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PHONEUTRIA NIGRIVENTER TOXIN 1 : A NOVEL, STATE-DEPENDENT INHIBITOR OF NEURONAL SODIUM CHANNELS WHICH INTERACTS WITH μ CONOTOXIN BINDING SITES *

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Running title : Tx1 inhibits neuronal sodium channels

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229 words in *Abstract*

324 words in *Introduction*

1444 words in *Discussion*

Abbreviations : BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N, N, N', N'-tetraacetic acid; CHO, chinese hamster ovary ; EDTA, ethylene diamine, N, N, N', N' tetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethane-sulfonic acid]; HPLC, high performance liquid chromatography;; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; $Na_v1.2$, brain II isoform of the Na^+ channel; Tx1, *Phoneutria nigriventer* toxin 1.

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ABSTRACT

A toxin was purified to homogeneity from the venom of the South American armed spider *Phoneutria nigriventer* and found to have a molecular mass of 8600 Da and a C-terminally amidated glycine residue. It appears to be identical to Toxin 1 (Tx1) previously isolated from this venom. Tx1 reversibly inhibited sodium currents in CHO cells expressing recombinant sodium ($Na_v1.2$) channels without affecting their fast biophysical properties. The kinetics of inhibition of peak sodium current varied with membrane potential, with on-rates increasing, and off-rates decreasing with more depolarized holding potentials in the (-100 to -50 mV) range. Thus the apparent affinity of Tx1 for the channel increases as the membrane is depolarized. A mono-[^{125}I] iodo Tx1 derivative displayed high affinity binding to a single class of sites (K_D = 80 pM, B_{max} = 0.43 pmol/mg protein) in rat brain membranes. Solubilized binding sites were immunoprecipitated by antibodies directed against a conserved motif in sodium channel α subunits. ^{125}I-Tx1 binding was competitively displaced by μ conotoxin GIIIB (IC_{50} = 0.5 μM), but not by 1 μM tetrodotoxin. However the inhibition of ^{125}I-Tx1 binding by μ conotoxin GIIIB was abrogated in the presence of tetrodotoxin (1 μM). Patch clamp and binding data indicate that *P. nigriventer* Tx1 is a novel, state-dependent sodium channel blocker that binds to a site in proximity to pharmacological site 1, overlapping μ conotoxin but not tetrodotoxin binding sites.

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INTRODUCTION

Voltage-gated sodium channels underlie the rapid depolarizing phase of the action potential and play a crucial role in the propagation of electrical signals in neurons, and in cardiac and skeletal muscle. Sodium channels consist of a pore-forming α subunit associated with either one or two β1-4 auxiliary subunits. Mammals express at least ten sodium channel genes and the co-expression of different combinations can confer distinct electrical properties on neurons and myocytes (Catterall et al., 2003). Sodium channels constitute a major target for therapeutic drugs used in the treatment of epilepsy, pain and cardiac arrhythmias. Many naturally occurring neurotoxins also bind to sodium channels and modify their properties (Cestèle and Catterall, 2000; Terlau and Olivera, 2004; Li and Tomaselli, 2004; Barbier et al., 2003). Their specificity and high affinity makes them ideal probes for defining the different pharmacological sites located on the channel, dissecting the allosteric interactions between these sites and mapping structure-function relationships. Five distinct pharmacological sites have been mapped within the sodium channel sequence (Cestèle and Catterall, 2000). Sites 1-5 are typically defined with tetrodotoxin, veratridine, alpha (Old World) scorpion toxins, beta (New World) scorpion toxins and brevetoxin respectively.

Spider venoms are a rich source of agents that target voltage-gated ion channels and we have thus examined the pharmacological action of peptides from the venom of the South American spider *Phoneutria nigriventer*. *P. nigriventer*, known as the "armed" spider, causes most of the severe human envenomations by spider bite in South-East Brazil (Gomez et al., 2002). Experiments with whole venom and partially

characterized fractions have suggested that toxicity principally involves voltage-gated sodium currents (Fontana and Vital-Brazil, 1985; Love and Cruz-Höfling, 1986; Araùjo et al., 1993).

We have purified *P. nigriventer* Tx1 and evaluated its mode of action by patch clamp and binding studies. Our results indicate that Tx1 is a novel antagonist of neuronal sodium channels that binds in proximity to site 1 and displays increasing affinity as the membrane potential is depolarized.

MATERIALS AND METHODS

Purification and biochemical characterization of Tx1 - Wild *P. nigriventer* were collected in the area of Santa Barbara, near Belo Horizonte, MG, Brazil, and kept at the Fundação Ezequiel Dias (FUNED), Belo Horizonte. Venom was obtained by electrical stimulation of anesthetized spiders. Tx1 was first purified from fractions lethal in mice using the method of Diniz et al. (1990), producing a fraction (designated sample R 21) which was lyophilized. As sample R21 proved to contain two peptides (see Results), it was submitted to an additional HPLC step using a reverse-phase Vydac C18 (218TP54) analytic column (0.46 x 25 cm) eluted with a linear gradient of acetonitrile (0-60%, 60 min) in 0.1% trifluoroacetic acid. Purity was assessed by MALDI TOF mass spectrometry and sequencing. This fraction was designated Tx1 sample R41.

N-terminal sequencing was performed by Edman degradation using a 476A automatic pulsed liquid sequencer (Applied Biosystems). Amino acid analyses were carried out on a 6300 Beckman Analyser. For peptide mass fingerprinting, 8.6 nanomoles of native Tx1 were reduced with dithiothreitol (60-fold excess over SH, 20 hours, 40°C, under N_2) in 0.25 M Tris 6 M guanidine buffer and alkylated with iodoacetamide (1.5-fold excess over SH, 1 hour at 25°C). The reaction was stopped with citric acid, then alkylated Tx1 was desalted by HPLC. Complete alkylation was assessed by MALDI-TOF MS. Alkylated Tx1 (1.46 nmol) was digested with Lys-C protease (final buffer : 25 mM Tris HCl, 1mM EDTA, 10% enzyme / protein (*w*/*w*), 22 hours, 37°C). The solution was acidified and submitted to C18 HPLC. Each peak was collected for mass measurement and / or sequencing.

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Cell culture - The stable CHO line CNaIIA (West et al., 1992) kindly provided by Prof. W.A. Catterall (Seattle), expressing rat brain $Na_v1.2$ was cultured in RPMI supplemented with 5% fetal calf serum, 2 mM glutamine, 10 mM glucose, penicillin (100 U/ml), streptomycin (0.1 g/l), and geneticin (0.2 g/l), at 37°C in a 5% CO_2 atmosphere.

Electrophysiology - Whole-cell currents were recorded from isolated cells using wide-tipped patch pipettes (1.0-1.8 MΩ). pClamp6 & 8 and Sigmaplot softwares were used for experimental protocols and analysis. Whole-cell currents (inward current traces downwards) were digitized (40-100 kHz) after low-pass filtering (5 kHz). The voltage stimulus used to elicit sodium currents is indicated in the Figure legends. A voltage protocol used for leak subtraction was applied after each stimulus (P/n protocole from the holding potential (E_H) with the same polarity as stimulus, n = 3 & 5 in Figs. 1 & 2 respectively). The peak sodium current value (peak I_{Na}) was measured from the zero current level in leak subtracted traces and plotted against the episode starting time. The pipette solution contained (in mM): 135 CsCl, 1 $MgCl_2$, 10 HEPES, 10 BAPTA, pH adjusted to 7.2 with CsOH. The extracellular solution contained (in mM): 150 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 15 glucose, 10 HEPES, pH adjusted to 7.4 with NaOH. Tx1 or tetrodotoxin were added manually near the cell as a single dose left to diffuse for the time indicated by the bars. In experiments shown in Fig. 2, before and after toxin applications a continuous extracellular solution flow was applied to the cell using a local superfusion system (70-100 µl/min).

