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Ciguatoxin enhances quantal transmitter release from frog motor nerve terminals

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1 Ciguatoxin (CTX), a marine toxin produced by the benthic dinoflagellate *Gambierdiscus toxicus*, is responsible for a complex endemic disease in man known as ciguatera fish poisoning. In the present study we have investigated the effects of purified CTX extracted for *Gymnothorax javanicus* moray-eel liver on frog isolated neuromuscular preparations with conventional electrophysiological techniques.

2 CTX (1–2.5 nM) applied to cutaneous pectoris nerve-muscle preparations induced, after a short delay, spontaneous fibrillations of the muscle fibres that could be suppressed with 1 μM tetrodotoxin (TTX) or by formamide to uncouple excitation-contraction.

3 In preparations treated with formamide, CTX (1–2.5 nM) caused either spontaneous or repetitive muscle action potentials (up to frequencies of 60–100 Hz) in response to a single nerve stimulus. Recordings performed at extrajunctional regions of the muscle membrane revealed that during the repetitive firing a prolongation of the repolarizing phase of the action potential occurred. At junctional sites the repetitive action potentials were triggered by repetitive endplate potentials (e.p.ps).

4 CTX (2.5 nM) caused a TTX-sensitive depolarization of the muscle membrane.

5 In junctions equilibrated in solutions containing high Mg^{2+} + low Ca^{2+}, addition of CTX (1.5 nM) first induced an average increase of 239 ± 36% in the mean quantal content of e.p.ps. Subsequently CTX reduced and finally blocked nerve-evoked transmitter release irreversibly.

6 CTX (1.5–2.5 nM) increased the frequency of miniature endplate potentials (m.e.p.ps) in junctions bathed either in normal Ringer, low Ca^{2+}-high Mg^{2+} medium or in a nominally Ca^{2+}-free solution containing EGTA. Extensive washing with toxin-free solutions did not reverse the effect. Furthermore, Cd^{2+} (0.1 mM), a potent calcium channel blocker, neither antagonized nor abolished the increase in transmitter release caused by CTX.

7 TTX (1 μM) completely prevented the effect of CTX (2.5 nM) on m.e.p.p. frequency. This effect was independent of the presence of extracellular Ca^{2+}. TTX, when added after CTX (2.5 nM) exposure, antagonized the increase in m.e.p.p. frequency. The antagonism was complete in Ca^{2+}-free medium. These results strongly suggest that increased permeability of the nerve terminal to Na^+ is responsible for the increase in m.e.p.p. frequency caused by CTX. It is likely that CTX may trigger calcium release from internal stores due to an increase of intraterminal Na^+ concentration.

8 It is concluded that CTX exerts, in the nanomolar concentration range, a selective action on sodium channels of the neuromuscular junction causing both pre- and postsynaptic effects.

Introduction

It has long been known that the eating of certain coastal fishes which inhabit sub-tropical and tropical seas causes, in man, a complex endemic disease known as ciguatera fish poisoning (Bagnis, 1968; Bagnis *et al.*, 1979; Withers, 1982; Anderson & Lobel, 1987).

Ciguatoxin (CTX) and maitotoxin (MTX), both produced by the benthic dinoflagellate *Gambierdiscus toxicus* (Yasumoto *et al.*, 1977; Bagnis, *et al.*, 1977) are the two main toxins transmitted to fish through the marine food chain (Taylor, 1979; Bagnis, 1981) which are responsible for ciguatera poisoning. Both CTX and MTX are among the most potent marine toxins known (Tachibana *et al.*, 1987; Yasumoto, 1980). While the effects of MTX are well characterized on excitable membranes (Freedman *et al.*, 1984; Miyamoto *et al.*, 1984; Kobayashi *et al.*, 1985), secretory cells (Takahashi *et al.*, 1983; Login, *et al.*, 1985; Schettini *et al.*, 1984) and at the neuromuscular junction (Kim *et al.*, 1985), the pharmacological actions of CTX (reviewed by Legrand & Bagnis, 1984) are less well known mainly due to difficulties in obtaining adequate quantities of chromatographically pure toxin.

CTX, first purified by Scheuer *et al.* (1967), is a complex lipid-soluble compound. Although H^1 nuclear magnetic resonance data suggest a molecular weight of 1111.7 ± 0.2 daltons, its molecular structure has not yet been completely established (Tachibana *et al.*, 1987). CTX has been shown to affect selectively sodium channels inducing membrane depolarization and spontaneous action potentials in neuroblastoma cells and myelinated axons (Bidart *et al.*, 1984; Benoit *et al.*, 1986).

In the present study we have investigated the effects of CTX at the frog neuromuscular junction in order to establish its effects on spontaneous and evoked quantal transmitter release from motor nerve endings. A preliminary account of part of this work has been previously communicated at the Joint Meeting of Association Française des Pharmacologistes and Nederlandse Vereniging voor Farmacologie (Molgó *et al.*, 1989).

Methods

All experiments were performed on isolated cutaneous pectoris nerve-muscle preparations removed from male frogs (*Rana esculenta*). The normal Ringer solution had the following composition in mM: NaCl 110.0, KCl 2.1, $CaCl_2$ 1.8 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/NaOH 5.0 (to give a pH of 7.25). In some experiments the Ca^{2+} concentration was reduced and Mg^{2+} added to the solution in concentrations specified in the Results. In

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other experiments Ca^{2+} was substituted by Mg^{2+} (2 mM) and 1 mM ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid (EGTA) was added to the medium. When changes were made of the ionic composition of the bathing solution, osmolarity was maintained by changing NaCl concentration. The experiments were performed at ambient room temperature which ranged from 20–22°C.

