Phoneutria nigriventer Toxin 1: A Novel, State-Dependent Inhibitor of Neuronal Sodium Channels That Interacts with μ Conotoxin Binding Sites

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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) isolated previously from this venom. Tx1 reversibly inhibited sodium currents in Chinese hamster ovary 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.

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 10 sodium-channel genes, and the coexpression 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; Barbier et al., 2003; Li and Tomaselli, 2004; Terlau and Olivera, 2004). Their specificity and high affinity make 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 to 5 are typically defined with tetrodotoxin, veratridine, α (Old World) scorpion toxins, β (New World) scorpion toxins, and brevetoxin, respectively.

ABBREVIATIONS: Tx1, *Phoneutria nigriventer* toxin 1; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; Na_v1.2, brain II isoform of the Na^+ channel.

Spider venoms are a rich source of agents that target voltage-gated ion channels, and we 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 Southeast 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 purified *P. nigriventer* Tx1 and evaluated its mode of action by patch-clamp and binding studies. Our results indicate that Tx1 is a novel inhibitor 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, 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 R21) that was lyophilized. Because sample R21 proved to contain two peptides (see *Results*), it was submitted to an additional HPLC step using a reverse-phase Vydac C18 (218TP54; Grace Vydac, Hesperia, CA) analytic column (0.46 × 25 cm) eluted with a linear gradient of acetonitrile (0–60% for 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, Foster City, CA). Amino acid analyses were carried out on a 6300 Beckman Analyzer (Beckman Coulter, Fullerton, CA). For peptide mass fingerprinting, 8.6 nmol concentration of native Tx1 were reduced with dithiothreitol (60-fold excess over cysteine, 20 h, 40°C, under N_2) in 0.25 M Tris/6 M guanidine buffer and alkylated with iodoacetamide (1.5-fold excess over cysteine, 1 h at 25°C). The reaction was stopped with citric acid, and 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, 1 mM EDTA, and 10% enzyme/protein (w/w), 22 h, 37°C]. The solution was acidified and submitted to C18 HPLC. Each peak was collected for mass measurement and/or sequencing.

Cell Culture. The stable CHO line CNaIIA (West et al., 1992) kindly provided by Professor W. A. Catterall (Seattle, WA), expressing rat brain $Na_v1.2$, was cultured in RPMI 1640 medium 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 and -8 (Molecular Devices, Sunnyvale, CA) and Sigma Plot (SPSS Inc., Chicago, IL) softwares were used for experimental protocols and analysis. Whole-cell currents (inward current traces downward) 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 protocol from the holding potential (E_H) with the same polarity as stimulus, n = 3 and 5 in Figs. 1 and 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 con-

tained 135 mM CsCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM BAPTA, with pH adjusted to 7.2 with CsOH. The extracellular solution contained 150 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 15 mM glucose, and 10 mM HEPES, with pH adjusted to 7.4 with NaOH. Tx1 or tetrodotoxin was 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).

^{125}I-Tx1 Binding Experiments. Tx1 was radioiodinated as described for ω *P. nigriventer* toxin IIA (Gouvêa dos Santos et al., 2002), with minor modifications. Tx1 (0.2 nmol) was reacted for 2 min with 0.2 nmol of carrier-free sodium ^{125}I (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in the presence of lactoperoxidase and H_2O_2 in 50 mM phosphate buffer at pH 7.2. Monoiodotoxins were separated by reverse-phase liquid chromatography on a C18 column (Vydac; 0.46 × 25 cm, 5-μm particles). The column was eluted with a linear gradient of 19 to 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 described in Martin-Moutot et al., 1995) was measured at 30°C in 200 μl of binding buffer: 140 mM NaCl, 10 mM Tris base, and 0.1% bovine serum albumin, adjusted to pH 7.4. Binding was initiated by the addition of 5 μg of membrane protein, stopped by rapid filtration on glass fiber filters (GF/C; Whatman, Clifton, NJ) 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, France); veratridine, brevetoxin 2, bupivacaine, lidocaine, tetracaine, and pompilidotoxin were from Sigma (St. Louis, MO); and μ conotoxin GIIIB was from the Peptide Institute (Osaka, Japan). Deltamethrin was from Calbiochem (San Diego, CA), and *Leiurus quinquestriatus quinquestriatus* toxin V and *Centruroides suffusus suffusus* toxin VI were kindly supplied by Dr. M. F. Martin-Eauclaire (Marseille, France).

