J Neurophysiol 90: 3529–3537, 2003.
First published July 30, 2003; 10.1152/jn.00155.2003.

Promiscuous and Reversible Blocker of Presynaptic Calcium Channels in Frog and Crayfish Neuromuscular Junctions From *Phoneutria nigriventer* Spider Venom

Lanfranco R. P. Troncone,[1,2] John Georgiou,[1,3] Shao-Ying Hua,[1] Donald Elrick,[1] Ivo Lebrun,[2] Fabio Magnoli,[2] and Milton P. Charlton[1]

[1]*Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada;* [2]*Instituto Butantan, Laboratory of Pharmacology, Biochemistry and Immunochemistry, São Paulo, SP, 05503-900 Brazil; and* [3]*Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada*

Submitted 19 February 2003; accepted in final form 25 July 2003

Troncone, Lanfranco R. P., John Georgiou, Shao-Ying Hua, Donald Elrick, Ivo Lebrun, Fabio Magnoli, and Milton P. Charlton. Promiscuous and reversible blocker of presynaptic calcium channels in frog and crayfish neuromuscular junctions from *Phoneutria nigriventer* spider venom. *J Neurophysiol* 90: 3529–3537, 2003. First published July 30, 2003; 10.1152/jn.00155.2003. Peptide channel blockers found in venoms of many predators are useful pharmacological tools and potential therapeutic agents. The venom of the Brazilian spider *Phoneutria nigriventer* contains a fraction, ω-phonetoxin-IIA (ω-Ptx-IIA, 8360 MW), which blocks Ca^{2+} channels. At frog neuromuscular junctions (NMJ) bathed in normal Ca^{2+} (1.8 mM) saline, ω-Ptx IIA did not affect spontaneous transmitter release but reversibly reduced evoked transmitter release by 75 and 95% at 12 and 24 nM, respectively. In contrast, toxin effects were irreversible in low-Ca^{2+} (0.5 mM) saline. Ca^{2+} imaging in normal-Ca^{2+} saline showed that ω-Ptx-IIA partially blocked stimulus-dependent presynaptic Ca^{2+} signals, and the blockade was almost completely reversible. Increases in spontaneous release frequency induced by high extracellular K^+ were blocked by ω-Ptx-IIA. Therefore ω-Ptx-IIA blocks N-type Ca^{2+} channels, which admit Ca^{2+} that triggers transmitter release at the frog NMJ. Additional evidence predicts that ω-Ptx-IIA binds to N-type Ca^{2+} channels at a different site from that of ω-Conotoxin-GVIA. ω-Ptx-IIA also gave a low-affinity partial blockade of transmitter release and presynaptic Ca^{2+} signals at crayfish NMJs where P-type channels are blocked by ω-agatoxin-IVA. The Ca^{2+}-dependent reversibility and promiscuity of this toxin may make it highly useful experimentally and therapeutically.

INTRODUCTION

Natural toxins are useful as blockers of various types of Ca^{2+} channels. For instance, N-type Ca^{2+} channels can be blocked by ω-conotoxin-GVIA (ω-CgTx-GVIA), a toxin from the marine snail *Conus geographus*, and P-type channels are inhibited by ω-agatoxin-IVA (ω-Aga-IVA), a toxin from the funnel-web spider *Agelenopsis aperta*. These ω-toxins bind irreversibly or are slowly reversible (see review in Olivera et al. 1994). These toxins block transmitter release at the frog neuromuscular junction (NMJ) and at the mammalian NMJ, respectively, where N- and P-type channels admit Ca^{2+} to trigger transmitter release (see review in Meir et al. 1999). Because they have affinity for distinct types of Ca^{2+} channels, each toxin alone cannot eliminate Ca^{2+} influx at synapses where there are multiple types of Ca^{2+} channels. For instance, although one of either N- or P/Q- type Ca^{2+} channels usually contributes at each type of nerve terminal, it is known that many nerve terminals possess more than one type of Ca^{2+} channel (for reviews, see Fisher and Bourque 2001; Meir et al. 1999). In the study of Ca^{2+}-dependent processes, it would thus be advantageous to employ a nonselective, as well as reversible and impermeant, channel blocker. This study describes physiological effects on transmitter release of such a Ca^{2+}channel blocker obtained from the venom of the South American armed spider, *Phoneutria nigriventer*.

The venom of *P. nigriventer* contains many peptides with distinct pharmacological actions that can block Na^+, K^+, and Ca^{2+} channels (Araujo et al. 1993; Figueiredo et al. 1995; and reviewed in Gomez et al. 2002). A fraction of the venom called PF3 blocked brain dopamine release (Troncone et al. 1995) and blocked acetylcholine (ACh) release at the mouse NMJ (Souccar et al. 1995). The effect of PF3 at mouse NMJs was antagonized by increased concentration of extracellular Ca^{2+}, and it was hypothesized that PF3 blocks presynaptic Ca^{2+} channels. Two other toxins, which have been isolated from the same spider venom and are likely to be the same as PF3, are known as PhTx3–4 and ω-phonetoxin-IIA (ω-Ptx-IIA). Henceforth we will refer to PF3 as ω-Ptx-IIA. PhTx3–4 blocks ACh release and PhTx3–4/ω-Ptx-IIA blocks N-type Ca^{2+} channels in rat dorsal root ganglion neurons and L-type channels in rat pancreatic β cells (Cassola et al. 1998; Cordeiro et al. 1993; Gomez et al. 1995). While ω-Ptx-IIA blocks Ca^{2+} channels in neurons, the mechanism of blockade of synaptic transmission is unknown.

The present study characterizes the mechanism of ω-Ptx-IIA toxin action at intact synapses. We demonstrate that ω-Ptx-IIA partially blocks stimulus-dependent Ca^{2+} signals in presynaptic terminals containing either N- or P-type Ca^{2+} channels. At

Address for reprint requests and other correspondence: M. P. Charlton, Dept. Physiology, MSB 3308, University of Toronto, 1 King's College Circle, Toronto, ON M5S 1A8, Canada (E-mail: milton@spine.med.utoronto.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

the frog NMJ, the block of presynaptic N-type channels by ω-Ptx-IIA was quickly reversible when the physiological saline contained 1.8 mM Ca^{2+}, but was practically irreversible under low extracellular Ca^{2+} (0.5 mM) conditions. ω-Ptx-IIA did not block binding of ω-CgTx-GVIA. Finally, we show that ω-Ptx-IIA also blocks presynaptic P-type channels in an arthropod.

METHODS

Venom and toxin

Purification of the ω-Ptx-IIA toxin originally called PF3 is described elsewhere (Troncone et al. 1995). Briefly, spider venom was obtained in the Laboratory of Arthropods of the Butantan Institute (São Paulo, Brazil) by electric milking, desiccated, resuspended in 2% (vol/vol) acetic acid, centrifuged to remove solids and applied to a Sephadex G50f chromatographic column. Fractions carrying the characteristic flaccid paralysis activity were pooled and lyophilized, resuspended in water and submitted to RP-HPLC using a μC18 Boundapack column with linear gradient of trifluoroacetic acid (0.1% in water, vol/vol) and acetonitrile (90% in phosphoric acid, vol/vol). The gradient ran from 5–35% of acetonitrile in 20 min. The ω-Ptx-IIA/PF3 toxin obtained showed only one chromatographic peak and one amino-terminal amino acid. Further chemical identification by mass spectrometry resulted in the characteristic values of m/z of 1394, 1195, and 1046 corresponding to 6–8 protons, respectively, compatible with a molecular weight of 8,360. Cassola et al. (1998) described the full sequence of this toxin and called it ω-Ptx-IIA. The primary sequence of ω-Ptx-IIA is identical to PF3 (amino acids 15 and 19 were mistyped in Troncone et al. 1995). Furthermore, electron-spray mass spectrometry revealed the same molecular mass obtained by Cassola et al. (1998), and we now refer to PF3 as ω-Ptx-IIA. While ω-Ptx-IIA and ω-Aga-IIIA have 49% amino acid identity, ω-Ptx-IIA has 14 cysteines, probably forming seven disulfide bridges whereas ω-Aga-IIIA has only six disulfide bridges (Cassola et al. 1998; Venema et al. 1992). Another toxin that is probably identical to ω-Ptx-IIA is called Tx3–4 (Cordeiro et al. 1993; see Cassola et al. 1998). For information on availability of ω-Ptx-IIA please contact L.R.P. Troncone.