In some cases the mechanical activity of cutaneous pectoris muscles was uncoupled from the membrane surface depolarization by equilibrating the preparation in Ringer solution containing 2 M formamide (see del Castillo & Escalona de Motta, 1978). Excitation-contraction uncoupling occurred 12–15 min after the addition of formamide. After this treatment preparations were bathed in normal Ringer solution and the resting membrane potential of the muscle fibres was depolarized by 20–30 mV. With a few exceptions, preparations were not used until complete recovery of the resting membrane potential of the muscle fibres, which occurred within 2–4 h of continuous washing with normal Ringer solution.

Membrane potential was recorded with intracellular glass capillary microelectrodes filled with 3 M KCl of 8–12 MΩ resistance. Recordings were made either at endplate regions, visually identified by locating terminal nerve branches where miniature endplate potentials (m.e.p.ps) had rise times of 1 ms or less, or at extrajunctional areas of the muscle fibres. The nerve was stimulated with supramaximal current pulses of 0.05–0.1 ms duration through a suction electrode at frequencies of 0.5 Hz.

In order to follow the time course of changes in m.e.p.p. frequency two protocols were generally used: (i) continuous recording at an individual junction; (ii) multiple sampling of different junctions from the same muscle.

Data were collected by an IBM-AT microcomputer equipped with a TM 100 Labmaster analogue interface board (Scientific solutions, U.S.A.) and modified pClamp software (Axon Instruments, U.S.A.). An event detector (AI 2020, Axon Instruments) was used to detect random events. Synaptic potentials were also stored on video tape with the aid of a modified digital audio processor (Sony PCM 701 ES) and a video cassette recorder for later analysis if needed.

The mean quantal content (m) of e.p.ps was calculated either by the failure method (m = ln Nt/No) in which Nt is the total number of stimuli delivered to the motor nerve and No the number of failures of release or by the direct method (m = e.p.p. amplitude/m.e.p.p. amplitude). Usually 30-100 e.p.ps and 30–100 consecutive m.e.p.ps were used in the calculations.

Ciguatoxin was extracted for *Gymnothorax javanicus* moray-eel liver and purified by column chromatography by use of silicic acid, Florisil, DEAE-cellulose, Sephadex LH_{20} and C_{18} reversed phase silica successively (Legrand *et al.*, 1989). Pure CTX thus obtained had a mouse LD_{50} (intraperitoneally) of 0.4 μg kg^{-1} with a mean survival time of 10 ± 1.4 h (Legrand *et al.*, 1988). The toxin sample was dissolved in ethanol and divided into several fractions. The ethanol was then evaporated under nitrogen flux. The fractions were kept at −18°C and diluted immediately before the experiment in Ringer solution to give final toxin concentrations of 1–2.5 nM.

Tetrodotoxin, formamide and (+)-tubocurarine were obtained from Sigma (St. Louis, MO, U.S.A.) and all salts were of analytical grade.

Statistical analysis of data was performed with Student's *t* test (two tailed). Values are expressed as mean ± s.e.mean with the number of observations (*n*) in parentheses. Data were considered significant at $P < 0.05$.

Results

Effects of ciguatoxin on neuromuscular transmission

Addition of CTX (1–2.5 nM) to neuromuscular preparations bathed in normal Ringer solution induced within seconds visible spontaneous uncoordinated contractions of the muscle fibres. This spontaneous activity occurred in random isolated groups of muscle fibres but with much less intensity in Ca^{2+}-free medium and in the presence of 30–60 μM (+)-tubocurarine. The contractile activity was not observed when excitation-contraction was uncoupled with formamide (see Methods) and was either prevented or suppressed by 1 μM tetrodotoxin (TTX). These results suggest that the contractile activity induced by CTX is mediated both pre- and post-synaptically.

Intracellular recordings in preparations previously treated with formamide rapidly showed, upon addition of CTX (1–2.5 nM) to the normal Ringer solution, bursts of spontaneous muscle action potentials at rates varying from a few Hz to tens of Hz. In this condition, a single nerve stimulus elicited trains of repetitive action potentials of variable frequency and duration at extrajunctional recording sites (Figure 1A). During repetitive nerve stimulation at 0.5 Hz, action potentials usually appeared as short trains in a given fibre (Figure 1Aa). With continued stimulation the trains became longer (Figure 1Ab, Ac) and the firing reached frequencies of about 60 to 100 Hz. The time course of long trains usually exhibited two phases. During the first repetitive action potentials appeared at a faster rate than during the second (Figure 1Ab, Ac). Analysis of the time course of the action potentials within the second phase of the train revealed that their decay times increased with little change in the amplitudes or the times to peak (Figure 1Bb and Bc). As these changes evolved during the second phase of a train the firing rate decreased and the train finally ceased. The trains were evoked by nerve stimulation only during 1 to 2 min following application of the toxin because the resting membrane potential depolarized. As shown in Table 1, CTX (2.5 nM) caused depolarization of skeletal muscle fibres bathed either in a normal Ringer solution or in a Ca^{2+}-free medium. The depolarization was antagonized by 1 μM TTX. Thus when muscles were bathed in TTX (1 μM) subsequent addition of CTX (2.5 nM) did not elicit depolarization of the muscle fibres (Table 1).