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 X-100 in 300 μl of solubilization buffer (10 mM HEPES and 0.1 M KCl, pH 7.4, containing Sigma protease inhibitor cocktail). After centrifugation at 100,000*g*, aliquots of supernatant containing 8 fmol of ^{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. After centrifugation, the pellet was washed with 0.5% Triton X-100 and 0.1% bovine serum albumin in solubilization buffer, and the immunoprecipitated radioactivity was counted.

Results

Purification of Tx1. Tx1 was purified (sample R21) from *P. nigriventer* venom using a method published previously (Diniz et al., 1990). Two molecular masses were detected at 8600 and 4920 Da in this fraction, and two sequences were assigned by N-terminal Edman sequencing. Whereas the major component (AELTSXFPVG) seemed to be the N-terminal sequence of Tx1 (Diniz et al., 1990), the minor one, accounting for approximately 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 (data not shown). However, these results suggest that previous reports concerning the pharmacology of Tx1 may involve the 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 i.e., less than 0.5%). We found that 8600 Da was 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. To confirm that sample R41 was identical with Tx1, the peptide MS fingerprint was established after 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 to 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.

***P. 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 the addition of Tx1 (10 μl, 1 μM) near the cell (Fig. 1A), yielding approximately 85% inhibition of sodium current within 10 min (Fig. 1, A and B).

Tx1 did not modify the fast biophysical properties of $Na_v1.2$ channels. First, the kinetics of inward current rise and fall during a 2-ms step depolarization were not modified after application of Tx1, although inhibition of peak I_{Na} developed from 0 to 85%, suggesting that Tx1 did not alter activation and fast inactivation. Second, conductance-voltage and inactivation characteristics were established using standard voltage protocols in the 1- to 100-ms range (Sarkar et al., 1995) from a holding potential of −100 mV. Each test peak I_{Na} value was normalized to a reference peak I_{Na} measured during a pulse (2 ms to 0 mV) applied a few milliseconds or tens of milliseconds 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). Finally, in addition, complete inhibition was observed at high Tx1 concentrations in favorable conditions (see below). These results suggest that in the presence of a nonsaturating 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. To measure a pool of functional channels, at holding potentials positive to −100 mV, a deinactivating prepulse of 200 ms to −100 mV was used to reverse the fast inactivation of sodium channels before the test. Under 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 (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, the 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 washout 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 washout). Tetrodotoxin produced full, reversible inhibition 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 washout 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 (data 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 (i.e., 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 presence of a large excess of unlabeled Tx1 (nonspecific binding). The difference between these curves yields the saturable binding component. A Scatchard plot of specific binding (Fig. 3A, inset)

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 were collected for mass spectrometry. The masses measured for three proteolytic peptides (4633.78, 1374.56, and 1491.63 Da) were within 100 ppm of the masses predicted for Tx1 proteolytic peptides 1 to 39, 42 to 53, and 56 to 67, as indicated above the published *P. nigriventer* toxin 1 sequence (Diniz et al., 1993; accession number P17727). The last mass measured (1046.31 Da) 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 78NH₂

