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Blockade of acetylcholine release at the motor endplate by a polypeptide from the venom of *Phoneutria nigriventer*

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1 The mechanisms underlying the muscle relaxation effect of a fraction (PF3) isolated from the *Phoneutria nigriventer* spider venom were assessed on mouse diaphragm and chick biventer cervicis muscle preparations.

2 PF3 (0.25-4 $\mu g\ ml^{-1}$) produced a concentration-dependent blockade of the nerve-elicited muscle twitch of the mouse diaphragm ($IC_{50} = 0.8\ \mu g\ ml^{-1}$) without affecting the directly induced muscle twitch. In similar preparations, the crude venom (1-10 $\mu g\ ml^{-1}$) produced muscle contracture and blocked both the direct and indirectly induced muscle twitches.

3 In the chick biventer cervicis muscle, PF3 (1-5 $\mu g\ ml^{-1}$) blocked the nerve stimulated muscle twitch ($IC_{50} = 1.26\ \mu g\ ml^{-1}$), but did not alter the postjunctional response to exogenous acetylcholine (ACh, 10 μM-10 mM).

4 PF3 (2-8 $\mu g\ ml^{-1}$) reduced the frequency of miniature endplate potentials (m.e.p.ps) recorded intracellularly from the mouse diaphragm muscle fibres by 58 to 64%, and diminished the amplitude of m.e.p.ps by 20 to 40% of control. The relationship between log m.e.p.p. frequency and log $[Ca^{2+}]_o$ was shifted rightwards in the presence of 4 $\mu g\ ml^{-1}$ PF3.

5 Raising the frequency of m.e.p.ps with high K^+ medium or theophylline (3 mM) did not prevent the toxin-induced depression of spontaneous ACh release.

6 The quantal content of e.p.ps (*m*), determined in cut-diaphragm muscle fibres, was reduced by 53% and 77% of control by 1 and 4 $\mu g\ ml^{-1}$ PF3, respectively. At 1 $\mu g\ ml^{-1}$ the toxin shifted the relationship between log *m* and log $[Ca^{2+}]_o$ towards higher values without apparent change of the slope.

7 E.p.p. trains elicited at 10 to 50 Hz in the presence of PF3 (1 $\mu g\ ml^{-1}$) exhibited irregular amplitudes and facilitation related to the frequency of nerve stimulation.

8 It is concluded that PF3 blocks neuromuscular transmission by acting prejunctionally and reducing the nerve-evoked transmitter release. The effect was related to a diminished Ca^{2+} entry into the nerve terminal associated with inhibition of exocytosis.

Keywords: *Phoneutria nigriventer*; motor nerve terminal; excitation-secretion coupling; ACh release

Introduction

Phoneutria nigriventer (family Ctenidae) is an agressive South American spider responsible for frequent human accidents in the state of São Paulo. Subcutaneous injections of the crude venom (0.2-1 mg kg^{-1}) in dogs and mice produce local pain, lacrimation, sialorrhoea, ataxia, priapism, dyspnoea, convulsions and death (Schenberg & Pereira Lima, 1971; 1976). Pharmacological studies showed that the venom caused membrane depolarization, increased the rate of spontaneous acetylcholine release and blocked neuromuscular transmission in rat diaphragm muscles. These effects were attributed to activation of voltage-dependent sodium channels in both nerve and muscle fibres (Fontana & Vital-Brasil, 1985). Morphological changes produced by the venom in peripheral nerves of mice were also reported (Cruz-Hofling *et al.*, 1985; Love *et al.*, 1986).

Early chemical studies revealed the presence of polypeptides with molecular weights ranging from 5000 to 7000 as the active components in the *P. nigriventer* venom (Schenberg & Pereira Lima, 1971; 1976). Rezende *et al.* (1991) isolated three neurotoxic fractions from the venom, PhTx1, PhTx2, PhTx3, and a fraction inducing contractions of the guinea-pig ileum smooth musculature. Upon intracerebroventricular administration, PhTx1 produced central excitatory effects in mice, associated with spastic paralysis. PhTx2 reproduced most effects reported for the crude venom, and was recently shown to inhibit inactivation of voltage-dependent sodium channels in frog skeletal muscles (Araújo *et al.*, 1993). PhTx3 caused skeletal muscle relaxation, and was reported to contain six peptides with sequences of amino acids similar to neurotoxins derived from venoms of other spider species (Cordeiro *et al.*, 1993).

The present study investigated the mechanisms accounting for muscle relaxation in mammalian and avian muscles induced by a fraction chemically identical to PhTx3, isolated from the *Phoneutria nigriventer* spider venom. The results show that this fraction, here termed PF3, blocks the neuromuscular transmission of skeletal muscles by acting prejunctionally to depress the nerve-evoked transmitter release. Some of these data have been presented in abstract form (Souccar *et al.*, 1994).