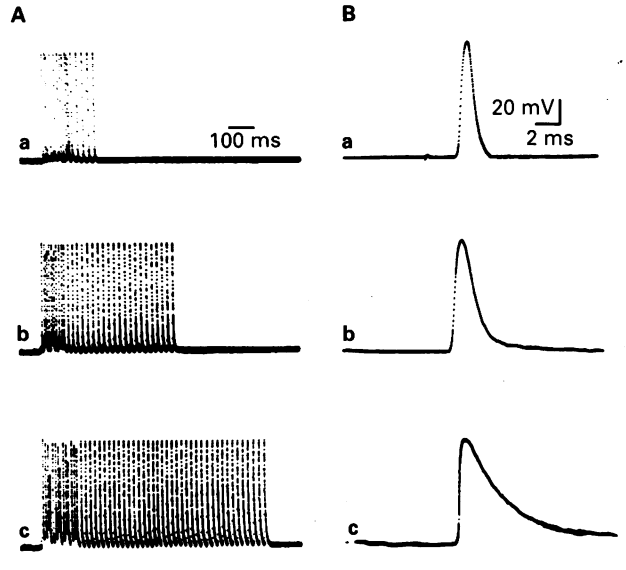

Figure 1 (A) Trains of repetitive muscle action potentials evoked by stimulation of the motor nerve at 0.5 Hz in the presence of ciguatoxin (CTX) (2.5 nM). (Aa–Ac) Action potential trains recorded at extrajunctional sites of a single fibre, 30–60 s after the addition of CTX to normal Ringer solution. Note the two phase firing pattern in (Ab) and (Ac). (B) Fast sweep recording of the action potentials during a single train such as those shown in (A). (Ba) First action potential of the train. (Bb) At about mid train and (Bc) just before the train stopped. Note the slowed repolarization in (Bb) and (Bc) as compared to (Ba). In (A) and (B) formamide was used to uncouple excitation from contraction. Vertical calibration applies to all recordings. Horizontal calibration in (Aa) applies to (Ab) and (Ac), and in (Ba) to (Bb) and (Bc). Resting membrane potential was −75 mV.

Table 1 Effect of ciguatoxin (CTX, 2.5 nM) on the resting membrane potential of frog cutaneous pectoris muscle fibres and the effect of tetrodotoxin (TTX, 1 μM)

Experimental condition	*Resting Membrane potential* (mV) *Controls*	*CTX*	*TTX + CTX*
Normal Ringer	−88.7±1.0 (49)	−57.4±2.2* (44)	−87.2±2.2 (15)
Ca^{2+}-free	−77.5±1.5 (27)	−56.6±2.0* (41)	−79.7±1.9 (16)

*$P < 0.001$ from respective controls. The numbers in parentheses represent number of fibres sampled in 3 different muscles. Note that TTX was applied 15 min before CTX perfusion. Values were obtained after 1 h of CTX exposure.

Recordings made at junctional sites of the fibres showed the classical synaptically-triggered action potential when the motor nerve (0.5 Hz) was stimulated in formamide-treated preparations. Application of CTX produced firing of action potentials in trains upon single nerve stimulation, as in the previous experiments. Soon after train activity was obtained, 2–5 μM (+)-tubocurarine was added to the perfusion medium. These experiments revealed that the trains of action potentials were triggered by repetitive e.p.ps. The timing of the application of (+)-tubocurarine was critically important because the effects of CTX were not long lived. The train firing in the presynaptic terminal revealed by recorded e.p.ps from a typical fibre are shown in Figure 2. The repetitive e.p.ps (Figure 2 b-h) usually exhibited some facilitation with respect to the first e.p.p. in each train and were separated by intervals ranging from 5 to 20 ms.

Effect of CTX on the quantal content of e.p.ps

In junctions equilibrated for 1 h in Ringer solution containing either 0.5 mM Ca^{2+} and 5 mM Mg^{2+} or 0.9 mM Ca^{2+} and 8 mM Mg^{2+}, CTX (1.5 nM) reduced the number of failures of release and increased the average amplitude of e.p.ps. The increase in quantal content of e.p.ps with respect to controls was 239±35.7% (range 135–350%, $n = 4$). In the junctions examined the increase in quantal content of e.p.ps reached a plateau in about 15–20 min and then declined during the subsequent 15–30 min. Concomitant with the decrease in quantal content of e.p.ps the frequency of spontaneous m.e.p.ps greatly increased, as shown in Figure 3. In most junctions examined

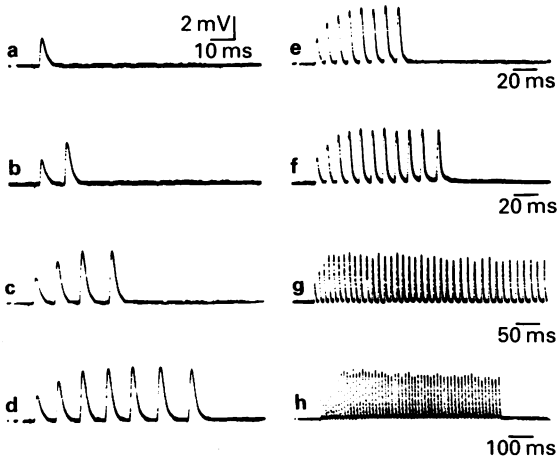

Figure 2 Sequential recording (a–h) obtained at a single junction 90 s after addition of ciguatoxin (CTX, 1.7 nM) to normal Ringer solution containing 3.5 μM (+)-tubocurarine. Note that in all records, a single nerve impulse applied to the motor nerve every 2 s elicited repetitive endplate potentials (e.p.ps) except in (a), which was recorded just before the repetitive firing occurred. Preparation treated with formamide.

