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Selective depolarization of the muscle membrane in frog nerve-muscle preparations by a chromatographically purified extract of the dinoflagellate *Ostreopsis lenticularis*

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1 The actions of a chromatographically identified extract of the marine dinoflagellate *Ostreopsis lenticularis*, named ostreotoxin-3 (OTX-3), were studied on frog isolated neuromuscular preparations.

2 OTX-3 (1–10 μg ml^{-1}) applied to cutaneous pectoris nerve-muscle preparations depolarized skeletal muscle fibres and caused spontaneous contractions. The depolarization was neither reversed by prolonged washing nor by (+)-tubocurarine.

3 OTX-3 decreased the amplitude of miniature end plate potentials (m.e.p.ps) but did not affect their frequency.

4 Extracellular recording of compound action potentials revealed that OTX-3 affected neither excitability nor conduction along intramuscular nerve branches.

5 End-plate potentials (e.p.ps) elicited by nerve stimulation were reduced in amplitude by OTX-3 and even showed reversed polarity in junctions deeply depolarized by the toxin.

6 Membrane depolarization induced by OTX-3 was decreased about 70% in muscles pretreated for 30 min with 10 μM tetrodotoxin. In contrast, muscles pretreated with 5 μM μ-conotoxin GIIIA were completely insensitive to OTX-3-induced depolarization.

7 OTX-3 did not affect e.p.p. amplitude and the quantal content of e.p.ps in junctions in which muscle depolarization was abolished by μ-conotoxin GIIIA.

8 OTX-3 is a novel type of sodium-channel activating toxin that discriminates between nerve and skeletal muscle membranes.

Keywords: *Ostreopsis lenticularis*; neuromuscular junction; sodium channels; tetrodotoxin; μ-conotoxin GIIIA

Introduction

Toxins produced by marine dinoflagellates are among the most potent non-proteinaceous poisons known. Twenty-one dinoflagellate species are known to produce toxins that include both water and lipid soluble moieties that have haemolytic, neurotoxic and gastrointestinal inflammatory activities (Steidinger & Baden, 1984; Kaul & Daftari, 1986; Wu & Narahashi, 1988; Yasumoto & Murata 1993). Dinoflagellate toxins have been implicated in human intoxications resulting from the consumption of poisonous fish and shellfish (Yasumoto *et al*., 1979; Anderson & Lobel, 1987; Tosteson, 1995).

In the Caribbean *Ostreopsis lenticularis* is the predominant toxic benthic dinoflagellate being found along the Southwest coast of Puerto Rico (Tosteson *et al*., 1986; 1989; 1992; Ballantine *et al*., 1988). The toxic benthic dinoflagellates *O. lenticularis* and *Gambierdiscus toxicus*, vectors of ciguatera fish poisoning have both been shown to produce multiple toxins (Holmes *et al*., 1991; Legrand *et al*., 1992; Mercado *et al*., 1995). Ostreotoxin-1 (OTX-1), the major polar chromatographic peak found in methanolic extracts of cultured *O. lenticularis* has been shown to act on nicotinic cholinoceptors (Escalona de Motta *et al*., 1992). Chromatographic analyses of methanolic extracts of *O. lenticularis* in which the dinoflagellate cells were initially extracted with acetone and subsequently the residual, insoluble material then extracted with methanol revealed the presence of a different major peak, ostreotoxin-3 (OTX-3), less polar than OTX-1 (Mercado *et al*., 1994). The presence of OTX-3 in these *O. lenticularis* extracts was correlated with significant increases in the toxicity of mice, as compared to those extracts prepared with methanol alone (T.R. Tosteson, personal communication). In addition, the acetone pre-extracted methanol extracts elicited muscle contractions and depolarized amphibian skeletal muscle fibres (Mercado *et al*., 1995). Experiments in chick embryo sympathetic neurones demonstrated that such extracts also affected sodium currents by shifting toward more negative potentials the voltage-dependencies of activation and steady-state inactivation (Rivera-Rentas *et al*., 1995). These results provided the first evidence that extracts of *O. lenticularis* contained a toxin that affected voltage-dependent sodium channels.

In the work presented here we have studied the action of the OTX-3 extract at the frog neuromuscular junction. Results presented here indicate that this extract induces a sodium-mediated depolarization of the muscle membrane without affecting nerve conduction or neurotransmitter release from motor nerve endings. Therefore, OTX-3 represents a novel type of toxin that discriminates between muscle and nerve sodium channels.

