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Neuromuscular effects of candoxin, a novel toxin from the venom of the Malayan krait (*Bungarus candidus*)

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1 Candoxin (MW 7334.6), a novel toxin isolated from the venom of the Malayan krait *Bungarus candidus*, belongs to the poorly characterized subfamily of nonconventional three-finger toxins present in Elapid venoms. The current study details the pharmacological effects of candoxin at the neuromuscular junction.

2 Candoxin produces a novel pattern of neuromuscular blockade in isolated nerve-muscle preparations and the tibialis anterior muscle of anaesthetized rats. In contrast to the virtually irreversible postsynaptic neuromuscular blockade produced by curaremimetic α-neurotoxins, the neuromuscular blockade produced by candoxin was rapidly and completely reversed by washing or by the addition of the anticholinesterase neostigmine.

3 Candoxin also produced significant train-of-four fade during the onset of and recovery from neuromuscular blockade, both, *in vitro* and *in vivo*. The fade phenomenon has been attributed to a blockade of putative presynaptic nicotinic acetylcholine receptors (nAChRs) that mediate a positive feedback mechanism and maintain adequate transmitter release during rapid repetitive stimulation. In this respect, candoxin closely resembles the neuromuscular blocking effects of d-tubocurarine, and differs markedly from curaremimetic α-neurotoxins that produce little or no fade.

4 Electrophysiological experiments confirmed that candoxin produced a readily reversible blockade ($IC_{50} \sim 10$ nM) of oocyte-expressed muscle ($\alpha\beta\gamma\delta$) nAChRs. Like α-conotoxin MI, well known for its preferential binding to the α/δ interface of the muscle ($\alpha\beta\gamma\delta$) nAChR, candoxin also demonstrated a biphasic concentration–response inhibition curve with a high- ($IC_{50} \sim 2.2$ nM) and a low- ($IC_{50} \sim 98$ nM) affinity component, suggesting that it may exhibit differential affinities for the two binding sites on the muscle ($\alpha\beta\gamma\delta$) receptor. In contrast, curaremimetic α-neurotoxins have been reported to antagonize both binding sites with equal affinity.
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Keywords: *Bungarus candidus*; postsynaptic neurotoxin; three-finger toxin; neuromuscular junction; nicotinic acetylcholine receptors; train-of-four fade

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CBCM, chick biventer cervicis muscle; CCh, carbachol; d-TC, d-tubocurarine; DTNB, dithiobisnitrobenzoic acid; GPD, guinea-pig diaphragm; KCl, potassium chloride; MHD, mouse hemidiaphragm; nAChR, nicotinic acetylcholine receptors; TOF, train-of-four

Introduction

Snake venoms are complex mixtures of protein and polypeptide toxins that encompass an arsenal of lethal neurotoxins. These include curaremimetic or α-neurotoxins which target muscle ($\alpha\beta\gamma\delta$) nicotinic acetylcholine receptors (nAChRs) with high affinity (K_d $10^{-9}-10^{-11}$ M) to produce postsynaptic neuromuscular blockade (Endo & Tamiya, 1991; Servent & Menez, 2001; Hodgson & Wickramaratna, 2002). Based on the length of their polypeptide chains, α-neurotoxins have been classified as short-chain neurotoxins (e.g. erabutoxin-b (*Laticauda semifasciata*); toxin-α (*Naja nigricollis*)) that have 60–62 residues and four conserved disulphide bonds and long-chain neurotoxins (e.g. α-bungarotoxin (*Bungarus multicinctus*); α-cobratoxin (*Naja kaouthia*)) with 66–75 residues and five disulphide bonds, with the additional disulphide bridge located in the middle loop (loop II) (Endo & Tamiya, 1991). In common, these neurotoxins belong to the superfamily of three-finger proteins that are characterized by a common tertiary structure consisting of three loops extending from a globular core crosslinked by four conserved disulphide bonds (Tsetlin, 1999; Servent & Menez, 2001; Kini, 2002).

We have recently reported the isolation and purification of a novel three-finger toxin, candoxin, from the venom of the

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Malayan krait *Bungarus candidus* (Nirthanan *et al*., 2002a). Candoxin (MW 7334.6) consists of a single polypeptide chain of 66 amino acids with five disulphide bridges, including four that are conserved among all three-finger toxins (see Figure 6). The fifth disulphide bridge (Cys^6-Cys^{11}) in candoxin is located at the tip of loop I (N-terminus loop), instead of in loop II as present in conventional long-chain α-neurotoxins as well as in κ-neurotoxins that have a predilection for neuronal (α3β2) nAChRs. This cysteine motif is typical of the poorly characterized subfamily of nonconventional toxins, isolated exclusively from Elapid venoms (Servent & Menez, 2001; Nirthanan *et al*., 2003). This class of toxins is typically characterized by a lower order of toxicity (LD_{50} from ~5 to 80 mg kg^{-1}) as opposed to prototype α-neurotoxins (LD_{50} ~ 0.04 – 0.3 mg kg^{-1}) and, because of this, they have also been referred to as weak toxins (Utkin *et al*., 2001). Apart from toxicity studies, the nonconventional toxins have been poorly investigated in terms of their function or molecular targets. Recently, it has been reported that two nonconventional ('weak') toxins from cobra venoms (WTX from *Naja kaouthia* and Wntx-5 from *Naja sputatrix*) produced a weak and irreversible inhibition of both, muscle (αβγδ) and α7 nAChRs, in micromolar inhibitory concentrations (Utkin *et al*., 2001; Poh *et al*., 2002). In contrast, candoxin has been shown to be a potent antagonist of muscle (αβγδ) (IC_{50} ~ 10 nM) and α7 (IC_{50} ~ 50 nM) nAChRs in electrophysiological experiments (Nirthanan *et al*., 2002a). Clearly therefore, nonconventional (weak) toxins do not appear to be a functionally homogeneous class of toxins. The present study provides a detailed account of the effects of a nonconventional toxin, candoxin, from krait venom at the neuromuscular junction.

Methods

Animals

Swiss Albino mice, Sprague – Dawley rats and Hartley guinea pigs were purchased from the Laboratory Animals Centre, National University of Singapore, (Sembawang, Singapore) and housed in the Animal Holding Unit of the Department of Pharmacology, National University of Singapore, before use. Water and food (Glen Forrest Stockfeeders, WA, Australia) were provided *ad libitum* and a 12 h light – 12 h dark cycle was maintained. Paper pellet bedding was also purchased from Glen Forrest Stockfeeders, WA, Australia. Locally bred chicks were purchased from a farm. These were delivered on the day of the experiment. The animals were handled according to the Guidelines of the National Medical Ethics Committee (Singapore), which conform to the World Health Organization's International Guiding Principles for Animal Research (*The WHO Chronicle* (1985); **39**(2) 51 – 56: International Guiding Principles for Biomedical Research involving Animals), adapted by the Council for International Organizations of Medical Sciences in 1985.

Organ bath studies

Isolated tissue experiments were performed using conventional organ baths of various volumes (4, 6 or 8 ml) for different tissues. Kreb's physiological salt solution (pH 7.4) of the following composition (mM): NaCl (118); KCl (4.8); KH_2PO_4 (1.2); $CaCl_2$ (2.5); $NaHCO_3$ (25); $MgSO_4$ (2.4) and D- (+) glucose (11) was used in all isolated tissue experiments. The solution was prepared in deionized water and aerated continuously with 5% carbon dioxide in oxygen. The temperature of the organ bath was maintained at 37°C. Isolated tissues were mounted in the organ bath under a resting tension of ~1 g. The preparations were allowed to equilibrate for about 45 – 60 min with changes of Kreb's solution at 15 min intervals before beginning the experiments. Electrical field stimulation was carried out through platinum ring electrodes using a Grass stimulator S88 (Grass Instruments, MA, U.S.A.). The magnitude of the contractile responses of the tissues was measured in gram (g) tension. Data were recorded in a MacLab system/8 (AD Instruments, NSW, Australia) via a force – displacement transducer (model FT03, Grass Instruments, MA, U.S.A.).

Chick biventer cervicis muscle

Chicks (7 – 14 days old) were killed by exposure to 100% CO_2 and the chick biventer cervicis muscle (CBCM) isolated as described by Ginsborg & Warriner (1960) and mounted in an 8 ml organ bath. Maximal twitch responses of the muscle were evoked by stimulating the motor nerve by electrical field stimulation (7 – 10 V) at a frequency of 0.2 Hz in supramaximal rectangular pulses of 0.1 ms duration. For direct muscle stimulation, the electrodes were lowered over the belly of the muscle and twitch responses evoked by electrical field stimulation (20 – 30 V, 1 ms, 0.2 Hz). d-Tubocurarine (d-TC) (10 μM) was added to the preparation to block neuromuscular transmission during direct muscle stimulation. The effects of candoxin (0.1 – 100 μg ml^{-1}; 0.0136 – 13.6 μM) on the uninterrupted twitch responses of the CBCM to nerve or direct muscle stimulation were then investigated. The time taken to block the amplitude of control twitch responses by 90% (T_{90}) was calculated in order to provide a quantitative measure of neurotoxicity. For comparison, the effects of 0.005 – 20 μg ml^{-1} of erabutoxin-b (0.733 nM – 2.9 μM), α-bungarotoxin (0.625 nM – 2.5 μM) or α-cobratoxin (0.626 nM – 2.55 μM) on the nerve-evoked twitch responses of the CBCM were also investigated. Each preparation was exposed to one concentration of a test substance. Submaximal contractures to exogenously applied acetylcholine (ACh) 300 μM, carbachol (CCh) 8 μM or potassium chloride (KCl) 30 mM were obtained in the absence of electrical field stimulation. The contact time allowed for the agonists were 30, 60 and 90 s for ACh, KCl and CCh, respectively. The effects of candoxin (0.1 – 100 μg ml^{-1}; 0.0136 – 13.6 μM) on the responses of the CBCM to exogenous ACh, CCh or KCl were investigated immediately after complete blockade of nerve-evoked twitch responses was established.

