

# Binding sites and actions of Tx1, a neurotoxin from the venom of the spider *Phoneutria nigriventer*, in guinea pig ileum

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## Abstract

Tx1, a neurotoxin isolated from the venom of the South American spider *Phoneutria nigriventer*, produces tail elevation, behavioral excitation and spastic paralysis of the hind limbs after intracerebroventricular injection in mice. Since Tx1 contracts isolated guinea pig ileum, we have investigated the effect of this toxin on acetylcholine release, as well as its binding to myenteric plexus-longitudinal muscle membranes from the guinea pig ileum. [<sup>125</sup>I]-Tx1 binds specifically and with high affinity ( $K_d = 0.36 \pm 0.02$  nM) to a single, non-interacting ( $n_H = 1.1$ ), low capacity ( $B_{max}$  1.1 pmol/mg protein) binding site. In competition experiments using several compounds (including ion channel ligands), only PhTx2 and PhTx3 competed with [<sup>125</sup>I]-Tx1 for specific binding sites ( $K_{0.5 \text{ apparent}} = 7.50 \times 10^{-4}$  g/l and  $1.85 \times 10^{-5}$  g/l, respectively). PhTx2 and PhTx3, fractions from *P. nigriventer* venom, contain toxins acting on sodium and calcium channels, respectively. However, the neurotoxin PhTx2-6, one of the isoforms found in the PhTx2 pool, did not affect [<sup>125</sup>I]-Tx1 binding. Tx1 reduced the [<sup>3</sup>H]-ACh release evoked by the PhTx2 pool by 33%, but did not affect basal or KCl-induced [<sup>3</sup>H]-ACh release. Based on these results, as well as on the homology of Tx1 with toxins acting on calcium channels ( $\omega$ -Aga IA and IB) and its competition with [<sup>125</sup>I]- $\omega$ -Cono GVIA in the central nervous system, we suggest that the target site for Tx1 may be calcium channels.

## Key words

- *Phoneutria nigriventer*
- Spider toxins
- Binding
- Enteric nervous system
- Calcium channels
- Guinea pig ileum

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*Phoneutria nigriventer*, a very aggressive solitary spider, is responsible for most spider bites in humans in central, eastern and southern Brazil (1). Bites by *P. nigriventer* cause intense local pain, spastic paralysis, autonomic dysfunction, tonic convulsions, priapism, tachycardia and visual disturbances (2). *P. nigriventer* venom contains several toxins that exert important biological effects

such as voltage-dependent sodium channel activation (3-5), leading to neuromuscular blockade in phrenic nerve-diaphragm muscle preparations (3), or the release of acetylcholine (ACh) and norepinephrine from autonomic nerve endings in guinea pig atria (4), local edema formation *in vivo*, and vascular (6) and non-vascular (7,8) smooth muscle contractions.

Rezende Jr. et al. (9) isolated three neurotoxic fractions from *P. nigriventer* venom. The neurotoxin Tx1 ( $LD_{50} = 0.05$  mg/kg) produces tail elevation, behavioral excitation and spastic paralysis of the posterior limbs after intracerebroventricular injection in mice (9). The radioiodinated toxin binds with high affinity to rat brain synaptosomes (7). Since Tx1 contracts the isolated ileum (7,8), and since most neurons in the myenteric plexus-longitudinal muscle (MPLM) preparation are cholinergic (10), we have examined the effect of this toxin on basal [ $^3H$ ]-ACh release by MPLM. Tx1 showed a high affinity for the neuromuscular guinea pig ileum preparation but did not alter basal [ $^3H$ ]-ACh release. However, Tx1 did modify the release evoked by other fractions of *P. nigriventer* venom.

To study the interaction of Tx1 with guinea pig ileum, the toxin was iodinated using the lactoperoxidase method (11). Protein (usually 4.8  $\mu$ g) was labeled with 0.5 mCi of carrier-free Na[ $^{125}I$ ] (17.4 Ci/mg) and separated from free iodine by batchwise resuspension with Dowex 1-X8 (Merck, Darmstadt, Germany) anion exchanger. The presence of free iodine was determined by ascending chromatography on Whatman No. 1 paper using methanol as solvent. The specific radioactivity was 1100 cpm/fmol toxin.