Methods

Dinoflagellate extracts and chromatographic characterization

Ostreopsis lenticularis clone 223 was isolated from the coastal waters of Southwest Puerto Rico in November 1989

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and has been kept in continuous clonal culture since that time at the Marine Station of the University of Puerto Rico-Mayaguez. Clonal cultures of *Ostreopsis* were established in washed single dinoflagellate cells isolated from the surfaces of field sampled macro algae (Tosteson *et al*., 1989). *Ostreopsis* 223 cells were harvested from their respective cultures by filtration (Gelman, 0.2 μm pore size), following which the filters were briefly rinsed with distilled water and freeze dried (Virtis, Freezemobile 25SL). Freeze dried filters were initially extracted with distilled acetone, following which the extract suspensions were refiltered and the residual insoluble material on the filters extracted with distilled methanol (MeOH). MeOH was removed from the extracts by flash evaporation (Buchi, Rotavapor) and the residues taken to dryness under nitrogen (Figure 1a). A portion of the MeOH extract was dissolved in high performance liquid chromatographic (h.p.l.c.) grade 100% methanol (Burdick and Jackson, Diagnostic Inc., Muskegn, MI, U.S.A.) at a concentration of 4 mg ml^{-1} for isocratic reverse phase (C_{18}) h.p.l.c. analyses (Waters, equipped with a model 486 u.v. detector) with 100% methanol as the mobile phase. Figure 1b shows shows the chromatographic profile of the MeOH dinoflagellate extract measured at an absorbance of 220 nm, at a flow rate of 1.5 ml min^{-1} and sensitivity of 1.5 AUFS. A second portion of the *O. lenticularis* MeOH extract was suspended in known volumes of distilled water and stored in vacuum at 0°C for subsequent experiments.

Biological preparation and solutions

Experiments were performed at 20–22°C on cutaneous pectoris nerve-muscle preparations isolated from 20–25 g male frogs (*Rana esculenta*). The standard frog physiological solution used in these experiments 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) 5.0; buffered at pH 7.25. This solution was modified by adding $MgCl_2$ (13.5 mM) and reducing the $CaCl_2$ concentration (0.3–0.45 mM) in those experiments designed to study evoked transmitter release with low quantal output. Tetrodotoxin, (+)-tubocurarine and μ-conotoxin GIIIA, all obtained from Sigma (St. Louis, MO, U.S.A.), were added to the standard physiological solution at the final concentrations indicated in the text.

In some experiments, excitation-contraction was uncoupled by treating cutaneous pectoris neuromuscular preparations with 2 M formamide for 17–20 min (see del Castillo & Escalona de Motta, 1978). Subsequently, treated preparations were perfused with standard physiological solution for about 1 h and only used after resting membrane potentials recovered to normal values.

Electrophysiological recordings

Membrane potentials, miniature endplate potentials (m.e.p.ps) and end-plate potentials (e.p.ps) were recorded with intracellular glass capillary microelectrodes filled with 3 M KCl (8–12 ΩM resistance) by use of conventional intracellular recording techniques. In some experiments the motor nerve was taken up into a glass capillary suction electrode and stimulated at a rate of 0.5 Hz with current pulses (50 μs duration) of sufficient strength to maximally elicit e.p.ps. The quantal content (m) of e.p.ps was estimated by the direct method (m = mean amplitude of e.p.ps/mean amplitude of m.e.p.ps) (del Castillo & Katz, 1954). Usually, 100 e.p.ps evoked at 0.2 Hz and 25 spontaneous m.e.p.ps recorded on the same junction were used in the calculations. No corrections were made for the effects of non-linear summation on e.p.p. amplitude (Martin, 1955).

Electrical recordings from superficial intramuscular motor nerve branches were performed with 1 M NaCl-filled microelectrodes (4–6 MΩ resistance) inserted in the perineural space of nerve bundles composed of several axons, as previously described (Molgó & Mallart, 1985).