Electrophysiology

All frog (*Rana pipiens*) experiments were performed on the *cutaneus pectoris* nerve-muscle preparation. Animals were maintained and killed (by double pithing) in accordance with the recommendations outlined in the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals (www.ccac.ca). Electrophysiology was conducted in normal Ringer solution (normal saline) containing (in mM) 120 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $NaHCO_3$, and 5 HEPES. In some experiments, a low-Ca^{2+} saline was used that contained 0.5 mM $CaCl_2$ and 5 mM $MgCl_2$. The pH was adjusted to 7.2 with NaOH. The muscle-selective Na^+ channel blocker μ-conotoxin-GIIIA (10 μg/ml; RBI or Bachem) was sometimes used to avoid contractions in the normal (1.8 mM)-Ca^{2+} saline (Robitaille et al. 1993). Transmitter release was assayed by measuring endplate potentials (EPPs) evoked by nerve stimulation (twin pulses at 100 Hz every 5 s). Standard electrophysiological techniques using intracellular microelectrodes (3 M KCl, resistance: 10–20 MΩ) were employed to record electrical responses in individual muscle cells caused by transmitter release. Spontaneous transmitter release was estimated by measuring miniature EPPs (MEPPs). Responses were acquired by a Labmaster DMA interface and averaged in groups of 6 or 10 by TOMAHACQ, a PC-based data-acquisition program. MEPPs were recorded onto tape using a VR10B digitizer (Instrutech) and later analyzed using ANMEPP software. Both programs were written by T. A. Goldthorpe (Univ. of Toronto). The dose dependency of ω-Ptx-IIA blockade was determined by pooling together data from separate experiments; each increase in toxin concentration was applied long enough to reach equilibrium as established by on-line graphing features in the acquisition software.

The compound EPP (CEPP) from frog muscle was recorded via two platinum recording wires at the bottom of the physiological chamber. The dorsal side of the muscle was oriented directly over the recording wires. These electrodes were connected to a differential AC amplifier (Grass P15) and the CEPP signal was amplified and further processed by computer. After initially setting up the preparation in continuous saline flow (1 pass, 0.5 ml/min), flow was stopped and the bath saline was recirculated with a peristaltic pump. This assured constant mixing of solution without disturbing microelectrode recordings as the toxins were added directly to the bath.

Evoked transmitter release from crayfish (*Procambarus clarkii*) NMJs was measured as excitatory junction potential (EJP) amplitude (Hua et al. 1998). Experiments were conducted on the opener muscle of the first walking leg of small (5 cm) crayfish (Wojtowicz and Atwood 1984). This muscle is innervated by a single excitatory axon and a single inhibitory axon, the former of which was separated from the rest of the nerve bundle at the meropodite segment. All other nerves were cut to ensure that only the selected axon was stimulated. Crayfish saline contained (in mM) 205 NaCl, 5.4 KCl, 13.5 $CaCl_2$, 2.7 $MgCl_2$, 10 D-glucose, and 10 HEPES (pH adjusted to 7.4 with NaOH). In low-Ca^{2+} (2.0 mM) crayfish saline, equinormal NaCl was substituted for $CaCl_2$, and when applied, the bathing saline volume was exchanged several times to ensure equilibration. In recordings of spontaneous miniature EJPs (MEJPs), 10 μM picrotoxin (Sigma) was added to block GABA receptor-mediated miniature inhibitory potentials (Takeuchi and Takeuchi 1969).

Nerve terminal Ca^{2+} imaging

Excised frog nerve-muscle preparations were pinned into specially adapted 25-mm petri dishes containing two wells, one 15-mm square and the other 4-mm square, both 4 mm deep. The wells were formed by two plastic blocks inserted into the uncured silicone elastomer (Sylgard; Dow Corning) poured into a petri dish. After the Sylgard cured, the plastic blocks were removed thus leaving the two wells. The muscle was pinned into the larger well containing frog Ringer solution, and the attached cut end of the nerve was submerged in the adjacent well containing a 5 mM solution of fluorescent Ca^{2+} indicator Ca^{2+} Green-1 dextran 3000 MW (K_d 260 nM; Molecular Probes, Eugene, OR). The end of the nerve was cut again while submerged in this solution. The petri dish was covered and nerve terminals were allowed to load with fluorescent indicator overnight (14 h at 15°C) by forward (anterograde) filling. Frog muscle contractions were eliminated using the acetylcholine receptor blocker α-bungarotoxin (5 μg/ml). For crayfish preparations, Ca^{2+} Green-1 dextran was microinjected directly into excitatory axons and allowed to diffuse into synaptic boutons (Hua et al. 1998). Ca^{2+} responses were obtained over a range of stimulation frequencies (20, 40, and 60 Hz for frog NMJs and 5, 10, 20, and 30 Hz for crayfish NMJs), and within these frequency ranges the response size increased with frequency. Thus our conclusions can be made from the lower frequency data with the assurance that the indicator was not saturated.

Nerve terminals were examined with a water immersion objective (×40, 0.55 NA, Nikon) on a Biorad 600 confocal laser-scanning microscope configured to deliver 488 nm wavelength excitation and to detect emission >500 nm. Photomultiplier gain and black level settings were held constant between repetitions. Fluorescence intensity was averaged for the area of interest, and Ca^{2+} signals were expressed as a relative change in fluorescence intensity using the formula $\Delta F/F = (F_{stimulation} - F_{rest})/F_{rest}$.

Data analysis

Data are presented as means ± SE. Normalization of data were based on baseline measurements obtained over a 5- to 10-min period

prior to start of each experiment. Statistical significance was evaluated using Student's *t*-test ($P < 0.05$).

RESULTS

ω-Ptx-IIA blocks neurotransmission at frog synapses

Presynaptic N-type Ca^{2+} channels are responsible for transmitter release at frog NMJs (Robitaille et al. 1990). To determine whether *Phoneutria* toxin ω-Ptx-IIA acts at frog NMJs, we performed electrophysiological recordings on excised cutaneus pectoris nerve-muscle preparations. ω-Ptx-IIA did not affect the muscle resting membrane potential, which remained between −70 and −90 mV. ω-Ptx-IIA blocked the EPP in a dose-dependent fashion, with an apparent EC_{50} of ~11 nM (Fig. 1*A*; $n = 5$–7). When ω-Ptx-IIA was applied at concentrations >30 nM, responses were vastly reduced within 5 min or were eliminated altogether. High-frequency nerve stimulation (100 Hz) failed to reverse the blockade (data not shown).

The blockade of synaptic responses was reversible under certain conditions. Application of 60 nM ω-Ptx-IIA in normal saline (containing 1.8 mM Ca^{2+} and μ-conotoxin-GIIIA to block muscle contractions) eliminated EPPs; on subsequent wash with fresh saline lacking μ-conotoxin-GIIIA, EPPs reappeared and when responses reached a level (>5 mV) sufficient to generate muscle contractions, recordings were lost (data not shown). We next performed similar experiments in low-Ca^{2+} (0.5 mM) saline, a condition in which transmitter release is reduced and in which muscle contractions do not occur. Figure 1*B* shows data from a typical experiment performed in low-Ca^{2+} saline in which EPPs were largely eliminated within a few minutes of ω-Ptx-IIA addition (120 nM) but the blockade did not reverse after washing with fresh low-Ca^{2+} saline ($n = 5$). In contrast, the blockade was relieved after a wash with normal saline (1.8 mM Ca^{2+}). Similar results were obtained in three experiments and in similar experiments using extracellular recordings of the CEPP (Fig. 4*A* and Table 2). Thus at frog neuromuscular synapses, ω-Ptx-IIA is a high-affinity blocker of synaptic transmission, the actions of which can be reversed by washing with saline containing sufficient (1.8 mM) Ca^{2+}.