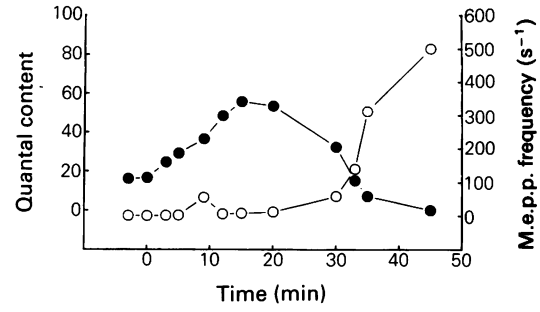

Figure 3 Effect of ciguatoxin (CTX, 2 nM) on the mean quantal content of endplate potentials (e.p.ps) (●) and on miniature endplate potential (m.e.p.p.) frequency (○). Data obtained on the same junction bathed in a Ringer solution containing low Ca^{2+} (1 mM) and 8 mM Mg^{2+}. The quantal content of e.p.ps was estimated by the direct method. Preparation treated with formamide.

after 45–60 min in CTX nerve stimulation did not evoke e.p.ps. Extensive washing of the preparation with CTX-free solution did not reverse either the blockade of evoked transmitter release or the increase in m.e.p.p. frequency.

Effect of CTX on spontaneous quantal transmitter release

CTX (1.5–2.5 nM) increased spontaneous m.e.p.p. frequency when added to normal Ringer solution (Table 2) in a time-dependent way. The increase in m.e.p.p. frequency usually developed after an initial delay of 20–40 min and was sustained at more than 100 times the basal level for up to 2 h and then declined towards zero within 3 h exposure in the continued presence of CTX. Extensive washing of the preparations (2 h) with a CTX-free solution did not reverse the effect of the toxin after it had been applied for 30 min.

Although CTX clearly increased spontaneous quantal release, the mechanism by which this occurred was uncertain. If CTX depolarized the nerve terminals, it might increase transmitter output by enhancing Na^+ and/or Ca^{2+} entry. This could be tested by blockade of either the influx of Na^+ with TTX, a highly specific blocker of voltage-dependent sodium channels (Narahashi, 1974), or the influx of Ca^{2+} by Cd^{2+}, a potent calcium channel blocker (Guan *et al.*, 1987).

In preparations equilibrated for 15 min with normal Ringer solution containing 1 μM TTX, addition of CTX (2.5 nM) caused no detectable change in m.e.p.p. frequency (Table 2). A similar lack of effect of 2.5 nM CTX in the presence of 1 μM TTX was observed after prolonged exposure (3 h). These results indicate that TTX prevents the presynaptic action of

Table 2 Miniature endplate potential frequency (s^{-1}) recorded under different experimental conditions just before and 45-60 min after addition of ciguatoxin (CTX)

Ionic medium	*Control*	*CTX* (1.4 nM)	*CTX* (2.5 nM)
Normal Ringer	1.1±0.1 (17/5)	157.5±34.0* (4/1)	208.6±4.2* (18/5)
Normal Ringer +TTX (1 μM)	—	—	0.8±0.07 (10/3)
Ca^{2+} (1 mM) Mg^{2+}(8 mM)	0.5±0.09 (7/2)	—	253.7±46.8* (3/1)
Ca^{2+}-free	0.3±0.07 (22/4)	—	233.2±49.5* (8/3)
Ca^{2+}-free +TTX(1 μM)	—	—	0.3±0.04 (19/3)

*$P < 0.001$ from respective controls. The figures in parentheses represent number of junctions sample and number of muscles. Note that TTX was applied before the addition of CTX.

CTX on spontaneous quantal release by blocking Na^+ entry through voltage-dependent channels.

It was of interest to study whether TTX antagonized the fully developed effect of CTX on m.e.p.p. frequency. As shown in a typical experiment (Figure 4A), although 1 μM TTX reduced the CTX-increased m.e.p.p. frequency by more than 90%, it did not suppress the effect of CTX completely.

In order to study the contribution of external Ca^{2+} to the increase in m.e.p.p. frequency caused by CTX, experiments were performed either on junctions bathed in normal Ringer solution to which Cd^{2+} (0.1 mM) was added or on junctions equilibrated for 60 min in a nominally Ca^+-free Ringer solution containing 1 mM EGTA and 2 mM Mg^{2+}. As shown by typical recordings of m.e.p.ps obtained in a junction bathed in normal Ringer solution containing 2.5 nM CTX (Figure 4Bb), addition of 0.1 mM Cd^{2+} did not antagonize the CTX-increase in m.e.p.p. frequency (Figure 4Bc). In addition, CTX (2.5 nM) also enhanced m.e.p.p. frequency in nominally Ca^{2+}-free medium (Table 2). Figure 5 shows representative experiments of the time course of CTX (2.5 nM) action on spontaneous m.e.p.p. frequency in a Ca^{2+}-free medium and the effect of TTX. As shown in Figure 5a, TTX (1 μM) completely antagonized the effect of CTX. However, removal of TTX by perfusion with a CTX-free and TTX-free solution induced the reappearance of an increased rate of m.e.p.ps, which indicates that the effect of TTX is more easily reversible than that of CTX. It is interesting to note that no reversal of CTX action was observed under those conditions, even after 2 h washing, and that subsequent addition of TTX could always reverse the CTX-induced increase on m.e.p.p. frequency (data not shown). As shown in Figure 5b, prior treatment with 1 μM TTX completely prevented the effect of CTX in a Ca^{2+}-free medium.