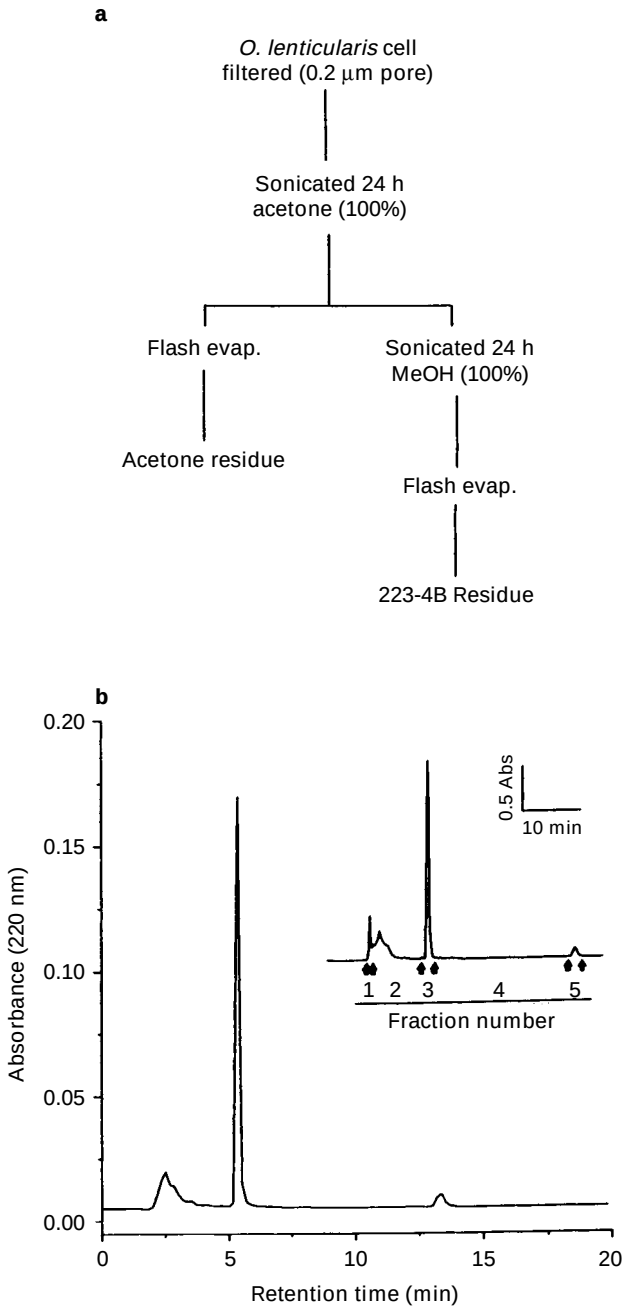

Figure 1 Preparation and chromatographic evaluation of the methanolic extract from *O. lenticularis* laboratory culture 223–4B. Flow chart in (a) summarizes the procedure used to prepare the methanolic extract. Chromatographic profile in (b) was obtained by analysing a 10 μg extract sample in a reverse phase C_{18} column at a flow rate of 1 ml min^{-1} with isocratic 100% methanol as solvent. A Waters h.p.l.c. system equipped with a u.v. detector was used. Insert in (b) shows fraction separation achieved with a semipreparative C_{18} column.

Electrical signals after conventional amplification were displayed on a digital oscilloscope and simultaneously recorded on video tape with the aid of a modified digital audio processor (Sony PCM 701 ES) and a video cassette recorder (Sony SLC9F). Data were collected with the aid of a microcomputer (Olivetti 386C) equipped with an analogue and digital I/O interface board (DT 2821, Data Translation MA, U.S.A.) at a sampling rate of 5–25 kHz. Computerized data analysis was performed with the SCAN program kindly provided by Dr John Dempster (University of Strathclyde, Scotland).

Data analysis

Statistical analysis of data was performed by use of Student's *t* test (two tailed). All values are expressed as the mean ± s.e.-mean and P values < 0.05 were considered significant.

Results

Depolarization of skeletal muscle fibres

Exposure of frog cutaneous pectoris nerve-muscle preparations to the OTX-3 extract induced spontaneous contractions or twitches and caused a depolarization of the muscle membrane that was dependent on both exposure time and concentration. As shown in Figure 2a, 10 μg ml^{-1} OTX-3 decreased the resting membrane potential of cutaneous pectoris muscle fibres to about 10% of matched control values. The typical time course of the changes in resting membrane potential produced in treated muscles are illustrated in Figure 2b. The depolarization induced by OTX-3 affected not only the endplate region but also the entire length of the muscle fibres and was not reversed by prolonged washing. In four different nerve-muscle preparations neither blockade of acetylcholine (ACh) receptors of the endplate region with (+)-tubocurarine (5 μM) nor of excitation-contraction uncoupling with formamide (see Methods) prevented or suppressed the muscle depolarization induced by OTX-3 (10 μg ml^{-1}).