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^{125}I-Tx1 binding experiments - Tx1 was radio-iodinated as described for ω *Phoneutria nigriventer* toxin IIA (Gouvêa dos Santos et al., 2002), with minor modifications. 0.2 nanomoles Tx1 was reacted for 2 min with 0.2 nanomoles carrier-free Na ^{125}I (Amersham Biosciences) in the presence of lactoperoxidase and H_2O_2 in 50 mM phosphate buffer at pH 7.2. Monoiodo-toxins were separated by reverse-phase liquid chromatography on a C18 column (Vydac, 0.46 x 25 cm, 5 µm particles). The column was eluted with a linear gradient of 19-27 % acetonitrile in 0.1 % trifluoroacetic acid for 65 min at a flow rate of 1 ml/min. Native toxin was eluted after 27 min (22 % acetonitrile), and two radioactive peaks corresponding to the two possible monoiodo-Tx1 derivatives were detected at 28 and 31 min. The second peak was used in binding assays. The binding of ^{125}I-Tx1 to rat brain synaptosomal membranes (prepared as in Martin-Moutot et al., 1995) was measured at 30 °C in 200 µl binding buffer: 140 mM NaCl, 10 mM Tris base, 0.1 % bovine serum albumin, adjusted to pH 7.4. Binding was initiated by addition of 5 µg membrane protein, stopped by rapid filtration on glass fiber filters (GF/C, Whatman) pretreated with 0.3 % polyethyleneimine and washed three times with 2 ml of ice-cold binding buffer. Bound ligand was measured by γ-counting. Tetrodotoxin was purchased from Latoxan (Valence, Fr), veratridine, brevetoxin 2, bupivacaine, lidocaine, tetracaine, pompilidotoxin were from Sigma, and µ conotoxin GIIIB was from the Peptide Institute (Osaka, Jp). Deltamethrin was from Calbiochem, *Leiurus quinquestriatus quinquestriatus* toxin V and *Centruroides suffusus suffusus* toxin VI were kindly supplied by Dr Martin-Eauclaire (Marseille).

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Immunoprecipitation - Synaptosomal membranes (2 mg) were incubated with 0.2 nM ^{125}I-Tx1 overnight at 4 °C in 6 ml of binding buffer, washed by centrifugation, and solubilized in 1.5 % Triton-X100 in 300 µl solubilisation buffer (HEPES 10 mM, KCl 0.1 M, pH 7.4, containing Sigma protease inhibitor cocktail). After centrifugation at 100,000x*g*, aliquots of supernatant containing 8 femtomoles ^{125}I-Tx1 were incubated with antibodies in a final assay volume of 50 µl. After 4 h at 4 °C, immune complexes were recovered by mixing for 30 min with Protein A-Sepharose CL4B. Following centrifugation, the pellet was washed with 0.5 % Triton, 0.1 % bovine serum albumine in solubilisation buffer, and the immunoprecipitated radioactivity was counted.

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RESULTS

Purification of Tx1

Tx1 was purified (sample R21) from *P. nigriventer* venom using a published method (Diniz et al., 1990). Two molecular masses were detected at 8600 and 4920 Da in this fraction, and two sequences assigned by N-terminal Edman sequencing. While the major component (AELTSXFPVG) appeared to be the N terminal sequence of Tx1 (Diniz et al., 1990), the minor one accounting for about 5 % (XGXAQAYKS) showed a clear homology to the N terminal segment of the Tx3-3 neurotoxin (GCANAYKS; Cordeiro et al., 1993), if we assume that Xs are half cystines. A supplementary chromatographic step was thus introduced to isolate the two peptides. The contaminating Tx3-3-like peptide (4920 Da) had no effect on sodium currents (not shown). However these results suggest that previous reports concerning the pharmacology of Tx1 may involve effects of a contaminating peptide (see Discussion).

The homogeneity of the purified major component (sample R41) was assessed by MALDI-TOF MS (8600.4 ± 1.6 Da) and N-terminal sequencing (AELTSXFPVG, without any detectable contamination ie. less than 0.5 %). 8600 Da is significantly higher than the 8557 Da calculated from the published Tx1 sequence (Diniz et al., 1990). However a discrepancy has been reported between the C-terminal sequence of Tx1 determined by peptide sequencing (Diniz et al., 1990) and that predicted by the cDNA (Diniz et al., 1993) : -RREC versus -RRNCGG respectively. In order to confirm that sample R41 was identical to Tx1, the peptide MS fingerprint was established following Lys-C protease digestion (Table 1). Alkylated peptides yielded

masses in good agreement with those predicted, except for the C-terminal peptide. This peptide was identified as KPCRRNCG, which is the 71–78 sequence predicted by the cDNA (Diniz et al., 1993), but with a single terminal glycine residue and C-terminal amidation. The corresponding mass predicted for Tx1 (8598.83 Da) is in good agreement with the observed value.

Initial electrophysiological experiments were performed using Tx1 sample R21 with the contaminating peptide (4920 Da). The 8600 Da Tx1 component was subsequently purified to homogeneity (sample R41). Tx1 samples R21 and R41 were found to have identical effects on sodium currents, thus no distinction was made between these two preparations in our patch clamp studies.

Phoneutria nigriventer Tx1 inhibits voltage-gated sodium currents

Voltage-gated sodium currents were recorded in CHO cells expressing $Na_v1.2$ channels using the whole cell configuration. Repetitive step depolarizations evoked transient inward currents that were reduced by addition of Tx1 (10 µl, 1 µM) near the cell (Fig. 1A), yielding approximately 85 % inhibition of sodium current within 10 minutes (Fig. 1A, B).

Tx1 did not modify the fast biophysical properties of $Na_v1.2$ channels. i/ The kinetics of inward current rise and fall during a 2 ms step depolarization, were not modified following application of Tx1 although inhibition of peak I_{Na} developed from 0 to 85%, suggesting that Tx1 did not alter activation and fast inactivation. ii/ Conductance - voltage and inactivation characteristics were established using standard voltage protocols in the 1 – 100 ms range (Sarkar et al., 1995) from a holding potential of –100 mV. Each test peak I_{Na} was normalized to a reference peak I_{Na} measured

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during a pulse (2 ms to 0 mV) applied a few ms or tens of ms before each conditioning pulse / test pulse pair. Experiments done in the absence of toxin, or in the presence of 100 nM Tx1 in conditions leaving 20 to 40% residual current, showed that conductance - voltage and fast inactivation characteristics were unaffected in the presence of Tx1 (data not shown). iii/ In addition, complete inhibition was observed at high Tx1 concentrations in favourable conditions (see below). These results suggest that in the presence of a non saturating concentration of Tx1, individual channels either function with unmodified fast biophysical properties, or are fully blocked.

