al.*, 1995). The highest concentrations of CTX-1 applied in these experiments caused a marked constriction of the tail artery.

The effects of CTX-1 on the tail artery were fully reversed by application of TTX indicating that CTX-1 acts on TTX-sensitive Na^+ channels. At the concentration used, TTX does not normally affect the RMP or the active or passive properties of vascular smooth muscle (Itoh *et al.*, 1983). It was notable that TTX always abolished the effects of CTX-1 prior to (i.e. more readily than) block of the transmurally evoked e.j.p. A similar observation was made in guinea-pig isolated ganglia (Hamblin *et al.*, 1985), in which low concentrations of TTX blocked the activity induced by CTX-1, without affecting the evoked excitatory synaptic potential or the somatic action potential. These data suggest that low concentrations of the toxin affect a sub-population of TTX-sensitive Na^+ channels on presynaptic axons. A similar suggestion has been made by Bidard *et al.* (1984) to explain the failure to detect any alteration by CTX-1 of the TTX-sensitive uptake of $^{22}Na^+$ into cultured neuroblastoma cells. Alternative possibilities are that CTX-1-modified Na^+ channels have a greater sensitivity to TTX, as demonstrated for some other Na^+ channel activating toxins (Catterall, 1992), or that blockade of a few Na^+ channels by TTX at the site of initiation of spontaneous discharge is sufficient to prevent excitation. The latter might imply that CTX-1 acts at a region of axon with a particularly low activation threshold, i.e. Na^+ channel distribution is non-uniform in unmyelinated axons.

The actions of CTX-1 are similar to those of the brevetoxins (see Baden, 1989), with which the ciguatoxins share a common receptor binding site (neurotoxin binding site 5) on the neuronal voltage-dependent Na^+ channel (Lombet *et al.*, 1987; Lewis *et al.*, 1991). Both toxins shift the voltage-dependence of neuronal Na^+ channel activation to more negative potentials with little effect on inactivation (Benoit *et al.*, 1986; Lewis & Endean, 1986; Catterall, 1992), thereby causing membrane depolarization and spontaneous and stimulus-evoked repetitive discharges in nerves (Benoit *et al.*, 1986; Bade, 1989). The precise way in which CTX-1 modifies Na^+ channel gating is not yet understood.

The actions of CTX-1 on the rat tail artery observed here might therefore be explained as follows: (i) at very low concentrations, there is depolarization of the postganglionic nerve terminals insufficient to initiate action potentials. This would

be expected to accelerate the spontaneous release of quanta of transmitter (e.g. Liley, 1956). This effect could not be reversed completely by washing. (ii) At slightly higher concentrations of CTX-1, larger s.e.j.ps and bursts of s.e.j.ps of similar amplitudes probably result from the firing of individual postganglionic axons. Consistent with this idea, s.e.j.p amplitudes facilitate during the bursts, suggesting that each burst arises from repetitive activation of same axon (see also Hamblin *et al.*, 1985). (iii) single stimuli in the presence of low concentrations of CTX-1 evoke e.j.ps with prolonged and irregular decay phases, often showing fast s.e.j.ps superimposed. These records presumably reflect repetitive discharges in some perivascular axons (see also Molgó *et al.*, 1990; Hamblin *et al.*, 1995). (iv) At higher concentrations of CTX-1, both the numbers of axons responding and the frequencies of repetitive discharge in individual axons increase and the muscle depolarizes. This maintained depolarization is probably due to a prolonged decrease in membrane K^+ conductance like that which follows short trains of electrical stimuli (see Cassell *et al.*, 1988; Jobling & McLachlan, 1992b). The receptors responsible for the maintained depolarization are likely to be α_2-adrenoceptors because the slow depolarization following short trains of stimuli and the depolarization evoked by exogenously applied NA (0.1 to 10 μM) are both blocked selectively by the α_2-adrenoceptor antagonist idazoxan (Jobling *et al.*, 1992). It might be predicted that higher concentrations of CTX-1 would further increase the observed frequency of s.e.j.ps. However, when the membrane depolarizes, individual s.e.j.ps are reduced in amplitude due to the decrease in driving force (Finkel *et al.*, 1984) and slower in time course because of the increased membrane time constant (Cassell *et al.*, 1988). Consequently, the membrane potential appears unstable at a depolarized level. When suramin is applied, it abolishes the instability of the membrane potential without producing significant change in the most negative level of the membrane potential. This presumably reflects block of the s.e.j.ps induced by CTX-1, consistent with the idea that these result from neurally-released ATP. (v) With application of the highest concentrations of CTX-1 studied (200–400 pM), frank and relatively rapid muscle depolarization from RMP sometimes initiates action potentials. However these do not recur, presumably because of inactivation of the Ca^{2+} channels giving rise to these regenerative responses (Hirst & Edwards, 1989). This implies that the transient Ca^{2+} channels which are responsible for the muscle action potential probably do not contribute to Ca^{2+} influx during the maintained depolarization produced by CTX-1. (vi) In these higher concentrations of CTX-1, e.j.ps fail to be elicited by stimuli applied via an electrode positioned 1–2 mm proximal to the recording site, although release can still be activated by local direct stimulation of the terminals. This effect might reflect Na^+ entry and depolarization of the perivascular axons sufficient to induce conduction block, as in motor nerve terminals at the frog neuromuscular junction (Molgó *et al.*, 1990).