Methods

All experiments were performed on the phrenic-diaphragm muscle preparation of mice or rats. F1 mice (25-30 g), the hybrid resulting from a cross between inbred C57B1/6 female

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and Balb/C male, and female Wistar rats (180–200 g) were used. The chick biventer cervicis muscle of 10 to 15 days old animals was used in a few experiments. Muscle preparations were isolated from animals anaesthetized with ether and kept in oxygenated (95%O_2:5%CO_2) physiological solution with the following composition (in mM): NaCl 135, $NaHCO_3$ 15, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1 and glucose 11, pH 7.3–7.4. In experiments where the concentrations of K^+ ($[K^+]_o$) or Ca^{2+} ($[Ca^{2+}]_o$) in the physiological solution were changed, osmolarity of the solution was maintained by adjustment of the NaCl concentration.

Venom and purified fraction (PF3)

Venom from *P. nigriventer* was obtained upon electric stimulation of the venom apparatus of living spiders at the Instituto Butantan, State of São Paulo, Brazil. The material was collected with a capillary pipette, desiccated and stored at −20°C. Chemical purification was carried out as described by Rezende *et al.* (1991); in short 100 mg of desiccated venom was diluted in 2 ml of 2% acetic acid and chromatographed on a Sephadex G50F column previoulsy equilibrated with 2% acetic acid, at 4°C. The peaks eluted were monitored at 280 nm and those active in mice were pooled, freeze-dried and stored at −24°C. The active fractions were further purified by high performance liquid chromatography using a μC18 Boundapack column eluted with a linear gradient of 0.1% trifluoroacetic and 90% acetonitrile in phosphoric acid.

Tension recording

The phrenic nerve-hemidiaphragm muscle was mounted under 1–2 g tension in an organ bath containing 2.5 ml of physiological solution continuously aerated. The nerve was stimulated via a bipolar platinum electrode with supramaximal pulses (0.5 ms, 0.1 Hz). In some experiments muscle preparations were submitted to alternate nerve and direct electrical stimulation (2 ms, 0.1 Hz and supramaximal voltage). Muscle twitches were isometrically recorded at room temperature (22–24°C) with a force displacement transducer FT03 (Grass Instruments) on a Beckman polygraph.

After 30 min equilibration, the resting tension was readjusted and the effects of the crude venom (CV) or purified fraction (PF3) were recorded. Only one concentration of CV or PF3 was tested in each preparation. The IC_{50} (median inhibitory concentration) values were determined from concentration-effect relationships as the concentrations that produced 50% inhibition of maximum contraction.

The chick biventer cervicis (BVC) muscle was suspended in a 10 ml organ bath containing the above physiological solution, and set up to a tension of 0.5–1 g. Isometric contractions of the BVC muscle were recorded at 35°C upon either nerve or direct muscle stimulation as described above. Cumulative dose-response curves to acetylcholine (ACh, 10 μM to 10 mM) were constructed prior to and after recording the effect of each concentration of PF3.

Intracellular recording

The left hemidiaphragm was pinned down slightly stretched on a Sylgard (Dow Corning) base in a 10 ml muscle chamber and continuously perfused with physiological salt solution. The resting membrane potentials (RMP) and miniature endplate potentials (m.e.p.ps) were recorded intracellularly at room temperature (22–24°C). Glass microelectrodes filled with 3 M KCl with 8–12 MOhm resistance were used. Membrane potentials were amplified with an Axoclamp 2A (Axon Instruments, U.S.A.) and the signals were filtered with a Bessel filter (Axon Instruments model 2040) set at 500 Hz, and further amplified. Data were collected using a TL-1 Labmaster system connected to a digital interface board (Scientific Solutions, U.S.A.) and Axotape software on an IBM-AT computer.

Endplate potentials (e.p.p.) with rise time of 1 ms or less, were recorded upon supramaximal nerve stimulation (0.5 Hz, 0.05 ms) from rat cut-muscle preparations as detailed before (Fann *et al.*, 1990). Trains of 30 e.p.ps were elicited by repetitive stimulation (10, 25 and 50 Hz) at intervals of 60–90 s. For quantal content determination at least 100 e.p.ps were recorded simultaneously to the m.e.p.ps. The amplitudes of e.p.ps were corrected for non-linear summation (McLachlan & Martin, 1981) assuming the reversal potential for ACh as 0 mV (Alemá *et al.*, 1981). The amplitudes of m.e.p.ps were corrected to a mean RMP of −45 mV as described by Fann *et al.* (1990). The quantal content of e.p.ps (*m*) was calculated by the direct method as the ratio of mean e.p.p. amplitude to the mean m.e.p.p. amplitude (Hubbard *et al.*, 1969).

Drugs

Drugs used were: acetylcholine chloride, theophylline, (+)-tubocurarine hydrochloride (Sigma Chem. Co). All muscle preparations were allowed to equilibrate for 30 min in the normal or modified solution before starting the experiments. Freshly prepared solutions of the crude venom or purified fraction were used in all experiments.