Mouse phrenic nerve hemidiaphragm

Male Swiss Albino mice (20 – 30 g) were killed by cervical dislocation after exposure to 100% CO_2. The mouse hemidiaphragm (MHD) with the associated phrenic nerve was isolated as described by Bulbring (1946) and mounted in a 4 ml organ bath. For indirect stimulation, the phrenic nerve was electrically stimulated at a frequency of 0.2 Hz in rectangular pulses of 0.2 ms duration and supra-maximal

voltage (7 – 10 V). Direct muscle stimulation was achieved by electrical stimulation (0.2 Hz, 2 ms, 20 – 30 V) in the presence of d-TC (10 μM). The effects of candoxin (3, 10, 30 and 100 μg ml^{-1}) on direct and indirect stimulation of the MHD were studied.

Train-of-four (TOF) stimulation

The effects of candoxin on the maximal twitch responses of the MHD evoked through TOF stimulation were also investigated. TOF stimulation (2 Hz for 2 s every 20 s) of the phrenic nerve was carried out as described by Cheah & Gwee (1988) and continued throughout the experiment. Control TOF twitches (T_1, T_2, T_3, T_4) were recorded for 20 min and conditions stabilized before the addition of candoxin (10 – 100 μg ml^{-1}; 1.36 – 13.6 μM). The time course changes of the first (T_1) and fourth (T_4) twitch were noted during the onset of and recovery from neuromuscular blockade produced by candoxin. The corresponding TOF ratios (T_4/T_1) at 10, 25, 50, 75 and 90% block for the first twitch (T_1) were calculated and the values obtained were plotted (T_1 *versus* T_4/T_1 relation). The effects of α-bungarotoxin (3 μg ml^{-1}; 0.37 μM), α-cobratoxin (3 μg ml^{-1}; 0.38 μM), erabutoxin-b (3 μg ml^{-1}; 0.44 μM) or d-TC (1 μM) on twitches evoked by TOF stimulation were also studied for comparison.

Guinea-pig diaphragm

Male Hartley guinea pigs (300 – 325 g) were killed by exposure to 100% CO_2 and exsanguination. The guinea-pig diaphragm (GPD) was prepared as described for the rat diaphragm by Wolthuis *et al*. (1981). This method was preferred over the conventional phrenic nerve-hemidiaphragm preparation since it could be accommodated in a smaller organ bath, thereby minimizing the requirement of candoxin. Longitudinal strips ($\sim$1.5 cm $\times$ 3 mm), cut parallel to the anatomical direction of the muscle fibres, were prepared from the isolated diaphragm. Ligatures were attached to the membranous and costal parts of the diaphragm strip and mounted vertically under 1 g tension, between two parallel platinum electrodes in a 6 ml organ bath. Twitch responses of the GPD were elicited by indirect field stimulation (7 – 10 V, 0.1 ms, 0.2 Hz). After an equilibration period of 30 min, d-TC (5 μM) was added to the preparation to confirm that the twitches elicited were as a result of nerve stimulation and not direct muscle stimulation (Hodgson & Wickramaratna, 2002). Twitches were then re-established by washing and following a further 45 min equilibration period, the effects of candoxin 3, 10, 30 and 100 μg ml^{-1} (in μM: 0.41, 1.36, 4.09 and 13.6) on the GPD were studied.

Reversal studies

The recovery of the CBCM, MHD and GPD from complete neuromuscular blockade produced by candoxin (10, 30 and 100 μg ml^{-1}; 1.36, 4.08 and 13.6 μM, respectively) was assessed. In the case of other neurotoxins, recovery from 90% blockade produced by 0.05, 1 and 3 μg ml^{-1} erabutoxin-b (7.25 nM, 0.145 and 0.435 μM), α-bungarotoxin (6.25 nM, 0.125 and 0.37 μM) or α-cobratoxin (6.26 nM, 0.13 and 0.38 μM) was assessed. The toxins were removed from the organ bath by washing by bath overflow (16 ml min^{-1} for the first 5 min followed by a slow drip-wash at a flow rate of $\sim$8 ml min^{-1}) with fresh Kreb's solution. The washing was continued until complete recovery or for a maximal period of 180 min. The effects of the anticholinesterase neostigmine 0.1, 1.0 or 3.0 μM on the reversal of neuromuscular blockade produced by candoxin or the effects of neostigmine added cumulatively up to 100 μM on the neuromuscular blockade produced by erabutoxin-b, α-bungarotoxin or α-cobratoxin were also studied.

In vivo *experiments on anaesthetized rats*

Experiments were designed to study the effects of candoxin (0.3 – 1 mg kg^{-1}) on the twitch responses of the tibialis anterior muscle evoked by nerve stimulation in anaesthetized rats. Male Sprague – Dawley rats weighing 300 – 375 g were anaesthetized by intraperitoneal injections of thiobutabarbitone sodium (Inactin™) in a dose of 100 mg kg^{-1} (i.p.) to the point of a loss of eyelid reflex and lack of withdrawal from painful stimuli. The depth of anaesthesia was assessed by the stability of blood pressure and heart rate, absence of eyelid reflexes and by an absence of a cardiovascular response to paw pinch. Supplementary doses of thiobutabarbitone sodium (10 – 25 mg kg^{-1}, i.v.) were given when necessary. The trachea was cannulated with a plastic endotracheal tube that was connected to a Harvard rodent ventilator (Model 683, Harvard Bioscience, MA, U.S.A.) and artificial ventilation provided throughout as described previously (Kleinman & Radford, 1964). For the i.v. administration of test substances, the right external jugular vein was cannulated with a Portex Fg2 nylon intravenous cannula that was connected to a three-way stopcock. For monitoring the blood pressure and heart rate, the left common carotid artery was cannulated with a Portex Fg2 nylon intravenous cannula and connected to a MacLab/8 recording system (AD Instruments, NSW, Australia) via a Gould Statham P23 pressure transducer.

Nerve-evoked maximal TOF twitches of the tibialis anterior muscle of the right lower limb of the rat were monitored throughout as described by Tran *et al.* (1982). The freed tendon of insertion of the tibialis anterior muscle was attached to a force – displacement transducer (model FT03, Grass Instruments), while the right lower limb was kept immobilized. The sciatic nerve in the popliteal space was stimulated (2 Hz for 2 s every 20 s) with a Harvard Dastre electrode connected to a Grass stimulator S88 to produce maximal twitches of the tibialis anterior muscle. The resting tension of the muscle was adjusted to give the greatest evoked twitch tension. Data were recorded on a MacLab/8 system. The effects of candoxin (0.3, 0.6 and 1 mg kg^{-1}; 40.9, 81.8 and 136.3 nmol kg^{-1}) ($n=4$), erabutoxin-b (100 μg kg^{-1}; 14.6 nmol kg^{-1}) ($n=3$) or dTC (10 nmol kg^{-1}) ($n=3$) on the twitch responses of the muscle were studied. The ability of the muscle to recover spontaneously from twitch blockade produced by candoxin and the effects of neostigmine 0.4, 4 or 40 μM on the twitch blockade produced by candoxin were also evaluated. The recovery of the fourth twitch (T_4) to its control twitch height was considered as complete recovery from neuromuscular blockade.

Assay for anticholinesterase activity

The effect of candoxin on the activity of acetylcholinesterase (AChE) was studied using the protocol of Ellman *et al.* (1961) as described in Nirthanan *et al.* (2002b). The initial rate of formation of thiocholine from acetylthiocholine by AChE was

measured by the increase in optical density at 412 nm resulting from the reaction of thiocholine with dithiobisnitrobenzoic acid (DTNB). The control assay mixture contained 1 U of AChE (electric eel enzyme type V-S), 50 mM of DTNB and 0.5 mM acetylthiocholine in 200 μl of 0.1 M phosphate buffer (pH 7.8). The toxin assay mixture contained, in addition to the above, 10, 30 or 100 μM of candoxin, which was preincubated with the AChE for 30 or 60 min prior to the addition of acetylthiocholine. The enzyme kinetics of AChE in all the assay mixtures was assessed spectrophotometrically over a period of 5 min. An assay where the candoxin was replaced with neostigmine (0.3, 1 and 3 μM) was carried out as a positive control. All assays were performed in triplicates and repeated thrice.