ω-Ptx-IIA acts at the presynaptic nerve terminal

To help determine the site of ω-Ptx-IIA action, we studied its effect on spontaneous neurotransmitter release. When spontaneous MEPPs were recorded in normal saline for 30 min before and after addition of ω-Ptx-IIA (120 nM), there was no change in MEPP frequency or amplitude (Fig. 2 and data not shown). The lack of effect of ω-Ptx-IIA on MEPPs is similar to that observed with ω-CgTx-GVIA (personal observations) and suggests that ω-Ptx-IIA does not affect the postsynaptic muscle cells and does not by itself alter intracellular [Ca^{2+}]. This observation contrasts with previous work on mouse NMJs by Souccar et al. (1995) showing that ω-Ptx-IIA reduced both amplitude and frequency of MEPPs. The difference in the effect on MEPP frequency may be due to differences in the membrane potential of the presynaptic terminals as discussed in Hua et al. (1998). To test further the hypothesis that ω-Ptx-IIA acts presynaptically, we examined its ability to interfere with nerve depolarization-induced increases in MEPP frequency. MEPP frequency was increased by the introduction to the saline of 8 mM KCl (with equimolar reduction in NaCl to maintain osmolarity) for 30 min and ω-Ptx-IIA was subsequently added to the bath for the same period. When K^+ concentration was increased, muscle cells were depolarized by ~30 mV and MEPP frequency increased nearly fivefold; subsequent addition of ω-Ptx-IIA reduced MEPP frequency to almost that observed originally in normal saline (Fig. 2; $n = 3$ for each comparison). The data suggest ω-Ptx-IIA blocks presynaptic voltage-dependent Ca^{2+} channels that were opened by the high extracellular K^+-induced presynaptic depolarization.

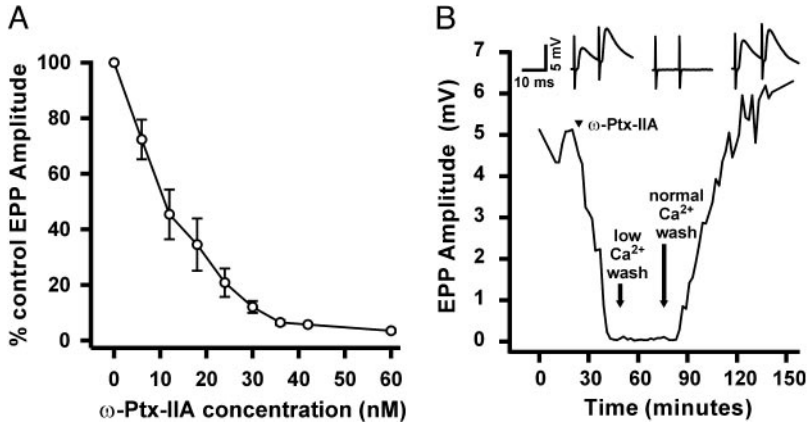

FIG. 1. Dose-dependent and reversible blockade of frog synapses by ω-phonetoxin-IIA (ω-Ptx-IIA). *A*: reduction in amplitude of evoked synaptic responses (endplate potential, EPP) by ω-Ptx-IIA at frog neuromuscular junctions (NMJs) in normal saline (containing 1.8 mM Ca^{2+}) and 3.8 μM μ-conotoxin-GIIIA to block Na^+ channel-dependent muscle contraction. Each experiment was normalized to the EPP amplitude in control saline. The averaged dose-effect curve represents 5–7 experiments in which increasing concentrations of ω-Ptx-IIA were applied. Each concentration was applied long enough to reach equilibrium. *B*: the blockade of synaptic transmission by ω-Ptx-IIA is partially reversible in normal- but not in low-Ca^{2+} saline. ω-Ptx-IIA (120 nM) was first applied in low-Ca^{2+} (0.5 mM) saline and EPPs were almost totally eliminated. Washing with fresh low-Ca^{2+} saline did not reverse the blockade ($n = 5$). However, EPPs were re-established by washing with normal saline (1.8 mM Ca^{2+}). Representative paired-pulse EPPs obtained before and after addition of ω-Ptx-IIA and after washout with normal saline are shown above the graph. No μ-conotoxin-GIIIA was used in this experiment, and the experiment was stopped when contractions appeared in normal Ca^{2+} saline. Similar results were obtained in 3 experiments (also see Fig. 4*A* and Table 2).

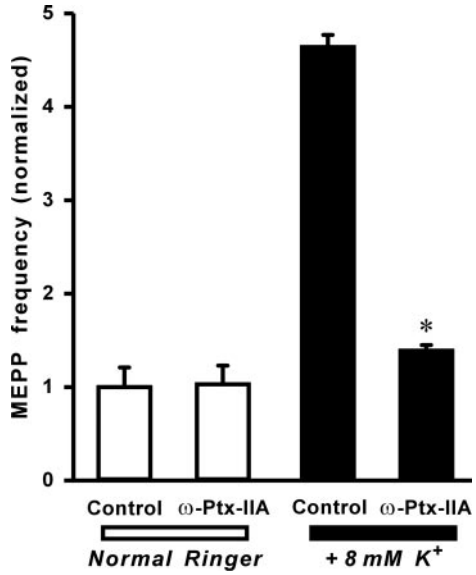

FIG. 2. Effect of ω-Ptx-IIA on miniature EPP (MEPP) frequency at frog synapses. Spontaneous neurotransmitter release frequency was normalized to control value in normal saline (containing 1.8 mM Ca^{2+}). Under these basal conditions, addition of ω-Ptx-IIA (120 nM) did not affect MEPP frequency (□). However, in another set of experiments (■), when asynchronous release rate was first increased (by ~5-fold) by application of saline containing 8 mM KCl, subsequent addition of ω-Ptx-IIA significantly (*t*-test, $P < 0.05$) reduced the MEPP frequency to near the level before application of K^+. Three experiments were performed for each comparison.

Nerve terminal Ca^{2+} signals are attenuated by ω-Ptx-IIA

To seek direct evidence of blockade of presynaptic Ca^{2+} influx, we imaged stimulus-dependent Ca^{2+} signals in nerve terminals. The Ca^{2+}-sensitive fluorescent dye Calcium Green-1 dextran was introduced directly into nerve terminals by anterograde loading through the cut end of the nerve. This method was superior to introduction of cell-permeant fluorescent dyes in that it offered clear and sensitive monitoring of Ca^{2+} in nerve terminals alone; the indicator does not appear in other cells such as perisynaptic glia and muscle cells which also take up the permeant indicators (Jahromi et al. 1992). Figure 3*A* (*left*) shows Calcium Green-1 fluorescence in a typical nerve terminal under resting conditions and during 60-Hz nerve stimulation; the graph shows the time course of the Ca^{2+} signal obtained with 20-, 40-, and 60-Hz stimulation (10 s each). After addition of ω-Ptx-IIA (120 nM), the stimulation-evoked responses in the same terminal were greatly reduced (Fig. 3*A*, *right*). Incomplete blockade of the Ca^{2+} signal is not due to insufficient ω-Ptx-IIA because higher concentrations also failed to eliminate the signal. The remaining signal was not further reduced by subsequent application of ω-CgTx-GVIA and could therefore be due to another channel type not blocked by either toxin.

Additional experiments allowed us to conclude that the remaining nerve terminal Ca^{2+} signals were likely due to influx of Ca^{2+} because they could be virtually eliminated in saline with no added Ca^{2+} (0 mM). The experiment shown in Fig. 3*B* reveals that application of ω-Ptx-IIA (120 nM) attenuated the Ca^{2+} responses, and the remaining signal was nearly eliminated on switching to zero Ca^{2+} saline (containing 120 nM ω-Ptx-IIA). In the same ω-Ptx-IIA-treated nerve terminals, the fluorescence signal was then restored to pretoxin control levels after washing with normal saline (Fig. 3*B*, *right*). A summary of the Ca^{2+} signal data appears in Table 1. We