Statistics

The results are expressed as means ± s.e.means, and differences among data were compared by Student's two tailed *t* test. Values of m.e.p.p. frequencies were presented as geometric means ± approximate s.e., and differences among data were detected by the Kolmogorov-Smirnov two sample test (Sokal & Rohlf, 1981). Differences between data were considered significant at $P<0.05$.

Results

Effect on the muscle twitch

Exposure of the mouse phrenic-diaphragm muscle preparations to the crude venom of *P. nigriventer* (CV; 1–10 μg ml^{-1}) caused a transient contracture followed by a progressive blockade of both the direct and nerve-elicited muscle twitch (Figure 1a). The effect was related to the concentration and was not significantly altered after increasing the bath tem-

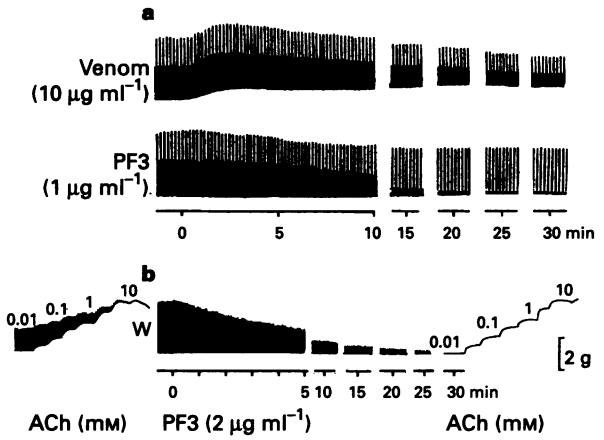

Figure 1 (a)Time course of the effects of the crude venom of the spider *Phoneutria nigriventer* (above) and its fraction PF3 (below) on the direct and indirect muscle twitches of the mouse diaphragm elicited at 0.1 Hz. Muscles twitches were recorded at room temperature (22–24°C), and the toxins incubated at the indicated concentrations at 0 min. (b) Responses of the chick biventer cervicis muscle to nerve stimulation, at 0.1 Hz, and to cumulative addition of acetylcholine (ACh), at the concentrations indicated, before (left) and after (right) exposure to PF3. Muscle twitch and contracture were recorded at 35°C, and PF3 was incubated after washout (W) of the agonist at 0 min.

perature to 30°C (Figure 2). The contracture tensions induced by 1, 5 and 10 μg ml^{-1} of CV on the nerve-induced muscle twitch were respectively, 0.16±0.07 g, 0.20±0.02 g and 0.32±0.07 g (n=4 to 7), corresponding to 1/5 to 1/4 of the control twitch amplitude.

Intraperitoneal injection of the purified fraction (PF3, 3 μg ml^{-1}) in mice produced ataxia, muscle relaxation and dyspnoea leading to death of the animal within 30 min. In mouse isolated diaphragm preparations PF3 (0.25 μg ml^{-1}) did not cause contracture, but caused a concentration-dependent blockade of the nerve-elicited muscle twitch without altering the directly-induced muscle twitch (Figure 1a). The IC_{50} values of both the venom and PF3 in these experiments were 1.2 μg ml^{-1} and 0.8 μg ml^{-1}, respectively (Figure 2).

In the chick biventer cervicis muscle (BVC), the venom (1–5 μg ml^{-1}) produced spontaneous twitching, sustained contracture and blockade of the nerve-induced muscle twitch after 30 min incubation. In parallel preparations PF3 (2, 5 and 10 μg ml^{-1}) blocked the nerve-evoked muscle twitch in a concentration-dependent manner within 30 min, without significant change of the muscle resting tension. The IC_{50} value determined in these experiments after 5 min incubation of PF3 was 1.26 μg ml^{-1}. In both mouse diaphragm and BVC muscle preparations, the neuromuscular blockade induced by PF3 was partially reversed after 60 min of continuous wash with the physiological salt solution.

Concentration-response curves constructed to ACh (10 μM–10 mM) at the same time as the nerve-elicited muscle twitch recordings produced contractures of the BVC muscle proportional to the agonist concentration (EC_{50}=15.5 mM, CL: 5.8–41.6; n=16). After complete blockade of the muscle twitch by PF3 (2 μg ml^{-1}), the response of the BVC muscle to addition of ACh did not differ from that recorded under control conditions (Figure 1b). In similar experiments, the nicotinic receptor antagonist, (+)-tubocurarine (2–10 μM), caused a parallel rightward shift of the concentration-response curves to ACh by 3 to 10 fold.

Effect on the spontaneous ACh release

Exposure of the mouse diaphragm muscles to CV (1 μg ml^{-1}) caused spontaneous twitching of the muscle fibres, and reduced the resting membrane potential (RMP) from −76±1 mV (n=30) to −65±2 mV (n=26) after 30 min. The frequency of m.e.p.ps in these muscle fibres was slightly increased (from 0.83±0.11 s^{-1} to 1.23±0.23 s^{-1}) (Figure 3).