| Tx1 | AELTSCFPVG | HECDGDASNC | NCCGDDVYCG | CGWGRWNCKC | KVADQSYAYG | ICKDKVNCPN | RHLWPAKVCK | KPCRRNCGG |

shows a single class of sites with $K_D = 80$ pM and $B_{max} = 0.43$ pmol/mg cell protein. The kinetics of ^{125}I-Tx1 association and dissociation from membrane binding sites are shown in Fig. 3, B and C. The slopes of the linear semilogarithmic plots (Fig. 3C, inset) gives the apparent association rate constant $(k_{app}) = -43 \times 10^{-4}$/s and the dissociation rate constant $(k_{-1}) = 11 \times 10^{-4}$/s. The equation $k_{+1} = (k_{app} - k_{-1})/[^{125}$I-Tx1] yields the association rate constant $k_{+1} = 32 \times 10^{6}$ M^{-1}/s. The equilibrium dissociation constant K_D calculated from the kinetic data ($K_D = k_{-1}/k_{+1}$) was 33 pM.
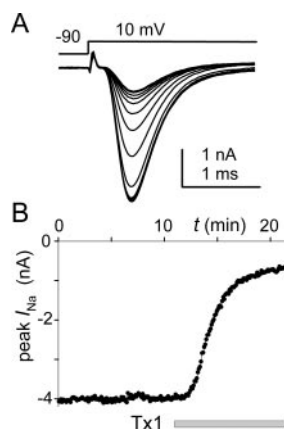

Fig. 1. Effect of *P. nigriventer* toxin 1 on sodium currents recorded from a CHO cell expressing recombinant Na$_v$1.2 channels. A, superimposed current traces recorded before and after the addition of Tx1 (10 μl, 1 μM) near the cell. A 5-ms step depolarization from -90 to 10 mV was applied every 6 s. The illustrated traces are separated by 60 s, and 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 (▦).

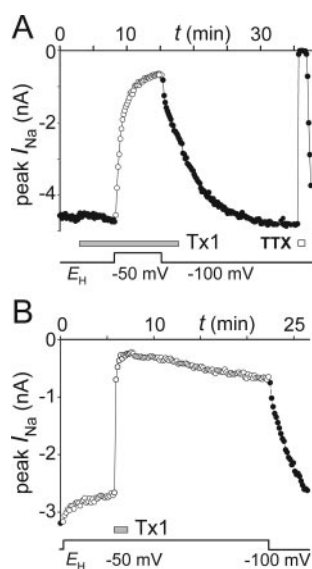**Fig. 2.** Effect of the holding potential on the peak current reduction by *P. nigriventer* toxin 1. Sodium currents from CHO cells expressing Na$_v$1.2 were elicited by test pulses (2 ms to 0 mV, 0.1 Hz) in the absence and in the presence of Tx1 (10 μl, 1 μM; ▦) or tetrodotoxin (TTX, 10 μl, 10 μM; □), 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 deinactivating prepulse (200 ms to -100 mV). A, E_H was shifted from -100 to -50 mV for 420 s in the presence of Tx1 and then back to -100 mV 180 s before washout. The small (0.2 nA) reduction observed between times 2 and 3 min was due to the arrest of perfusion 0.6 min before the 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.