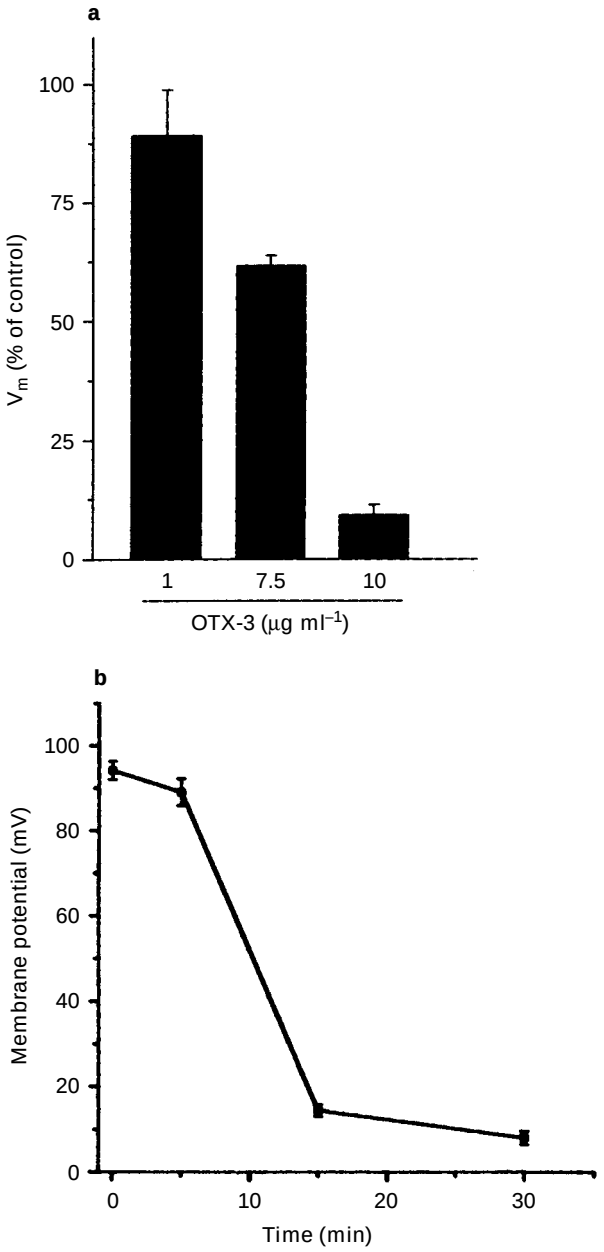

Figure 2 Depolarizing effect of OTX-3 in frog cutaneous pectoris nerve-muscle preparation. (a) The extent of muscle membrane depolarization produced by 30 min incubations with increasing concentrations of OTX-3, all compared to matched controls. (b) The time course of the depolarizing action of 10 μg ml^{-1} OTX-3. All values are the mean of at least 10 membrane potential measurements in each of 3 different muscles; vertical lines show s.e.mean.

Spontaneous miniature endplate potential amplitude and frequency

Treatment of neuromuscular preparations with OTX-3 (7.5 μg ml^{-1}) Strongly depolarized the muscle membrane and reduced the amplitude of the recorded spontaneous m.e.p.ps (Figure 3a) by about 66%. However, no change was observed in m.e.p.p. frequency (Figure 3b).