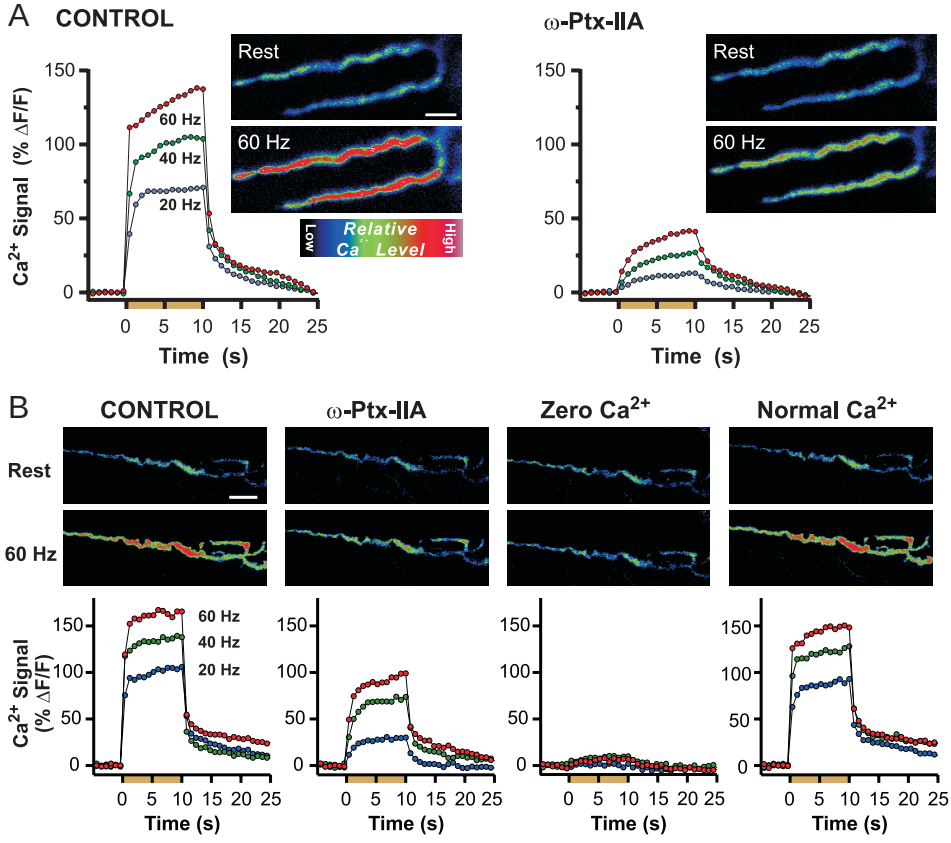

FIG. 3. ω-Ptx-IIA reversibly reduces nerve terminal Ca^{2+} signals at frog synapses. Confocal images of a nerve terminal filled with fluorescent Ca^{2+} indicator Ca^{2+}-Green-1 dextran. Fluorescence appears in pseudocolor, where blue, green, and red correspond to increasing Ca^{2+} levels. Bar, 10 μm. *A*, *left*: image of Ca^{2+} indicator fluorescence before and after 60-Hz nerve stimulation for 10 s in normal (1.8 mM Ca^{2+}) saline. Plotted below the images is a graph of the Ca^{2+} signal in response to 20-, 40-, and 60-Hz stimulation (blue, green, and red circles, respectively). *A*, *right*: corresponding images and plot of Ca^{2+} signals as in left side, after addition of ω-Ptx-IIA (120 nM, 20 min). Note that ω-Ptx-IIA did not affect the resting Ca^{2+} fluorescence but reduced the nerve stimulation-evoked signals. Similar results were obtained in four separate nerve-muscle preparations. *B*: recovery of Ca^{2+} signals after application of ω-Ptx-IIA. Images show fluorescence changes to 60-Hz nerve stimulation in control normal saline, saline containing 120 nM ω-Ptx-IIA, after washing with 0 mM [Ca^{2+}] saline and after return to normal Ca^{2+} saline (20 min each). Graphs below the images show measurements of Ca^{2+} signals obtained with 20-, 40-, and 60-Hz stimulation in the conditions mentioned in the preceding text. These data show almost complete recovery of Ca^{2+} signals after washout of the toxin in normal Ca^{2+} saline. See Table 1 for data summary.

TABLE 1. *Recovery of calcium signals in frog terminals from ω-Ptx-IIA*

Treatment	Calcium Signals by Stimulation Frequency			
	20 Hz	40 Hz	60 Hz	Repetitions (*n*)
Control responses, normal saline, %Δ*F*/*F*	64 ± 10	94 ± 9	114 ± 9	8
ω-Ptx-IIA, normal saline, % control	37 ± 5	46 ± 5	51 ± 6	8
ω-Ptx-IIA + zero Ca^{2+} saline, % control	2 ± 1	4 ± 1	4 ± 1	2
Recovery in normal saline, % control	81 ± 6	93 ± 5	97 ± 5	5

Summary of Ca^{2+} imaging experiments at frog nerve terminals. Muscle contractions were blocked by pre-application of α-bungarotoxin, and Ca^{2+} signals were evoked by successive trains of electrical nerve stimulation at 20, 40, and 60 Hz (10 s each delivered 2 min apart). The first row shows the average size of the Ca^{2+} signal change obtained in normal (1.8 mM Ca^{2+}) saline. All subsequent rows reveal the relative size of the Ca^{2+} responses under various conditions, compared to the initial size of control responses. Concentration of ω-phonetoxin-IIA (ω-Ptx-IIA) was 120 nM and time between treatments was 20 min. Recovery was assessed after washout of toxin by multiple volume exchanges with fresh normal saline.

conclude from these data that ω-Ptx-IIA blocks neurotransmission by reversibly interfering with Ca^{2+} influx at presynaptic nerve terminals.

Do ω-Ptx-IIA and ω-CgTx-GVIA bind to the same site?

To ask whether ω-Ptx-IIA and ω-CgTx-GVIA occupy the same site on the Ca^{2+} channel, we performed a competition experiment. The experimental design relied on the differential recovery of the two toxins, namely that the blockade by ω-Ptx-IIA, but not that of ω-CgTx-GVIA, can be washed off in normal saline. If both toxins occupy the same site, then ω-Ptx-IIA should reduce the ability of ω-CgTx-GVIA to block neurotransmission irreversibly. For these experiments, we used extracellular recordings of the CEPP because this is more suitable for long experiments than intracellular recordings. The CEPP is an average response from many endplates. First, a muscle was exposed to a very high concentration of ω-Ptx-IIA (1.2 μM) for 30 min; this is longer than necessary to achieve a complete blockade of the CEPP. Then, an equimolar concentration of ω-CgTx-GVIA was added to the bath, and both toxins were allowed to compete for 2 h. Subsequently, the muscle was washed for ≥2 h with a constant flow of normal saline. Nerve stimulation was maintained during the experiment at 5-s intervals. A control muscle was incubated in the same conditions but only ω-Ptx-IIA was added, and a second control muscle was incubated with only ω-CgTx-GVIA. These initial experiments were performed in normal (1.8 mM Ca^{2+}) saline (Fig. 4*A*). Application of ω-Ptx-IIA alone produced a blockade of synaptic responses that recovered on washout. In contrast, addition of ω-CgTx-GVIA alone blocked responses irreversibly. When ω-CgTx-GVIA was applied after ω-Ptx-IIA, there was no additional decrease in the amplitude of the CEPP, and there was no recovery after prolonged washing (see Table 2, 2nd column, for summary). This result indicates that ω-CgTx-GVIA was able to bind to channels in the presence of ω-Ptx-IIA as if the two toxins do not share the same binding site. However, the fact that effects of ω-Ptx-IIA are readily reversible in normal saline (1.8 mM Ca^{2+}) raises the possibility that ω-Ptx-IIA constantly binds and unbinds under these conditions. If ω-Ptx-IIA and ω-CgTx-GVIA share a binding site, then ω-CgTx-GVIA may have the opportunity to bind irreversibly every time ω-Ptx-IIA unbinds.

To examine binding sites in more detail, we performed similar experiments in low-Ca^{2+} saline conditions, in which