Because a previous report suggested that Tx1 acts on N-type (Ca$_v$2.2) calcium channels (Gouvêa dos Santos et al., 1999), immunoprecipitation experiments were performed with solubilized ^{125}I-Tx1-labeled binding sites and antibodies directed against conserved sequences in the Na$_v$1 or Ca$_v$2 families of proteins (pan anti-Na$_v$, antibody directed against a conserved sequence in domain III-IV of the α subunit and an antibody against a conserved sequence in the N-terminal domain of Ca$_v$2). The results (data not shown) indicated that anti-Na$_v$ antibodies recognized the ^{125}I-Tx1/channel complex and that immunoprecipitation was blocked by the cognate peptide. In contrast, anti-Ca$_v$2 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.
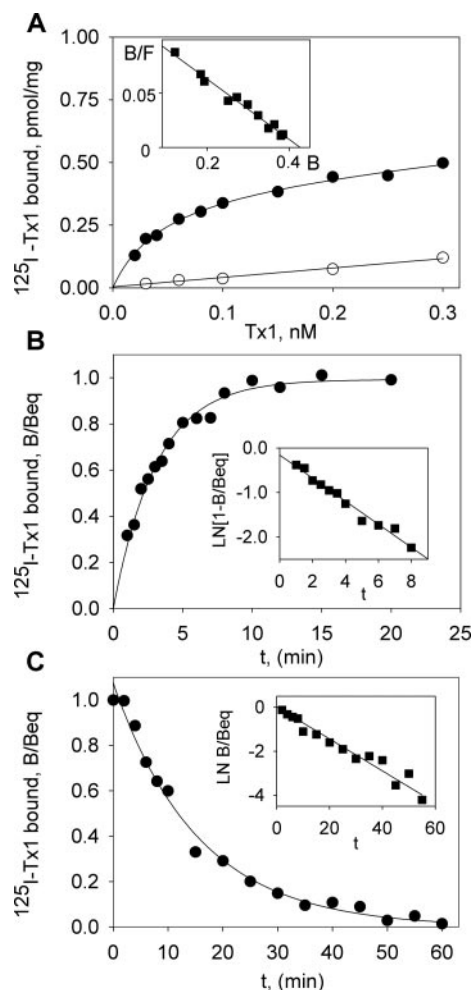
Fig. 3. Binding of ^{125}I-tagged *P. nigriventer* toxin 1 to rat brain membranes. A, rat brain membranes were incubated with the indicated concentrations of ^{125}I-Tx1 for 30 min at 30°C in the absence (●, total binding) or presence (○, nonspecific binding) of 50 nM unlabeled Tx1 and then filtered and washed. Bound toxin was measured by γ counting. Inset, Scatchard plot of the specific binding component (total minus nonspecific). 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 semilogarithmic 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 measured by filtration at the indicated times to yield dissociation kinetics. Inset, linear semilogarithmic plot.

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 (1 μM veratridine, 0.2 μM *Leiurus quinquestriatus quinquestriatus* toxin V and *Centruroides suffusus suffusus* toxin VI, and 0.1 μM brevetoxin, respectively) nor local anesthetics (bupivacaine, lidocaine, and 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 (Barbier et al., 2003; Li and Tomaselli, 2004; Terlau and Olivera, 2004) displaced 75% of specific ^{125}I-Tx1 binding. Assays performed to determine the concentration dependence for displacement (Fig. 4A) gave an IC$_{50}$ value of 300 pM for native Tx1, yielding a calculated K_i value of 75 pM, and showed that radioiodination 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 1 μM 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.

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 K_D = 30 pM, B_{max} = 520 fmol/mg protein in the absence of μ conotoxin GIIIB, and K_D = 140 pM and 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 behavioral 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$_v$2.2 (N-type) channels (Gouvêa dos Santos et al., 1999). Our results suggest that the contaminating 4920-Da peptide in the initial Tx1 sample R21 could be toxin Tx3-3 based on its partial N-terminal sequence. The Tx3 fraction includes several toxins that are antagonists of high-voltage-activated calcium channels with a preference for Ca$_v$2 channels (Cassola et al., 1998; Leão et al., 2000; Gouvêa dos Santos et al., 2002; 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$_v$1.2 channels indicate that Tx1 inhibits neuronal voltage-gated sodium channels in a reversible manner. Its effect resembles the tetrodotoxin effect, in that it can induce full inhibition and does not affect the fast biophysical properties of the residual current in nonsaturating 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 nonfunctional. 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
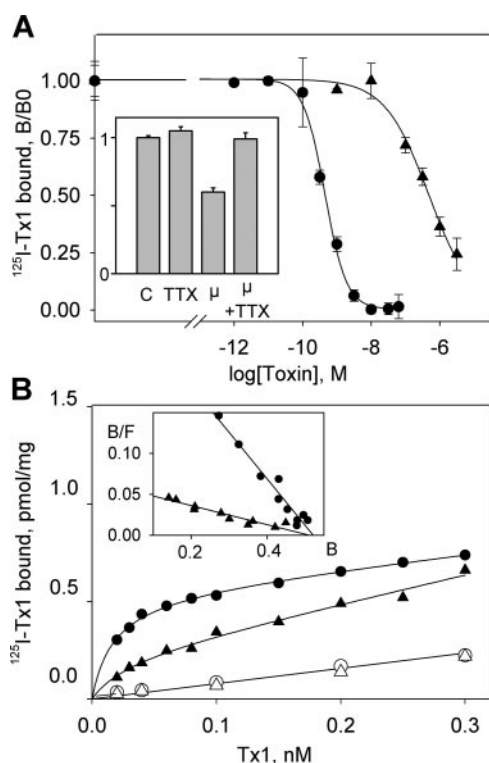
Fig. 4. Interactions between ^{125}I-tagged *P. nigriventer* toxin 1 and μ conotoxin binding sites in rat brain membranes. A, ^{125}I-Tx1 (0.1 nM) was incubated with rat brain membranes for 30 min at 30°C in the presence of the indicated concentrations of unlabeled Tx1 (●) or μ conotoxin GIIIB (▲). Specific binding was measured by filtration. Inset, specific binding of ^{125}I-Tx1 as indicated above (C = control) was measured in the presence of 1 μM tetrodotoxin (TTX) or 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 (▲) or absence (●) of 3 μM μ-conotoxin GIIIB. Inset, Scatchard plot of specific binding in the presence (▲) or absence (●) of μ conotoxin GIIIB.