The experiments illustrated in Fig. 2 were performed to assess the effects of the holding potential on inhibition of sodium currents by Tx1. In order to measure a pool of functional channels, at holding potentials positive to –100 mV a de-inactivating prepulse of 200 ms to –100 mV was used to reverse the fast inactivation of sodium channels before test. In these conditions, in the absence of Tx1 a slow decrease of peak I_{Na} was observed, corresponding to a slow voltage-dependent inactivation of $Na_v1.2$ channels (see Fig. 2B before Tx1 application). Switching the holding potential from –100 to –50 mV in the absence of toxin resulted in a 15 % decrease in peak I_{Na} within 5 min. In Fig. 2A, currents were evoked initially from a holding potential of –100 mV. In these conditions addition of Tx1 had no effect within 5 min. However shifting the holding potential to –50 mV in the presence of Tx1 rapidly produced inhibition, reaching > 85 % reduction within 7 min. Inhibition was reversed by returning to a holding potential of –100 mV in the presence of Tx1, and toxin wash out at –100 mV had no effect on the kinetics of peak I_{Na} recovery (slow τ_{off} = 201 s before, τ_{off} = 238 s after wash-out). Tetrodotoxin produced full, reversible inhibition

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when applied at –100 mV. In Fig. 2B, Tx1 (10 µl, 1 µM) was applied at a holding potential of –50 mV, inducing a 92 % reduction of peak I_{Na} within 110 s . After wash-out of Tx1, inhibition was only slowly reversible at –50 mV (τ_{off} > 35 min) but recovery accelerated significantly when the holding potential was hyperpolarized to –100 mV (τ_{off} = 172 s). These results, added to those of experiments at E_H intermediate between –100 and –50 mV (not shown), are consistent with the view that depolarizing the holding potential both increases the on-rate and decreases the off-rate for sodium channel inhibition, *ie*. the $Na_v1.2$ sodium channel displays relatively higher apparent affinity for Tx1 at more depolarized membrane potentials.

^{125}I-Tx1 binding properties

Results of an equilibrium binding experiment demonstrating saturable binding of Tx1 to synaptosomal brain membranes are shown in Fig. 3A. Increasing concentrations of ^{125}I-Tx1 were added to membranes in the absence (total binding) or in the presence of a large excess of unlabeled Tx1 (non-specific binding). The difference between these curves yields the saturable binding component. A Scatchard plot of specific binding (Fig. 3A, *inset*) shows a single class of sites with a dissociation constant K_D = 80 pM and a binding capacity B_{max} = 0.43 pmol/mg of cell protein. The kinetics of ^{125}I-Tx1 association and dissociation from membrane binding sites are shown in Fig. 3B and C. The slopes of the linear semilogarithmic plots (Fig.3, C *inset*) gives the apparent association rate constant (k_{app}) = -43 x 10^{-4} s^{-1} and the dissociation rate constant (k_{-1}) = 11 x 10^{-4} s^{-1}. The equation $k_{+1} = (k_{app} - k_{-1}) / [^{125}I\text{-}Tx1]$ yields the association rate constant k_{+1} = 32 x 10^6 $M^{-1}s^{-1}$. The equilibrium dissociation constant K_D calculated from the kinetic data ($K_D = k_{-1}/k_{+1}$) was 33 pM.

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As a previous report suggested that Tx1 acts on N-type ($Ca_v2.2$) calcium channels (Gouvêa dos Santos et al., 1999), immunoprecipitation experiments were performed with solubilized ^{125}I-Tx1-labelled binding sites and antibodies directed against conserved sequences in the Na_v1 or Ca_v2 families of proteins (pan anti-Na_v, antibody directed against a conserved sequence in domain III-IV of the α subunit and an antibody against a conserved sequence in the N-terminal domain of Ca_v2). The results (not shown) indicated that anti-Na_v antibodies recognized the ^{125}I-Tx1 / channel complex and that immunoprecipitation was blocked by the cognate peptide. In contrast anti-Ca_v2 antibodies that recognize N-type channels only precipitated background amounts of ^{125}I-Tx1 / channel complexes. These results are consistent with our patch clamp data and indicate that Tx1 binding sites are associated with sodium channels.

Competition experiments were performed to determine whether Tx1 interacts with any of the defined pharmacological sites on sodium channels. At the indicated concentrations, none of the drugs or toxins active at sodium channel sites 2, 3, 4, or 5 (veratridine 1 µM, *Leiurus quinquestriatus quinquestriatus* toxin V and *Centruroides suffusus suffusus* toxin VI 0.2 µM, and brevetoxin 0.1 µM respectively) nor local anaesthetics (bupivacaine, lidocaine, tetracaine, 10 µM), the wasp venom peptide pompilidotoxin (0.1 µM), nor the pyrethroid deltamethrin (10 µM) significantly modified ^{125}I-Tx1 binding. In contrast although 1 µM tetrodotoxin had no effect, µ conotoxin GIIIB (3 µM), a peptide active at site 1 (Terlau and Olivera, 2004; Li and Tomaselli, 2004; Barbier et al., 2003) displaced 75 % of specific ^{125}I-Tx1 binding. Assays performed to determine the concentration dependency for displacement

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(Fig. 4A) gave an IC_{50}= 300 pM for native Tx1 yielding a calculated K_i= 75 pM, and showing that radio-iodination does not affect the affinity of Tx1. μ conotoxin GIIIB displaced 50% of ^{125}I-Tx1 binding at 0.5 μM. μ conotoxin GIIIB and tetrodotoxin are known to display binding interactions, thus there is an apparent discrepancy in the fact that ^{125}I-Tx1 binding was displaced by μ conotoxin GIIIB but not by tetrodotoxin. Experiments designed to address this issue (Fig. 4A, *inset*), indicated that 1μM tetrodotoxin completely reverses inhibition of ^{125}I-Tx1 binding by 0.3 μM μ conotoxin GIIIB. Thus ^{125}I-Tx1 and tetrodotoxin bind to two distinct sites but μ conotoxin GIIIB overlaps both.

In order to assess the nature of the interaction between the two ligands ^{125}I-Tx1 saturation curves were determined in the presence or absence of μ conotoxin GIIIB (3 μM; Fig. 4B). Scatchard plots (Fig. 4B, *inset*) show a single class of sites in both conditions with a dissociation constant K_D = 30 pM, B_{max} = 520 fmol/mg protein in the absence, and K_D = 140 pM, B_{max} = 520 fmol/mg in the presence of μ conotoxin GIIIB. Inhibition of binding in the presence of μ conotoxin GIIIB is essentially due to an apparent decrease in affinity with no significant modification of binding site capacity. These data are consistent with competitive interaction between Tx1 and μ conotoxin GIIIB.

DISCUSSION

Tx1 was initially purified and sequenced in 1990 (Diniz et al., 1990). Intracerebroventricular injection of this toxin in mice was reported to produce behavioural excitation and spastic paralysis. Our analysis of fractions prepared using the original protocol indicated a major component of 8600 Da and a minor contaminant of 4920 Da. Peptide fingerprinting, mass spectrometry and amino-acid sequencing have unequivocally identified the 8600 Da peptide as Tx1. Tx1 differed from the sequence predicted by the cDNA (Diniz et al., 1993) only at the C-terminus and our data are consistent with post-translational maturation of Tx1 which involves carboxypeptidase cleavage of a glycine residue and α-amidation.