Analysis of the amplitude distribution of m.e.p.ps in the presence of CTX revealed that the distribution was monomodal as in controls. The coefficient of variation (mean $\pm$ s.d.) of the m.e.p.p. amplitude distribution in the presence of 1.5 nM CTX was 0.20 ± 0.01 ($n = 4$) and did not significantly differ from values obtained in control junctions (0.22 ± 0.018; $n = 4$). However, despite the similar distribution of m.e.p.p. amplitudes, in most junctions analysed there was a decrease in the mean m.e.p.p. amplitude with CTX as compared to controls (Figure 6). The reduction in amplitude can be accounted for by a reduction in the acetylcholine driving force through the activated channels of the endplate based on the assumption that the equilibrium potential for acetylcholine is 0 mV before and after CTX action.

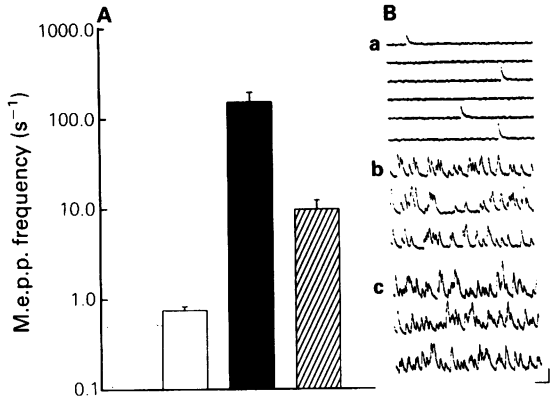

Figure 4 (A) Increase in miniature endplate potential (m.e.p.p.) frequency produced by 2.5 nM ciguatoxin (CTX) (solid column) over controls (open column) and the inability of tetrodotoxin (TTX, 1 μM) to reverse completely the effect of CTX (hatched column). Preparation bathed in a normal Ringer solution and previously treated with formamide. Each column represents values obtained by sampling 8–12 different junctions. CTX was applied for 30 min in normal Ringer solution before recording, while TTX was applied for 25 min in the continuous presence of CTX. Note the logarithmic scale of the ordinate. (B) Examples of m.e.p.ps recorded at a single junction just before (a), 60 min after the addition of 2.5 nM CTX (b), and after 15 min of 0.1 mM Cd^{2+} addition (c) to the preparation. Note that the addition of Cd^{2+} did not antagonize the effect of CTX. Vertical calibration in (c) is 1 mV and applies to all recordings. Horizontal calibration is 50 ms in (a) and 20 ms in (b) and (c).

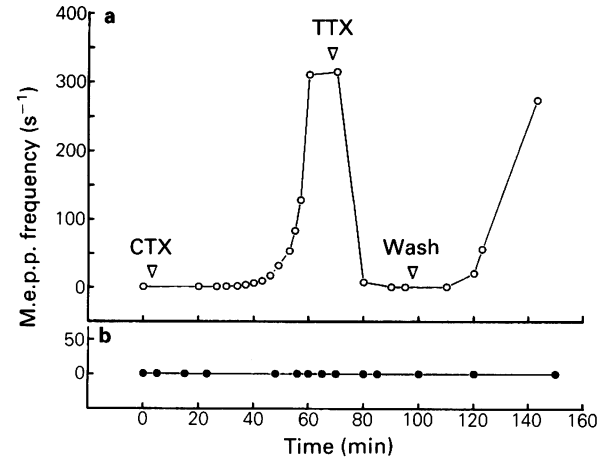

Figure 5 (a) Time course of the increase in miniature endplate potential (m.e.p.p.) frequency produced by 2.5 nM ciguatoxin (CTX, ○) in a Ca^{2+}-free Ringer solution and the ability of tetrodotoxin (TTX, 1 μM) to reverse completely and rapidly the effect of CTX. CTX was applied for 68 min and then the preparation was perfused with the same Ringer with TTX (1 μM) added. Note that washing with TTX-free and CTX-free solution markedly enhanced m.e.p.p. frequency. Arrow heads indicate changes in bathing solutions. All data were obtained from the same junction. (b) Failure of CTX (2.5 μM) to increase the rate of occurrence of m.e.p.ps (●) in another preparation exposed for 1 h to 1 μM TTX in a calcium-free Ringer solution. Data obtained by multiple sampling of different junctions in the same preparation.

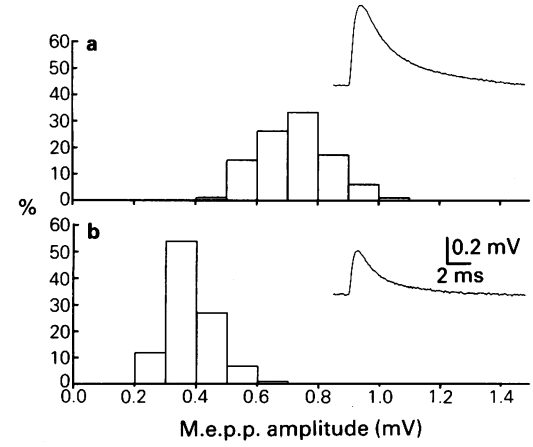

Figure 6 Histograms showing the distribution of miniature endplate potential (m.e.p.p.) amplitudes recorded before (a) and after (b) the action of 1.5 nM ciguatoxin (CTX). Values are expressed as a percentage of the total number of m.e.p.ps counted from a single junction. Preparation bathed in normal Ringer solution. Insert in (a) and (b) averaged digitized records of 67 m.e.p.ps, calibration in insert (b) applies also to insert (a). Resting membrane potential was −81.7 mV in controls and −45.5 mV during CTX action.