PF3 (1–8 μg ml^{-1}) did not affect the RMP values of the diaphragm muscle fibres compared to control values (−77±1 mV, n=54). The frequency of m.e.p.ps (0.79±0.03 s^{-1}) was also unaltered in presence of 1 μg ml^{-1} PF3, but it was reduced to 0.30±0.04 s^{-1}, 0.33±0.06 s^{-1} and 0.28±0.05 s^{-1} after 30 min exposure to 2, 4 and 8 μg ml^{-1} of the toxin, respectively (Figure 3). The amplitudes of these m.e.p.ps were reduced by 20% and 40% of control (1.05±0.04 mV, n=180). Washing the preparations with physiological solution for 60 min partially reversed the toxin effect on both the frequency and amplitude of m.e.p.ps.

Effect of $[K^+]_o$ and theophylline

Increase in the extracellular concentration of K^+ ($[K^+]_o$) is known to increase the spontaneous release of ACh through a sustained depolarization of the presynaptic membrane raising voltage-gated Ca^{2+} influx (Liley, 1956; Csillik, 1993). In control diaphragm muscles bathed in a medium containing 15 mM K^+, the frequency of m.e.p.ps increased from 0.85±0.07 s^{-1} (n=52 cells) to 6.32±1.29 s^{-1} (n=36 cells). Addition of PF3 (2 μg ml^{-1}) to muscle preparations pretreated with a high-K^+ solution reduced the m.e.p.p. frequency to 0.48±0.08 s^{-1} (n=31 cells) after 30 min (Figure 3). This value was slightly higher than that obtained in the presence of an

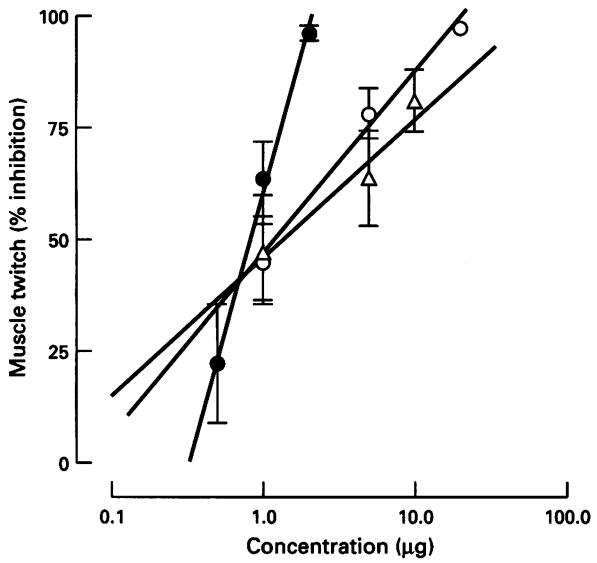

Figure 2 Concentration-effect relationships for the twitch block of mouse diaphragm muscles obtained after 10 min incubation of the crude venom of *Phoneutria nigriventer* at room temperature (○) or at 30°C (△), and the fraction inducing muscle relaxation (PF3, ●) at room temperature (22°C). Muscle twitches were elicited by nerve stimulation and expressed (ordinates) as percentage inhibition of the maximal twitch amplitude recorded before incubation of the venom and PF3. Symbols are means±s.e. of 4–7 muscle preparations.

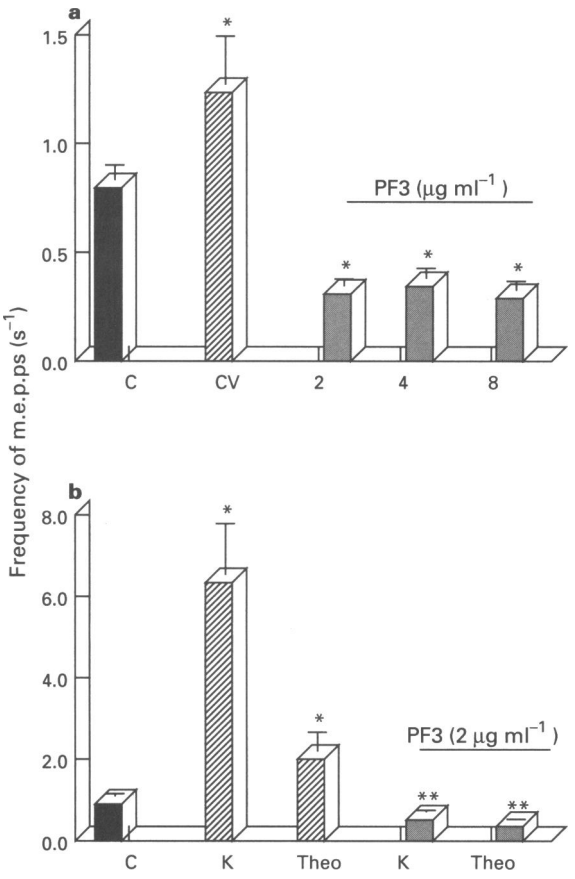

Figure 3 (a) Frequency of miniature endplate potentials (m.e.p.ps) recorded from mouse diaphragm muscle fibres in control conditions (C) and after exposure to the crude venom of *Phoneutria nigriventer* (CV, 1 μg ml^{-1}) or its purified fraction (PF3: 2, 4 and 8 μg ml^{-1}). (b) Frequency of m.e.p.ps recorded from mouse diaphragm muscle fibres in control conditions (C), and in presence of physiological solution containing 15 mM K^+ (K) or 3 mM theophylline (Theo), before and after incubation of PF3 (2 μg ml^{-1}). Columns are geometric means ±approximate s.e. of 31 to 52 muscle fibres in 4 muscles.