Guinea pigs (300-500 g) of either sex were anesthetized with  $CO_2$  before exsanguination. A segment of ileum was removed and the lumen flushed with the following solution: 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 1 mM  $MgCl_2$  and 2.5 mM  $CaCl_2$ , pH 7.4, supplemented with 10  $\mu$ g morphine/ml to inhibit spontaneous contractions. Longitudinal muscle with adherent Auerbach's plexus was obtained as described by Paton and Zar (12). The tissue was sliced with a tissue chopper (McIlwain-Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, England) and homogenized (0.15 g/ml) in Tris-HCl buffer, pH 7.4. All manipulations were performed at 4°C. The homogenate was cen-

trifuged for 30 min at 30,000 g (Sorvall Centrifuge - RC5C, Newtown, CT, USA). The pellet was resuspended in Tris buffer and centrifuged again under the same conditions. The final pellet was resuspended (0.3 g/ml) in incubation buffer containing: 25 mM HEPES, 10 mM glucose, 140 mM choline chloride, 5.4 mM KCl, 0.8 mM  $MgSO_4$  and 1.8 mM  $CaCl_2$ , pH 7.4.

MPLM membranes (100  $\mu$ g protein/ml) were incubated with [ $^{125}I$ ]-Tx1 (0.1 nM) at 37°C for 40 min, in a final volume of 500  $\mu$ l of incubation buffer supplemented with 0.2% BSA. Saturation experiments were performed in the presence of increasing concentrations of  $^{125}I$ -Tx1 (0.1 pM-10  $\mu$ M). Nonspecific binding was measured in parallel experiments using an excess of unlabeled toxin and the values were subtracted from the total binding. Competition experiments were performed using ion channels and cholinergic receptors ligands. These ligands were tested at concentrations varying from 0.1 pM to 10  $\mu$ M. Incubation was terminated by centrifugation (14,500 g, 5 min) in a microfuge (Marathon, Pittsburgh, PA, USA). The pellets were rinsed twice with 1 ml of ice-cold rinsing buffer, pH 7.4, containing: 5 mM HEPES, 10 mM glucose, 140 mM choline chloride, 5.4 mM KCl, 0.8 mM  $MgSO_4$ , 1.8 mM  $CaCl_2$  and 0.5% BSA. Bound radioactivity was estimated using an LKB gamma counter. All assays were run in triplicate.

The release of [ $^3H$ ]-ACh into the incubating medium was studied after labeling tissue ACh with methyl [ $^3H$ ]-choline chloride (15 Ci/mmol), as described by Suzski and Toth (13). Initially, the ACh stores were depleted by incubation in Krebs solution for 15 min in the presence of 50 mM  $K^+$ . Subsequently, slices of MPLM (approximately 40 mg) were incubated in Krebs solution containing 0.67  $\mu$ Ci of methyl [ $^3H$ ]-choline chloride for 30 min to label endogenous pools of ACh. The slices were then washed with cold choline (1.0  $\mu$ M) and then incubated for 30 min with or without the drugs or toxins of interest. The

incubation solution contained: 136 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5.5 mM glucose, 10 mM Trizma base and 20 μM p-nitrophenyl phosphate (Paraoxon, Sigma Chemical Co., St. Louis, MO, USA) to prevent the hydrolysis of ACh. The pH was adjusted to 7.4. To characterize the radioactivity released, [<sup>3</sup>H]-choline and [<sup>3</sup>H]-acetylcholine were separated from the supernatants as described by Gordon et al. (14). Using this method, [<sup>3</sup>H]-ACh accounted for 80% of the total radioactivity.

The intracerebroventricular toxicity of Tx1 labeled with cold iodide was identical to that of native toxin (data not shown). [<sup>125</sup>I]-Tx1 (0.1 nM) bound to specific sites on MPLM in a saturable manner (Figure 1A). Specific binding was dependent on the tissue protein concentration (data not shown). Scatchard analysis of the saturation isotherm (Figure 1A, inset) produced a linear plot over the concentration range used, indicating the existence of a single class of non-interacting binding sites ( $n_H = 1.1$ ). The dissociation constant ( $K_d$ ) was  $0.36 \pm 0.02$  nM, showing that these were high affinity binding sites. The  $B_{max}$  was  $1.15 \pm 0.06$  pmol/mg of protein.

The competition between [<sup>125</sup>I]-Tx1 and other fractions and toxins from *P. nigriventer* venom is shown in Figure 1B. PhTx2 and PhTx3 competed partially with [<sup>125</sup>I]-Tx1 for binding sites on MPLM membranes ( $K_{0.5 \text{ apparent}} = 7.50 \times 10^{-4}$  g/l and  $1.85 \times 10^{-5}$  g/l, respectively).

Unlike PhTx2, Tx2-6, an isoform purified from fraction PhTx2 which inactivates sodium channels (5), did not affect the binding of [<sup>125</sup>I]-Tx1 to MPLM (data not shown).