Discussion

The results presented here provide evidence that CTX, in the nanomolar range, affects the skeletal neuromuscular junction by a combination of pre- and post-junctional effects. Postsynaptically the toxin induces membrane depolarization and causes spontaneous contractile activity. Since both effects are prevented or suppressed by TTX, it is likely that an increase in Na^+ permeability is involved in these responses. Furthermore, a prolongation of the repolarizing phase of indirectly-

elicited action potentials was found to occur during repetitive firing. In this respect, the action of CTX is similar to that previously found with sea anemone, *Anemonia sulcata* toxin II (ATX-II) on frog skeletal muscle fibres (Khan *et al.*, 1986). However, in contrast to ATX-II we have been unable to detect action potentials with a long plateau in the presence of CTX. Further studies are required in order to understand direct effects of CTX on voltage-dependent ionic currents of the muscle membrane responsible for action potential generation.

One striking prejunctional effect of CTX is its ability to produce repetitive transmitter release, which triggers repetitive action potentials in the muscle fibre in response to a single nerve stimulus. These action potentials might be in part responsible for the spontaneous uncoordinated muscle contractions observed with CTX.

Several drugs and toxins, such as tetraethylammonium, guanidine, aminopyridines, dendrotoxins and ATX II, which affect presynaptic K^+ or Na^+ channels, induce repetitive e.p.ps at the neuromuscular junction (Koketsu, 1960; Matthews & Wickelgren, 1977; Molgó, 1982; Molgó & Mallart, 1985; Anderson & Harvey, 1988). However, to our knowledge the repetitive firing of the motor nerves produced by these agents did not attain the high frequency observed with CTX. At the frog node of Ranvier, it has been shown by Benoit *et al.* (1986) that CTX induces spontaneous action potentials at a frequency of about 100 Hz, with no modification of their time course. Such spontaneous activity has been attributed to a specific modification of a fraction of sodium channels which in the presence of CTX are activated at the normal resting potential and which fail to inactivate.

CTX in about the same range of concentrations used in the present study has been shown to stimulate the initial rate of TTX-sensitive $^{22}Na^+$ uptake by neuroblastoma cells and rat muscle cells in the presence of veratridine (Bidart *et al.*, 1984).

The present experiments show that CTX has a dual action on evoked transmitter release. The facilitatory effect on the mean quantal content of the e.p.p is not maintained and is followed, first, by a reduction, and finally by the irreversible blockade of nerve-evoked transmitter release. It is likely that the CTX-facilitatory effect on evoked transmitter release is not due to enhanced calcium entry during the presynaptic action potential, but to an increase in intraterminal calcium concentration due to sodium entry (see below). The reduction and finally the block of evoked transmitter release that follows the facilitatory action of CTX could be due both to a reduction in the driving force for Ca^{2+} entry and to the depolarization of the terminals caused by Na^+ entry. The blockade of evoked transmitter release can be explained neither by a depletion of transmitter stores in the nerve terminal nor by a change in the sensitivity to acetylcholine of the postsynaptic receptors, since at the time stimulation failed to elicit evoked release, m.e.p.ps could still be recorded and their frequency was markedly increased.

CTX increased the spontaneous quantal release of acetylcholine measured electrophysiologically as an increase in m.e.p.p. frequency. Nerve terminal calcium channel blockade with Cd^{2+} did not antagonize this effect. Furthermore, the acceleration of m.e.p.p. rate caused by CTX occurred independently of extracellular Ca^{2+} since it was observed even in a nominally Ca^{2+}-free solution containing EGTA. These results seem to rule out the possiblity that CTX enhances Ca^{2+} influx through voltage-sensitive Ca^{2+} channels of the nerve terminal, in contrast to the hydrosoluble MTX, whose stimulating effect on spontaneous transmitter release depends upon Ca^{2+} entry into motor endings (Kim *et al.*, 1985).

TTX, in the presence of extracellular Ca^{2+}, did not completely antagonize the increase in m.e.p.p. frequency caused by CTX. The reason for this may be that, even when TTX is able to block Na^+ influx through voltage-sensitive channels, the toxin does not prevent Ca^{2+} uptake through the reversed operation of the Na^+-Ca^{2+} exchange system that normally uses the Na^+ gradient to extrude Ca^{2+} (Blaustein & Nelson, 1982). However, the present study shows that application of CTX following TTX pretreatment failed to increase m.e.p.p. frequency and that TTX completely inhibited the CTX-induced increase in m.e.p.p. frequency in a Ca^{2+}-free medium. These results suggest that increased permeability of the nerve terminal to Na^+ is responsible for the enhancement of m.e.p.p. frequency. In synaptoneurosomes increases of intracellular Na^+ concentration can elicit phosphoinositide breakdown and stimulate phosphatidylinositol systems which are capable of mobilizing Ca^{2+} from internal stores (Gusovsky *et al.*, 1986). In addition, in various secretory systems increases in intracellular Na^+ may mobilize intracellular Ca^{2+} and support transmitter or hormone release (Lowe *et al.*, 1976; Rahamimoff *et al.*, 1980; Melinek *et al.*, 1982). Whether the enhancement of spontaneous quantal transmitter release by CTX is related to increases in phosphoinositide breakdown, intraterminal calcium mobilization or to other mechanism remains to be determined.