Electrophysiological experiments

Electrophysiological experiments were carried out using the oocyte expression system as described in Nirthanan *et al.* (2002a). The effects of candoxin on currents evoked by ACh in the muscle ($\alpha\beta\gamma\delta$) nAChR were studied. Concentration–response curves were adjusted using the empirical Hill equation: $Y = 1/(1 + (x/IC_{50})\hat{}nH)$ where Y = the fraction of remaining current, IC_{50} = concentration of half inhibition, nH = the apparent cooperativity, x = antagonist concentration. The inhibition curve for muscle ($\alpha\beta\gamma\delta$) nAChRs was fitted with a two-component Hill equation, and for comparison, a one-component Hill equation. When two Hill equations were employed, the sum of two identical equations was computed.

Drugs and chemicals

The drugs used in the pharmacological studies: acetylcholine iodide, AChE (electric eel enzyme type V-S), carbamylcholine chloride (CCh), d-Tc, neostigmine bromide and thiobutabarbitone (Inactin™) were obtained from Sigma Chemicals, St Louis, MO, U.S.A. All chemicals and reagents and α-conotoxin MI were also purchased from Sigma (St Louis, MO, U.S.A.). α-Bungarotoxin, α-cobratoxin and erabutoxin-b were from Latoxan (Valence, France). Candoxin was purified as described in Nirthanan *et al.* (2002a) from *Bungarus candidus* venom obtained from Venom Supplies Pvt. Ltd. (Tanunda, SA, Australia). The stock solutions of drugs and toxins were freshly made in deionized water and diluted to the required concentration with Kreb's solution for *in vitro* experiments or 0.9% saline for *in vivo* experiments.

Statistics

The data are expressed as mean ± standard error of mean (s.e.m.) of at least four to six experiments unless stated otherwise. The experiments that required 100 μM or higher concentrations of candoxin were repeated thrice. Paired *t*-tests (two-tailed) were used to determine significance and $P < 0.05$ was considered significant. The data were analysed using Fig P software version 2.2a (Fig P Software Corporation, Durham, NC, U.S.A.) or GraphPad Prism software (GraphPad Software Inc., San Diego, CA, U.S.A.).

Results

Effects of candoxin on neuromuscular transmission

Chick biventer cervicis muscle In control experiments, the twitch responses of the CBCM were 92 ± 6% of control twitch height after 180 min of uninterrupted nerve stimulation at 0.2 Hz (mean ± s.e.m., $n = 7$). The twitches evoked by direct muscle stimulation were 96 ± 3% of control twitch height after 180 min of uninterrupted stimulation ($n = 4$). The responses to exogenously applied ACh, CCh and KCl were 95 ± 3, 93 ± 4 and 97 ± 2% of control response height, respectively, at the end of 300 min of uninterrupted nerve stimulation ($n = 7$). The muscle tone remained at baseline throughout this period of time. Candoxin produced a time-dependent blockade of the twitch responses of the CBCM to nerve stimulation as well as complete blockade of the responses to exogenously applied ACh and CCh (Figures 1a, b). Neither the twitch responses elicited by direct muscle stimulation nor the responses to exogenously applied KCl were affected by candoxin even at a concentration of 300 μg ml^{-1} (40.8 μM) ($n = 2$). No contracture (or increase in muscle tone) was observed at any concentration of candoxin tested for up to ~180 min following the addition of candoxin. α-Bungarotoxin, erabutoxin-b and α-cobratoxin also produced time-dependent blockade of nerve-evoked, but not directly stimulated, twitch responses of the CBCM as well as blockade of the responses elicited by ACh and CCh but not KCl. However, these neurotoxins were about 7–10-fold more potent than candoxin in producing neuromuscular blockade in the CBCM (Figure 1b).

Mouse phrenic nerve-hemidiaphragm

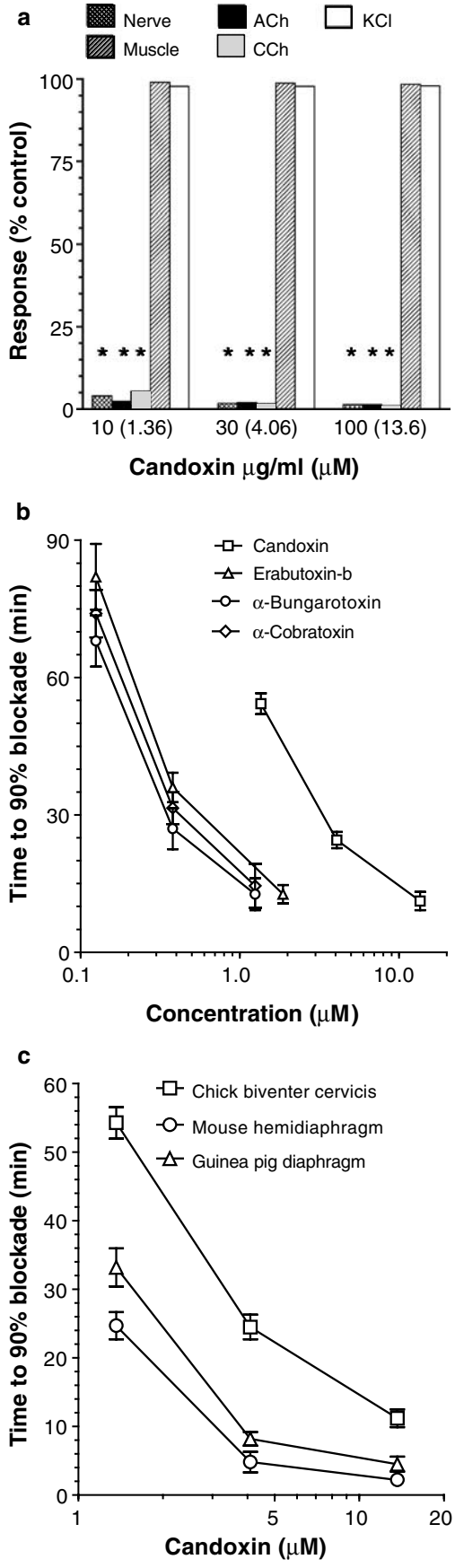
In control experiments, the twitch responses of the MHD were 97 ± 1% ($n = 5$) and 98 ± 2% ($n = 4$) of control twitch height after 90 min of uninterrupted nerve or direct muscle stimulation, respectively. The muscle tone remained at baseline throughout the entire duration of nerve or direct muscle stimulation. Candoxin 3, 10, 30 and 100 μg ml^{-1} (in μM: 0.41, 1.36, 4.09 and 13.6) produced complete blockade of nerve-evoked, but not directly stimulated, twitch responses in the MHD in a time-dependent manner (Figure 1c).

Guinea-pig diaphragm

In control experiments, the twitch responses of the GPD were 96 ± 4% of control twitch height after 60 min of uninterrupted electrical field stimulation ($n = 4$). The muscle tone remained at baseline throughout the entire duration of field stimulation. Nerve-evoked twitch responses of the GPD were completely blocked by candoxin 3, 10, 30 and 100 μg ml^{-1} (in μM: 0.41, 1.36, 4.09 and 13.6) in a time-dependent manner (Figure 1c).

Reversal of nerve-evoked twitch blockade produced by candoxin in vitro

The twitch responses of the CBCM were rapidly and completely restored by washing out candoxin with fresh Kreb's solution following the blockade of nerve-evoked twitch responses produced by candoxin 3, 10, 30 and 100 μg ml^{-1} (in μM: 0.41, 1.36, 4.09 and 13.6) (Figures 2a, c).



In another series of experiments, neostigmine 0.1, 1 and 3 μM produced complete reversal of the candoxin-induced blockade of nerve-evoked twitches in the CBCM in a concentration-dependent manner (Figures 2b, d). Likewise, the MHD and GPD were also able to recover rapidly and completely from the blockade of nerve-evoked twitch responses produced by candoxin (Figure 2c). Neostigmine 0.1, 1 and 3 μM also produced complete reversal of the twitch blockade produced by candoxin in the MHD and GPD (Figure 2d). In contrast, the twitch blockade produced by 0.05, 1 and 3 μg ml^{-1} α-bungarotoxin, erabutoxin-b and α-cobratoxin in the CBCM were virtually irreversible, even after prolonged washing for 180 min ($n=3$). The addition of the neostigmine (cumulatively up to 100 μM) failed to reverse the blockade of nerve-evoked twitches produced by α-bungarotoxin, erabutoxin-b or α-cobratoxin, once complete neuromuscular blockade had been established in the CBCM ($n=3$).

