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ORIGINAL ARTICLE

Harry I. Wilson · Graham M. Nicholson

Presynaptic snake β-neurotoxins produce tetanic fade and endplate potential run-down during neuromuscular blockade in mouse diaphragm

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Abstract The present study investigated the ability of a number of presynaptic snake neurotoxins (snake β-neurotoxins) to produce nerve-evoked train-of-four fade, tetanic fade and endplate potential run-down during the development of neuromuscular blockade in the isolated mouse phrenic-hemidiaphragm nerve-muscle preparation. All the snake β-neurotoxins tested, with the exception of notexin, produced train-of-four and tetanic fade of nerve-evoked isometric muscle contractions. Train-of-four fade was not present during the initial depressant or facilitatory phases of muscle tension produced by the snake β-neurotoxins but developed progressively during the final depressant phase that precedes complete neuromuscular blockade. The 'non-neurotoxic' bovine pancreatic phospholipase A_2 and the 'low-toxicity' phospholipase A_2 from *Naja naja atra* venom failed to elicit train-of-four fade, indicating that the phospholipase activity of the snake β-neurotoxins is not responsible for the development of fade.

Intracellular recording of endplate potentials (EPPs) elicited by nerve-evoked trains of stimuli showed a progressive run-down in EPP amplitude during the train following incubation with all snake β-neurotoxins except notexin. Again this run-down in EPP amplitude was confined to the final depressant phase of snake β-neurotoxin action. However when EPP amplitude fell to near uniquantal levels (<3 mV) the extent of toxin induced-fade was reduced.

Unlike postjunctional snake α-neurotoxins, prejunctional snake β-neurotoxins interfere with acetylcholine release at the neuromuscular junction during the development of neuromuscular blockade. This study provides further support for the hypothesis that fade in twitch and tetanic muscle tension is due to an underlying rundown in EPP amplitude resulting from a prejunctional alteration of transmitter release rather than a use-dependent block of postjunctional nicotinic receptors.

Key words Presynaptic · Snake neurotoxin · β-neurotoxin · Neuromuscular junction · Endplate potential run-down · Train-of-four fade · Tetanic fade · Tubocurarine

H.I. Wilson · G.M. Nicholson (✉)
Department of Health Sciences, University of Technology, Sydney, City Campus, P.O. Box 123, Broadway NSW 2007, Australia

Introduction

Non-depolarising nicotinic antagonists such as (+)-tubocurarine produce both a competitive antagonism of postjunctional nicotinic receptors (Colquhoun and Sheridan 1982) and a waning or 'fade' of tetanic muscle contractions (Naess 1952). Tubocurarine-induced fade of muscle tension produced by tetanic or train-of-four nerve stimulation is caused by a run-down of successive endplate potentials (EPPs) or endplate currents (EPCs) during repetitive stimulation (Glavinovic 1979; Gibb and Marshall 1984). The phenomenon of fade and run-down has been ascribed to a progressive reduction in the release of acetylcholine from the motor nerve terminal (Bowman 1980; Bowman et al. 1986; Chang et al. 1988; Apel et al. 1995). Bowman and co-workers (1986) have suggested that this prejunctional phenomenon arises because (+)-tubocurarine and similar competitive nicotinic antagonists block a prejunctional nicotinic autoreceptor responsible for a positive feedback mechanism to mobilise 'reserve' stores of acetylcholine to a 'releasable' store and thus maintain transmitter release during physiological high-frequency nerve activity. Blockade of this autoreceptor would inhibit the feedback control of acetylcholine release resulting in run-down or 'fade' in EPPs and muscle tension respectively. This prejunctional mechanism is further supported by the work of Hong and Chang (1991) who showed, using (+)-tubocurarine and cobratoxin, that the quantal response (MEPP) of the postjunctional acetylcholine receptor was not depressed in parallel with the run-down of EPP amplitude during

tetanic stimulation. In addition (+)-tubocurarine depresses the responses of trains of acetylcholine applied iontophoretically without any run-down (Gibb and Marshall 1984). In addition Apel et al. (1995) have shown that (+)-tubocurarine causes a reduction in the nerve evoked ^{3}H-acetylcholine release from rat nerve terminals. These studies strongly argue against a postjunctional mechanism involving use-dependent failure of nicotinic receptors.

Other postjunctional competitive blockers have similar actions in producing fade and run-down, however, the extent of fade or run-down and the phase of drug or toxin action during which fade develops differs between antagonists. This is most prominent with postsynaptic α-neurotoxins isolated from snake venoms such as erabutoxin-b, α-bungarotoxin and cobratoxin. These snake α-neurotoxins cause the blockade of postjunctional nicotinic receptors without inducing fade or marked run-down (Gibb and Marshall 1984; Bowman et al. 1986) but upon washout fade becomes prominent and persists after the complete recovery from the postjunctional inhibitory effect (Bradley et al. 1987, 1990; Chang and Hong 1987; Cheah and Gwee 1988). This indicates that tetanic fade or EPP run-down, can be dissociated from the blockade of postjunctional nicotinic receptors suggesting that snake α-neurotoxins bind at a prejunctional receptor with slow association/dissociation kinetics (Bradley et al. 1987, 1990; Chang and Hong 1987; Cheah and Gwee 1988; Hong and Chang 1991). Interestingly however ^{3}H-acetylcholine release is unaffected by these agents arguing that snake α-neurotoxins do not block prejunctional nicotinic autoreceptors (Apel et al. 1995).