Effects on m.e.p.p. frequency similar to those obtained here with CTX have been observed previously with other marine toxins like ATX-II (Lemeignan *et al.*, 1981; Harris & Tesseraux, 1984; Molgó *et al.*, 1986). However, CTX is more potent than ATX-II on a molar basis. Among the toxins isolated from the dinoflagellate *Ptychodiscus brevis*, brevetoxin B has been shown to share a common receptor site with CTX on the neuronal voltage-dependent Na^+ channel protein (Lombet *et al.*, 1987) and T-17 toxin to increase the rate of occurrence of m.e.p.ps in frog and rat neuromuscular preparations (Atchison *et al.*, 1986). However, in contrast to CTX, Ca^{2+} in the external solution was found to be necessary to stimulate m.e.p.p. frequency in the presence of T-17 toxin.

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References

ANDERSON, A.J. & HARVEY, A.L. (1988). Effects of the potassium channel blocking dendrotoxins on acetylcholine release and motor nerve terminal activity. *Br. J. Pharmacol.*, **93**, 215–221.

ANDERSON, D.M. & LOBEL, P.S. (1987). The continuing enigma of ciguatera. *Biol. Bull.*, **172**, 89–107.

ATCHISON, W.D., LUKE, V.S., NARAHASHI, T. & VOGEL, S.M. (1986). Nerve membrane sodium channels as the target site of brevetoxins at neuromuscular junctions. *Br. J. Pharmacol.*, **89**, 731–738.

BAGNIS, R. (1968). Clinical aspects of ciguatera (fish poisoning) in French Polynesia. *Hawaii Med. J.*, **28**, 25–28.

BAGNIS, R. (1981). L'ichtyosarcotoxisme de type ciguatera: Processus biologiques connus et perspectives au seuil des anées 80. *Ann. Inst. océanogr.*, **57**, 5–24.

BAGNIS, R., CHANTEAU, S. & YASUMOTO, T. (1977). Un agent étiologique vraisemblable de la ciguatera. *C. R. Acad. Sci. Paris D*, **285**, 105–107.

BAGNIS, R., KUBERSKI, T. & LAUGIER, S. (1979). Clinical observations on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. *Am. J. Trop. Med. Hyg.*, **28**, 1067–1073.

BENOIT, E., LEGRAND, A.M. & DUBOIS, J.M. (1986). Effects of ciguatoxin on current and voltage clamped frog myelinated nerve fibre. *Toxicon*, **24**, 357–364.

BIDART, J.N., VIJVERBERG, H.P.M., FRELIN, C., CHUNGUE, E., LEGRAND, A.M., BAGNIS, R. & LAZDUNSKI, M. (1984). Ciguatoxin is a novel type of Na^+ channel toxin. *J. Biol. Chem.*, **259**, 8353–8357.

BLAUSTEIN, M.P. & NELSON, M.T. (1982). Sodium-calcium exchange: its role in the regulation of cell calcium. In *Calcium Transport Across Biological Membranes*. ed. Carafoli, E. pp. 217–236. New York: Academic Press.

DEL CASTILLO, J. & ESCALONA DE MOTTA, G. (1978). A new method for excitation-contraction uncoupling in frog skeletal muscle. *J. Cell Biol.*, **78**, 782–784.

FREEDMAN, S.B., MILLER, R.J., MILLER, D.M. & TINDALL, D.R. (1984). Interactions of maitotoxin with voltage-sensitive calcium channels in cultured neuronal cells. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 4582–4585.

GUAN, Y.Y., QUASTEL, D.M.J. & SAINT, D.A. (1987). Multiple actions of cadmium on transmitter release at the mouse neuromuscular junction. *Can. J. Physiol. Pharmacol.*, **65**, 2131–2136.

GUSOVSKY, F., HOLLINGSWORTH, E.B. & DALY, J.W. (1986). Regulation of phosphatidylinositol turnover in brain synaptoneurosomes: stimulatory effects of agents that enhance influx of sodium ions. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 3003–3007.

HARRIS, J.B. & TESSERAUX, I. (1984). Effects of sea anemone toxin (ATX-II) on the frequency of miniature endplate potentials at rat neuromusclar junctions. *Br. J. Pharmacol.*, **81**, 573–574.

KHAN, A.R., LEMEIGNAN, M. & MOLGO, J. (1986). Effects of toxin II from the sea *Anemonia sulcata* on contractile and electrical responses of frog skeletal muscle fibres. *Toxicon*, **24**, 373–384.

KIM, Y.I., LOGIN, I.S. & YASUMOTO, T. (1985). Maitotoxin activates quantal transmitter release at the neuromuscular junction: evidence for elevated intraterminal Ca^{2+} in the motor nerve terminal. *Brain Res.*, **346**, 357–362.

KOBAYASHI, M., OHIZUMI, Y. & YASUMOTO, T. (1985). The mechanism of action of maitotoxin in relation to Ca^{2+} movements in guinea-pig and rat cardiac muscles. *Br. J. Pharmacol.*, **86**, 385–391.

KOKETSU, K. (1960). Action of tetraethylammonium on neuromuscular transmission in frogs. *Am. J. Physiol.*, **193**, 213–218.

LEGRAND, A.M. & BAGNIS, R. (1984). Mode of action of ciguatera toxins. In *Sea food Toxins*. ed. Ragelis, E.P. pp. 217–223. Washington, DC: Am. Chem. Soc.

LEGRAND, A.M., LITAUDON, M., GENTHON, J.N., BAGNIS, R. & YASUMOTO, T. (1989). Isolation and some properties of ciguatoxin. *J. Appl. Phycol.*, (in press).