Tx1 was purified to homogeneity and our data indicate that this toxin is an inhibitor of neuronal voltage-gated sodium channels. Previous reports suggested that Tx1 targets calcium channels, mainly based on sequence homologies to agatoxins and on its ability to inhibit the binding of ^{125}I-ω conotoxin GVIA, a ligand specific for $Ca_v2.2$ (N-type) channels (Gouvêa dos Santos et al., 1999). Our results suggest that the contaminating 4920 Da peptide in the initial Tx1 sample R21 could be toxin Tx3-3, based on its partial N-terminal sequence. The Tx3 fraction includes several toxins that are antagonists of high voltage activated calcium channels with a preference for Ca_v2 channels (Leão et al., 2000; Gouvêa dos Santos et al., 2002; Cassola et al., 1998; Vieira et al., 2005). Thus the contaminating peptide may account for Tx1 initially being designated inappropriately as a calcium channel blocker.

Whole cell patch clamp recording from CHO cells expressing recombinant $Na_v1.2$ channels indicate that Tx1 inhibits neuronal voltage-gated sodium channels in

a reversible manner. Its effect resembles tetrodotoxin effect, in that it can induce full inhibition, and does not affect the fast biophysical properties of the residual current in non saturating conditions. These results suggest that in conditions of incomplete inhibition by Tx1, individual channels are either functional with unmodified properties or fully blocked. The simplest explanation would be that channels with bound Tx1 become non-functional. These properties contrasts with those reported for two *P. nigriventer* venom peptides shown to target sodium channel site 3 and slow down sodium channel inactivation in frog muscle (PnTx2-6; Matavel et al., 2002) and insect axon (Tx4(6-1); De Lima et al., 2002) respectively, and suggest that Tx1 may strongly contribute to lethality in human envenomation.

The kinetics of peak sodium current inhibition by Tx1 were voltage-dependent, with on-rates increasing, and off-rates decreasing with more depolarized holding potentials in the -100 to –50 mV range, corresponding to an apparent increase in the affinity of Tx1 for sodium channels at more depolarized membrane potentials. Whether the voltage-dependent inhibition is due to voltage effect on Tx1 or the channel may be questionned. A number of points do not support the hypothesis that the voltage-dependency is due to an effect of the electric field on the toxin molecule: i/ a 78 amino acid peptide with a molecular mass of 8600 Da is not likely to enter the channel pore; ii/ the net charge estimated for Tx1 at pH 7.4 from the published amino-acid sequence (Diniz et al., 1990; Cordeiro et al., 1993) and our data concerning the C-terminus (N76, and C-terminal amidation) is positive (~ +4.6). A positively charged open channel blocker would undergo a relief of block with depolarization, contrary to what is observed; iii/ the idea that a negatively charged arm of the toxin molecule

would behave as an open channel blocker is hardly compatible with the very slow on and off kinetics observed. Therefore, it is likely that the voltage-dependency of inhibition by Tx1 is due to voltage-dependent conformational changes of the sodium channel, making Tx1 a channel state-dependent inhibitor. Tx1 had no effect on sodium currents when applied at a hyperpolarized holding potential (-100 mV) in experiments with brief (2 ms) infrequent (0.1 Hz) depolarizing test pulses. This suggests that the deactivated, deinactivated sodium channel is not a target for Tx1, and that Tx1 rather binds one or several of the channel states reached through membrane depolarization.

Tetrodotoxin and saxitoxin are known to induce use-dependent block of neuronal sodium channels due to channel state-dependent binding (Lönnendonker, 1989a&b; Patton et Goldin, 1991). Using a combination of non stationary fluctuation analysis and use-dependent block analysis, Lönnendonker (Lönnendonker, 1989b) showed that Ranvier node sodium channel affinity for tetrodotoxin and saxitoxin is independent of the holding potential when channels are stimulated at 1 Hz, whereas unstimulated channels may have a lower affinity at more negative holding potentials. In the case of Tx1, the very slow kinetics of on and off effects on neuronal sodium currents allows direct demonstration of a change in apparent affinity at various holding potentials. Slow kinetics in the case of Tx1 may be an advantage for further mecanistic studies.

Slow voltage-dependency has not been studied for Huwentoxin-IV (Peng et al., 2002), the only other spider toxin known to block tetrodotoxin-sensitive sodium channels. However, properties similar to those of Tx1 may be expected for the

uncharacterized toxins 1A and 1B from *Phoneutria keyserlingi* which display 95 and 94% sequence identity respectively to Tx1 (see Table 2). The next nearest sequences found in data banks (34-38% identity to Tx1) correspond to spider toxins known to inhibit voltage-gated calcium channels (variants of omega-phonetoxins from *P. nigriventer* and omega-agatoxin III from *Agelenopsis aperta*).

A ^{125}I-Tx1 derivative displayed high affinity binding to a single class of sites in rat brain membranes. In keeping with the electrophysiological data, two lines of evidence indicate that these binding sites are associated with voltage-gated sodium channels. Firstly detergent-solubilized ^{125}I-Tx1 / binding site complexes were immunoprecipitated by antibodies directed against a conserved motif from sodium channel α subunits. Secondly ^{125}I-Tx1 binding was inhibited by μ conotoxin GIIIB, a sodium channel antagonist from the venom of the predatory marine gasteropod *Conus geographus*.

μ conotoxin GIIIB and the homologous peptide μ conotoxin GIIIA block sodium channels by interacting with the extracellular pore region and compete with tetrodotoxin for binding to site 1. Tetrodotoxin and μ conotoxin binding sites appear to overlap but are not identical (Cestèle and Catterall, 2000; Terlau and Olivera, 2004; Li and Tomaselli, 2004; Barbier et al., 2003). μ conotoxins are potent blockers of skeletal muscle ($Na_v1.4$) sodium channels but display relatively weak affinity for other sodium channel subtypes (Cruz et al., 1985). For example, μ conotoxin GIIIB inhibited ^{3}H-saxitoxin binding to sodium channel site 1 in rat muscle membranes by 80 % with an apparent K_D = 0.14 μM, but only displaced about 20% of binding to brain membranes (Moczydlowski et al., 1986). Our present data indicate that at the

highest concentration tested, μ conotoxin GIIIB inhibited ^{125}I-Tx1 binding to brain membranes by about 70% and that the interaction between the two ligands is competitive. These findings and the fact that Tx1 inhibited sodium currents but did not modify their biophysical properties, suggest that Tx1 acts in proximity to sodium channel site 1. However intriguingly ^{125}I-Tx1 binding was not inhibited by tetrodotoxin, nor were any interactions between ^{3}H-saxitoxin and Tx1 detected (not shown). Nevertheless tetrodotoxin did block the ability of μ conotoxin GIIIB to displace ^{125}I-Tx1 binding. These findings can be explained by assuming that Tx1 and tetrodotoxin bind to distinct sites and that μ conotoxins overlap both.