The actions of presynaptic snake phospholipase A_2 (PLA_2) neurotoxins (snake β-neurotoxins) on tetanic fade or EPP run-down are less well defined. β-Bungarotoxin (Lee 1972; Chang et al. 1973), textilotoxin (Su et al. 1983) and crotoxin (Chang and Lee 1977) have been reported to produce neuromuscular blockade without fade at low quantal contents. On the other hand Kamenskaya and Thesleff (1974) showed evidence at higher quantal contents (refer to Fig. 6 of their paper) that taipoxin induces fade with a characteristic reduction in EPP amplitude in response to tetanic stimulation at 50 Hz. However they made no further investigation of this observation.

Previous preliminary investigations in our laboratory have shown that the snake β-neurotoxin textilotoxin (Wilson et al. 1995) disrupts the ability of the nerve terminal to maintain normal rates of acetylcholine release during periods of high frequency nerve stimulation by displaying train-of-four fade (Nicholson et al. 1992). The aim of the present study was therefore to further investigate these actions of textilotoxin and to compare it with other snake β-neurotoxins. The effects of *Naja naja atra* and bovine pancreatic PLA_2 were also examined in order to determine if the PLA_2 enzymic activity, characteristic of snake β-neurotoxins, is required for these actions.

Materials and methods

Nerve-muscle preparations. Phrenic-hemidiaphragm nerve-muscle preparations isolated from adult Quakenbush-Swiss mice, of either sex (weighing 20–30 g), were used for both the twitch tension and intracellular experiments. Isolated nerve-muscle preparations were placed in organ baths and perfused with Tyrodes solution of the following composition (mM): NaCl 137; $NaHCO_3$ 23.8; KCl 3.5; NaH_2PO_4 0.6; $CaCl_2$ 1.8; D-Glucose 11.1; $MgCl_2$ 1.2; pH 7.4±0.1 and aerated with 95% O_2/5% CO_2. All experiments were conducted at ambient room temperature (23±1°C). Toxins, bovine pancreatic PLA_2 or (+)-tubocurarine, dissolved in the minimum amount of Tyrodes solution (20–50 μl), were applied by direct injection into the organ bath after at least a 30 min period of stable control recordings.

Muscle contraction experiments. Preparations were mounted horizontally in a 3 ml organ bath. The free end of the diaphragm muscle was attached to an isometric force-displacement transducer (UFI Dynamometer), and adjusted to 110% of its *in vivo* resting length. The output of the force transducer was displayed on a chart recorder (Neomedix NeoTrace 200ZEF) fitted with a variable gain DC amplifier. Twitch contractions of the diaphragm muscle were elicited by supramaximal stimulation of the phrenic nerve using bipolar platinum stimulating electrodes delivering 8-30 V square wave pulses of 0.05–0.1 ms duration.

The effect of snake β-neurotoxins on train-of-four fade was assessed using indirect twitch contractions of the muscle to short trains of stimuli evoked using a train-of-four stimulation protocol (2 Hz for 2 s every 20 s). The amplitude of the first twitch (t_1) in the train during the experiment was compared with twitch tension during the control period as a measure of the extent of neuromuscular blockade. The tension generated by the fourth twitch (t_4) in the train was compared against the first twitch (t_1) of the train as a measure of the amount of fade in the response. Fade was calculated using the following formula:

$$\%\ \text{train-of-four fade} = 100 \times \left(1 - \frac{t_4}{t_1}\right) \quad (1)$$

The % train-of-four fade was obtained from the average of three consecutive trials every ten minutes during the train-of-four stimulation and correlated with time after administration of toxin and the extent of neuromuscular blockade.

In separate experiments the ability of the mouse phrenic-hemidiaphragm nerve-muscle preparation to maintain tetanic contractions in response to a 3 s tetanic train of stimuli in the presence of snake β-neurotoxins was investigated. The phrenic nerve received supramaximal stimulation at 50 Hz for 0.1 s every 20 s for the duration of the experiment. Control tetanic responses were elicited by stimulating the nerve at 50 Hz for 3 s. These were at least 10 min apart, in order to preclude any contributing effects of post-tetanic potentiation (Hubbard 1963). Following addition of toxin to the bath a single 50 Hz, 3 s train was interspersed at 25%, 50% and 75% neuromuscular blockade. The amount of fade in the tetanic response was calculated by comparing the amplitude of the initial and final tension during the tetanic train. Tetanic fade was

$$\%\ \text{tetanic fade} = 100 \times \left(1 - \frac{t_{final}}{t_{initial}}\right) \quad (2)$$

where t_{final} and $t_{initial}$ represent amplitudes of the end and the beginning of the tetanic contraction respectively.

Intracellular experiments. Intracellular recordings of EPPs were made from isolated phrenic-hemidiaphragm nerve-muscle preparations mounted horizontally in a 4 ml organ bath and perfused with aerated Tyrodes solution at a rate of 1–2 ml/min. The phrenic

nerve was drawn into a tight-fitting suction electrode and EPPs elicited by supramaximal square wave pulses, as described above, and recorded using borosilicate glass microelectrodes filled with 3M KCl (15–30 MΩ). All recordings were made from focal positions of surface cells with rise times less than 1.5 ms. Muscle contractions were prevented by using 1.2–1.5 μM μ-conotoxin (Moczydlowski et al. 1986; Hong and Chang 1989). The effects of the snake β-neurotoxins could thus be investigated under conditions allowing normal neurotransmitter release. EPPs of up to 45 mV were regularly recorded using this procedure without initiating muscle contractions. EPPs were recorded using an AxoProbe-1A amplifier (Axon Instruments, USA). Data were filtered at 1 kHz and then amplified 100× for display and data acquisition. The amplified signal was digitised at between 10 and 20 kHz and stored on hard disk drive using the AxoTape© (Axon Instruments, Foster City, Calif., USA) or MacLab© (Analogue Digital Instruments, New Zealand) data acquisition systems. Data analysis was performed off-line using a modification of the Fetchan protocol of the pClamp© program.