Effects of candoxin on TOF twitch responses in the MHD

Figure 3a shows the typical effects of candoxin (100 μg ml^{-1}; 13.6 μM) on maximal twitch responses of the MHD evoked by TOF nerve stimulation. During the onset of blockade of nerve-evoked twitches, candoxin progressively depressed all the four twitches (T_1, T_2, T_3, T_4) in each train, but at different rates ($T_4 > T_3 > T_2 \gg T_1$), as evidenced by the twitch heights recorded (Figure 3a). During recovery from twitch blockade produced by candoxin, T_1 reached control levels faster than did T_4. Thus, various concentrations of candoxin (10, 30 and 100 μg ml^{-1}) (in μM: 1.36, 4.09 and 13.6) were found to produce significant TOF fade during the onset of and recovery from twitch blockade in the MHD (Figures 3c, d). The intensity of TOF fade produced by candoxin in the MHD was, however, less than that observed for d-TC (1 μM) at all stages of T_1 block. In contrast, 3 μg ml^{-1} α-bungarotoxin (0.37 μM), erabutoxin-b (0.45 μM) and α-cobratoxin (0.38 μM) did not show significant TOF fade during their virtually irreversible blockade of nerve-evoked twitch responses (Figure 3c). For comparison, the absence of a fade response during the blockade of nerve-evoked TOF twitch responses of the MHD by α-bungarotoxin (3 μg ml^{-1}) is shown in Figure 3b.

Figure 1 Pharmacological characterization of candoxin using the chick biventer cervicis muscle (a, b) and comparison of the blockade of nerve-evoked twitch responses produced by candoxin in various isolated nerve-muscle preparations (c). (a) The effects of candoxin (10, 30 and 100 μg ml^{-1}) on twitch responses evoked by nerve stimulation (Nerve); twitch responses evoked by direct muscle stimulation (Muscle) and contractures produced by ACh (300 μM), CCh (8 μM) and KCl (30 mM) in the chick biventer cervicis muscle. The responses of the muscle to candoxin are expressed as a percentage of the respective control values in the absence of candoxin. Values are mean $\pm$ s.e.m., $n=6$. Vertical bars represent the s.e.m. (b) Concentration-dependent blockade of nerve-evoked twitch responses of the chick biventer cervicis muscle produced by candoxin, erabutoxin-b, α-bungarotoxin and α-cobratoxin. The time taken to produce 90% twitch blockade is shown. Each data point is the mean of six experiments. The vertical bars represent the SEM. (c) The concentration-dependent blockade of nerve-evoked twitch responses produced by candoxin in the chick biventer cervicis muscle ($n=10$), mouse phrenic nerve hemidiaphragm ($n=8$) and GPD ($n=8$) is shown. The time to produce 90% twitch blockade in the respective nerve-muscle preparations is shown. Vertical bars represent the s.e.m. *$P<0.001$, significantly different from control.

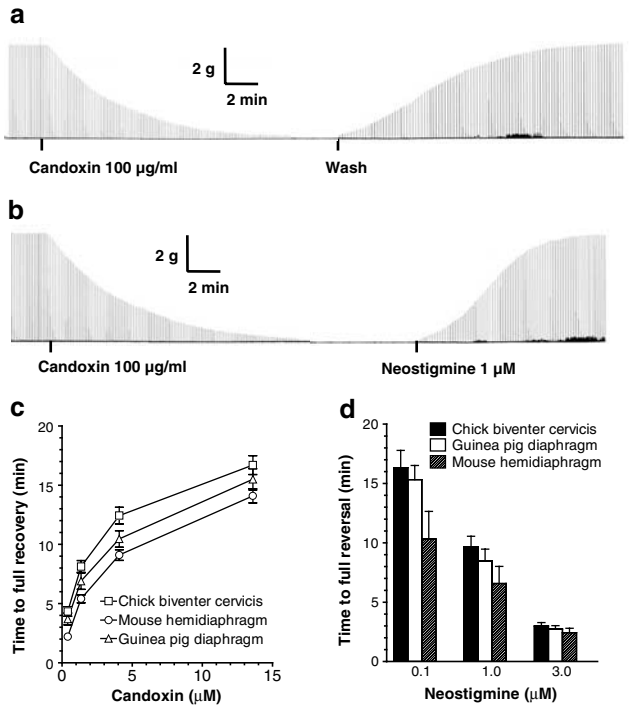


Figure 2 Reversibility of nerve-evoked twitch blockade produced by candoxin. (a) Segments of tracing showing the complete blockade of nerve-evoked twitches of the chick biventer cervicis muscle produced by candoxin (100 μg ml^{-1}; 13.6 μM). Candoxin was washed out at the point indicated. (b) Neostigmine (1 μM) was added to the bath at the point indicated. The vertical bar represents the magnitude of the twitch response in g tension. The horizontal bar depicts the time in min. The figures are representative of six experiments. (c) Comparison of the time to full recovery from complete blockade of nerve-evoked twitch responses produced by various concentrations of candoxin in the chick biventer cervicis muscle ($n=6$), mouse phrenic nerve hemidiaphragm ($n=4$) and GPD ($n=4$). Candoxin was washed out immediately following the establishment of complete twitch blockade. The time required for the restoration of nerve-evoked twitch responses to 100% of control twitch height is shown. (d) Comparison of the time to full reversal of blockade of nerve-evoked twitch responses produced by candoxin (100 μg ml^{-1}; 13.6 μM) following the addition of various concentrations of neostigmine. Neostigmine was added 5 min after complete twitch blockade was established. Data is the mean of four experiments for all tissues. Vertical bars represent the s.e.m.

Neuromuscular blockade produced by candoxin in vivo

Control twitch responses of the rat tibialis anterior muscle to nerve stimulation were 16 ± 1.2 g ($n=6$). Candoxin 0.3, 0.6 and 1 mg kg^{-1} produced complete blockade of the nerve-evoked twitch responses of the muscle in a time-dependent manner (Figure 4a). Furthermore, 1 mg kg^{-1} (136.3 nmol kg^{-1}) of candoxin produced complete neuromuscular blockade in the tibialis anterior muscle in about the same time ($\sim$4 min) as that mediated by 0.1 mg kg^{-1} (14.6 nmol kg^{-1}) of erabutoxin-b (Figure 4b). The twitch responses of the muscle recovered spontaneously and completely; the time to complete spontaneous recovery was longer with increasing doses of candoxin (Figure 4c). The blockade of twitch responses produced by 1 mg kg^{-1} candoxin was also completely reversed by the addition of neostigmine (0.4, 4 or 40 μg kg^{-1}) in a dose-dependent manner (Figure 4d). Candoxin (1 mg kg^{-1}; 136.3 nmol kg^{-1}) also produced significant TOF fade during the onset of and spontaneous recovery from twitch blockade *in vivo* (Figures 4e, f). Candoxin did not appear to affect the arterial blood pressure, heart rate and cardiac conductivity of the anaesthetized rat during neuromuscular blockade (data not shown).

Erabutoxin-b (0.1 mg kg^{-1}; 14.6 nmol kg^{-1}) produced rapid neuromuscular blockade in the tibialis anterior muscle that did not recover spontaneously. The injection of neostigmine, in bolus doses cumulatively up to 80 μg kg^{-1}, did not reverse the erabutoxin-induced blockade. No TOF fade was observed during the twitch blockade produced by erabutoxin-b (Figure 4e). In contrast, d-TC (10 nmol kg^{-1}) produced reversible twitch blockade accompanied by significant TOF fade during the onset of and spontaneous recovery from twitch blockade (Figures 4e, f).

Biochemical assay for anticholinesterase activity

The rate of change in optical density for the control assay was 7.14 ± 0.1 mAU min^{-1} ($n=9$). The rate of change in optical density for the candoxin assay with 10, 30 and 100 μM of candoxin was 7.05 ± 0.11, 6.96 ± 0.2 and 07.07 ± 0.14 mAU min^{-1}, respectively, after 30 min incubation and 6.97 ± 0.16, 7.11 ± 0.12 and 7.09 ± 0.08 mAU min^{-1}, respectively, after 60 min incubation. Thus, candoxin did not inhibit AChE activity. Neostigmine 1 μM completely inhibited the AChE activity.

Effects of candoxin on muscle ($\alpha\beta\gamma\delta$) nicotinic ACh receptors

Candoxin strongly inhibited ACh-evoked currents in the rat muscle ($\alpha\beta\gamma\delta$) nAChRs expressed on oocytes with a half inhibitory concentration (IC_{50}) of 10 nM. The inhibition of muscle ($\alpha\beta\gamma\delta$) nAChRs by candoxin was also rapidly and completely reversed by washing (Nirthanan *et al.*, 2002a). The inhibition concentration–response over a broad range of candoxin concentrations is shown in Figure 5a. Attempts to describe these points with a single Hill equation yielded a low Hill coefficient ($nH=0.64$) (Figure 5a, grey line), whereas the fit obtained with the sum of two Hill equations showed a high- (2.2 nM, $nH=1.6$) and a low-affinity (98 nM, $nH=1.4$) component. The high- and low-affinity components contributed almost equivalently to the inhibition (46 and 54%, respectively). The blockade of muscle ($\alpha\beta\gamma\delta$) nAChRs produced by α-conotoxin MI (which has high selectivity for the α/δ interface of mammalian muscle nAChRs), measured under the same experimental conditions, also yielded a biphasic concentration–response inhibition curve with a high (0.33 nM, $nH=1.2$) and low affinity (150 nM, $nH=1.2$) contributing to 88 and 12% to the blockade, respectively (Figure 5a).