Sequence comparisons between Tx1 and the μ conotoxin family provided some support for this hypothesis (Table 2). A 19 residue overlap between amino-acids 22–40 of Tx1, toxins 1A and 1B from *P. keyserlingi* and six μ conotoxins indicates overall similarity in the spacing of four conserved cysteines as well as a tryptophan present in the three toxins 1 and three μ conotoxins. Extensive structure-function studies with μ conotoxin GIIIA analogues have indicated an important role for basic residues at positions 13, 16, and 19 which are conserved in μ conotoxins (Li and Tomaselli, 2004; Huang and Miller, 1991; Bulaj et al., 2005; Sato et al., 1991). Arginine 13 is particularly critical for interaction with site 1 and the requirement for a guanidinium group in the channel blocking activity of tetrodotoxin, saxitoxin and the μ conotoxins has led to their classification as the "guanidinium" group of toxins. Interestingly the toxin 1 variants also carry basic residues that align with μ conotoxin residues 16 and 19, but position 13 is occupied by a glycine or an alanine. We may speculate that similarities in cysteine scaffolding and the position of two positive

charges may underlie competitive interactions between Tx1 and the μ conotoxins, while the absence of a basic residue equivalent to arginine 13 might preclude competition between Tx1 and tetrodotoxin. Further studies will be required to clarify the molecular mechanisms by which Tx1 produces state-dependent inhibition in proximity to sodium channel site 1.

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REFERENCES

Araùjo DA, Cordeiro MN, Diniz CR and Beirão PSL (1993) Effect of a toxic fraction, PhTx2, from the spider *Phoneutria nigriventer* on the sodium current. *Naunyn-Schmiedeberg's Arch Pharmacol* **347:**205–208.

Barbier J, Benoit E and Molgó J (2003) Conotoxins targeting voltage-sensitive Na+ channels, in *Bacterial, Plant and Animal Toxins* (Ascenti P, Polticelli F and Visca P eds) pp 127-145, Research Signpost, India.

Bulaj G, West PJ, Garrett JE, Marsh M, Zhang MM, Norton RS, Smith BJ, Yoshikami D and Olivera BM (2005) Novel Conotoxins from *Conus striatus* and *Conus kinoshitai* Selectively Block TTX-Resistant Sodium Channels. *Biochemistry* **44:**7259-7265.

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

Catterall WA, Goldin AL and Waxman SG (2003) International Union of Pharmacology. XXXIX. Compendium of voltage-gated ion channels: sodium channels. *Pharmacol Rev* **55:**575-578.

Cestèle S and Catterall WA (2000) Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie (Paris)* **82:**883-892.

Cordeiro MN, de Figueiredo SG, Valentim AC, Diniz CR, von Eickstedt VRD, Gilroy J and Richardson M (1993) 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.

Cruz LJ, Gray WR, Olivera BM, Zeikus RD, Kerr L, Yoshikami D and Moczydlowski E (1985) *Conus geographus* toxins that discriminate between neuronal and muscle sodium channels. *J Biol Chem* **260:**9280-9288.

De Lima ME, Stankiewicz M, Hamon A, de Figueiredo SG, Cordeiro MN, Diniz CR, Martin-Eauclaire M and Pelhate M (2002) The toxin Tx4(6-1) from the spider *Phoneutria nigriventer* slows down Na^+ current inactivation in insect CNS via binding to receptor site 3. *J Insect Physiol* **48:**53-61

Diniz CR, Cordeiro MN, Rezende LJr, Kelly P, Fischer S, Reimann F, Oliveira EB and Richardson M (1990) The purification and amino acid sequence of the lethal neurotoxin Tx1 from the venom of the Brazilian 'armed'spider *Phoneutria nigriventer FEBS Lett* **263:**251-253.

Diniz MRV, Paine MJI, Diniz CR, Theakston RDG and Crampton JM (1993) Sequence of the cDNA coding for the lethal neurotoxin Tx1 from the Brazilian "armed" spider *Phoneutria nigriventer* predicts the synthesis and processing of a preprotoxin. *J Biol Chem* **268:**15340-15342.

Fontana MD and Vital-Brazil O (1985) Mode of action of Phoneutria nigriventer spider venom at the isolated phrenic nerve-diaphragm of the rat. *Braz J Med Biol Res* **18:**557–565.

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

Gouvêa dos Santos R, Diniz CR, Cordeiro MN and De Lima ME (1999) Binding sites and actions of Tx1, a neurotoxin from the venom of the spider *Phoneutria nigriventer*, in guinea pig ileum. *Braz J Med Biol Res* **32:**1565-1569.

Gouvêa dos Santos R, Van Renterghem C, Martin-Moutot N, Mansuelle P, Cordeiro MN, Diniz CR, Mori Y, De Lima ME and Seagar M (2002) *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.

Huang X and Miller W (1991) A time-efficient, linear-space local similarity algorithm. *Adv Appl Math* **12:**337-357.

Leão RM, Cruz JS, Diniz CR, Cordeiro MN and Beirão PS (2000) Inhibition of neuronal high-voltage activated calcium channels by the ω-Phoneutria nigriventer Tx3-3 peptide toxin. *Neuropharmacology* **39:**1756-1767.

Li RA and Tomaselli GF (2004) Using the deadly mu-conotoxins as probes of voltage-gated sodium channels. *Toxicon* **44:**117-122.

Lönnendonker U (1989a) Use-dependent block of sodium channels in frog myelinated nerve by tetrodotoxin and saxitoxin at negative holding potentials. *Biochim Biophys Acta* **985:**153-160.

Lönnendonker U (1989b) Binding of tetrodotoxin and saxitoxin to Na+ channels at different holding potentials: fluctuation measurements in frog myelinated nerve. *Biochim Biophys Acta* **985:**161-167.

Love S and Cruz-Höfling MA (1986) Acute swelling of nodes of Ranvier caused by venoms which slow inactivation of sodium channels. *Acta Neuropathol (Berl)* **70:**1–9.

Martin-Moutot N, Lévêque C, Sato K, Kato R, Takahashi M and Seagar M (1995) Properties of ω conotoxin MVIIC receptors associated with α1A calcium channel subunits in rat brain. *FEBS Lett* **366:**21-25.

Matavel A, Cruz JS, Penaforte CL, Araùjo DAM, Kalapothakis E, Prado VF, Diniz CR, Cordeiro MN and Beirão PSL (2002) Electrophysiological characterization and molecular identification of the *Phoneutria nigriventer* peptide toxin PnTx2-6. *FEBS lett* **523:**219-223.

Moczydlowski E, Olivera BM, Gray WR and Strichartz GR (1986) Discrimination of muscle and neuronal Na-channel subtypes by binding competition between [^{3}H]saxitoxin and μ-conotoxins. *Proc Natl Acad Sci USA* **83:**5321-5325.

Patton DE and Goldin AL (1991) A voltage-dependent gating transition induces use-dependent block by tetrodotoxin of rat IIA sodium channels expressed in *Xenopus* oocytes. *Neuron* **7:**637-647.

Peng K, Shu Q, Zhonghua L and Liang S (2002) Function and solution structure of Huwentoxin-IV, a potent neuronal tetrodotoxin (TTX)-sensitive sodium channel antagonist from Chinese bird spider *Selenocosmia huwena*. *J Biol Chem* **277:**47564-47571. Correction in *J Biol Chem* (2003) **278:**5489.

Sarkar SN, Adhikari A and Sikdar SK (1995) Kinetic characterization of rat brain type IIA sodium channel alpha-subunit stably expressed in a somatic cell line. *J Physiol (Lond)* **488:**633-645.