Actions of the snake β-neurotoxins on EPP run-down were assessed using a train of ten EPPs following tetanic stimuli at 50 Hz applied for 200 ms once every 20 s. The amount of run-down in EPP amplitude was quantified by comparing the amplitude of the first EPP (EPP_1) with the amplitude of the tenth EPP (EPP_{10}) as follows:

$$\% \text{ EPP rundown} = 100 \times \left(1 - \frac{EPP_{10}}{EPP_1}\right) \quad (3)$$

Occasional spontaneous contractions during the enhancement phase of transmitter release produced by the snake β-neurotoxin action prevented the recording of EPPs from a single impaled muscle cell. Accordingly a group sampling method was adopted. Thus trains of EPPs were recorded from between fifteen to twenty endplates in each twenty minute period until complete blockade of transmission. The responses at all endplates in a group were then averaged and analysed. Both the extent of neuromuscular blockade, as determined from the changes in EPP_1 amplitude, and the extent of % EPP run-down were averaged from these cells over each 20 min period.

Statistical analysis. All data are presented as mean ± standard error of the mean (SEM). *n* refers to the total number of experiments. Comparisons of two sample means were made using an unpaired Welch's *t*-test. A test was considered to be significant when $P<0.05$.

Drugs and toxins. Supplies of μ-conotoxin GIIIB were obtained from Peptide Institute Inc. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., USA). Toxins were assayed for purity by the supplier using HPLC. β-Bungarotoxin, bovine pancreatic PLA_2, and (+)-tubocurarine chloride were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Lyophilised textilotoxin, taipoxin and notexin were purchased from Venom Supplies, P.O. Box 547, Tanunda, South Australia. These venoms were purified by gel filtration chromatography as previously described (Fohlmann et al. 1976; Su et al. 1983). *Naja naja atra* PLA_2 was a kind gift from Dr. C.C. Yang of Taiwan National University, Taipei. Lyophilised venoms were stored at –20°C.

Results

Train-of-four fade

To investigate the ability of snake β-neurotoxins to produce train-of-four fade, twitch contractions of the isolated mouse phrenic-hemidiaphragm nerve-muscle preparation were recorded in response to indirect nerve stimulation using a train-of-four stimulation protocol. Control experiments in the absence of toxins exhibited a small degree of train-of-four fade which ranged from 2 to 10% (mean ± SEM = 4.0±0.5%, $n = 25$). The extent of fade was, however, constant during control experiments of up to 3 h duration.

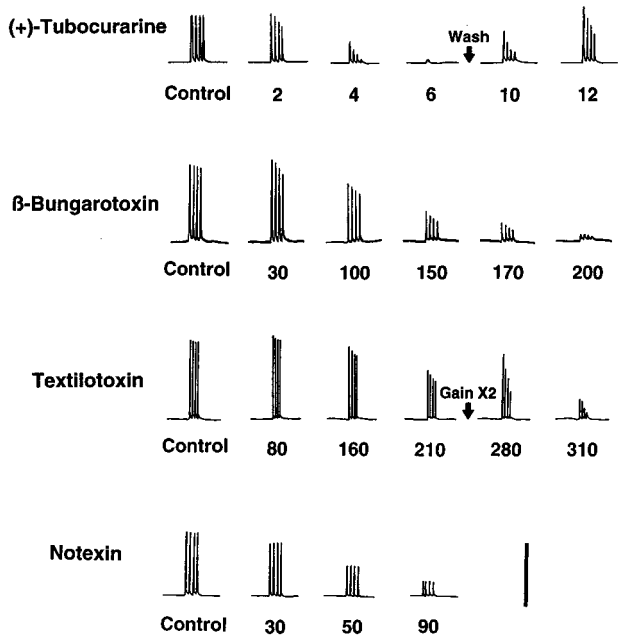

Fig. 1 Differenfial effects of snake β-neurotoxins on twitch contractions of the mouse phrenic-hemidiaphragm nerve-muscle preparation. Indirect contractions of the muscle were elicited using a train-of-four protocol of stimuli at 2 Hz for 2 s every 20 s. Numbers beneath each trace represents the time after the addition of drug or toxin. The force calibration represents 5 g tension. Traces represent typical experiments using 2 μM (+)-tubocurarine, 20 μg ml^{-1} β-bungarotoxin, 10 μg/ml textilotoxin and 10 μg/ml notexin

The classical postjunctional nicotinic antagonist (+)-tubocurarine produced a rapid reversible depression of initial twitch amplitude (t_1) accompanied by an increasing degree of train-of-four fade. Figure 1a illustrates this rapid depression of twitch contractions using 2 μM (+)-tubocurarine. At a concentration of 1.5 μM (+)-tubocurarine produced 95% neuromuscular blockade at 26±2 min ($n = 3$) after the addition of drug. 100% train-of-four fade could be seen by 6±1 min ($n = 3$). Upon washout of (+)-tubocurarine with drug-free Tyrodes solution, train-of-four fade was more prominent than during the development of neuromuscular blockade, despite the progressive recovery of the initial twitch contraction (see Fig. 1a).