Discussion

Neuromuscular blockade produced by candoxin

Candoxin produces a novel pattern of neuromuscular blockade: the onset of blockade is rapid and the offset is also rapid and completely reversible. In the CBCM, candoxin produced

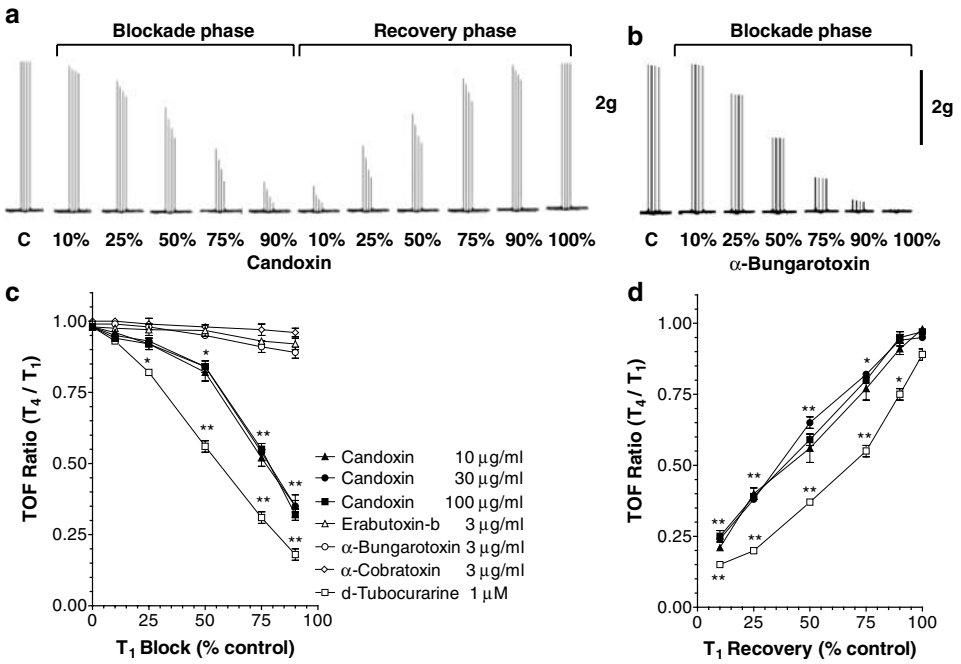


Figure 3 TOF fade produced by candoxin in the MHD. Segments of a typical tracing showing the effects of (a) candoxin ($100\,\mu g\,ml^{-1}$) and (b) α-bungarotoxin ($3\,\mu g\,ml^{-1}$) on the twitch responses elicited by TOF nerve stimulation in the mouse phrenic nerve hemidiaphragm. The TOF fade response at 10, 25, 50, 75 and 90% block and/or recovery (for the first twitch, T_1) is shown. The data presented in (a) and (b) is representative of six experiments. The vertical bar depicts the magnitude of the twitch response in g tension. The neuromuscular blockade produced by α-bungarotoxin was irreversible. (c, d) Comparison of the TOF fade produced by candoxin, d-tubocurarine and other α-neurotoxins *in vitro*. The fade response produced during the onset of nerve-evoked twitch blockade (c) and recovery from twitch blockade (d) by various concentrations of candoxin, d-tubocurarine, α-bungarotoxin, α-cobratoxin and erabutoxin-b during TOF stimulation of the mouse phrenic nerve hemidiaphragm. The nerve-evoked twitch blockade produced by α-bungarotoxin, α-cobratoxin and erabutoxin-b were irreversible. The TOF ratio (T4/T1) at 10, 25, 50, 75 and 90% block (for the first twitch, T_1) is compared. Each data point is the mean of six experiments. The vertical bars represent the s.e.m. $^*P< 0.01$; $^{**}P<0.001$, significantly different from control.

concentration- and time-dependent blockade of uninterrupted twitch responses of the muscle to nerve stimulation. This blockade can be attributed to a postsynaptic action since candoxin also blocks the contractile responses of the CBCM to the exogenously applied cholinoceptor agonists ACh and CCh. As candoxin did not block the twitch responses elicited by direct muscle stimulation or the responses elicited by KCl as a consequence of direct muscle depolarization, the postsynaptic activity of candoxin cannot be attributed to a direct inhibitory effect on muscle contractility but, rather, more specifically to its blockade of postsynaptic nAChRs at the neuromuscular junction. These effects closely resemble the neuromuscular blockade produced by curaremimetic α-neurotoxins such as erabutoxin-b, α-bungarotoxin and α-cobratoxin, which are well documented to have high selectivity and high affinity for postsynaptic nAChRs (Lee, 1972; Chang, 1979). However, the neuromuscular blockade produced by candoxin in the CBCM was $\sim 7-10$-fold less potent than that produced by erabutoxin-b, α-bungarotoxin or α-cobratoxin. A direct myotoxic effect of candoxin was further excluded by the observation that candoxin did not produce a slow-onset and irreversible contracture that is usually, but not invariably (Harris, 1991) seen as a characteristic feature of neuromuscular blockade associated with venom components such as phospholipase A_2 that cause myotoxicity (Rowan *et al*., 1989; Geh *et al*., 1992; Hodgson & Wickramaratna, 2002). Moreover, candoxin did not show any phospholipase A_2 activity in a reliable and sensitive biochemical assay described by Kawuachi *et al*. (1971) (data not shown).

Candoxin also produced concentration- and time-dependent neuromuscular blockade in some mammalian nerve-muscle preparations, including the phrenic nerve hemidiaphragm of the mouse (MHD) and the GPD. The MHD and GPD were more sensitive ($EC_{50}\sim 0.8\,\mu M$) to neuromuscular blockade by candoxin than the CBCM ($EC_{50}\sim 1.5\,\mu M$). It has been reported that the determinant residues for toxin binding are different between the ligand-binding domains of the α subunits of the chick and rat nicotinic receptors (Arias, 2000) and therefore, it may be likely that snake toxins have evolved to show species selectivity to effectively target their intended prey (Dufton *et al*., 1989), which in the case of *Bungarus candidus* consists of reptiles and rodents (Lim, 1990). The results obtained from *in vitro* experiments were corroborated by *in vivo* pharmacological studies performed on anaesthetized rats. Candoxin produced dose- and time-dependent blockade of twitch responses in the tibialis anterior muscle evoked by electrical stimulation of the sciatic nerve. Doses less than $0.3\,mg\,kg^{-1}$ produced only partial ($45-85\%$ of control) blockade, whereas 0.3, 0.6 and $1\,mg\,kg^{-1}$ candoxin produced rapid and complete neuromuscular blockade. Furthermore, like in the *in vitro* studies, erabutoxin-b was about 10-fold more potent than candoxin *in vivo* in producing neuromuscular lockade.