To examine the possible interaction of Tx1 with ion channels and cholinergic receptors, competition experiments were done using ligands for sodium channels (tetrodotoxin, brevetoxin, α-scorpion toxin TsTx, β-scorpion toxin TsVII and veratridine), potassium channels (kaliotoxin, charybdotoxin, apamin, 4-aminopyridine, tetraethylammo-

nium and dendrotoxin), calcium channels (diltiazem, nifedipine, verapamil, ω-conotoxin GVIA and ω-agatoxin IVA) and nicotinic cholinergic receptors (α-bungarotoxin, d-tubocurarine and 3-hemicholinium). None of these ligands affected [<sup>125</sup>I]-Tx1 binding to MPLM (data not shown).

Tx1 did not significantly increase basal [<sup>3</sup>H]-ACh release from MPLM (Figure 2A). However, Tx1 caused a 33% reduction in the release of [<sup>3</sup>H]-ACh elicited by PhTx2 pool (Figure 2B).

These results demonstrate a high affinity interaction ( $K_d = 0.36 \pm 0.02$  nM) between Tx1 and a neuromuscular preparation of guinea pig ileum and rule out a possible action of this toxin on basal ACh release.

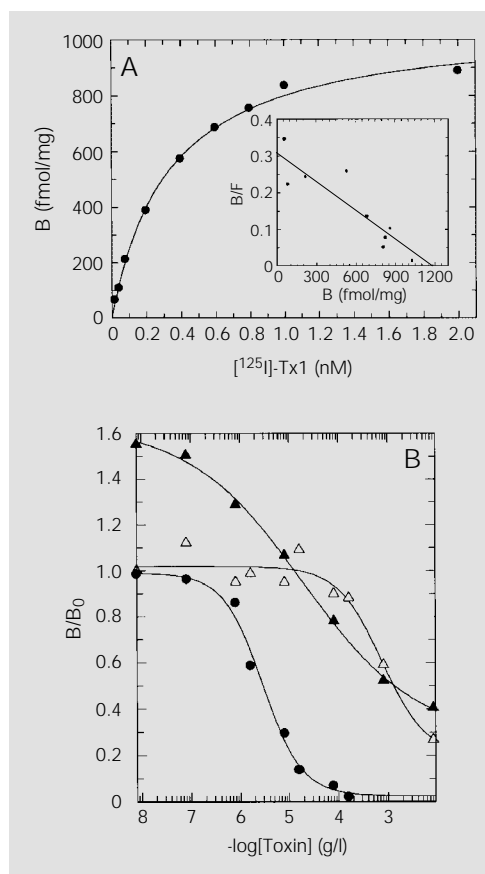


Figure 1 - Binding of [<sup>125</sup>I]-Tx1 to myenteric plexus-longitudinal muscle (MPLM) membranes. A, Saturation isotherm of [<sup>125</sup>I]-Tx1: aliquots (100 μg protein/ml) of MPLM were incubated for 40 min at 37°C with increasing concentrations (0.1 pM-2.0 nM) of [<sup>125</sup>I]-Tx1 in the absence (total binding) or presence of 0.2 μM unlabeled Tx1 for the determination of nonspecific binding. Specific binding (circles) is the difference between total and nonspecific binding. Inset, Scatchard transformation of the saturation isotherm:  $K_d = 0.35$  nM,  $B_{max} = 1.15$  pmol/mg of protein. Hill slope ( $n_H$ ) = 1.1 (data not shown). The data are for one experiment done in triplicate. Similar results were obtained in three independent experiments. B, Competition for high affinity binding sites. Aliquots of MPLM were incubated with 0.1 nM of [<sup>125</sup>I]-Tx1 in the presence of increasing concentrations of unlabeled Tx1 (circles), PhTx2 (open triangles) or PhTx3 (filled triangles). After 40 min, bound toxin was separated from free labeled toxin by centrifugation and quantified by γ-counting. PhTx2 and PhTx3 partially competed with [<sup>125</sup>I]-Tx1 binding sites ( $K_{0.5 \text{ apparent}} = 7.50 \times 10^{-4}$  g/l and  $1.85 \times 10^{-5}$  g/l, respectively). The data are for one experiment done in triplicate. Similar results were obtained in three independent experiments. The amount of [<sup>125</sup>I]-Tx1 bound in the presence of competing toxins (B) is reported relative to total binding ( $B_0$ ).

Since Tx1 contracts the isolated ileum (7,8), its site of interaction could be cholinergic receptors or ion channels. The first of these sites is improbable since the cholinergic ligands used did not compete with radioiodinated toxin and Tx1 (0.1  $\mu$ M) had no significant effect on basal ACh release. Tx1 partially blocked the [ $^3$ H]-ACh release evoked by PhTx2 (Figure 2B). This inhibitory effect, together with the binding data, further suggests a possible relationship between the Tx1 and PhTx2 binding sites.