Neuromuscular blockade with the snake β-neurotoxins developed slowly (Figs. 1 and 2). 95% Neuromuscular blockade was achieved after only 198±6 min with 20 μg/ml β-bungarotoxin ($n = 4$), 290±11 min for 10 μg/ml textilotoxin ($n = 3$), 163±2 min for 10 μg/ml taipoxin ($n = 3$), and 101±9 min for 10 μg/ml notexin ($n = 5$). Neuromuscular blockade was also characterised

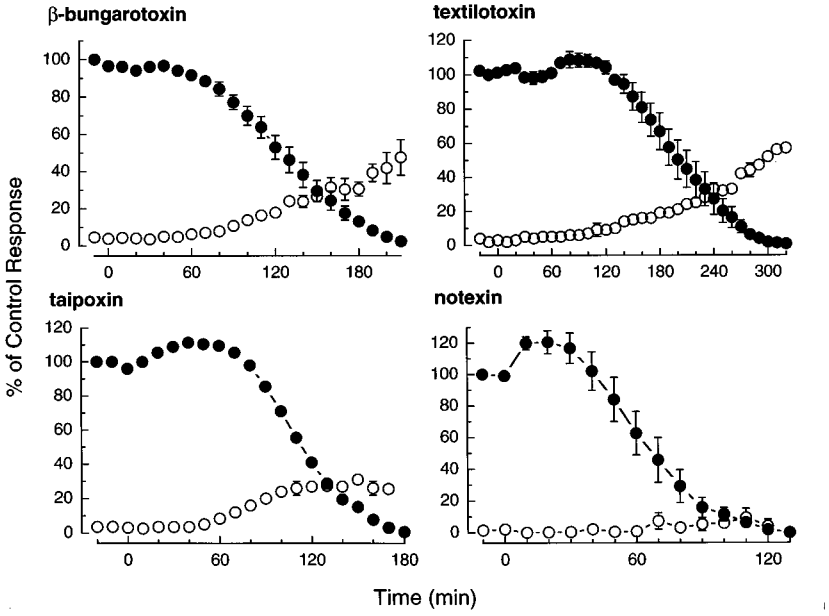

Fig. 2 Time course of train-of-four fade and neuromuscular blockade by snake β-neurotoxins in the mouse phrenic-hemidiaphragm nerve-muscle preparation. The phrenic nerve was stimulated at 2 Hz for 2 s every 20 s and % train-of-four fade (○) calculated from Eq. 1 (see Materials and methods) every 10 min. In addition neuromuscular blockade (●) was also determined from the % change in twitch contraction amplitude (t_1) every 10 min. Values represent the mean ± SEM after the addition of 20 μg/ml β-bungarotoxin (n = 4), 10 μg/ml textilotoxin (n = 4), 10 μg/ml taipoxin (n = 4), 10 μg/ml notexin (n = 5)

by triphasic changes in twitch tension amplitude (t_1) typical of the actions of these snake β-neurotoxins (Chang and Lee 1963; Harris et al. 1973; Kamenskaya and Thesleff 1974; Wilson et al. 1995). These phases were an initial depression of twitch tension, followed by a period of enhanced twitch contractions, and then an eventual reduction in muscle tension to complete neuromuscular blockade.

All the snake β-neurotoxins, with the exception of notexin, exhibited marked train-of-four fade during the development of neuromuscular blockade (see Fig. 1b–d). Significant train-of-four fade was not present during the initial depression phase or the subsequent enhancement of muscle tension produced by the snake β-neurotoxins but developed progressively during the final phase prior to complete neuromuscular blockade. Figure 2a–d shows the correlation between train-of-four fade and neuromuscular blockade. Textilotoxin produced the greatest degree of fade at 95% neuromuscular blockade (54±10%, n = 3), followed by β-bungarotoxin (48±10%, n = 4), and taipoxin (27±1%, n = 3). Notexin consistently failed to produce any significant increase in train-of-four fade from that seen during the control period, even in the final stages immediately prior to complete neuromuscular blockade.

To determine whether the actions of the snake β-neurotoxins to produce train-of-four fade were due to the PLA_2 enzymatic activity of the toxins, a comparison was made with the effects of two PLA_2 enzymes with relatively high enzymatic activity. The 'low-toxicity' PLA_2 isolated from *Naja naja atra* venom (20 μg/ml) produced 95% neuromuscular blockade after 167±9 min (n = 3) but at no time during the experiments did train-of-four fade increase over that recorded during the control period. The 'non-neurotoxic' bovine pancreatic PLA_2 was also examined at concentrations from 20 to as high as 300 μg/ml. In no experiment was there a depression of twitch tension or any development of fade.