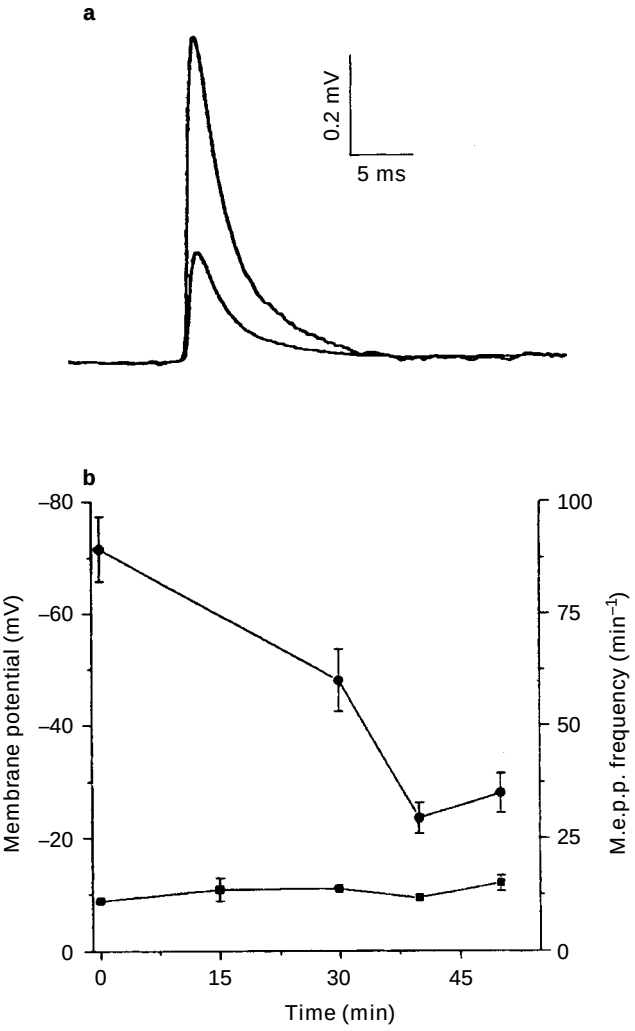

Figure 3 Effect of OTX-3 on the amplitude and frequency of spontaneous miniature endplate potentials (m.e.p.ps). Tracings in (a) are superimposed averaged spontaneous m.e.p.ps recorded under control conditions at a resting potential of −82 mV (top trace) and 30 min after the addition of 7.5 μg ml^{-1} OTX-3, resting potential −27 mV (bottom trace). Each trace is the average of 25 spontaneous events recorded intracellularly in standard frog physiological solution. (b) The increased depolarizing effect of 7.5 μg ml^{-1} OTX-3 observed with longer exposure periods (●) and the absence of significant changes in the frequency of m.e.p.ps (■) recorded under the same conditions. All values are the mean of at least 10 measurements in each of 3 different muscles; vertical lines show s.e.mean.

Intramuscular nerve excitability and conduction

Extracellular perineural recordings were made in intramuscular axons of toxin-treated muscles in order to determine whether OTX-3 ($10-30$ $\mu g\ ml^{-1}$) also affected nerve conduction. To accomplish this, single or paired supramaximal nerve stimuli were delivered at low frequencies (0.2 Hz) to the motor nerve trunk. Compound action potentials were effectively recorded in all the intramuscular branches tested despite profound depolarization of the muscle fibres. Figure 4 shows an example of the compound action potentials, elicited by paired nerve stimuli at different interstimulus intervals, recorded in an intramuscular nerve branch of a muscle depolarized to -18 mV by OTX-3. The extracellularly recorded potentials under these conditions exhibited a supernormal period (for interstimulus intervals between 2.8 and 8.0 ms) and a relative refractory period that was similar to that recorded in untreated preparations (results not shown).

Nerve-evoked endplate potentials

As nerve conduction seemed unaltered by muscle fibre depolarization in the presence of OTX-3 ($10-30$ $\mu g\ ml^{-1}$) it was of interest to determine whether synchronous transmitter release could still be elicited by nerve stimulation in deeply depolarized muscle fibres. Fibres with resting potentials in the range -5 to -7 mV still produced e.p.ps upon nerve stimulation though with an inverted polarity, as seen in the typical record shown in Figure 5.

Sodium channel blockers prevent OTX-3-induced muscle depolarization

Experiments were performed to test whether tetrodotoxin (TTX), an agent that blocks voltage-dependent sodium channels in electrically excitable membranes, was able to prevent the depolarization of the muscle membrane induced by OTX-3. Neuromuscular preparations were pretreated with 10 μM TTX for up to two hours before addition of 10 $\mu g\ ml^{-1}$ OTX-3. Figure 6a shows that this treatment abolished at most 70% of the induced membrane depolarization and that shorter incubation periods inhibited less than one half of the effect.

In contrast, incubation of a preparation for 30 min in 5 μM μ-conotoxin GIIIA (μ-CgTx GIIIA), a conotoxin that selectively blocks voltage-gated sodium channels in skeletal muscle (Cruz *et al*., 1985; Gray *et al*., 1988), completely prevented the OTX-3 (10 $\mu g\ ml^{-1}$)-induced muscle membrane depolarization. Dose-response curves were obtained for the inhibitory effects exerted by TTX and μ-CgTx GIIIA on OTX-3-induced depolarizations (Figure 6b). The respective IC_{50} values calculated from these data were 2.5×10^{-6} M for TTX and 5.7×10^{-8} M for μ-CgTx GIIIA, indicating that μ-CgTx GIIIA was at least fifty times more potent than TTX in preventing the depolarization of the muscle membrane by OTX-3.