Sato K, Ishida Y, Wakamatsu K, Kato R, Honda H, Ohizumi Y, Nakamura H, Ohya M, Lancelin J-M, Kohda D and Inagaki F (1991) Active site of μ-conotoxin GIIIA, a peptide blocker of muscle sodium channels. *J Biol Chem* **266:**16989-16991.

Terlau H and Olivera BM (2004) *Conus* venoms: a rich source of novel ion channel-targeted peptides. *Physiol Rev* **84:**41-68.

Vieira LB, Kushmerick C, Hildebrand ME, Garcia E, Stea A, Cordeiro MN, Richardson M, Gomez MV and Snutch TP (2005) Inhibition of High Voltage-Activated Calcium Channels by Spider Toxin PnTx3-6. *J Pharmacol Exp Ther* **314:**1370-1377.

West JW, Scheuer T, Maechler L and Catterall WA (1992) Efficient expression of rat brain type IIA Na^+ channel α subunits in a somatic cell line. *Neuron* **8:**59-70.

FOOT NOTES

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FIGURE LEGENDS

Fig. 1. **Effect of *P. nigriventer* toxin 1 on sodium currents recorded from a CHO cell expressing recombinant $Na_v1.2$ channels**. A. Superimposed current traces recorded before and after addition of Tx1 (10 µl, 1 µM) near the cell. 5 ms step depolarization from -90 mV to 10 mV was applied every 6 s. The illustrated traces are separated by 60 s, each trace is the average of leak subtracted currents recorded in 10 consecutive tests. B. Plot of the peak sodium current (peak I_{Na}) against test time, in the absence and in the presence of Tx1 (grey box).

Fig. 2. **Effect of the holding potential on the peak current reduction by *P. nigriventer* toxin 1**. Sodium currents from CHO cells expressing $Na_v1.2$ were elicited by test pulses (2 ms to 0 mV, 0.1 Hz) in the absence and in the presence of Tx1 (10 µl, 1 µM; grey bars) or tetrodotoxin (TTX, 10 µl, 10 µM; open bar), and peak I_{Na} was plotted against time. Two intertest holding potentials were used. At E_H = -50 mV, test pulses were preceded by a de-inactivating prepulse (200 ms to –100 mV). A. E_H was shifted from –100 mV to –50 mV for 420 s in the presence of Tx1, and then back to -100 mV 180 s before wash-out. The small (0.2 nA) reduction observed between times 2 and 3 min was due to arrest of perfusion 0.6 min before addition of Tx1. B. E_H was shifted from –100 to -50 mV in the absence of toxin. Then Tx1 was applied for 100 s, and washed out at E_H = -50 mV.

Fig. 3. **Binding of ^{125}I-*P. nigriventer* toxin 1 to rat brain membranes**. A. Rat brain membranes were incubated with the indicated concentrations of ^{125}I-Tx1 for 30 min at 30 °C, in the absence (black circles, total binding) or presence (open circles, non-specific binding) of 50 nM unlabeled Tx1 and then filtered and washed. Bound toxin was measured by gamma counting. Inset: Scatchard plot of the specific binding component (total minus non-specific). B. Rat brain membranes were incubated with 0.1 nM ^{125}I-Tx1 in the presence and absence of unlabeled Tx1. Incubations were stopped by filtration at the indicated time points and the kinetics of specific binding were plotted. Inset: Linear semi-logarithmic plot. C. Rat brain membranes were incubated with 0.1 nM ^{125}I-Tx1 for 30 min at 30 °C. At equilibrium 50 nM unlabeled Tx1 was added and the residual specific binding component was measure by filtration at the indicated times to yield dissociation kinetics. Inset: Linear semi-logarithmic plot.

Fig. 4. **Interactions between ^{125}I-*P. nigriventer* toxin 1 and μ conotoxin binding sites in rat brain membranes**. A. ^{125}I-Tx1 (0.1 nM) was incubated with rat brain membranes for 30 min at 30 °C in the presence of the indicated concentrations of unlabeled Tx1 (circles) or μ conotoxin GIIIB (triangles). Specific binding was measured by filtration. Inset: specific binding of ^{125}I-Tx1 as indicated above (C = control) was measured in the presence of 1μM tetrodotoxin (TTX), 0.3 μM μ conotoxin GIIIB alone (μ) or in the presence of 1 μM tetrodotoxin (μ + TTX). B. Saturation curves were established as in Fig. 3A, in the presence (open symbols) or absence (filled symbols) of 50 nM unlabeled Tx1, and in the presence (triangles) or

absence (circles) of 3 μM μ-conotoxin GIIIB. Inset: Scatchard plot of specific binding in the presence (triangles) or absence (circles) of μ conotoxin GIIIB.

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TABLE 1: Peptide fingerprint of *P. nigriventer* toxin 1

Tx1 was purified to homogeneity, reduced, alkylated and digested with Lys-C protease. Peptides were separated by C18 HPLC and peaks collected for mass spectrometry. The masses measured for three proteolytic peptides (4633.78, 1374.56, 1491.63 Da) were within 100 ppm of the masses predicted for Tx1 proteolytic peptides 1-39, 42-53, and 56-67, as indicated above the published *P. nigriventer* toxin 1 sequence (Diniz et al., 1993; accession number P17727). The last mass measured (1046.31) corresponds (within 192 ppm) to a C-terminal peptide with N at position 76, and a single, amidated terminal glycine.

Mass predicted	4634.04	1374.54	1491.76	1046.51
LysC	*1 39*	*42 53*	*56 67*	*71 78* NH_2
Tx1	AELTSCFPVG HECDGDASNC NCCGDDVYCG CGWGRWNCKC KVADQSYAYG ICKDKVNCPN RHLWPAKVCK KPCRRNCGG			

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TABLE 2 : Sequence similarities between *Phoneutria* toxins 1 and μ conotoxins

The sequence of Tx1 was initially aligned to that of *Conus stercusmuscarum*, μ conotoxin SmIIIA using the LALIGN programme (Huang and Miller, 1991) and then with μ conotoxins: SIIIA from *C. striatus*; KIIIA from *C. kinoshitai;* PIIIA from *C. purpurascens*, GIIIA and GIIIB from *C. geographus* using Table 1 in Bulaj et al. (2005) and *Phoneutria keyserlingi* toxins 1A and 1B (PkTx1A,B, accession numbers P84062 and P84063). Numbers on the left indicate the position of the illustrated motif in each sequence. Grey boxes indicate identity between toxins 1 and μ conotoxins, open boxes indicate conserved basic residues with demonstrated functional importance in GIIIA, numbered below according to their position in this toxin. O = hydroxyproline.