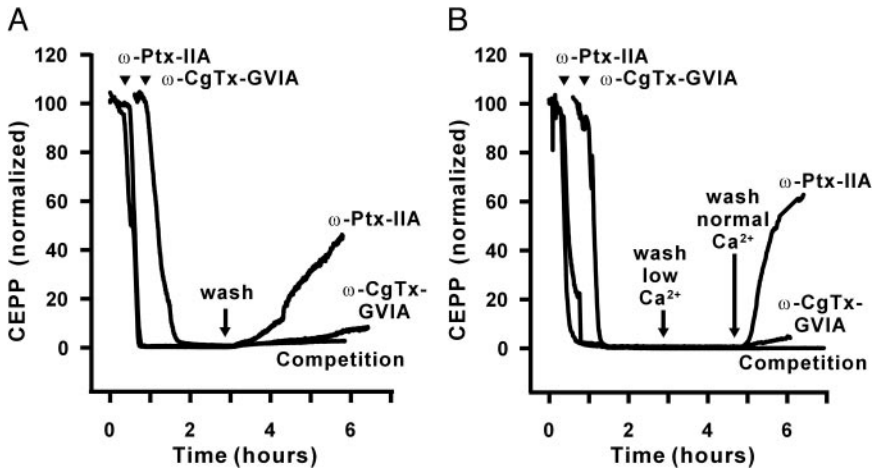

FIG. 4. ω-Ptx-IIA does not prevent action of ω-conotoxin-GVIA (ω-CgTx-GVIA) at frog synapses. Extracellular recordings of the compound EPP (CEPP, 0.2-Hz nerve stimulation) illustrating that blockade by ω-Ptx-IIA is reversible whereas that of ω-CgTx-GVIA is irreversible. *A*: for the 3 experiments shown, normal-Ca^{2+} saline was used, and time of toxin application and wash is shown with arrowheads. In 1 experiment, ω-Ptx-IIA (1.2 μM) was applied, CEPPs disappeared and when ω-Ptx-IIA was washed off synaptic responses reappeared. In another experiment, ω-CgTx-GVIA (1.2 μM) was applied; CEPPs disappeared but hardly recovered after washing. In the competition experiment, ω-CgTx-GVIA was added 30 min after ω-Ptx-IIA, and evoked responses did not recover even after prolonged wash. *B*: similar experiments as in *A* with the exception that saline contained low (0.5 mM) Ca^{2+} during baseline recordings, toxin application and 1st wash. With low Ca^{2+} washing, toxin effects did not reverse. When subsequently washed with normal (1.8 mM) Ca^{2+} saline, CEPPs returned in ω-Ptx-IIA-treated synapses but not in those treated with ω-CgTx-GVIA alone or with ω-Ptx-IIA + ω-CgTx-GVIA (competition). Data summary for part *A* and *B* appear in Table 2.

TABLE 2. *Recovery of neurotransmission from ω-Ptx-IIA and/or ω-CgTx-GVIA*

Toxin Treatment	Normalized Compound Endplate Potential Amplitude: Applied and Washed in Normal Ca^{2+} (1.8 mM), %	Normalized Compound Endplate Potential Amplitude: Applied and Washed in Low Ca^{2+} (0.5 mM) → Followed by Normal-Ca^{2+} Wash, %
ω-Ptx-IIA alone	48 (3)	1 → 65 (2)
ω-CgTx-GVIA alone	5 (2)	0 → 5 (1)
ω-Ptx-IIA + ω-CgTx-GVIA	3 (3)	0 → 0 (2)

Second column shows a summary of experiments shown in Fig. 4*A* in which toxin binding sites on N-type Ca^{2+} channels at frog neuromuscular junctions (NMJs) were studied. Numbers represent percent recovery of control amplitude. Data show effect of toxins on synaptic responses in normal (1.8 mM Ca^{2+}) saline, conditions in which ω-Ptx-IIA blocks reversibly. Either ω-Ptx-IIA (1.2 μM) alone, ω-conotoxin-GVIA (ω-CgTx-GVIA; 1.2 μM) alone, or ω-Ptx-IIA followed by ω-CgTx-GVIA were applied to block neurotransmission. Subsequent washout of toxins was performed by perfusing fresh normal saline. Third column shows a summary of experiments shown in Fig. 4*B* in which toxins were applied to NMJ preparations in low (0.5 mM)-Ca^{2+} saline and subsequently washed in low-Ca^{2+} saline that does not displace ω-Ptx-IIA from N-type channels. A final wash was performed in normal saline. Numbers represent percent recovery with respect to control amplitude established in low-Ca^{2+} saline. Note that for all experiments in the second and third columns, toxins reduced neurotransmission to <1% of initial control levels.

ω-Ptx-IIA blocking effects are irreversible. In these conditions, the ability of ω-CgTx-GVIA to find a binding site vacated by ω-Ptx-IIA would be vastly decreased. In these experiments, there was no recovery of synaptic responses after sequential application of both toxins and washing with low-Ca^{2+} saline (Fig. 4*B*; also see Table 2, 3rd column, summary). However, when washed with normal saline, which displaces the ω-Ptx-IIA, there again was little recovery of synaptic responses. This result indicates that ω-CgTx-GVIA was able to bind under conditions that prohibit unbinding of ω-Ptx-IIA, and therefore it is likely that the two toxins bind to different sites.

Effects of ω-Ptx-IIA at crayfish synapses

We next tested the effects of ω-Ptx-IIA at crayfish NMJs where neurotransmitter release is blocked by ω-agatoxin-IVA (Araque et al. 1994; Wright et al. 1996) and is likely to depend on P-type Ca^{2+} channels. In normal crayfish saline (13.5 mM Ca^{2+}), a 0.5 μM concentration of ω-Ptx-IIA reduced synaptic responses to 35% of control, but EJP amplitude was drastically reduced when the toxin was applied at 1.2 μM (Fig. 5*A*, $n = 8$). When applied at 1.2 μM, 50-Hz stimulation could not restore neurotransmission (Fig. 5*A*), but the effect of ω-Ptx-IIA was easily reversible by washing in toxin-free saline for 5 min (data not shown). Additional observations indicated that ω-Ptx-IIA did not affect the presynaptic action potentials or the amplitude of spontaneous miniature EJPs recorded from muscle ($n = 9$, 3 respectively, data not shown). These findings reveal that ω-Ptx-IIA works presynaptically in crayfish as it does at frog nerve terminals.

Although a higher concentration of toxin was required to block neurotransmission at crayfish synapses than at frog NMJs, the lower sensitivity could be due to the presence of P-type Ca^{2+} channels instead of N-type channels or could also be due to the higher Ca^{2+} concentration (13.5 mM) in the crayfish saline. To examine this further, we next constructed a dose-response curve in normal crayfish saline and in saline that contained only 2.0 mM Ca^{2+}. This analysis showed that the EC_{50} was 120 nM in 2.0 mM Ca^{2+} saline and 360 nM in 13.5 mM Ca^{2+} saline (Fig. 5*B*). The former value is ~10-fold greater than that observed at frog NMJs in similar extracellular Ca^{2+} conditions. Thus the lower sensitivity of crayfish synapses to ω-Ptx-IIA may be due to the presence of P-type Ca^{2+} channels instead of N-type channels at active zones.

To establish the mechanism of action of ω-Ptx-IIA at crayfish NMJs, we performed Ca^{2+} imaging experiments (Hua et al. 1998). The fluorescent indicator Calcium Green-1-dextran was introduced by microinjection directly into crayfish axons and allowed to accumulate at synaptic boutons (Fig. 6*A*). The increase in Calcium Green-1 fluorescence was monitored during short trains of stimuli at 5, 10, 20, and 30 Hz. Simultaneous electrophysiological recordings revealed that at all frequencies tested, the EJP was completely blocked during the tetani (Fig. 6*A*). The graph in Fig. 6*B* shows that ω-Ptx-IIA (1.2 μM) reduced Ca^{2+} signals at 5 Hz but had progressively smaller effects as frequency was increased to 30 Hz (data shown represents mean of 6 experiments). The Ca^{2+} signals were completely blocked when preparations were bathed in crayfish saline containing zero Ca^{2+} and 1 mM EGTA ($n = 5$). Therefore while ω-Ptx-IIA does block presynaptic Ca^{2+} signals, this block is incomplete and is frequency sensitive.

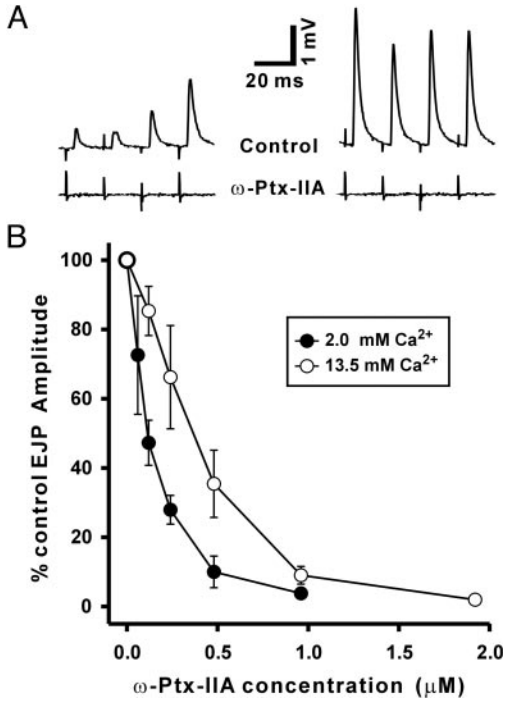

FIG. 5. ω-Ptx-IIA blocks crayfish neuromuscular synapses. *A*: 2 sets of representative data showing excitatory junction potentials (EJPs) evoked by 50-Hz nerve stimulation of crayfish NMJ under control conditions and during exposure to 1.2 μM ω-Ptx-IIA. EJPs were recorded at the beginning (*left*) and end (*right*) of a train of stimuli lasting several s. Facilitation of EJPs was evident in control saline (compare *left* to *right*) but EJPs were not detectable in the presence of ω-Ptx-IIA. Experiments were performed in normal crayfish saline containing 13.5 mM Ca^{2+}. *B*: dose-response curves comparing efficacy of ω-Ptx-IIA action at crayfish synapses in saline containing 2.0 and 13.5 mM Ca^{2+} ($n = 8$).