Tetanic fade

In order to examine if snake β-neurotoxins, particularly notexin, produce a greater degree of fade at more physiological frequencies of nerve stimulation, fade in muscle tension was investigated in response to a 3 s, 50 Hz tetanic train which placed greater demands on the process of transmitter release. Tetanic fade in controls was minimal in all experiments with a range of 4% to 13% (7±1%, n = 23). This lack of significant tetanic fade can be seen in the control traces of Fig. 3a–e. A triphasic pattern of alteration in muscle contractions, similar to that observed with the train-of-four fade twitch experiments, was also seen with the β-neurotoxins (data not shown). Typically after an initial 10–15 min delay, there was a decrease in muscle tension of approximately 10%, followed by an enhancement of muscle tension to 120–130% of control, and then a gradual decline to complete neuromuscular blockade. In addition contractions evoked by the short pulses immediately following the tetanic train showed post-tetanic potentiation, which was especially obvious in the later stages of neuromuscular blockade with β-neurotoxins.

Several differences were noted between the actions of (+)-tubocurarine and the snake toxins on tetanic fade and train-of-four fade. The time to reach 95% neuromuscular blockade in the presence of the snake β-neurotoxins was also significantly less with tetanic stimulation than with train-of-four stimulation. This is undoubtedly due to the higher stimulation rates employed in the tetanic fade experiments, given that the neuromuscular blocking actions of snake β-neurotoxins are frequency-dependent (Chang

et al. 1973; Kamenskaya and Thesleff 1974; Chang and Lee 1977; Lloyd et al. 1991). Also there was a greater extent of fade in the tetanic fade experiments compared to that seen in the train-of-four fade experiments. In the case of taipoxin, which produced the greatest degree of tetanic fade, this fade developed rapidly during the tetanic train and reached 92±4% (n = 5) at 75% neuromuscular blockade with tetanic fade reaching 100% at 75% neuromuscular blockade in one experiment. Textilotoxin and β-bungarotoxin also showed an increase in the magnitude, and a more rapid time course, of tetanic fade as compared with the train-of-four fade experiments (see Table 1). In contrast to the train-of-four fade experiments, notexin did produce some tetanic fade in tetanic tension particularly in the latter stages of experiments. The extent of tetanic fade with notexin was considerably less, however, than with the other β-neurotoxins tested, only reaching 53±9% at 75% neuromuscular blockade. Importantly, the absolute fall in tension from the beginning to the end of the train did not increase during the course of experiments with notexin (refer to Fig. 3e). In the presence of 1 μM (+)-tubocurarine the isometric tension rapidly faded to a plateau level (Fig. 3a). With β-bungarotoxin, textilotoxin and taipoxin tetanic fade developed gradually in the initial 500 ms to 1000 ms of the tetanic train to a plateau that was maintained to the end of the train (Fig. 3b–d). In contrast incubation with notexin caused the tetanic tension to continually decline to the end of the 3 s tetanic train (Fig. 3e).

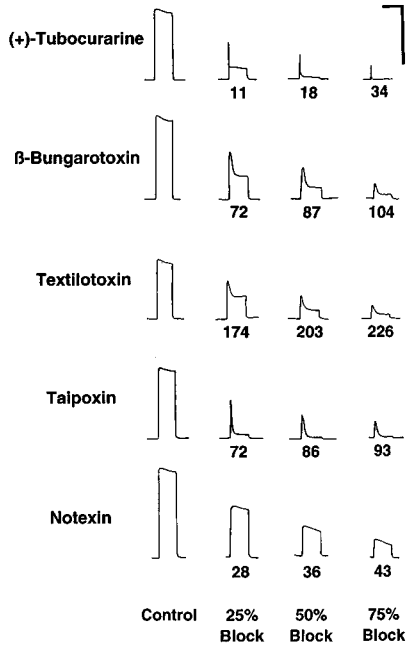

Fig. 3 Snake β-neurotoxins produce an increase in tetanic fade in the mouse phrenic-hemidiaphragm nerve-muscle preparation. The phrenic nerve was stimulated with a 50 Hz 3 s train. Representative muscle tension records showing the extent of tetanic fade at 25%, 50% and 75% neuromuscular blockade in response to (+)-tubocurarine (1 μM), 20 μg/ml β-bungarotoxin, 10 μg/ml textilotoxin, 10 μg/ml taipoxin and 10 μg/ml notexin. The force calibration represents 5 g tension and 3 s duration. Numbers beneath each trace indicate the time in minutes after the addition of the drug or toxin

EPP run-down

The very nature of isometric muscle tension experiments makes it impossible to examine the phenomenon of fade at low transmitter release levels immediately prior to complete neuromuscular blockade. This is because individual muscle fibres will not contract once the EPP amplitude falls below the threshold for sodium channel activation and subsequent muscle action potential generation. The actions of the snake β-neurotoxins were therefore further examined using intracellular recording techniques in the mouse phrenic-hemidiaphragm nerve-muscle preparation to assess if they produced a run-down in postjunctional potentials during tetanic stimulation. In addition it was necessary to examine acetylcholine release and the development of run-down in EPPs at normal quantal contents to enable the observation of small, graded changes in EPP amplitude. This would not be possible in situations where quantal content was reduced by, for example, the use of low Ca^{2+}/high Mg^{2+} physiological solutions.