equal concentration of PF3 in normal physiological solution, but still significantly lower than control, indicating that the toxin-induced depression of m.e.p.p. frequency was not counteracted by high $[K^+]_o$ (Figure 3).

Raising the cyclic AMP levels with the phosphodiesterase inhibitor, theophylline, was shown to increase m.e.p.p. frequency at motor nerve terminals (Dryden *et al.*, 1988). As expected, exposure of the diaphragm muscle fibres to theophylline (3 mM) also increased the frequency of m.e.p.ps from $0.63 \pm 0.06\ s^{-1}$ (control) to $1.97 \pm 0.48\ s^{-1}$ after 30 min. Addition of PF3 (2 $\mu g\ ml^{-1}$) to theophylline-treated muscles did not prevent the prejunctional inhibitory effect of the toxin alone, the m.e.p.p. frequency being reduced to $0.29 \pm 0.03\ s^{-1}$ after 30 min (Figure 3).

Effect of extracellular calcium

To determine the influence of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on the prejunctional inhibitory effect of PF3, the frequency of m.e.p.ps in toxin-treated and untreated muscle fibres was obtained in the presence of different $[Ca^{2+}]_o$. In control muscles, reducing $[Ca^{2+}]_o$ in the physiological solution to 0.5 and 1 mM decreased the frequency of m.e.p.ps to $0.24 \pm 0.02\ s^{-1}$ and $0.41 \pm 0.05\ s^{-1}$, respectively, whereas raising the $[Ca^{2+}]_o$ to 5 mM increased m.e.p.p. frequency to $1.06 \pm 0.08\ s^{-1}$ (Figure 4). After 30 min exposure to PF3 (4 $\mu g\ ml^{-1}$) the frequency of m.e.p.ps was reduced by 40% to 60% in presence of 0.5 to 5 mM $[Ca^{2+}]_o$. The relationships of the log of m.e.p.p. frequency to the log $[Ca^{2+}]_o$ determined in the absence and presence of PF3 were linear, being the latter shifted rightwards with no apparent change in the slope (Figure 4). The toxin thus appears to depress the spontaneous ACh release without interfering with the intracellular regulatory mechanisms of Ca^{2+}.

Effect on the evoked transmitter release

The effect of PF3 on the quantal content of e.p.ps (*m*) was also examined in the rat diaphragm cut-muscle preparation, a procedure that does not affect the nerve terminal function (Glavinovic, 1979). The mean RMP recorded from the 'cut-muscle' fibres was -42 ± 1 mV (56 fibres/11 muscles), and was not significantly altered after 30 min incubation of 1 and 4 g ml^{-1} PF3. These concentrations caused a proportional decrease of both the e.p.p. amplitude (by 55% and 78% of control) and *m* values (by 53% and 77% of control, respectively), without a significant change of the amplitude of m.e.p.ps (Table 1).

The toxin effect on the $[Ca^{2+}]_o$-dependence of *m* was also assessed. In control conditions, changing $[Ca^{2+}]_o$ from 1 to 5 mM increased *m* values from 13.64 ± 3.08 to 21.56 ± 1.52 (Figure 4). After 30 min exposure to PF3 (1 $\mu g\ ml^{-1}$) the quantal content of e.p.ps was reduced by 57% to 40% of control, respectively, shifting the relationship of log *m* against log $[Ca^{2+}]_o$ rightwards with no apparent change of the slope (Figure 4). These experiments indicated that the toxin decreases the Ca^{2+}-dependent transmitter release without altering the cooperativity of Ca^{2+} in binding to its receptor.

The onset of the quantal content depression was slow ($\approx$20 min), but it was accelerated by either reducing $[Ca^{2+}]_o$ or upon continuous nerve stimulation. In toxin-free muscles, e.p.p. trains elicited at 10–50 Hz presented an early transitory facilitation followed by a decrease of the e.p.p. amplitude to a plateau after the tenth to the fiftieth pulse (Figure 6). The mean amplitude of the first e.p.p. elicited at 10, 25 and 50 Hz in these muscles was 8.71 ± 0.81 mV; 12.63 ± 1.05 mV and 11.77 ± 1.19 mV, respectively. In toxin-treated muscles, the amplitude of the first e.p.p. elicited at 10 to 50 Hz was reduced by 50 to 70% and by 70 to 90% of control in presence of 1 and 2 $\mu g\ ml^{-1}$ PF3 respectively. Unlike control recordings, the profile of the response obtained in presence of PF3 was irregular (Figure 5) and exhibited eventual failures to nerve stimulation at high concentrations (2 and 4 $\mu g\ ml^{-1}$). Nevertheless, despite intense depression of the first e.p.p. amplitude, the toxin facilitated the transmitter release proportionately to the frequency of nerve stimulation (Figures 5 and 6).