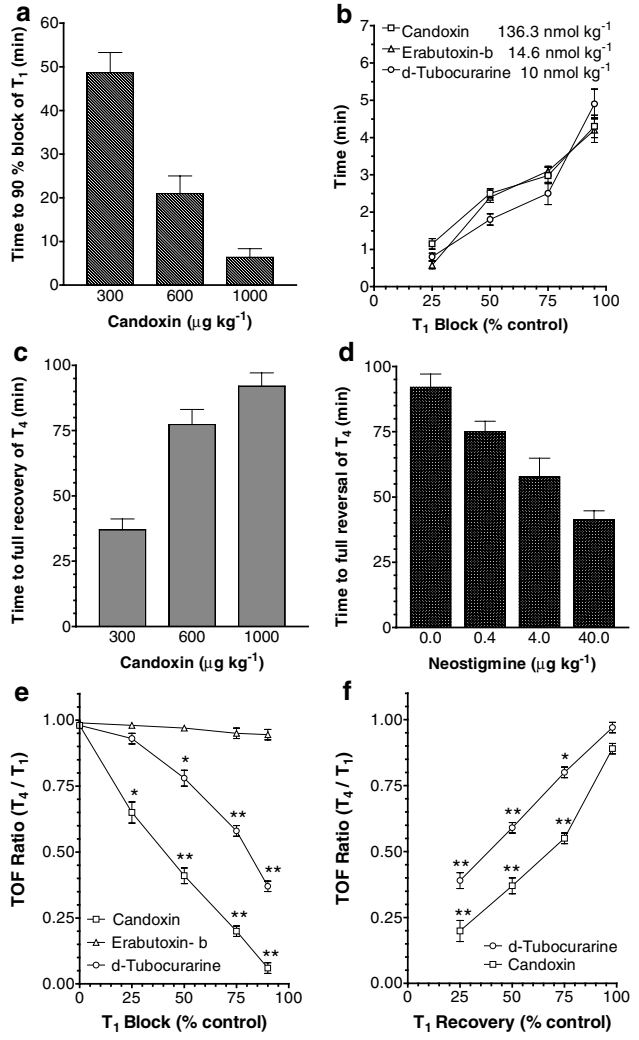


Figure 4 Neuromuscular blockade produced by candoxin *in vivo*. (a) Concentration-dependent candoxin-induced blockade of twitch responses evoked by TOF nerve stimulation in the tibialis anterior muscle in the anaesthetized rat. The time taken to produce 90% blockade of the first twitch (T_1) is shown. (b) Comparison of the blockade of nerve-evoked twitch responses of the tibialis anterior muscle produced by candoxin (1 mg kg^{-1}; 136.3 nmol kg^{-1}), erabutoxin-b (100 μg kg^{-1}; 14.6 nmol kg^{-1}) and d-tubocurarine (10 nmol kg^{-1}). The time taken to produce 25, 50, 75 and 90% blockade of the first twitch response (T_1) is shown. (c) Time-dependent spontaneous recovery of the fourth twitch response (T_4) of the tibialis anterior muscle from the nerve-evoked twitch blockade produced by candoxin 0.3, 0.6 or 1 mg kg^{-1}. The time to complete recovery of the fourth twitch response (T_4) is shown. (d) Dose-dependent reversal of nerve-evoked twitch blockade produced by candoxin (1 mg kg^{-1}) in the anterior tibialis muscle by neostigmine (0.4, 4 or 40 μg kg^{-1}). The time to complete recovery of the fourth twitch response (T_4) is shown. All values are mean $\pm$ s.e.m. of four experiments. Vertical bars indicate the s.e.m. (e, f) Comparison of the TOF fade produced by candoxin, d-tubocurarine and erabutoxin-b *in vivo*. The fade response produced during the onset of nerve-evoked twitch blockade (e) and recovery from twitch blockade (f) by candoxin, d-tubocurarine and erabutoxin-b during TOF stimulation of the tibialis anterior muscle in the anaesthetized rat. The nerve-evoked twitch blockade produced by erabutoxin-b was irreversible. The TOF ratio (T4/T1) at 25, 50, 75 and 90% block (for the first twitch, T_1) is compared. Each data point is the mean of four experiments. The vertical bars represent the s.e.m. $^*P<0.01$; $^{**}P<0.001$, significantly different from control.

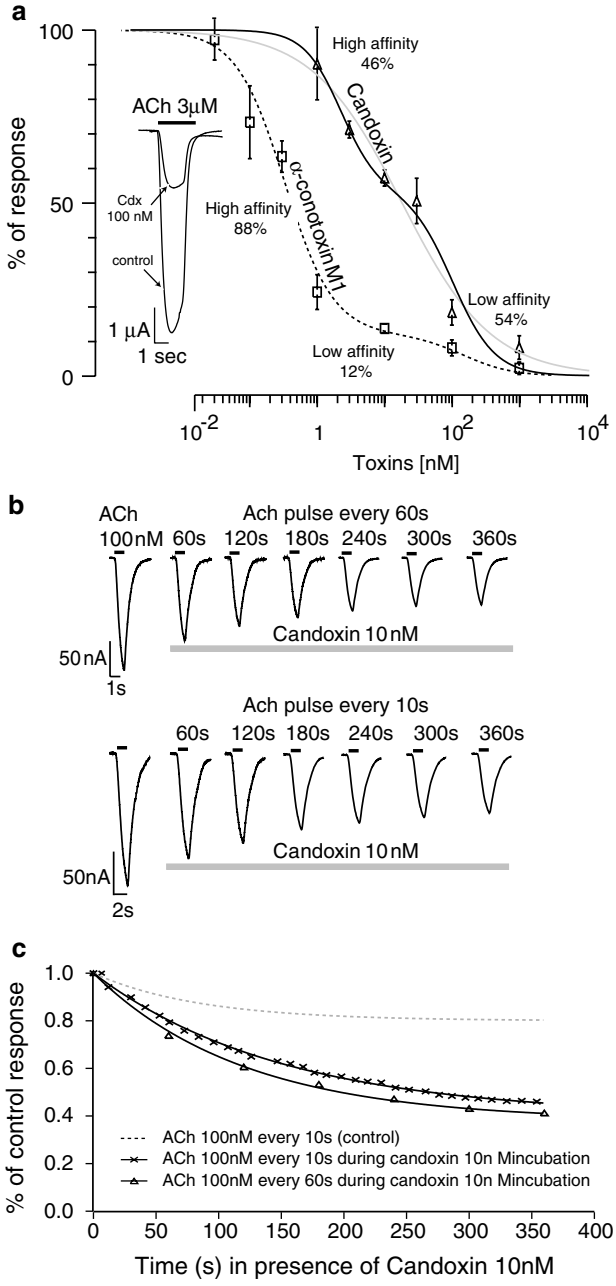


Figure 5 Inhibition of ACh currents in oocyte-expressed muscle ($\alpha\beta\gamma\delta$) nicotinic ACn receptors by candoxin and α-conotoxin M1. The inhibition curve of candoxin was fitted by the sum of two Hill equations, with a high (2.2 nM, $nH=1.6$) and a low affinity (98 nM, $nH=1.4$) component, contributing to 46 and 54%, respectively. For comparison, the fit using a single Hill equation that yielded a low Hill coefficient (IC_{50} of 18 nM, $nH=0.64$) is also shown (grey line). The inhibition curve of α-conotoxin M1 was also biphasic with a high- (0.33 nM, $nH=1.2$) and a low-affinity (150 nM, $nH=1.2$) component, contributing to 88 and 12%, respectively. Inset: typical current evoked by 3 μM ACh before and after 30 min exposure to 100 nM candoxin. (b) Candoxin blockade is use independent. Currents evoked by 100 nM ACh were recorded at successive intervals of either 60 (upper traces) or 10 (lower traces) seconds in presence of 10 nM candoxin. Values above the traces indicate the incubation time in candoxin. (c) Plot of the current amplitude as a function of incubation time yields a single exponential decay that is fitted by an exponential in the form $y=a*e^{-t/\tau}+b$, where $a=0.6$, $\tau=140$ and $b=0.4$ for the 10 s interval and $a=0.5$, $\tau=120$ and $b=0.5$, for the 60 s interval.

Reversibility of neuromuscular blockade induced by candoxin

In contrast to most α-neurotoxins that produce virtually irreversible neuromuscular blockade (Lee, 1972; Chicheportiche *et al*., 1975; Lee *et al*., 1977; Chang, 1979; Harris, 1984), candoxin produced a blockade of nerve-evoked twitch responses *in vitro* and *in vivo* that was rapidly and completely reversed by washing or by the addition of the anticholinesterase neostigmine. Hence, the neuromuscular blockade produced by candoxin closely resembled the neuromuscular effects of d-TC, a reversible and competitive antagonist of ACh acting selectively at postsynaptic nAChRs. The rapid recovery of the candoxin-induced neuromuscular blockade can be attributed to the rapid removal of the toxin from its postsynaptic target sites by washing. The reversal of neuromuscular blockade by neostigmine is likely because of AChE inhibition and ACh preservation at the synapse, possibly resulting in competitive displacement of candoxin.

α-Conotoxins MI and GI, short (~12–30 residues) disulphide-rich peptides from marine cone snail venoms, are well known to produce reversible postsynaptic neuromuscular blockade *in vitro* and *in vivo* (Marshall & Harvey, 1990). There are also a few other neurotoxins and neurotoxin homologues isolated from snake venoms that reportedly exhibit reversible or partially reversible neuromuscular blockade in isolated nerve-muscle preparations. These include neurotoxins: LSIII (a postsynaptically acting neurotoxin isolated from the venom of the sea snake *Laticauda semifasciata*; Maeda & Tamiya, 1974), fasciatoxin (a weak postsynaptic neurotoxin from *Bungarus fasciatus*; Liu *et al*., 1989) and pseudonajatoxin b (a highly lethal neurotoxic polypeptide from the Australian common brown snake *Pseudonaja textilis*; Tyler *et al*., 1987). Neurotoxin homologues CM10, CM12 (*Naja haje annulifera*; Joubert, 1975) and S_5C_{10} (*Dendroaspis jamesoni*; Joubert & Taljaard, 1979) are proteins that are structurally similar to neurotoxins, but with greatly reduced toxicity ($LD_{50} \sim 5-60\,mg\,kg^{-1}$) (Dufton & Hider, 1983). Candoxin showed very little sequence homology to all these 'reversible' toxins as well as to other more conventional neurotoxins (Figure 6).