Tx1	(22-40)	C	C	G	D	D	V	Y	C	G	C	G	W	G	R	W	N	C	K	C
PkTx1A	(22-40)	C	C	G	D	D	V	Y	C	A	C	G	W	G	R	W	N	C	K	C
PkTx1B	(22-40)	C	C	G	D	D	V	Y	C	A	C	G	W	G	R	W	N	C	K	C
SmIIIA	(3-21)	C	C	N	G	R	R	G	C	S	S	R	W	C	R	D	H	S	R	C
SIIIA	(3-19)	C	C	N	G	-	-	G	C	S	S	K	W	C	R	D	H	A	R	C
KIIIA	(1-15)	C	C	N	-	-	-	-	C	S	S	K	W	C	R	D	H	S	R	C
PIIIA	(4-21)	C	C	G	F	O	K	S	C	R	S	R	Q	C	K	O	H	-	R	C
GIIIA	(3-20)	C	C	T	O	O	K	K	C	K	D	R	Q	C	K	O	Q	-	R	C
GIIIB	(3-20)	C	C	T	O	O	R	K	C	K	D	R	R	C	K	O	M	-	K	C
												13			16				19	

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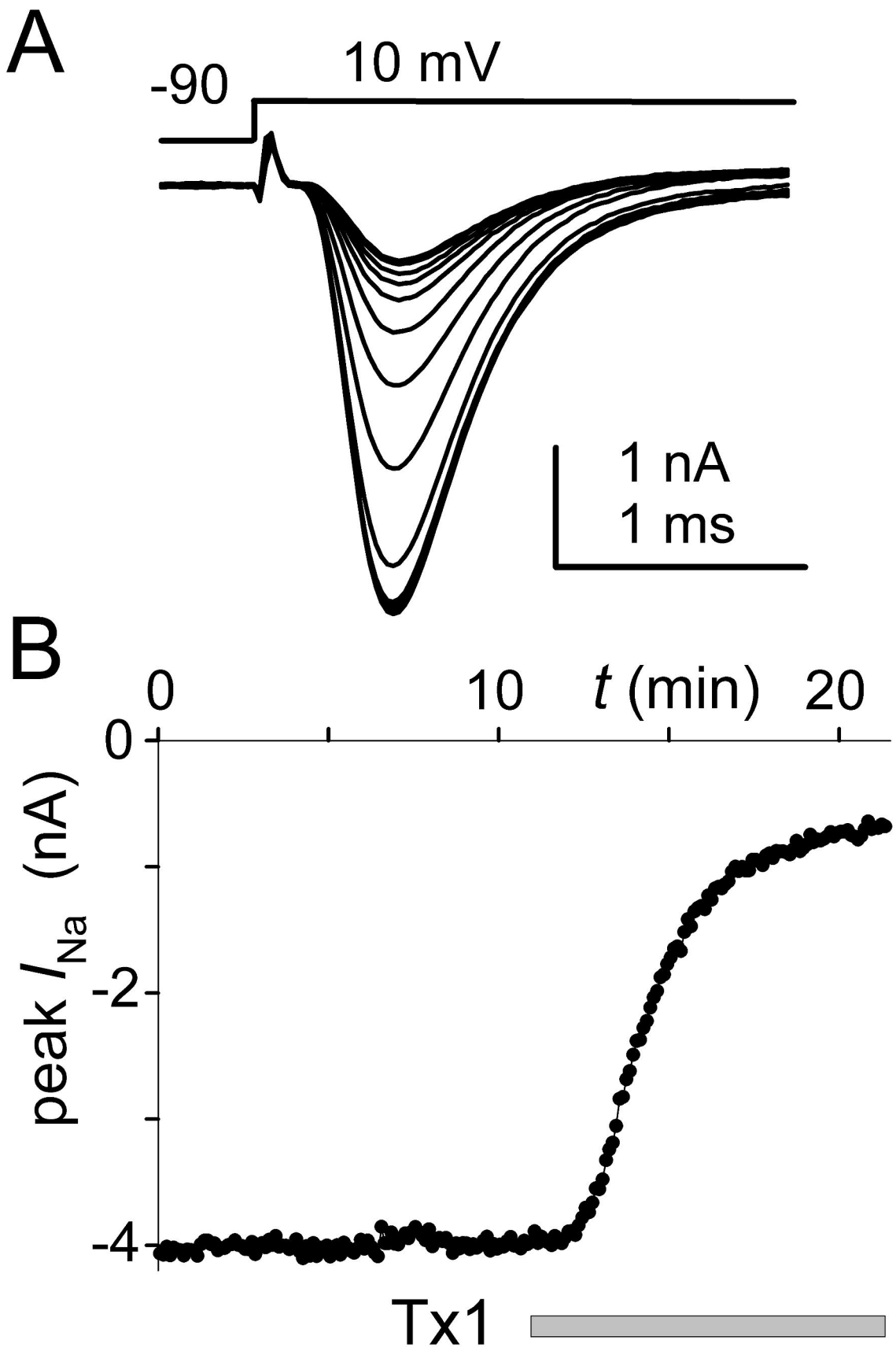

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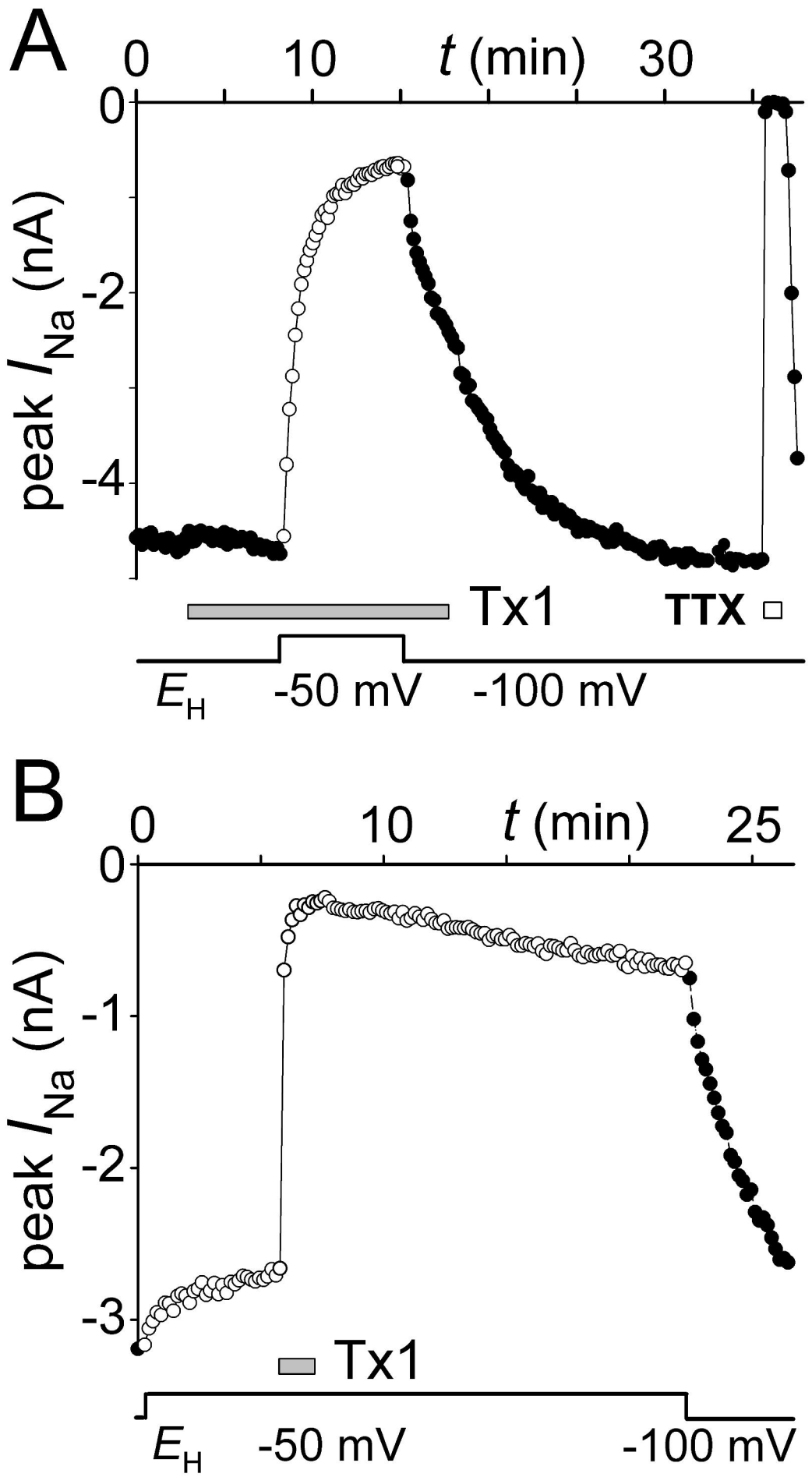