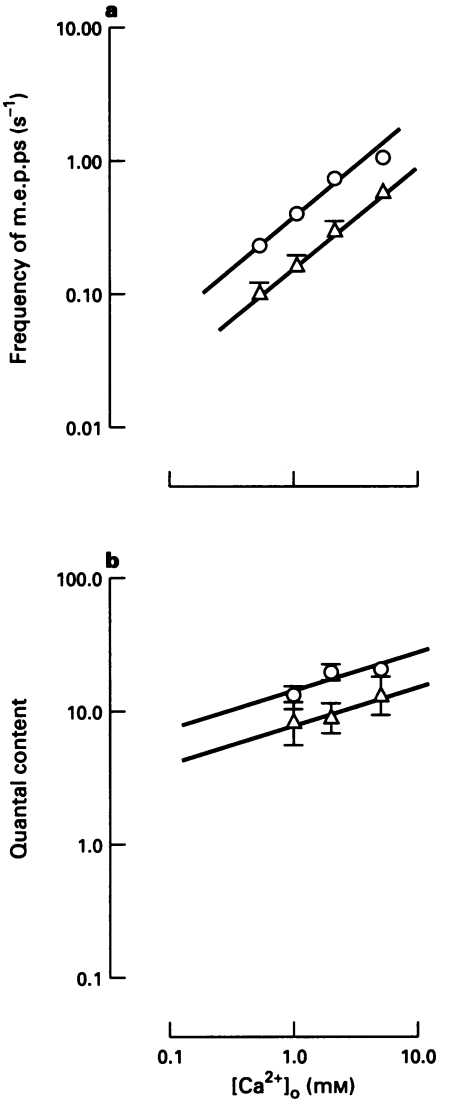

Figure 4 (a) Frequency of miniature endplate potentials (m.e.p.ps) as a function of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) recorded from mouse diaphragm muscle fibres at room temperature (22–25°C), in control preparations (○) and after 30 min exposure to PF3 4 $\mu g\ ml^{-1}$ (△). Symbols are geometric means ± approximate s.e. of 15–24 fibres in 3 muscles. (b) Quantal content of e.p.ps as a function of $[Ca^{2+}]_o$ determined in the 'cut' rat diaphragm muscle fibres in the absence (○) and presence of PF3 1 $\mu g\ ml^{-1}$ (△). Symbols are means ± s.e. of 15 to 22 cells in 3 to 7 muscles.

Table 1 Effect of PF3 on the amplitude of e.p.ps, m.e.p.ps, and the quantal content of e.p.p.s (*m*) determined in the rat 'cut' diaphragm muscle fibres

Condition	*e.p.p. amplitude* (mV)	*m.e.p.p. amplitude* (mV)	m
Control	8.20 ± 0.57	0.36 ± 0.03	23.36 ± 1.95
PF3 (1 $\mu g\ ml^{-1}$)	3.58 ± 0.81*	0.43 ± 0.04	9.39 ± 2.39*
PF3 (4 $\mu g\ ml^{-1}$)	1.75 ± 0.45*	0.38 ± 0.02	4.51 ± 0.96*

Data are means ± s.e.means obtained from 12 to 22 muscle fibres in 3 to 7 preparations. *Significantly different from control ($P < 0.05$).

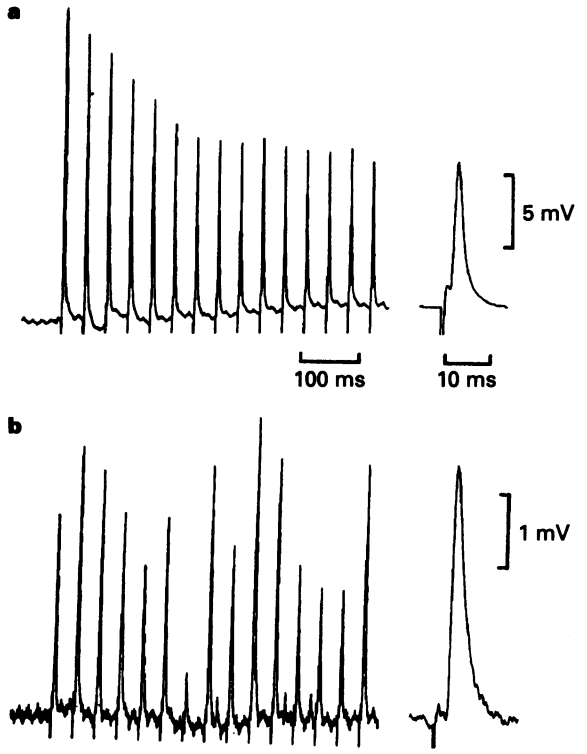

Figure 5 Representative responses to tetanic nerve stimulation of the rat cut diaphragm muscle fibres obtained in control conditions (a) and after exposure to PF3 1 $\mu g\,ml^{-1}$ (b). Shown are the initial 15 responses of endplate potentials (e.p.ps) trains evoked at 25 Hz. The 50th e.p.p. is also shown at a fast sweep recording. Horizontal calibrations are the same for the e.p.p. train and single e.p.p. in (a) and (b).