The neuromuscular blockade induced by toxin LSIII (*Laticauda semifasciata*) in the CBCM was reported to be significantly reversed by washing (Maeda *et al*., 1974; Harvey & Rodger, 1978). However, the time to recovery from neuromuscular blockade produced by LSIII increased with increasing concentrations of the toxin used for blockade. In contrast, the recovery of twitch responses of the CBCM or MHD or GPD from blockade produced by low (3–10 $\mu g\,ml^{-1}$) or high (100 $\mu g\,ml^{-1}$) concentrations of candoxin occurred well within 15–20 min. The concentration-dependent pattern of recovery shown by LSIII is unusual, since it has been proposed that although a higher concentration of toxin spreads faster into the neuromuscular junction within the muscle and produces a more rapid twitch blockade, once the

a

Toxin	Accession #	Amino acid sequence		Identity %
		Loop I / Loop II / Loop III		
Candoxin	P81783	MKCKICNFDTCRAGELKVCASGEKYCFKES-WRE---ARGTRIERGCAATCPKGSVYGLYVLCCTTDDCN--------	66	
WTX	P82935	LTCLNCPEMFC--GKFQICRNGEKICFKKLHQRR---PLSWRYIRGCADTCPVGKPYEM-IECCSTDKCNR-------	65	38%
α-Cobratoxin	P01391	IRCFITPDI-----TSKDCPNG-HVCYTKTWCDAFCSIRGKRVDLGCAATCPTVKTG-VDIQCCSTDNCNPFPTRKRP	71	30%
α-Bungarotoxin	P01378	IVCHTTATSP---ISAVTCPPGENLCYRKMWCDAFCSSRGKVVELGCAATCPS-KKPYEEVTCCSTDKCNPHPKQRPG	74	37%
κ-Bungarotoxin	809178	RTCLISPSS-----TPQTCPNGQDICFLKAQCDKFCSIRGPVIEQGCVATCPQFRLNYRSLLCCTTDNCNH-------	66	33%
Toxin-α	P01426	LECHNQQSSQP--PTTKTCPG-ETNCYKKVWRD----HRGTIIERGCG--CPTVKP-GIKLNCCTTDKCNN-------	61	39%
NmmI	P01431	LECHNQQSSEP--PTTTRCSGGETNCYKKRWRD----HRGYRTERGCG--CPTVKK-GIELNCCTTDRCNN-------	62	37%
Erabutoxin b	230845	RICFNHQSSQP--QTTKTCSPGESSCYHKQWSD----FRGTIIERGCG--CPTVKP-GIKLSCCESEVCNN-------	62	35%

b

Toxin	Accession #	Amino acid sequence		Identity (%)
		Loop I / Loop II / Loop III		
Candoxin	P81783	MKCKICNFDTCRAGELKV--CASGEKYCFKESWRE----ARGTRIERGCAATCPKGSVYGLYVLCCTTDDCN-------	66	
CM10	P01420	MICYKQQSLQFPI---TTV-CPGEKN-CYKKQWSG----HRGTIIERGCG--CPS-VKKGIEINCCTTDKCNR------	61	42%
CM12	P01421	MICYKQRSLQFPI---TTV-CPGEKN-CYKKQWSG----HRGTIIERGCG--CPS-VKKGIEINCCTTDKCNR------	61	40%
Pseudonajatoxin B	P13495	RTCFITPD--VKS---KP--CPPGQEVCYTETWCDGFCGIRGKRVELGCAATCPTPKKTGIDIQCCSTDDCNTFPLRP-	71	37%
LSIII	P01379	RECYLNP---HDT---QT--CPSGQEICYVKSWCNAWCSSRGKVLEFGCAATCPS-VNTGTEIKCCSADKCNTYP----	66	35%
S5C10	P01419	RICYNHQS-NTPA---TTKSCVENS--CYKSIWAD----HRGTIIKRGCG--CPR-VKS--KIKCCKSDNCNL------	58	34%
Fasciatoxin	P14534	LKCHKAQ---FPN—IETQ--CKWQTL-CFQRDVKP---HPSSMIVLRGCTSSCGK------GAMCCATDLCNGPSTPST	61	24%

Figure 6 Comparison of the amino-acid sequence of candoxin with the sequences of other (a) neurotoxins and (b) 'reversible' neurotoxins and neurotoxin homologues. The number of amino-acid residues in each sequence is indicated at the end of the respective sequence. The percentage (%) identity of each sequence to the sequence of candoxin is also shown. The cysteine residues are shaded in grey. The four conserved disulfide linkages and the segments contributing to the three loops are outlined. The species names are as follows: WTX (nonconventional toxin) (*Naja kaouthia*), α-cobratoxin (*Naja kaouthia*), α-bungarotoxin (*Bungarus multicinctus*), κ-bungarotoxin (*Bungarus multicinctus*), toxin α (*Naja nigricollis*), NmmI (*Naja mossambica mossambica*), erabutoxin b (*Laticauda semifasciata*), CM10 and CM12 (*Naja haje annulifera*), pseudonajatoxin B (*Pseudonaja textilis*), LSIII (*Laticauda semifasciata*), S5C10 (*Dendroaspis jamesoni*) and fasciatoxin (*Bungarus fasciatus*). The protein database accession numbers are also stated for each toxin.

preparation is completely blocked, the 'effective' toxin concentration is similar and independent of the initial concentration added to the bath, and consequently, the pattern of recovery is similar (Harvey & Rodger, 1978). Functional studies on the neurotoxin homologues, CM10, CM12 and S_5C_{10} showed that these proteins also exhibit a postsynaptic site of action as do curaremimetic α-neurotoxins, but with reduced affinity and that in addition, the neuromuscular blockade produced by CM10, CM12 and S_5C_{10} were easily reversed by washing or by the addition of neostigmine (Harvey *et al*., 1984). The nature of the reversibility of neuromuscular blockade induced by fasciatoxin (*Bungarus fasciatus*) and by pseudonajatoxin b (*Pseudonaja textilis*) has not been investigated in detail.

It could be argued that the reversibility of neuromuscular blockade induced by some toxins, as opposed to the irreversible blockade attributed to others, may just result from their weak binding affinity to the receptors. However, in electrophysiological studies, α-bungarotoxin produced an irreversible block of muscle ($\alpha\beta\gamma\delta$) receptors with an IC_{50} of ~5 nM (Johnson *et al*., 1995), which is just two-fold lower than the IC_{50} (~10 nM) of candoxin, which produced reversible blockade of the same receptor (Nirthanan *et al*., 2002a). Utkin *et al*. (2001) have also found that WTX (*Naja kaouthia*), a toxin that is structurally similar to candoxin but a 1000-fold weaker antagonist at muscle ($\alpha\beta\gamma\delta$) receptors, is almost irreversible in its action. Clearly, therefore, the reversibility of toxin action at the neuromuscular junction is not always a reflection of their binding affinity to the receptor. It has already been suggested that reversibility of neurotoxin action may be associated with a specific area of interaction on the toxin molecule, distinct from the receptor recognition site (Harvey & Rodger, 1978). For instance, it was recently reported that the mutation of a single residue (Phe^{38}) in a three-finger toxin m1-Toxin 1 (*Dendroaspis angusticeps*) that binds specifically and irreversibly to M_1 muscarinic receptors, resulted in completely reversible binding of the toxin to M_1 receptors (Krajewski *et al*., 2001). Harvey *et al*. (1984) also made the observation that in contrast to most α-neurotoxins, an aspartate at position 31 was conspicuously absent in both CM10 and CM12, as well as in toxin LSIII, all of which were reported to be reversible in their action. The authors postulated that the absence of an aspartate at position 31 may be associated with easy reversibility of neuromuscular blockade produced by these toxins. Interestingly, candoxin also lacks an aspartate at a position homologous to position 31 in CM10 and CM12.

Effects of candoxin on oocyte-expressed muscle (αβγδ) nAChRs

Candoxin effectively blocked currents evoked by ACh in muscle ($\alpha\beta\gamma\delta$) nAChRs expressed in *Xenopus* oocytes with a low half-inhibitory concentration of ~10 nM (Nirthanan *et al*., 2002a). In agreement with the neuromuscular blockade produced by candoxin in *in vitro* and *in vivo*, complete recovery from the candoxin-induced block of muscle ($\alpha\beta\gamma\delta$) nAChRs was observed following washing. In contrast, the functional block of oocyte-expressed muscle ($\alpha\beta\gamma\delta$) nAChRs produced by α-bungarotoxin (Johnson *et al*., 1995), erabutoxin-a (Servent *et al*., 1997) and WTX (Utkin *et al*., 2001) were virtually irreversible. Candoxin is therefore functionally distinct from other curaremimetic short- and long-chain α-neurotoxins as well as other nonconventional toxins from cobra venoms.

The inhibition concentration – response curve of muscle ($\alpha\beta\gamma\delta$) receptors by candoxin was fitted by the sum of two Hill equations with a high- (2.2 nM, $nH = 1.6$) and low- (98 nM, $nH = 1.4$) affinity component that contributed almost equivalently, 46 and 54%, respectively, to the inhibition. Attempts to fit the data points using a single Hill equation yielded a low Hill coefficient (IC_{50} of 18 nM, $nH = 0.64$), which cannot adequately describe the cooperativity between the subunits in the allosteric model of the nAChR (Changeux & Edelstein, 1998). Even if candoxin showed equal affinity for the two binding sites and acted independently upon them, the Hill coefficient would be expected to be close to one. Taking this into consideration, we were of the opinion that using the sum of two Hill equations to describe the inhibition curve of candoxin for the muscle receptor was a better representation of physiological reality. Furthermore, this apparent dual affinity inhibition of muscle $\alpha\beta\gamma\delta$ nAChRs by candoxin also revealed the presence of a plateau phase between the high- and low-affinity dose – response inhibition curves. These data suggested that candoxin might exhibit different affinities for the two nonidentical binding sites present on muscle $\alpha\beta\gamma\delta$ nAChRs although further experiments are needed to confirm this finding. The existence of two binding sites, a high-affinity site at the α/γ subunit interface and a low-affinity site at the α/δ subunit interface, in the mammalian muscle and *Torpedo* receptors has been previously established based on the ability of d-TC to bind to the two sites with dissociation constants that differed by ~100-fold (Neubig & Cohen, 1979; Pedersen & Cohen, 1990). This difference has been attributed to the influence of the nonequivalent subunits (i.e. γ and δ) on the conformation of the binding sites at their respective interfaces with the α subunit (Neubig & Cohen, 1979).