LEGRAND, A.M., LOTTE, C., QUOD, J.P., DE DECKKER, F., GENTHON, J. N., LECHAT, I., YASUMOTO, T. & BAGNIS, R. (1988). Acute toxicity in mice of ciguatoxin from *Gymnothorax javanicus* moray-eels and maitotoxin from wild *Gambierdiscus toxicus*. *Proceedings of the Japanese Association of Mycotoxicology, Suppl. 1*, Mycotoxins and Phycotoxins, pp. 17–18.

LEMEIGNAN, M., MOLGÓ, J. & TAZIEFF-DEPIERRE, F. (1981). Effects of *Anemonia sulcata* toxin II on normal and chronically denervated mammalian neuromuscular junctions. *Br. J. Pharmacol.*, **72**, 546P.

LOGIN, I.S., JUDD, A.M., CRONIN, M.J., KOIKE. K., SCHETTINI, G., YASUMOTO, T. & MACLEOD, R.M. (1985). The effects of maitotoxin on $^{45}Ca^{2+}$ flux and hormone release in GH_3 rat pituitary cells. *Endocrinolgy*, **116**, 622–627.

LOMBET, A., BIDART, J.N. & LAZDUNSKI, M. (1987). Ciguatoxin and brevetoxins share a common receptor site on the neuronal voltage-dependent Na^+ channel. *FEBS Lett.*, **219**, 355–359.

LOWE, A.D., RICHARDSON, B.P., TAYLOR, P. & DONATSCH, P. (1976). Increasing intracellular sodium triggers calcium release from bound pools. *Nature*, **260**, 337–338.

MATTHEWS, G. & WICKELGREN, W.O. (1977). Effects of guanidine on transmitter release and neuronal excitability. *J. Physiol.*, **266**, 69–89.

MELINEK, R., LEV-TOV, A., MEIRI, H., ERULKAR, S.D. & RAHAMIMOFF, R. (1982). Regulatory role of intracellular sodium ions in neurotransmitter secretion. *Israel J. Med. Sci.*, **18**, 37–43.

MIYAMOTO, T., OHIZUMI, Y., WAHIO, H. & YASUMOTO, T. (1984). Potent excitatory effect of maitotoxin on Ca channels in the insect skeletal muscle. *Pflügers Arch.*, **400**, 439–441.

MOLGÓ, J. (1982). Effects of aminopyridines on neuromusclar transmission. In *Aminopyridines and Similarly Acting Drugs: Effects on Nerves, Muscles and Synapses*. ed. Lechat, P., Thesleff, S. & Bowman, W.C. *Advances in the Biosciences* Vol. 35, pp. 95–116. Oxford: Pergamon Press.

MOLGÓ, J., COMELLA, J.X. & LEGRAND, A.M. (1989). Ciguatoxin enhances spontaneous quantal transmitter release at the skeletal neuromuscular junction. *Abstracts of the Joint Meeting of Association Française des Pharmacologistes and Nederlandse Vereniging voor Farmacologie*, 28P.

MOLGÓ, J., LEMEIGNAN, M. & TAZIEFF-DEPIERRE, F. (1986). Enhancement by *Anemonia sulcata* toxin II of spontaneous quantal transmitter release from mammalian motor nerve terminals. *Toxicon*, **25**, 441–450.

MOLGÓ, J. & MALLART, A. (1985). Effects of *Anemonia sulcata* toxin II on presynaptic currents and evoked transmitter release at neuromuscular junctions of the mouse. *Pflügers Arch.*, **405**, 349–353.

NARAHASHI, T. (1974). Chemicals as tools in the study of excitable membranes. *Physiol. Rev.*, **54**, 813–889.

RAHAMIMOFF, R., LEV-TOV, A. & MEIRI, H. (1980). Primary and secondary regulation of quantal transmitter release: calcium and sodium. *J. Exp. Biol.*, **89**, 5–18.

SCHETTINI, G., KOIKE, K., LOGIN, I.S., JUDD, A.M., CRONIN, M.J., YASUMOTO, T. & MACLEOD, R.M. (1984). Maitotoxin stimulates hormonal release and calcium flux in rat anterior pituitary cells *in vitro*. *Am. J. Physiol.*, **247**, E520–E525.

SCHEUER, P.J., TAKAHASHI, W., TSUTSUMI, J. & YOSHIDA, T. (1967). Ciguatoxin isolation and chemical nature. *Science*, **155**, 1267–1268.

TACHIBANA, K., NUKINA, M., JOH, Y. & SCHEUER, P. (1987). Recent developments in the molecular structure of ciguatoxin. *Biol. Bull.*, **172**, 122–127.

TAKAHASHI, M., TATSUMI, M., OHIZUMI, Y. & YASUMOTO, T. (1983). Ca^{2+} channel activation function of maitotoxin, the most potent marine toxin known, in clonal rat pheochromocytoma cells. *J. Biol. Chem.*, **238**, 10944–10949.

TAYLOR, F.J.R. (1979). A description of the benthic dinoflagellate associated with maitotoxin and ciguatoxin, including observations on Hawaiian material. In *Toxic Dinoflagellate Blooms*, ed. Taylor, D.L. & Seliger, H.H. pp. 47–56, New York: Elsevier Science Publishing Co.

WITHERS, N.W. (1982). Ciguatera fish poisoning. *Ann. Rev. Med.*, **33**, 97–111.

YASUMOTO, T. (1980). Ciguatera. *Igaku no Ayumi*, **112**, 886–892.

YASUMOTO, T.I., NAKAJIMA, I., BAGNIS, R. & ADACHI, R. (1977). Finding a dinoflagellate as a likely culprit of ciguatera. *Bull. Jpn. Soc. Sci. Fish*, **43**, 1021–1026.

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