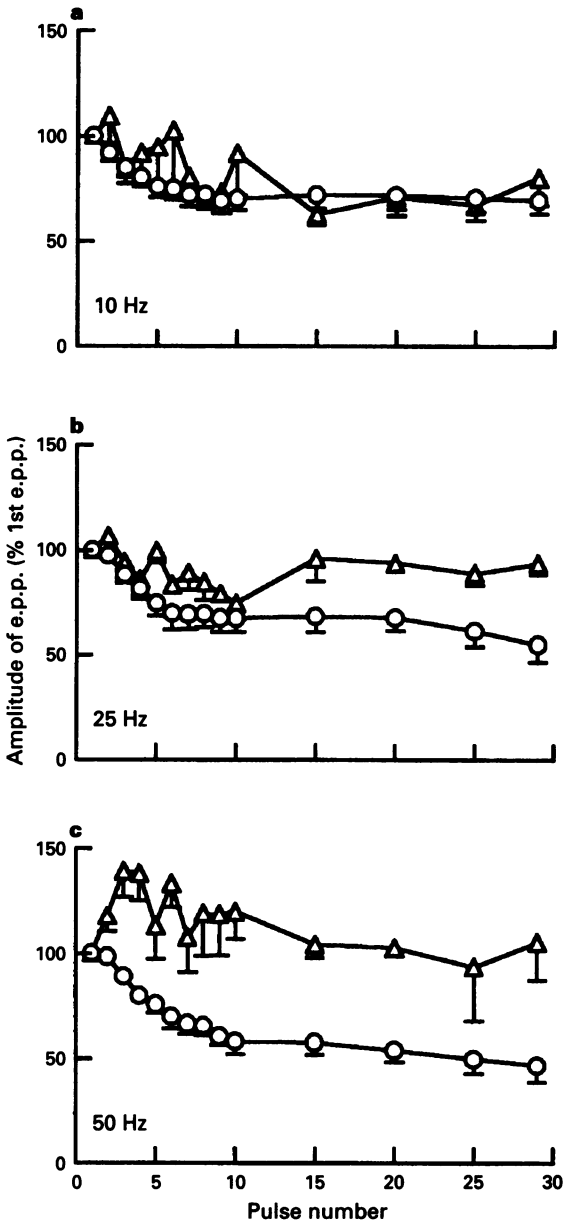

Figure 6 Time course of the decay of endplate potentials (e.p.ps) evoked by nerve stimulation at 10 (a), 25 (b) and 50 Hz (c) of cut diaphragm muscle fibres of the rat, obtained in the absence (○) and presence of PF3 1 $\mu g\,ml^{-1}$ (△). The amplitude of e.p.ps is expressed as a percentage of the first e.p.p. amplitude. Symbols are means of 8 to 15 fibres in 5 muscles.

Discussion

The mechanism involved in muscle relaxation produced by fraction PF3 purified from the *Phoneutria nigriventer* spider venom was investigated on the mouse diaphragm and chick biventer cervicis muscle preparations. PF3 inhibited the single muscle twitch elicited by nerve stimulation of the mouse diaphragm without affecting that induced by direct muscle stimulation, indicating blockade of neuromuscular transmission. In similar preparations, the crude venom produced a dose-related muscle contracture accompanied by blockade of both the direct and nerve-induced muscle twitches. Although the IC_{50} values of PF3 and the crude venom were of the same order of magnitude, the slopes of both dose-response relationships were different (Figure 2) indicating distinct mechanisms underlying the neuromuscular blockade induced by the venom and the purified fraction.

Membrane depolarization, spontaneous muscle twitching, and increase of m.e.p.p. frequency were associated with the neuromuscular blockade induced by the venom in accordance with other studies (Fontana & Vital-Brasil, 1985). Some of these effects may be related to those reported for PhTx2, a fraction that produces most toxic effects of the venom and prevents inactivation of the voltage-dependent sodium channels (Araujo *et al.*, 1993).

The presented data showed that PF3 blocked the neuromuscular transmission in the chick biventer muscle without affecting the postjunctional response to exogenous ACh, indicating a prejunctional effect of the toxin. As observed in the intracellular studies, PF3 produced a long lasting depression of both the spontaneous and nerve-evoked transmitter release without changing the resting membrane potential.