More recently, α-conotoxin MI (*Conus magus*) has been reported to display unique specificity for the α/δ site over the α/γ site (Jacobsen *et al*., 1999). The current electrophysiological study on α-conotoxin MI demonstrated that the presence of a high- and a low-affinity site on muscle $\alpha\beta\gamma\delta$ nAChRs can be functionally revealed by the observation of a biphasic concentration – response inhibition curve with a plateau phase. Specifically, α-conotoxin MI revealed the existence of a high- and a low-affinity site for which it displayed IC_{50} values of ~0.33 nM ($nH = 1.2$) and ~150 nM ($nH = 1.2$), respectively. In contrast, α-neurotoxins such as erabutoxin-b, α-bungarotoxin and α-cobratoxin have been reported to antagonize both binding sites with equal affinity (Taylor *et al*., 1998; Jacobsen *et al*., 1999) and such a dual affinity curve with a plateau phase has not been observed in their binding or functional studies. However, site-directed mutagenesis studies on α-cobratoxin revealed that the mutation of Lys^{23} and Lys^{49} to Glu^{23} and Glu^{49}, respectively, caused a differential lowering of binding affinity at the two binding sites of the muscle $\alpha\beta\gamma\delta$ nAChRs (Antil-Delbeke *et al*., 2000). This observation was verified for a short-chain α-neurotoxin *Naja mossamica mossambica* (NmmI), whereby the mutation of Lys^{27} to Glu^{27} affected binding at the α/γ site more than the α/δ site (Ackermann & Taylor, 1997). Interestingly, position 29 in candoxin (homologous to Lys^{23} and Lys^{27} in α-cobratoxin and NmmI, respectively) is occupied by a glutamic acid (Glu^{29}) instead of a lysine (Figure 6). The possible role of Glu^{29} in candoxin in

conferring differential selectivity for the two binding sites of the muscle $\alpha\beta\gamma\delta$ nAChR warrants further investigation.

Effects of candoxin on twitch responses evoked by TOF stimulation

Candoxin, like d-TC, produced significant TOF fade during the rapid onset of and recovery from neuromuscular blockade in the mouse diaphragm *in vitro* and in anaesthetized rats. In contrast, under the same experimental conditions, erabutoxin-b, α-cobratoxin and α-bungarotoxin did not produce such a fade during neuromuscular blockade. It has long been recognized that d-TC and related compounds, in addition to producing neuromuscular blockade, also produced an independent 'fade' phenomenon characterized by rapidly waning tetanic tension or rundown in the successive twitch responses to TOF nerve stimulation (Paton & Zaimis, 1952; Cheah and Gwee, 1988; Gibb & Marshall, 1986; Bowman *et al.*, 1988). In contrast, curaremimetic α-neurotoxins such as α-bungarotoxin do not display prominent fade response during TOF stimulation (Gibb & Marshall, 1986; Bowman *et al.*, 1988; Cheah & Gwee, 1988; Blount *et al.*, 1992; Wilson & Nicholson, 1997; Oliveira & Oliveira, 1999). Nonetheless, it has been reported that under certain experimental conditions, α-cobratoxin (Chang & Hong, 1987; Hong & Chang, 1991) and erabutoxin-b (Bradley *et al.*, 1987), but not α-bungarotoxin (Bradley *et al.*, 1990), also produced fade when tested in low (<5 nM) concentrations and after prolonged ($>5-8$ h) incubation periods, the fade being more pronounced following the washout of the toxin (Bradley *et al.*, 1990).

The phenomenon of TOF fade has been ascribed to a presynaptic event at the neuromuscular junction involving putative autofacilitatory nAChRs. Bowman *et al.* (1986; 1988) hypothesized that these prejunctional nicotinic receptors serve as 'autoreceptors' that sustain a positive feedback mechanism, which mobilizes 'reserve' stores of ACh to a 'releasable' store and thus maintain adequate transmitter release during high-frequency nerve activity. Consequently, blockade of this autoreceptor would inhibit the positive feedback control of ACh release resulting in a fade in muscle tension during rapid, repetitive nerve stimulation (Wessler *et al.*, 1986; Gibb & Marshall, 1987; Bowman *et al.*, 1988; Prior *et al.*, 1995; Singh & Prior, 1998). Other studies have reported that d-TC, but not snake α-neurotoxins, produced a reduction in nerve-evoked ^{3}H-ACh release from rat nerve terminals during rapid and repetitive nerve stimulation, providing direct evidence that a presynaptic modulation of ACh release may be involved in the fade response produced by d-TC (Vizi & Somogyi, 1989; Wessler, 1992; Apel *et al.*, 1995).

An alternate hypothesis suggests that the presynaptic nicotinic receptors normally function to reduce ACh release via a *negative* feedback mechanism and consequently, a block of these presynaptic receptors would *enhance* ACh release (Wilson, 1982). Subsequently, it has been suggested that another distinct population of prejunctional 'inhibitory' nicotinic autoreceptors may coexist with the 'facilitatory' autoreceptors involved in positive feedback modulation of transmitter release (Tian *et al.*, 1994; 1997). The negative feedback mechanism, however, has been proposed to act to reduce the number of vesicles docked at the release sites during passive leakage or spontaneous release of transmitter from the nerve terminal (Prior *et al.*, 1995). Therefore, it is unlikely that these inhibitory autoreceptors, which operate at low frequencies of motor nerve stimulation, are involved in contributing to the fade response observed during rapid nerve stimulation.

Other workers have attributed the fade response to a use-dependent failure of postsynaptic receptor function under conditions of repetitive nerve stimulation (Colquhoun *et al.*, 1979; Bradley *et al.*, 1990). Specifically, it was suggested that the fade-producing nicotinic receptor antagonists exhibit slow dissociation rates from the postsynaptic nicotinic receptors with resultant accumulation of blocked channels during repetitive stimulation. Consequently, fewer receptors are available for activation with each consecutive depolarization, thereby producing a fade in endplate currents. To assess if the TOF fade produced by candoxin could be attributed to a progressive blockade of the postsynaptic receptors, use-dependent experiments were designed at neuromuscular junction receptors expressed in *Xenopus* oocytes. Application of low ACh test pulses at periodic intervals (10 s) showed little time dependence when recorded in control conditions. In contrast, incubation with 10 nM candoxin caused a progressive reduction of the ACh-evoked currents (Figure 5b, c) and the time course of the blockade was independent of the frequency of the ACh test pulses (Figure 5c). Comparable results were obtained in five cells (data not shown). This clearly indicates that the fade produced by candoxin cannot be attributed to an open-channel blocking effect of the toxin.

Taken together, the present data strongly suggest that, in addition to its established competitive antagonistic action at postsynaptic nAChRs, candoxin also mediates a presynaptic action at the neuromuscular junction as evidenced by the significant TOF fade produced during the onset of and offset from neuromuscular blockade. This is a novel and unusual functional characteristic of candoxin, not previously reported for snake toxins that exert a curaremimetic effect at the neuromuscular junction.

Effect of candoxin at other cholinergic sites

We also carried out a biochemical assay to investigate the effects of candoxin on AChE since it was important to ensure that candoxin did not possess an intrinsic anticholinesterase activity such as that shown by fasciculin, a three-finger toxin isolated from *Dendroaspis* spp. (Cervenansky *et al.*, 1991). Such an inherent anticholinesterase activity of candoxin would result in a 'self-cancellation' effect (Kenakin, 1993) as a consequence of its neuromuscular blocking action being opposed by the inherent anticholinesterase activity. Studies using an established *in vitro* biochemical assay showed that candoxin did not produce inhibition of AChE activity even at concentrations as high as 100 μM. Furthermore, candoxin, like other snake α-neurotoxins, did not mediate any effects on muscarinic receptors in isolated tissue preparations such as the guinea-pig ileum, rat anococcygeus muscle and rat isolated atria nor block ganglionic transmission mediated by ganglionic nAChRs in the guinea-pig ileum in concentrations that were 250-fold higher than that required to produce complete neuromuscular blockade in the GPD (data not shown).

Conclusions

In summary, candoxin produces a novel pattern of neuromuscular blockade at the neuromuscular junction, not usually

associated with curaremimetic neurotoxins from snake venoms: (1) the blockade of nerve-evoked twitches was rapidly and completely reversed by washing or by the addition of an anticholinesterase, (2) a significant TOF fade was observed during the onset of and recovery from neuromuscular blockade, and (3) the inhibition of the muscle ($\alpha\beta\gamma\delta$) nAChR in electrophysiological studies was characterized by different apparent affinities for the two distinct binding sites on the receptor.

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