In order to record large amplitude EPPs, muscle contractions were abolished by preventing the generation of

Table 1 Extent and time course of tetanic fade and neuromuscular blockade produced by snake β-neurotoxins

Toxin	Concentration	Time to 95% NMB[a]	Fade[b] in Control	Fade at 25% NMB[c]	Fade at 50% NMB	Fade at 75% NMB
(+)-Tubocurarine	1μM	not reached	5±1	62±8**	88±7**	91±7**
β-Bungarotoxin	20μg/ml	188±30	12±3	47±6*	62±6**	73±4**
Textilotoxin	10μg/ml	252±38	8±1	48±4*	57±3**	63±2**
Taipoxin	10μg/ml	156±3	8±1	80±7**	87±5**	92±4**
Notexin	10μg/ml	70±9	6±1	15±6	33±7*	53±9*

[a] Time to 95% neuromuscular blockade (NMB) of muscle contractions

[b] Tetanic fade was evoked by a 50 Hz train of pulses for 3 s and % tetanic fade calculated using Eq. 2 (see Materials and method)

[c] The amount of tetanic fade was assessed at 25%, 50% and 75% neuromuscular blockade (NMB)

n, number of experiments, * P<0.05 vs control fade, ** P<0.01 vs control fade determined by unpaired Welch's t-test

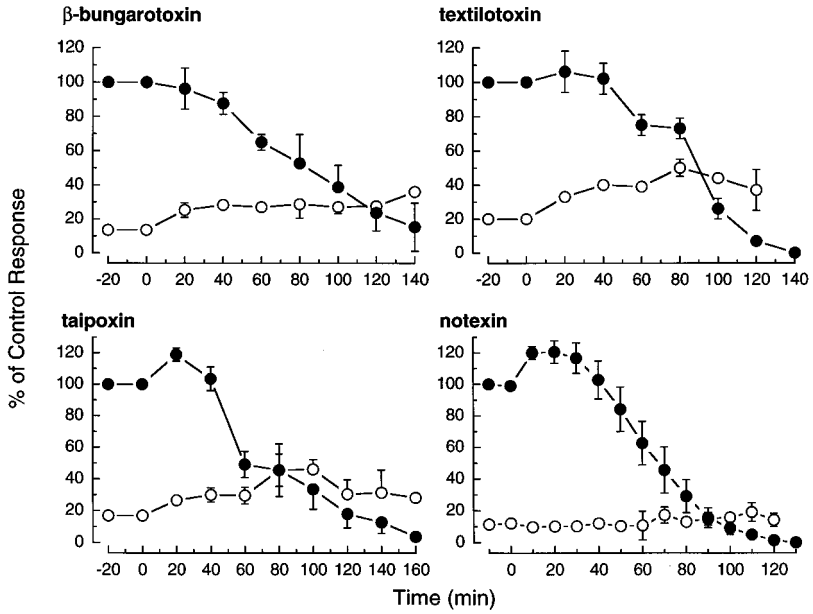

Fig. 4 Time course of EPP run-down and neuromuscular blockade by snake β-neurotoxins in the mouse phrenic-hemidiaphragm nerve-muscle preparation. The phrenic nerve was stimulated at 50 Hz for 200 ms every 20 s and % EPP run-down (○) calculated from Eq. 3 (see Materials and methods) by averaging data within a 20 min period. Neuromuscular blockade (●) was also determined from the average reduction of the first EPP amplitude (EPP_1) during the same 20 min period. Values represent the mean ± SEM following the addition of 20 μg/ml β-bungarotoxin (n = 4), 10 μg/ml textilotoxin (n = 4), 10 μg/ml taipoxin (n = 5), and 10 μg/ml notexin (n = 5)

muscle action potentials with μ-conotoxin, a specific antagonist of muscle sodium channels. This 22-amino acid peptide toxin binds to neurotoxin receptor site 1 of the sodium channel and has been reported to show a 1,000 fold specificity for skeletal muscle over neuronal sodium channel subtypes (Moczydlowski et al. 1986) although later reports have suggested a much lower selectivity of approximately threefold (Hong and Chang 1989). Undistorted EPPs without muscle contractions could therefore be evoked by perfusion with 1.2–1.5 μM μ-conotoxin. Control experiments were necessary to ascertain any contribution made by μ-conotoxin to EPP run-down. In the presence of μ-conotoxin the average EPP amplitude, the extent of EPP run-down and the resting membrane potential did not change significantly over the course of 2–3 h. Initially EPP run-down was 16±1% (n = 3) whilst after 1 h it was 16±3% (n = 3), and was 16±4% after 2 h (n = 3). This lack of an effect of μ-conotoxin on EPP run-down is in agreement with the findings of Hong and Chang (1989).

The effects of the snake β-neurotoxins on the time course of neuromuscular blockade and EPP run-down are presented in Fig. 4. As in the tetanic fade tension experiments, 95% neuromuscular blockade developed more rapidly than in the train-of-four tension experiments due to the frequency-dependent nature of toxin action. In the presence of the snake β-neurotoxins it was also found that the responses of neighbouring cells in the same muscle were often quite different in terms of both the extent of neuromuscular blockade, and the amount of EPP run-down present. A small proportion of cells were more resistant to the actions of the snake β-neurotoxins on transmitter release. The response of different cells became increasingly variable as neuromuscular blockade developed such that it was always possible to find some cells whose endplates still showed EPPs after the majority of cells were blocked. For example, particularly during the latter stages of the enhancement phase of toxin action some cells exhibited EPPs up to 45 mV in amplitude, whilst neighbouring cells had EPPs of only 2–5 mV. The influence of these resistant cells may have prolonged the final stages to complete neuromuscular blockade. This was most evident with textilotoxin which caused the most cell-to-cell variability.