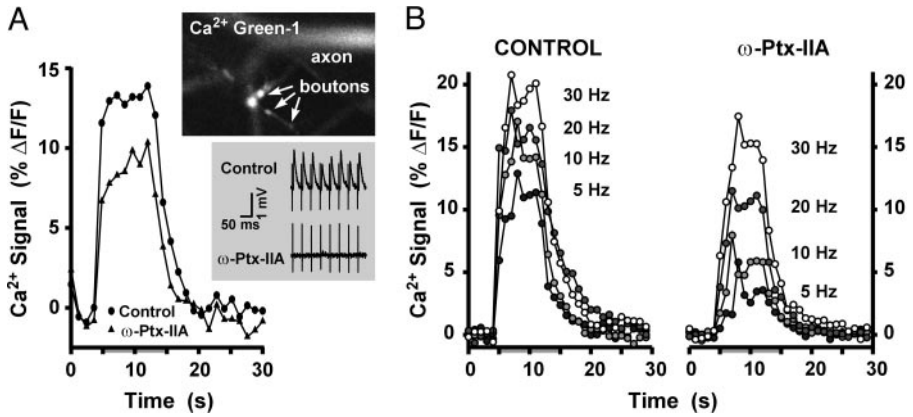

FIG. 6. ω-Ptx-IIA Reduces Ca^{2+} signals in crayfish nerve terminals. *A*: graph shows Ca^{2+} signal obtained during 20-Hz nerve stimulation under control conditions (●) and after addition of 1.2 μM ω-Ptx-IIA (▲). *Top inset*: a representative image of fluorescent Ca^{2+} indicator Calcium Green-1-dextran at crayfish presynaptic boutons loaded via direct injection into axons. *Bottom inset*: simultaneously obtained electrophysiological recordings from same muscle fiber before and after toxin application. Note absence of detectable EJPs in the presence of ω-Ptx-IIA (*bottom records*); all that remain are the stimulus artifacts. All data are from the same muscle fiber bathed in normal crayfish saline. *B*: frequency-dependent attenuation of Ca^{2+} signals by ω-Ptx-IIA. Ca^{2+} responses caused by presynaptic nerve stimulation for 10 s at the indicated frequencies were measured before and after application of 1.2 μM ω-Ptx-IIA. The effect of ω-Ptx-IIA was about threefold greater at 10 than at 30 Hz ($n = 6$).

DISCUSSION

The present data show that ω-Ptx-IIA blocks neurotransmitter release by blocking stimulus-dependent Ca^{2+} entry in intact presynaptic terminals. A direct measurement of intracellular Ca^{2+} by fluorescent imaging showed great reduction in stimulus-dependent signals in presynaptic terminals. Furthermore, ω-Ptx-IIA did not block presynaptic action potentials because after application of ω-Ptx-IIA to block transmitter release, these were unaffected in crayfish NMJs and partial Ca^{2+} signals were still detected in frog terminals. Because ω-Ptx-IIA did not alter the amplitude of spontaneous synaptic potentials, it does not block postsynaptic ACh (frog NMJ) or glutamate (crayfish NMJ) receptors. There could be other effects of this toxin on Ca^{2+} signaling that could not be detected with our assays.

Previous work showed that ω-Ptx-IIA is effective in mammals. For instance intraperitoneal injection of 3 μg of ω-Ptx-IIA in 25 g mice causes flaccid paralysis and death (Rezende et al. 1991). At mouse phrenic-hemidiaphragm NMJ (Souccar et al. 1995) and rat brain striatal tissue (Troncone et al. 1995), ω-Ptx-IIA caused strong blockade of neurotransmitter release. Both of these effects are likely to be due to blockade of P-type Ca^{2+} channels that normally cause transmitter release at the mammalian NMJ (Katz et al. 1997; Meir et al. 1999; Protti et al. 1996; Sugiura et al. 1995) as well as in brain tissue (Carvalho et al. 1995; Kimura et al. 1995). In addition to N- and P-type channels, ω-Ptx-IIA blocks L-type Ca^{2+} channels expressed in cultured undifferentiated PC12 cells (Wolff et al. 1997) and in rat dorsal root ganglion cells (Cassola et al. 1998). Although ω-Ptx-IIA/Tx3–4 partially blocked $^{45}Ca^{2+}$ influx in synaptosomes depolarized by *Tityus* scorpion toxin (Miranda et al. 2001), it is not clear that this influx is connected to Ca^{2+} channels that trigger exocytosis or that would be opened by action potentials.

Reversible action of ω-Ptx-IIA at frog neuromuscular synapses

To characterize the potency of ω-Ptx-IIA at frog NMJs, a dose-response relationship was constructed by adding increasing amounts of ω-Ptx-IIA to normal saline. An approximate EC_{50} of 11 nM was obtained (Fig. 1*A*). Similar EC_{50} values for ω-CgTx-GVIA at frog NMJ have been obtained by others (Katz et al. 1995; Sano et al. 1987; Zengel et al. 1993). Thus ω-Ptx-IIA is a high-affinity blocker of N-type Ca^{2+} channels. ω-Ptx-IIA did not alter spontaneous release at frog NMJs but blocked most of the fourfold increase in frequency induced by 8 mM K^+. This observation suggests that ω-Ptx-IIA blocks Ca^{2+} channels opened by depolarization but is not displaced by depolarization.

The blockade of transmitter release by ω-Ptx-IIA was partially reversed after a few minutes of washing in normal Ca^{2+} saline but was irreversible in low-Ca^{2+} saline. Owing to the onset of contractions we could not follow recovery in normal-Ca^{2+} saline to quantitate the maximal extent of recovery. However, using Ca^{2+} imaging and α-bungarotoxin to block muscle contraction, we found that there was 81–97% recovery of the Ca^{2+} signal when ω-Ptx-IIA was washed off with normal-Ca^{2+} saline. Therefore ω-Ptx-IIA has reversible action at the frog NMJ.

Toxins reveal multiple binding sites on N-type Ca^{2+} channels

Application of ω-Ptx-IIA in low-Ca^{2+} saline conditions, in which its effects were irreversible, did not prevent the irreversible binding of ω-CgTx-GVIA; reversal of blockade, as expected for ω-Ptx-IIA alone (Fig. 4), did not occur when ω-CgTx-GVIA was also applied. This indicates that binding of ω-Ptx-IIA cannot prevent binding of ω-CgTx-GVIA and that therefore the two toxins may bind to different sites on the channel. On the other hand, Dos Santos et al. (2002) showed that ω-Ptx-IIA can totally occlude binding of ω-CgTx-GVIA to recombinant Ca^{2+} channels expressed in baby hamster kidney cells. The comparison of binding studies on recombinant channels with our physiological studies is difficult and may be complicated by differences between channels in different animals.

Because the amino acid sequences of ω-Ptx-IIA and ω-Aga-IIIA are similar, it may be useful to compare them in regard to

binding-site specificity. In binding studies with radiolabeled toxins and rat brain membranes, Adams et al. (1993) demonstrated that ω-Aga-IIIA occludes the binding of ω-CgTx-GVIA and ω-conotoxin-MVIIC but not ω-Aga-IVA. Furthermore McDonough et al. (2002) showed that ω-Aga-IIIA blocked the ability of ω-CgTx-GVIA to block channels in rat sympathetic neurons that otherwise would have been blocked; the authors suggested that ω-CgTx-GVIA and ω-Aga-IIIA bind to a single site on N-type channels to block the channel pore (McDonough et al. 2002). Therefore our results with ω-Ptx-IIA and ω-CgTx-GVIA competing for the N-type voltage-operated calcium channel at frog NMJ suggest differences between ω-Ptx-IIA and ω-Aga-IIIA or differences between rat and frog N-type Ca^{2+} channels. It must also be remembered that our assay was for evoked transmitter release and not binding. Owing to nonlinearities between Ca^{2+} channel function and transmitter release, comparisons of these assays are difficult (see review in Meir et al. 1999). The unbinding of ω-Ptx-IIA from frog N-type channels was highly Ca^{2+} dependent. Effects of extracellular divalent ions such as Ca^{2+} on toxin binding and unbinding have been noted for several toxins including ω-CgTx-GVIA (Liang and Elmslie 2002; Wagner et al. 1988; Witcher et al. 1993).