Raising the extracellular concentration of calcium caused a proportional increase of spontaneous ACh release in both control and toxin-treated muscles. The relationship between log m.e.p.p. frequency and log $[Ca^{2+}]_o$ obtained in presence of the toxin, however, was shifted towards higher values of $[Ca^{2+}]_o$ without apparent change in the slope (Figure 4). Spontaneous ACh release has been related to the free intraterminal calcium concentration (Katz & Miledi, 1969), which is determined by the ion influx and regulatory systems of intracellular Ca^{2+} compartments (Blaustein, 1988). Our results therefore indicate that the toxin-induced decrease in m.e.p.p. frequency is unrelated to changes in Ca^{2+} mobilization from intraterminal stores. Thus, it is likely that the toxin acts on a step beyond this event leading to a decrease of exocytosis.

Cyclic AMP is believed to enhance spontaneous transmitter release in motor nerve terminals by regulating the concentration of intracellular calcium and the exocytosis of transmitter (Standaert & Dretchen, 1979). Increase of cyclic AMP with the phosphodiesterase inhibitor theophylline raised the frequency of m.e.p.ps in control diaphragm muscle fibres as reported before (Singer & Goldberg, 1970; Dryden *et al.*, 1988), but failed to counteract the prejunctional effect of PF3. These re-

sults agree with those obtained in the presence of different $[Ca^{2+}]_o$ indicating that PF3 diminishes the spontaneous ACh release by acting on presynaptic events somewhere between the intracellular mobilization of Ca^{2+} and exocytosis. Potential sites of action would include synaptic vesicle phosphoproteins, plasma membrane and cytoplasmic proteins (Greengard *et al.*, 1993).

PF3 also reduced the frequency of m.e.p.ps in muscle preparations depolarized with a high-K^+ medium, which promotes Ca^{2+} influx into the nerve terminals through voltage-gated calcium channels (Csillik, 1993). This observation suggested that besides lowering the rate of spontaneous ACh release, the toxin might block the neuronal Ca^{2+} current as well and affect the evoked-transmitter release.

The effect of PF3 on transmitter released upon nerve stimulation was more intense than on that released spontaneously. Indeed, a significant decrease of the quantal content was detected at a toxin concentration (1 $\mu g\ ml^{-1}$) that did not affect the frequency of m.e.p.ps. As observed on m.e.p.p. frequency, PF3 shifted the relationship between log m and log $[Ca^{2+}]_o$ to the right of that obtained in control conditions without changing the slope, suggesting that the cooperativity of calcium action in the release process was not altered. These data are in accord with those obtained in K^+-depolarized muscles indicating that PF3 inhibits the voltage-dependent Ca^{2+} influx into the nerve terminals, which is required for the nerve stimulated ACh release (Csillik, 1993).

PF3 produced a concentration-dependent depression of the quantal content of e.p.p. The effect was exacerbated upon continuous nerve stimulation at low frequency (0.5 Hz), suggesting a use-dependent action. When trains of e.p.ps were elicited at high frequencies of nerve stimulation, however, facilitation of the e.p.p. amplitude related to the frequency of stimulation was noted. Similar facilitation of transmitter release induced by tetanic stimulation obtained in a low-calcium medium (Mallart & Martin, 1968; Katz & Miledi, 1968) was attributed to residual calcium provided by a previous pulse (Katz & Miledi, 1968; Zengel & Magleby, 1982). This observation favours the hypothesis that PF3 also acts prejunctionally by reducing the Ca^{2+} influx in the nerve terminal. As a consequence, the nerve-evoked transmitter release is depressed and irregular, but is facilitated upon high frequencies of nerve-stimulation.

Reduction of the nerve-elicited ACh release similar to that obtained with PF3 was also reported in mouse muscles exposed to omega-Agatoxin (ω-Aga-IVA), a peptide derived from the venom of the funnel web spider *Agelenopsis aperta* that blocks presynaptic Ca^{2+} currents (Protti & Uchitel, 1993; Uchitel & Protti, 1994). Similar patterns of deficient quantal transmitter release were also reported in mice treated with immunoglobulin G from patients with Lambert-Eaton myasthaenic syndrome, an autoimmune disease characterized by a reduced nerve-evoked transmitter release caused by autoantibodies against the voltage-dependent calcium channel (Lang *et al.*, 1984; Sher *et al.*, 1991).

In conclusion, fraction PF3 purified from the venom of a South American spider, *Phoneutria nigriventer* blocked the neuromuscular transmission in mammalian and avian muscles producing muscle relaxation. The effect was related to a blockade of the nerve-evoked quantal transmitter release, with no significant influence on the postjunctional nicotinic ACh receptors. PF3 also blocked the spontaneous ACh release without an apparent change of Ca^{2+} mobilization from intracellular stores. The results indicated that PF3 inhibited the nerve stimulated ACh release by reducing the intraneuronal influx of Ca^{2+} through voltage-gated calcium channels, and by affecting presynaptic events at a stage beyond calcium mobilization from intraneuronal stores causing a reduced exocytosis.

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