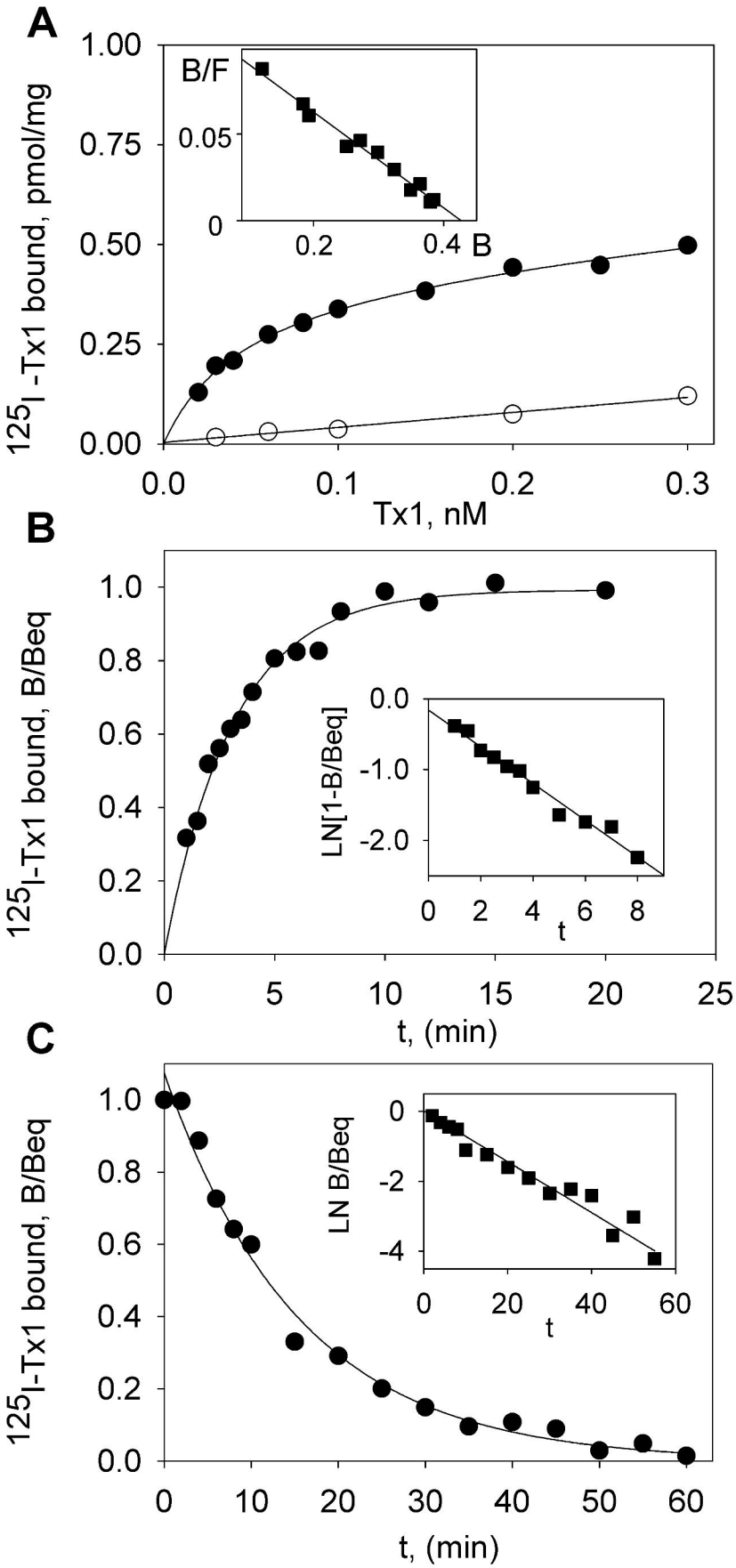

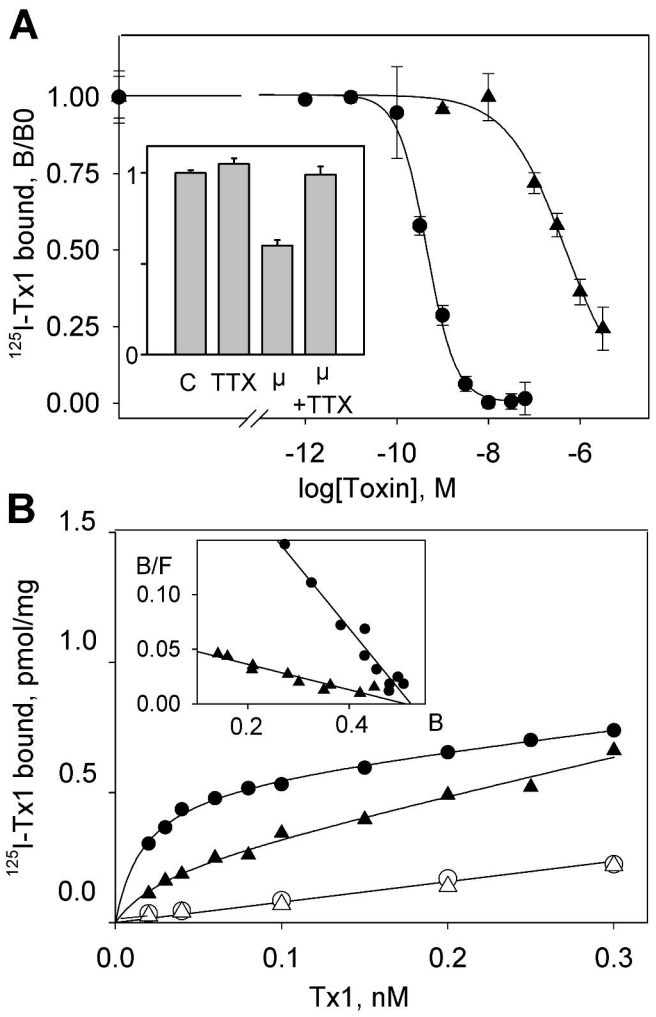
A
1.00
0.75
0.50
0.25
0.00
^{125}I-Tx1 bound, B/B0
1
0
C
TTX
μ
μ
+TTX
-12
-10
-8
-6
log[Toxin], M
B
1.5
1.0
0.5
0.0
^{125}I-Tx1 bound, pmol/mg
B/F
0.10
0.05
0.00
0.2
0.4
B
0.0
0.1
0.2
0.3
Tx1, nM