Action at crayfish synapses

The EC_{50} for ω-Ptx-IIA at crayfish NMJ was 120 nM in 2.0 mM Ca^{2+} saline and 360 nM in 13.5 mM Ca^{2+} saline (Fig. 5*B*). Araque et al. (1994) found 80% blockade of transmitter release at crayfish NMJs with 300 nM ω-Aga-IVA while Wright et al. (1996) found 100% blockade of presynaptic Ca^{2+} current and transmitter release by 100 nM ω-Aga-IVA. It thus appears that ω-Ptx-IIA is a somewhat weaker blocker of crayfish presynaptic Ca^{2+} channels than is ω-Aga-IVA. ω-Aga-IIIA, which is structurally similar to ω-Ptx-IIA, does not affect transmission at NMJs of houseflies but does block Ca^{2+} channels in thoracic ganglion neurons of locusts (Bindokas et al. 1991; Ertel et al. 1994; Pocock et al. 1992). Therefore both toxins can block some Ca^{2+} channels in arthropods.

Calcium signals and multiple channel types

The blockade of presynaptic Ca^{2+} channels by ω-Ptx-IIA was demonstrated by Ca^{2+} imaging. While the blockade of evoked transmitter release at the frog NMJ was complete, the blockade of stimulation-induced fluorescence induced by ω-Ptx-IIA was incomplete (Fig. 3). Similar results were obtained with ω-CgTx-GVIA; when this toxin completely blocked transmitter release the Ca^{2+}-triggered fluorescence signal was still significant (unpublished observations). In our previous studies (Robitaille et al. 1993) using the cell-permeant indicator fluo3-AM, the Ca^{2+} signal was blocked when transmitter release was blocked. This difference may be due to our use of anterogradely loaded Calcium-Green-1-dextran, which has a higher affinity (260 nM) than the fluo3 (390 nM) employed in the earlier study; this would have facilitated the detection of the remaining small Ca^{2+} signals in the present study.

Katz et al. (1995) tested the effects of three different toxins on neurotransmission at frog NMJs. The funnel-web spider toxin FTX (a polyamine), and ω-CgTx-GVIA blocked evoked transmitter release but ω-Aga-IVA had no effect. The effectiveness of FTX to block transmitter release and to block partially perineural Ca^{2+} currents at the frog NMJ (Katz et al. 1995) indicates that there is a small proportion of Ca^{2+} channel types in addition to N channels at these synapses (see also Thaler et al. 2001). In our experiments, the persistence of Ca^{2+} signals when evoked transmitter release had been blocked by ω-Ptx-IIA or ω-CgTx-GVIA indicates by a different method that these presynaptic terminals may possess multiple channel types. However, it is also possible that nonlinearities in the relation between active zone Ca^{2+} concentration and transmitter release combined with the low affinity of the Ca^{2+} sensor for transmitter release and partial channel block in normal Ca^{2+} saline could explain these observations. Similarly, at the crayfish NMJ, we found that presynaptic Ca^{2+} signals persisted when transmitter release had been blocked by ω-Ptx IIA. Rathmayer et al. (2002) found that there are at least two types of channels sensitive to ω-Aga-IVA and ω-CgTx-GVIA that contribute to evoked transmitter release at crab NMJs. Similarly at crayfish motorneuron cell bodies, Hong and Lnenicka (1997) found that when Ba^{2+} replaced extracellular Ca^{2+}, ω-Aga-IVA blocked only 33% of the current, but the remainder was not blocked by ω-CgTx-GVIA or nifedipine. However, at presynaptic terminals of crayfish neuromuscular junctions, Wright et al. (1996) found no effect of ω-CgTx-GVIA on presynaptic Ca^{2+} current or transmitter release but total blockade with 100 nM ω-Aga-IVA. Therefore participation of multiple channel types at crayfish NMJs remains somewhat controversial.

Summary

The work here is the first direct demonstration that ω-Ptx-IIA blocks presynaptic Ca^{2+} channels associated with triggered exocytosis. The results presented here indicate that in addition to its known effects in mammals, ω-Ptx-IIA is also active in lower vertebrates and arthropods. Therefore ω-Ptx-IIA is a broad-spectrum reversible Ca^{2+} channel blocker. This toxin might be useful in pain or stroke treatment where blockade of multiple channel types could be beneficial (see reviews by McIntosh and Jones 2001; Scott et al. 2002). In basic science experiments, this toxin may be useful to block reversibly transmitter release or ion currents that are evoked by action of multiple Ca^{2+} channel types (see review by Fisher and Bourque 2001).

Physiological experiments were performed by L.R.P. Troncone, S.-Y. Hua, D. Elrick, and J. Georgiou in the laboratory of M. P. Charlton at University of Toronto.

DISCLOSURES

L.R.P. Troncone was supported by a postdoctoral fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brasilia, Brazil, No. 200538/95-0. J. Georgiou was supported by a Neuroscience Network Studentship and currently holds a fellowship from the Canadian Neurotrauma Research Program/Medical Research Council of Canada. S.-Y. Hua and D. Elrick were supported by a Canadian Institute of Health Research grant to M. P. Charlton.

Present address of S.-Y. Hua: Department of Biological Science, Barnard College, New York, NY, 10027.

REFERENCES

Adams ME, Myers RA, Imperial JS, and Olivera BM. Toxityping rat brain Ca^{2+} channels with omega-toxins from spider and cone snail venoms. *Biochemistry* 32: 12566–12570, 1993.

Araque A, Clarac F, and Buño W. P-type Ca^{2+} channels mediate excitatory and inhibitory synaptic transmitter release in crayfish muscle. *Proc Natl Acad Sci USA* 91: 4224–4228, 1994.

Araujo DA, Cordeiro MN, Diniz CR, and Beirao PS. Effects of a toxic fraction, PhTx2, from the spider *Phoneutria nigriventer* on the sodium current. *Naunyn-Schmiedebergs Arch Pharmacol* 347: 205–208, 1993.

Bindokas VP, Venema VJ, and Adams ME. Differential antagonism of transmitter release by subtypes of omega-agatoxins. *J Neurophysiol* 66: 590–601, 1991.

Carvalho CM, Ferreira IL, Duarte CB, Malva JO, Tretter L, Adam-Vizi V, and Carvalho AP. Relation of $[Ca^{2+}]_i$ to dopamine release in striatal synaptosomes: role of Ca^{2+} channels. *Brain Res* 669: 234–244, 1995.

Cassola AC, Jaffe H, Fales HM, Castro Afeche S, Magnoli F, and Cipolla-Neto J. omega-phonetoxin-IIA: a calcium channel blocker from the spider *Phoneutria nigriventer*. *Pfluegers* 436: 545–552, 1998.

Cordeiro MdN, de Figueiredo SG, Valentim AdC, Diniz CR, von Eickstedt VR, Gilroy J, and Richardson M. Purification and amino acid sequences of six Tx3 type neurotoxins from the venom of the Brazilian "armed" spider *Phoneutria nigriventer* (Keys). *Toxicon* 31: 35–42, 1993.

Dos Santos RG, von Renterghem C, Martin-Moutot N, Mansuelle P, Cordeiro MN, Diniz CR, Mori Y, de Lima ME, and Seagar M. *Phoneutria nigriventer* ω-phonetoxin IIA blocks the Ca_v2 family of calcium channels and interacts with ω-conotoxin-binding sites. *J Biol Chem* 277: 13856–13862, 2002.

Ertel EA, Warren VA, Adams ME, Griffin PR, Cohen CJ, and Smith MM. Type III ω-agatoxins: a family of probes for similar binding sites on L- and N- type calcium channels. *Biochemistry* 33: 5098–5108, 1994.