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 of question. A number of points do not support the hypothesis that the voltage-dependence is due to an effect of the electric field on the toxin molecule: 1) a 78-amino acid peptide with a molecular mass of 8600 Da is not likely to enter the channel pore; 2) 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 ($\sim +4.6$); a positively charged open-channel blocker would undergo a relief of block with depolarization contrary to what is observed; and 3) 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-dependence 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 and Goldin, 1991). Using a combination of nonstationary fluctuation analysis and use-dependent block analysis, Lönnendonker (1989a) 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 mechanistic studies.

Slow voltage-dependence 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 (Table 2). The next nearest sequences found in databanks (34–38% identity to Tx1) correspond to spider toxins known to inhibit voltage-gated calcium channels (variants of ω-phonetoxins from *P. nigriventer* and ω-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. First, detergent-solubilized ^{125}I-Tx1/binding site complexes were immunoprecipitated by antibodies directed against a conserved motif from sodium channel α subunits. Second, ^{125}I-Tx1 binding was inhibited by μ conotoxin GIIIB, a sodium channel antagonist from the venom of the predatory marine gastropod *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 seem to overlap but are not identical (Cestèle and Catterall, 2000; Barbier et al., 2003; Li and Tomaselli, 2004; Terlau and Olivera, 2004). 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 approximately 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 approximately 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, ^{125}I-Tx1 binding was not inhibited by tetrodotoxin, nor were any interactions between [^{3}H]saxitoxin and Tx1 detected (data 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

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 *P. 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. Bold letters indicate the identity between toxins 1 and μ conotoxins, and bold and underlined letters indicate conserved basic residues with demonstrated functional importance in GIIIA, numbered below according to their position in this toxin.

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	

O, hydroxyproline.

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 to 40 of Tx1, toxins 1A and 1B from *Phoneutria keyserlingi*, and six μ conotoxins indicates overall similarity in the spacing of four conserved cysteines and a tryptophan present in the three toxins 1 and three μ conotoxins. Extensive structure-function studies with μ conotoxin GIIIA analogs have indicated an important role for basic residues at positions 13, 16, and 19 that are conserved in μ conotoxins (Huang and Miller, 1991; Sato et al., 1991; Li and Tomaselli, 2004; Bulaj et al., 2005). 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. 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, whereas the absence of a basic residue equivalent to arginine 13 might preclude competition between Tx1 and tetrodotoxin. Further studies are required to clarify the molecular mechanisms by which Tx1 produces state-dependent inhibition in proximity to sodium channel site 1.

Acknowledgments

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