Nerve-evoked transmitter release is not affected by OTX-3 in junctions treated with μ-CgTx GIIIA

As shown above, OTX-3 did not seem to modify spontaneous or evoked neurotransmitter release despite its depolarizing effect on the muscle membrane. To confirm these observations experiments were performed in the presence of 5 μM μ-CgTx GIIIA which completely abolished the depolarizing effect of OTX-3. E.p.ps elicited by nerve stimulation under these conditions were of similar amplitude and time course before and after addition of 10 $\mu g\ ml^{-1}$ OTX-3 to the μ-CgTx GIIIA containing media (Figure 7). Furthermore, in 4 different nerve-muscle preparations equilibrated for 30 min with a low calcium (0.45 mM) and high magnesium (13.5 mM) physiological solution supplemented with μ-CgTx GIIIA (5 μM) the mean quantal content of e.p.ps was 11.64 ± 1.09 before, and 11.86 ± 1.23 after, exposure to OTX-3. These results further support the conclusion that OTX-3 does not significantly affect the quantal content of e.p.ps evoked by nerve impulses.

Discussion

The effects obtained in the present study indicate that OTX-3 contains a toxin that depolarizes the skeletal muscle membrane without affecting nerve excitability and conduction in the axonal membrane or motor nerve terminals. Spontaneous and evoked synaptic potentials were reduced in amplitude in junctions depolarized by OTX-3, as expected by the reduction in the driving force for acetylcholine (ACh) which depends on the resting membrane potential and the equilibrium potential for ACh (reviewed by Ginsborg & Jenkinson, 1976). Furthermore, e.p.ps could even be recorded with inverted polarity when the membrane potential of the junctional membrane was

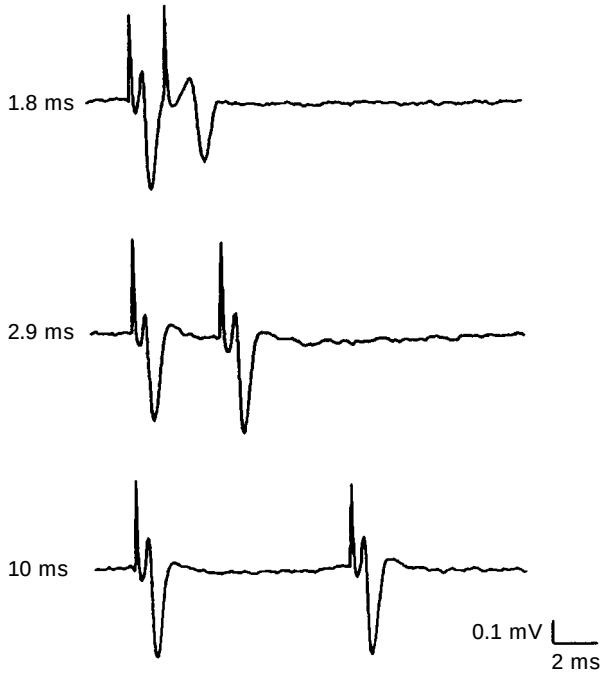

Figure 4 Compound action potentials recorded extracellularly by a microelectrode inserted within the perineural space of an intramuscular nerve branch from a muscle depolarized by 30 $\mu g\ ml^{-1}$ OTX-3. Action potentials were triggered by paired stimuli (0.2 Hz). Interstimulus intervals were as indicated in the upper, middle and lower records. Presence of a classical supranormal period is indicated by the larger peak amplitude of the second spike in the middle record. During the experiment the registered muscle membrane potential was -18 mV.

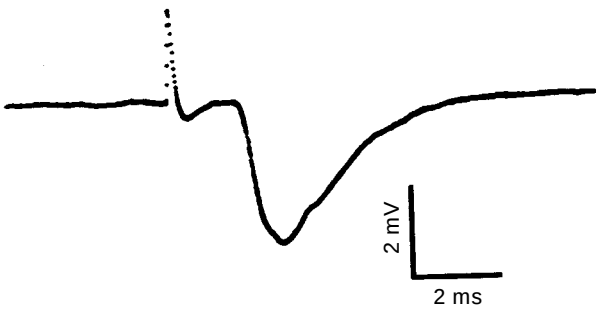

Figure 5 Inverted endplate potential evoked by nerve stimulation in an OTX-3-depolarized neuromuscular junction. The e.p.p. illustrated was recorded intracellularly from a junction after treatment for 30 min with 30 $\mu g\ ml^{-1}$ OTX-3. The resting membrane potential of the muscle fibre was -7 mV. Note that the direction of the potential change (after the stimulus artefact) was inverted due to the change in electrochemical driving force.