Figueiredo SG, Garcia ME, Valentim AC, Cordeiro MN, Diniz CR, and Richardson M. Purification and amino acid sequence of the insecticidal neurotoxin Tx4(6-1) from the venom of the "armed" spider *Phoneutria nigriventer* (Keys). *Toxicon* 33: 83–93, 1995.

Fisher TE and Bourque CW. The function of Ca^{2+} channel subtypes in exocytotic secretion: new perspectives from synaptic and non-synaptic release. *Prog Biophys Mol Biol* 77: 269–303, 2001.

Gomez RS, Casali TA, Romano-Silva MA, Cordeiro MN, Diniz CR, Moraes-Santos T, Prado MA, and Gomez MV. The effect of PhTx3 on the release of ^{3}H-acetylcholine induced by tityustoxin and potassium in brain cortical slices and myenteric plexus. *Neurosci Lett* 196: 131–133, 1995.

Gomez MV, Kalapothakis E, Guatimosim C, and Prado MA. *Phoneutria nigriventer* venom: a cocktail of toxins that affect ion channels. *Cell Mol Neurobiol* 22: 579–588, 2002.

Hong SJ and Lnenicka GA. Characterization of a P-type calcium current in a crayfish motoneuron and its selective modulation by impulse activity. *J Neurophysiol* 77: 76–85, 1997.

Hua SY, Raciborska DA, Trimble WS, and Charlton MP. Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. *J Neurophysiol* 80: 3233–3246, 1998.

Jahromi BS, Robitaille R, and Charlton MP. Transmitter release increases intracellular calcium in perisynaptic Schwann cells in situ. *Neuron* 8: 1069–1077, 1992.

Katz E, Ferro PA, Cherksey BD, Sugimori M, Llinas R, and Uchitel OD. Effects of Ca^{2+} channel blockers on transmitter release and presynaptic currents at the frog neuromuscular junction. *J Physiol* 486: 695–706, 1995.

Katz E, Protti DA, Ferro PA, Rosato Siri MD, and Uchitel OD. Effects of Ca^{2+} channel blocker neurotoxins on transmitter release and presynaptic currents at the mouse neuromuscular junction. *Br J Pharmacol* 121: 1531–1540, 1997.

Kimura M, Yamanishi Y, Hanada T, Kagaya T, Kuwada M, Watanabe T, Katayama K, and Nishizawa Y. Involvement of P-type calcium channels in high potassium-elicited release of neurotransmitters from rat brain slices. *Neuroscience* 66: 609–615, 1995.

Liang H and Elmslie KS. Rapid and reversible block of N-type calcium channels (Ca_V 2.2) by omega-conotoxin GVIA in the absence of divalent cations. *J Neurosci* 22: 8884–8890, 2002.

McDonough SI, Boland LM, Mintz I, and Bean BP. Interaction among toxins that inhibit N-type and P-type calcium channels. *J Gen Physiol* 119: 313–328, 2002.

McIntosh JM and Jones RM. Cone venom–from accidental stings to deliberate injection. *Toxicon* 39: 1447–1451, 2001.

Meir A, Ginsburg S, Butkevich A, Kachalsky SG, Kaiserman I, Ahdut R, Demirgoren S, and Rahamimoff R. Ion channels in presynaptic nerve terminals and control of transmitter release. *Physiol Rev* 79: 1019–1088, 1999.

Miranda DM, Romano-Silva MA, Kalapothakis E, Diniz CR, Cordeiro MN, Moraes-Santos T, De Marco L, Prado MA, and Gomez MV. Spider neurotoxins block the beta scorpion toxin-induced calcium uptake in rat brain cortical synaptosomes. *Brain Res Bull* 54: 533–536, 2001.

Olivera BM, Miljanich GP, Ramachandran J, and Adams ME. Calcium channel diversity and neurotransmitter release: the omega-conotoxins and omega-agatoxins. *Annu Rev Biochem* 63: 823–867, 1994.

Pocock JM, Venema VJ, and Adams ME. Omega-agatoxins differentially block calcium channels in locust, chick and rat synaptosomes. *Neurochem Int* 20: 263–270, 1992.

Protti DA, Reisin R, Mackinley TA, and Uchitel OD. Calcium channel blockers and transmitter release at the normal human neuromuscular junction. *Neurology* 46: 1391–1396, 1996.

Rathmayer W, Djokaj S, Gaydukov A, and Kriesel S. The neuromuscular junctions of the slow and the fast excitatory axon in the closer of the crab *Eriphia spinifrons* are endowed with different Ca^{2+} channel types and allow neuron-specific modulation of transmitter release by two neuropeptides. *J Neurosci* 22: 708–717, 2002.

Rezende L Jr, Cordeiro MN, Oliveira EB, and Diniz CR. Isolation of neurotoxic peptides from the venom of the "armed" spider *Phoneutria nigriventer*. *Toxicon* 29: 1225–1233, 1991.

Robitaille R, Adler EM, and Charlton MP. Strategic location of calcium channels at transmitter release sites of frog neuromuscular synapses. *Neuron* 5: 773–779, 1990.

Robitaille R, Adler EM, and Charlton MP. Calcium channels and calcium-gated potassium channels at the frog neuromuscular junction. *J Physiol Paris* 87: 15–24, 1993.

Sano K, Enomoto K, and Maeno T. Effects of synthetic omega-conotoxin, a new type Ca^{2+} antagonist, on frog and mouse neuromuscular transmission. *Eur J Pharmacol* 141: 235–241, 1987.

Scott DA, Wright CE, and Angus JA. Actions of intrathecal omega-conotoxins CVID, GVIA, MVIIA, and morphine in acute and neuropathic pain in the rat. *Eur J Pharmacol* 451: 279–286, 2002.

Souccar C, Goncalo MdC, Lapa AJ, Troncone LR, Lebrun I, and Magnoli F. Blockade of acetylcholine release at the motor endplate by a polypeptide from the venom of *Phoneutria nigriventer*. *Br J Pharmacol* 116: 2817–2823, 1995.

Sugiura Y, Woppmann A, Miljanich GP, and Ko CP. A novel omega-conopeptide for the presynaptic localization of calcium channels at the mammalian neuromuscular junction. *J Neurocytol* 24: 15–27, 1995.

Takeuchi A and Takeuchi N. A study of the action of picrotoxin on the inhibitory neuromuscular junction of the crayfish. *J Physiol* 205: 377–391, 1969.

Thaler C, Li W, and Brehm P. Calcium channel isoforms underlying synaptic transmission at embryonic *Xenopus* neuromuscular junctions. *J Neurosci* 21: 412–422, 2001.

Troncone LR, Lebrun I, Magnoli F, and Yamane T. Biochemical and pharmacological studies on a lethal neurotoxic polypeptide from *Phoneutria nigriventer* spider venom. *Neurochem Res* 20: 879–883, 1995.

Venema VJ, Swiderek KM, Lee TD, Hathaway GM, and Adams ME. Antagonism of synaptosomal calcium channels by subtypes of ω-agatoxins. *J Biol Chem* 267: 2610–2615, 1992.

Wagner JA, Snowman AM, Biswas A, Olivera BM, and Snyder SH. Omega-conotoxin GVIA binding to a high-affinity receptor in brain: characterization, calcium sensitivity, and solubilization. *J Neurosci* 8: 3354–3359, 1988.

Witcher DR, De Waard M, and Campbell KP. Characterization of the purified N-type Ca^{2+} channel and the cation sensitivity of omega-conotoxin GVIA binding. *Neuropharmacology* 32: 1127–1139, 1993.

Wojtowicz JM and Atwood HL. Presynaptic membrane potential and transmitter release at the crayfish neuromuscular junction. *J Neurophysiol* 52: 99–113, 1984.

Wolff P, Troncone LRP, Lebrun I, Magnoli F, Raw I, and Ho PL. Further biochemical and pharmacological characterization of PF3, a neurotoxin peptide from the "armed" spider *Phoneutria nigriventer*. *J Nat Toxins* 6: 211–219, 1997.

Wright SN, Brodwick MS, and Bittner GD. Presynaptic calcium currents at voltage-clamped excitor and inhibitor nerve terminals of crayfish. *J Physiol* 496: 347–361, 1996.

Zengel JE, Sosa MA, and Poage RE. omega-Conotoxin reduces facilitation of transmitter release at the frog neuromuscular junction. *Brain Res* 611: 25–30, 1993.