Although the ion channel ligands used did not affect [ $^{125}$ I]-Tx1 binding, we cannot exclude the possibility that this toxin interacts with another site in ion channels. Indeed, two neurotoxic fractions from *P. nigriventris* venom, PhTx2 and PhTx3, partially competed for the [ $^{125}$ I]-Tx1 binding site. PhTx2 contains toxins that act on sodium channels (5). However, the isotoxin Tx2-6, which is also active on sodium channels, did not compete with [ $^{125}$ I]-Tx1. Thus, other

components of fraction PhTx2, with pharmacological activities distinct from those of Tx2-6, must compete with Tx1 (Figure 1B). Fraction PhTx2 causes a rapid increase in the intracellular calcium concentration and stimulates the release of glutamate and ACh (15). The lack of sufficient quantities of isotoxins from PhTx2 and PhTx3, known to be active on sodium and calcium channels, precluded further competition experiments with Tx1.

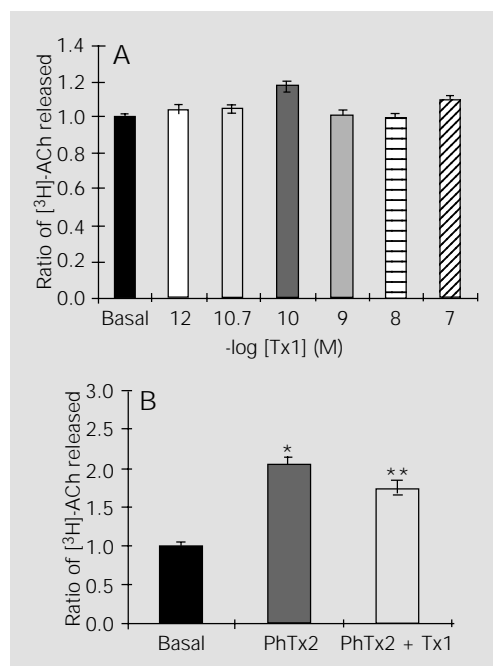
Gomez et al. (16) showed that PhTx3 decreased KCl-elicited ACh release in MPLM and suggested that PhTx3 acted on calcium channels. Guatimosim and co-workers (17) have shown that Tx3-3, one of the isoforms from fraction PhTx3, blocks the exocytosis of synaptic vesicles. This effect was thought to result from the inhibition of  $\omega$ -conotoxin GVIA-sensitive calcium channels associated with the control of exocytosis in rat cortex cerebral synaptosomes.

A peptide recently isolated from *P. nigriventris* venom, omega-phonotoxin-IIA, blocks L- and N-type calcium currents (18). This peptide has an amino acid sequence very similar to that of Tx1 and  $\omega$ -agatoxin IIIA. Although we have shown here that  $\omega$ -conotoxin GVIA did not compete with [ $^{125}$ I]-Tx1, preliminary experiments have demonstrated that Tx1 competes with [ $^{125}$ I]- $\omega$ -conotoxin GVIA (De Lima ME, Done SC, Santos RG, Cordeiro MN and Diniz CR, unpublished observations). The report that Tx1 shares homology with  $\omega$ -agatoxins (IA, IB and II-type) from *Agelenopsis aperta* (19,20), which are active on calcium channels, further suggests that Tx1 may interact with calcium channels.

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Figure 2 - A, Effect of Tx1 on basal [ $^3$ H]-ACh release by myenteric plexus-longitudinal muscle (MPLM) from guinea pig ileum. Increasing concentrations of Tx1 (1 pM-0.1 mM) were incubated for 35 min at 37°C with slices of MPLM (40 mg) previously loaded with 1  $\mu$ Ci of [ $^3$ H]-ACh. The incubation solution contained 136 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl<sub>2</sub>, 5.5 mM glucose, 10 mM Trizma base, pH 7.4, and 20  $\mu$ M p-nitrophenyl phosphate. The basal radioactivity released (basal) was 258.4  $\pm$  16.4 cpm/mg tissue (N = 3). [ $^3$ H]-ACh release is reported as the ratio of the counts (cpm/mg tissue) in the presence and absence of different concentrations of Tx1 (+Tx1/-Tx1). The column heights indicate the mean  $\pm$  SEM for three experiments. B, Effect of Tx1 on PhTx2-evoked [ $^3$ H]-ACh release. Slices of MPLM (40 mg), previously loaded with [ $^3$ H]-Ch (1  $\mu$ Ci), were incubated in the absence (basal) or presence of PhTx2 (8  $\times$  10<sup>-4</sup> g/l) or in the presence of both Tx1 (0.1  $\mu$ M) and PhTx2 (8  $\times$  10<sup>-4</sup> g/l) for 35 min, as indicated. The basal radioactivity released was 216.5  $\pm$  43.0 cpm/mg tissue. The column heights indicate the mean  $\pm$  SEM for three experiments. \*P<0.001 compared to basal, \*\*P<0.02 compared to basal and PhTx2 alone.



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