All snake β-neurotoxins, except notexin, also showed significant increases in EPP run-down over controls. Interestingly however the extent of EPP run-down declined during the latter stages of each experiment. This was most evident with taipoxin and textilotoxin (see Fig. 5). During the latter stages of neuromuscular blockade, there were a large number of endplates with few quanta being released (EPPs around 0.5–2.5 mV). These endplates tended to show far less EPP run-down than some cells that, at the same time, had higher quantal release. This can be seen in Fig. 5d from an experiment using 10 μg/ml textilotoxin. When EPP_1 amplitude fell to near uniquantal level, subsequent EPPs showed marked variations in amplitude, and fade was not evident. Similar results were seen in experiments using taipoxin, but were not as marked in experiments with β-bungarotoxin.

Discussion

The present study represents the first systematic investigation of tetanic fade and EPP run-down with snake β-neurotoxins, and shows that snake β-neurotoxins, along with nicotinic antagonists such as (+)-tubocurarine and cobratoxin, can produce marked fade in muscle tension during neuromuscular blockade resulting from a run-down in EPP amplitude. These snake β-toxins have previously been shown to produce triphasic alterations in transmitter release by way of a prejunctional rather than a postjunctional mechanism (Chang et al. 1973; Kamens-

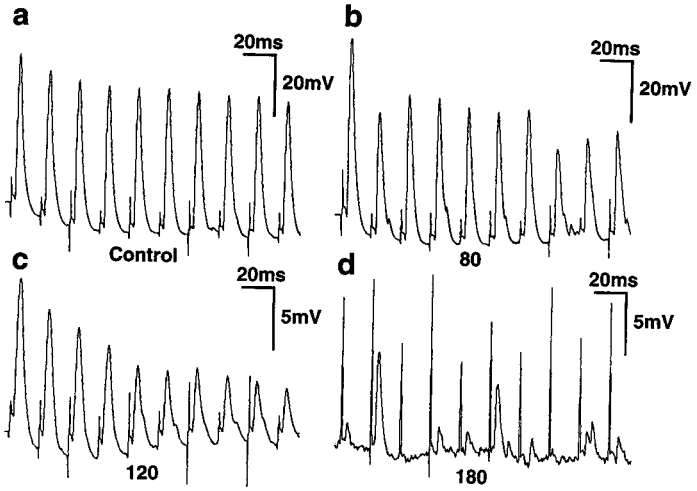

Fig. 5a–d Typical effects of textilotoxin on EPP run-down in the mouse phrenic-hemidiaphragm nerve-muscle preparation. Muscle contractions were paralysed with 1.2 μM μ-conotoxin. EPPs were indirectly evoked by trains of pulses at 50 Hz for 200 ms. **a** Control, **b** 80 min, **c** 120 min, **d** 180 min after the addition of 10 μg/ml textilotoxin. Resting membrane potential was –80 mV in **a** and –85 mV in **b**, **c** and **d**

kaya and Thesleff 1974; Wilson et al. 1995). This further supports a prejunctional origin for the phenomena of EPP run-down and fade in muscle responses.

Since train-of-four and tetanic fade are believed to arise from an underlying run-down in EPP amplitude (Bowman et al. 1986; Chang and Hong 1987) we investigated the ability of snake β-neurotoxins to produce run-down using the selective skeletal muscle sodium channel blocker μ-conotoxin to directly inhibit muscle contractions allowing us to examine the effects of the snake β-neurotoxins on EPPs of normal quantal content. The present study shows that snake β-neurotoxins produce EPP run-down during the final depressant phase, before complete neuromuscular blockade. In addition the resting membrane potential did not significantly change during the 50 Hz trains nor did it change during the course of the experiment, backing up previous studies using the same snake β-neurotoxins (Harris et al. 1973; Kamenskaya and Thesleff 1974; Livengood et al. 1978; Wilson et al. 1995). Thus EPP run-down produced by snake β-neurotoxins is not the result of changes in membrane potential during the 50 Hz train which may progressively decrease the driving force on permeant ions through the postjunctional nicotinic receptor.

Several objections have been raised to the idea of a prejunctional origin of the phenomena of fade and EPP run-down. Firstly Ginsbourg and Jenkinson (1976) speculated that (+)-tubocurarine induces tetanic fade as a result of unmasking an inherent run-down in acetylcholine release during repetitive nerve stimulation. Normally, this run-down is concealed since EPP amplitude approaches the reversal potential for acetylcholine and is nonlinear due to the high transmitter release levels and the profuse numbers of postjunctional acetylcholine receptors. Thus when quantal content is reduced, with postjunctional blockers such as (+)-tubocurarine, EPP run-down and subsequent fade in muscle contractions become evident. The presynaptic hypothesis (Bowman 1980) conversely would predict that if few quanta are released the size of the 'releasable' pool should not be significantly reduced and thus EPP run-down and subsequent fade should be less evident. In the present study all the snake β-neurotoxins tested showed less EPP run-down at low quantal release (see Fig. 4d) consistent with the prejunctional hypothesis of Bowman (1980). Several previous studies, however, have failed to show EPP run-down with snake β-neurotoxins but, importantly, these studies were all performed at low quantal contents in high $[Mg^{2+}]_o$/low $[Ca^{2+}]_o$ solutions in order to enable intracellular recording (Chang et al. 1973; Chang and Lee 1977; Hawgood and Smith 1977). Indeed in a study using taipoxin which was performed under conditions of higher quantal content EPP run-down was clearly evident (Kamenskaya and Thesleff 1974).