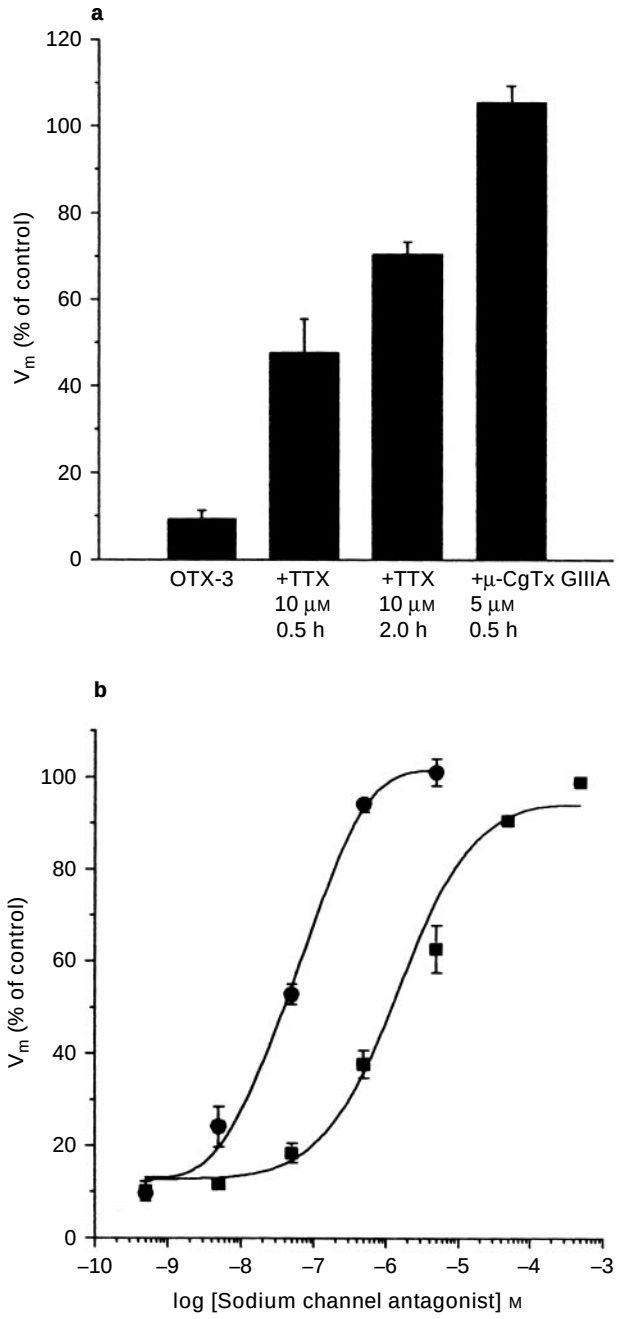

Figure 6 Inhibition of muscle membrane depolarization by OTX-3 in the presence of toxins that block the voltage-gated sodium channels. As shown in (a), 10 μg ml^{-1} OTX-3 were applied for 30 min to neuromuscular preparations before and after incubation in 10 μM TTX (30 and 120 min) or 5 μM CgTx GIIIA (30 min), ($n=3$, each treatment). Dose-response curves in (b) demonstrate that μ-CgTx GIIIA (●) is a more specific inhibitor of the depolarizing effect of OTX-3 than TTX (■). the calculated IC_{50} values for these two antagonists were 5.7×10^{-8} and 2.5×10^{-6} M, respectively.

more positive than the equilibrium potential for ACh. Thus, the decrease in amplitude of m.e.p.ps and e.p.ps observed with OTX-3 was due to a change in the driving force for ACh and not to a decrease in the amount of ACh released spontaneously or in response to nerve impulses. This conclusion was confirmed when both e.p.p. amplitudes and the quantal content of e.p.ps, were compared, in junctions in which the depolarizing action of OTX-3 was prevented by blocking muscle sodium channels with μ-CgTx GIIIA.