Although many of these effects of CTX-1 are identical to those observed at synapses in sympathetic ganglia (Hamblin *et al.*, 1995), there were a number of differences: (i) The artery was sensitive to concentrations of toxin at least 10 times lower than produced detectable responses in ganglion cells. (ii) Most of the effects of CTX-1 on postganglionic axons were easily reversed after removal of the toxin, whereas in ganglia the effects of the toxin were long-lasting even after brief periods of exposure. (iii) There was evidence of propagation failure in postganglionic but not in preganglionic axons. (iv) Exposure to TTX prior to application of CTX-1 blocked all of the toxin's effects in ganglion cells even after TTX was washed out. Prior exposure of the artery to TTX did not have this prolonged protective effect against the actions of CTX-1.

These observations are difficult to explain but they might reflect, e.g. differences between pre- and postganglionic axons in the affinity of CTX-1 for the binding site. There do not appear to be species or tissue differences in the sensitivity of Na^+ channels on postganglionic nerves to CTX-1, as responses to CTX-1 occur in preparations of guinea-pig saphenous and mesenteric and rat mesenteric arteries at similar concentrations (J.A. Brock and E.M. McLachlan, unpublished observations).

Although the s.e.j.ps within the bursts induced by CTX-1 occurred at much higher frequencies than the discharges of individual vasoconstrictor neurones recorded *in vivo* (Jänig, 1988), the overall effect on the artery of asynchronous postganglionic discharge produced by the toxin are probably similar to the effects of natural nerve activity in the intact animal. In a study of anaesthetized rats with normal levels of blood pressure (Bryant *et al.*, 1985), the mean membrane potential in the tail artery was found to be close to −40 mV, which is similar to the values obtained with the highest concentrations of CTX-1 used in the present study; this value is depolarized relative to the levels recorded *in vitro* (see Jobling & McLachlan, 1992b). Although there may be additional effects of distension by the blood pressure within the vessel (Bryant *et al.*, 1985, cf. Surprenant, 1980) a major factor contributing to maintained depolarization *in vivo* is likely to be activity in the perivascular nerves. Further, the findings of the present study suggest that most of this maintained depolarization is due to activation of α-adrenoceptors, in contrast to the responses evoked by brief trains of electrical stimuli which are largely resistant to α-adrenoceptor blockade (Cassell *et al.*, 1988). Thus studying the mechanisms underlying the responses to CTX-1 under controlled conditions *in vitro* may help to clarify the neural control of vascular smooth muscle without the problems of movement artefacts associated with the arterial pressure wave (see Neild & Keef, 1985) or the complications introduced by circulating vasoactive substances.

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