Another potential postjunctional mechanism for the development of EPP run-down and fade is a use-dependent failure of the postjunctional receptor ion channel. For example the ganglion-blocking drug trimetaphan can produce a run-down in EPCs via a use-dependent blockade of the postjunctional nicotinic receptor ionophore (Gibb and Marshall 1982). It is unlikely, however, that fade seen with the snake β-neurotoxins is due to a postjunctional open-channel block since there is no evidence that these toxins reduce the amplitude of MEPPs and all snake β-neurotoxins act to produce a triphasic change in muscle tension through a prejunctional mechanism (Chang et al. 1973; Chang and Lee 1977; Wilson et al. 1995). In support of a prejunctional mechanism even classical nicotinic antagonists which cause fade, such as (+)-tubocurarine, whilst reducing MEPP amplitude importantly do not cause progressive waning of MEPP amplitude recorded during trains of nerve stimulation (Hong and Chang 1991).

The results of the present study therefore suggest that the EPP run-down observed with snake β-neurotoxins is likely to be prejunctional in origin. There exist several mechanisms by which this fade in transmitter release may occur. Firstly snake β-neurotoxins have been shown to block potassium channels in the nerve terminal which results in a prolongation of the terminal action potential and a subsequent increase in transmitter release (Dreyer and Penner 1987; Rowan and Harvey 1988). This leads to the enhancement phase of transmitter release prior to neuromuscular blockade. However it is unlikely that an

interaction with prejunctional potassium channels underlies this EPP run-down since even if there were a use-dependent dissociation of the snake β-neurotoxins from the potassium channel as occurs with another potassium channel blocker 4-aminopyridine (Ogata and Tatebayashi 1993) the resultant EPP run-down should also be present during the enhancement phase. In the present study, the development of fade always occurred considerably after the enhancement phase of muscle tension.

Another mechanism involves the model proposed by Bowman (1980) where the blockade of a prejunctional nicotinic autoreceptor leads to a fade in transmitter release. This is thought to be due to a reduction in the size of the immediately available pool of vesicles rather than any change in the probability of release (Tian et al. 1994). This indicates that fade may be due to a disruption of the process that mobilises vesicles from a 'reserve store', rather than any disruption of the process of exocytosis from a 'releasable' store. Whilst our data does not exclude the possibility that fade seen with snake β-neurotoxins is due to a block of these autoreceptors, the slower time course and different pattern of tetanic fade and e.p.p. run-down seen with snake β-neurotoxins, compared to (+)-tubocurarine, seem to indicate much slower kinetics more like snake α-neurotoxins. In addition there is as yet no evidence that snake β-neurotoxins interact with any pre- or postjunctional nicotinic receptor. Therefore any involvement of the prejunctional nicotinic autoreceptor in the fade produced by snake β-neurotoxins is at this stage purely circumstantial.

At present the most likely possibility is that the run-down in transmitter release may result from an alteration in the activity of terminal proteins, such as synapsin I, involved in the translocation of synaptic vesicles from the 'reserve store' to the 'releasable' store. In support of this hypothesis recent studies have shown that β-bungarotoxin inhibits phosphorylation of synapsin I to inhibit ^{3}H-acetylcholine release from synaptosomes (Chapell and Rosenberg 1996; Ueno and Rosenberg 1990, 1992 and 1995). Phosphorylation of synapsin I by calcium/calmodulin-dependent protein kinase II (Ca^{2+}/CaM KII) leads to dramatic decreases in the affinity of synapsin I for synaptic vesicles (Schiebler et al. 1986) and a loss of binding affinity for actin in the cytoskeleton (Llinas et al. 1985; Benfenati et al. 1991). This causes synapsin I to dissociate from the vesicle and cytoskeleton, enabling the vesicle to release its contents via exocytosis. Inhibition of synapsin I phosphorylation by β-bungarotoxin (Ueno and Rosenberg 1990, 1992) would be expected to decrease the number of synaptic vesicles in the 'releasable store' and therefore decrease neurotransmitter release (Llinas et al. 1985; Greengard et al. 1993). Since protein kinase C (PKC) can directly modulate the activity of Ca^{2+}/CaM KII it is believed that β-bungarotoxin may inhibit Ca^{2+}/CaM KII activity either directly or via inhibition of PKC thus leading to a reduction of synaptic vesicle mobilisation (Ueno and Rosenberg 1995). Whilst this model has been proposed to describe normal transmitter release a similar mechanism is no doubt involved in the movement of vesicles from the 'reserve' store to the 'releasable' store to maintain transmitter release during high nerve stimulation rates. This may provide the intraterminal event causing run-down in transmitter release seen with snake β-neurotoxins.

The ability of snake β-neurotoxins to induce fade does not appear to be related to their phospholipase A_2 activity. 'Low-toxicity' *Naja naja atra* and 'non-neurotoxic' bovine pancreatic PLA_2, both of which have high PLA_2 enzymic activity, failed to elicit any fade, even during protracted incubation periods. In support Ueno and Rosenberg (1995) have shown that β-bungarotoxin, but not *Naja naja atra* PLA_2, blocks both the basal and phorbol-ester stimulated PKC-dependent phosphorylation of synapsin I as well as other terminal proteins.

In conclusion this is the first study showing that snake β-neurotoxins which act prejunctionally to block transmitter release can produce EPP run-down and fade and lends further support to the hypothesis that fade occurs via a prejunctional not postjunctional mechanism. This has implications for the mechanisms underlying the mobilisation of synaptic vesicles during transmitter release.

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