The depolarization induced by OTX-3 was inhibited by the sodium channel blockers TTX and μ-CgTx GIIIA. A comparison of the action of these two sodium channel blockers revealed

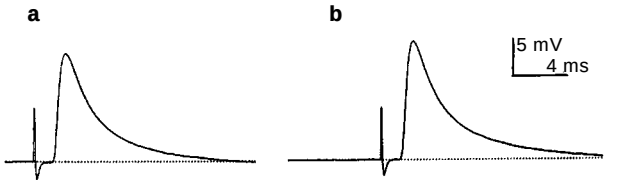

Figure 7 Endplate potentials evoked by nerve stimulation (0.5 Hz) in a μ-CgTx GIIIA-treated frog neuromuscular junction before and after application of OTX-3. Tracings are the averaged e.p.ps recorded during 1 min of nerve stimulation at 0.5 Hz in a muscle bathed in frog physiological solution containing 5 μM μ-CgTx GIIIA before (a) and 30 min after the addition of 10 μg ml^{-1} OTX-3 (b). Note the similarity in e.p.p. amplitudes and time course. The corresponding resting membrane potentials recorded during the experiment were −85 in (a) and −86 mV in (b).

that μ-CgTx GIIIA was about 50 times more potent than TTX in preventing the depolarizing effect of OTX-3. Therefore, these results implicate sodium channels sensitive to μ-CgTx GIIIA as possible targets for OTX-3 action in skeletal muscle fibres. A large number of naturally occurring highly specific toxins, from a wide range of origins, have provided essential tools for the characterization of electrophysiological and molecular properties of sodium channels. Toxins that alter sodium channels may affect one or more of the three essential properties of these channels: voltage-dependent activation, inactivation and selectivity (for reviews see Catterall, 1980; 1992; Strichartz *et al*., 1987). In chick embryo cultured sympathetic neurones, OTX-3 shifted the voltage-dependencies of activation and steady-state inactivation of sodium currents toward more negative potentials and μ-CgTx GIIIA completely prevented these effects (Mercado *et al*., 1994; Rivera-Rentas *et al*., 1995). It is worth noting that in these cultured neurones a fraction of the sodium channels is TTX-resistant but μ-CgTx GIIIA-sensitive (A.L. Rivera-Rentas, personal communication).

In adult skeletal muscle, voltage-sensitive sodium channels are specifically inhibited by TTX and saxitoxin (STX) (Catterall, 1980). However, in foetal skeletal mammalian muscle *in vivo* (Harris & Marshall, 1973) and in denervated adult skeletal muscle (Harris & Thesleff, 1971; Pappone, 1980) there are sodium channels which are relatively insensitive to inhibition by these toxins. However, these TTX-insensitive sodium channels have not been found in denervated frog muscle (Nasledov & Thesleff, 1974). A comparison of the inhibition and binding kinetics of TTX, STX and μ-CgTx GIIIA in mammalian skeletal muscles has led to the discovery of sodium channel subtypes (Gonoi *et al*., 1987), μ-CgTx GIIIA has been shown to bind competitively with TTX and STX to high affinity sodium channels in amphibian and mammalian skeletal muscles (Cruz *et al*., 1985; Moczydlowski *et al*., 1986) and protein expression studies have indicated that the α-subunits form the receptor sites for TTX, STX and μ-CgTx GIIIA (reviewed by Trimmer & Agnew, 1989; Catterall, 1992). Mutagenesis of the adult μI skeletal muscle sodium channel has shown that mutation of glutamate 403 to glutamine prevents the blockade by TTX and STX but the mutant channel retains its sensitivity to blockade by μ-CgTx GIIIA, suggesting that the binding sites are not identical for TTX, STX and μ-CgTx GIIIA (Stephan *et al*., 1994). The fact that μ-CgTx GIIIA preferentially inhibited the depolarization caused by OTX-3 in skeletal muscle is indicative that its selective action is due to an interaction with sodium channels present in muscle which have a greater affinity for μ-CgTx GIIIA than for TTX. Although the essential properties of sodium channels affected by OTX-3 in skeletal muscle fibres remain unknown, we suggest, on the basis of the present results, that OTX-3 may affect muscle sodium channel activation.

Dinoflagellates other than *O. lenticularis* are known to produce sodium-channel activator toxins like brevetoxins and ciguatoxins (Baden, 1989; Legrand *et al*., 1989; Murata *et al*., 1990; Holmes *et al*., 1991). However, in contrast to OTX-3 isolated from *O. lenticularis*, brevetoxins and ciguatoxins have

been shown to affect sodium channels in both nerve and muscle membranes and to increase quantal neurotransmitter release from motor nerve endings (Atchinson *et al*., 1986; Baden, 1989 Wu & Narahashi, 1988; Bidard *et al*., 1984; Benoit *et al*., 1986; Molgó *et al*., 1990; 1992). To our knowledge this is the first demonstration of a dinoflagellate toxin which depolarizes skeletal muscle without affecting motor nerves in isolated skeletal nerve-muscle preparations.

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