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PURIFICATION AND N-TERMINAL SEQUENCING OF TWO PRESYNAPTIC NEUROTOXIC PLA_2, NEUWIEDITOXIN-I AND NEUWIEDITOXIN-II, FROM *Bothrops neuwiedi pauloensis* (JARARACA PINTADA) VENOM

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ABSTRACT: Two presynaptic phospholipases A_2 (PLA_2), neuwieditoxin-I (NeuTX-I) and neuwieditoxin-II (NeuTX-II), were isolated from the venom of *Bothrops neuwiedi pauloensis* (BNP). The venom was fractionated using molecular exclusion HPLC (Protein-Pak 300SW column), followed by reverse phase HPLC (μBondapak C18 column). Tricine-SDS-PAGE in the presence or absence of dithiothreitol showed that NeuTX-I and NeuTX-II had a molecular mass of approximately 14 kDa and 28kDa, respectively. At 10μg/ml, both toxins produced complete neuromuscular blockade in indirectly stimulated chick biventer cervicis isolated preparation without inhibiting the response to acetylcholine, but NeuTX-II reduced the response to KCl by 67.0±8.0% (n=3; $p<0.05$). NeuTX-I and NeuTX-II are probably responsible for the presynaptic neurotoxicity of BNP venom *in vitro*. In fact, using loose patch clamp technique for mouse phrenic nerve-diaphragm preparation, NeuTX-I produced a calcium-dependent blockade of acetylcholine release and caused appearance of giant miniature end-plate potentials (mepps), indicating a pure presynaptic action. The N-terminal sequence of NeuTX-I was DLVQFGQMILKVAGRSLPKSYGAYGCYCGWGGRGK (71% homology with bothropstoxin-II and 54% homology with caudoxin) and that of NeuTX-II was SLFEFAKMILEETKRLPFPYYGAYGCYCGWGGQGQPKDAT (92% homology with Basp-III and 62% homology with crotoxin PLA2). The fact that NeuTX-I has Q-4 (Gln-4) and both toxins have F-5 (Phe-5) and Y-28 (Tyr-28) strongly suggests that NeuTX-I and NeuTX-II are Asp49 PLA_2.

KEY WORDS: chick biventer cervicis, loose patch clamp, nerve-muscle preparation, neuromuscular junction, neurotoxicity, PLA_2 neurotoxin, presynaptic action, *Bothrops neuwiedi pauloensis,* Neuwieditoxin-I, Neuwieditoxin-II.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Out of the four genera of venomous snakes in Brazil (*Bothrops*, *Crotalus*, *Lachesis* and *Micrurus*), only *Crotalus* and *Micrurus* cause failure of the neuromuscular junction, producing peripheral muscular weakness. However, the main complications in lethal envenomations by *Bothrops* species are acute renal failure, shock and sepsis (6, 41). Respiratory failure is also observed sometimes but the mechanism is not well understood. Since *Bothrops* venoms produce no signs of neurotoxicity after snakebite, respiratory failure has been related to pulmonary edema (41).

The neuromuscular actions of various *Bothrops* venoms have been extensively investigated in recent years. Cogo *et al*. (9) reported the neurotoxicity of *Bothrops insularis* venom on mice and chicks and on mouse phrenic nerve-diaphragm (MPND) and chick biventer cervicis (CBC) isolated preparations. After a lethal intramuscular (i.m.) dose of venom, chicks showed flaccidity followed by head-drop, dyspnea, apnea, convulsions and death. The venom produced irreversible blockade of MPND and CBC, and increased the frequency of mepps in mouse diaphragm. Subsequently, a neurotoxic presynaptic PLA_2 fraction, which blocked indirectly-evoked twitches in CBC but did not affect the responses to acetylcholine (ACh) and KCl, was isolated from this venom (10).

Zamuner *et al*. (53) reported that *B. neuwiedi* venom caused head-drop, loss of balance and respiratory failure in chicks, after an i.m. dose of 0.55 mg/kg. The venom also inhibited the twitch-tension responses in CBC preparations, but did not decrease the contractures to exogenous ACh or KCl, thus suggesting a presynaptic action. Borja-Oliveira *et al*. (5) subsequently investigated the neurotoxicity of several lots of *B. neuwiedi* venoms on CBC and observed that not all *B. neuwiedi* venoms exhibited neurotoxicity. Furthermore, analysis of the neurotoxic action of a sample of *B. neuwiedi pauloensis* (BNP) venom on CBC showed that the presynaptic action of the venom was temperature-dependent (4). More recently, we have observed that the presynaptic neurotoxicity of BNP on MPND is Ca^{2+}-dependent and accompanied by a pronounced increase in the frequency of mepps and the presence of giant mepps (12).

PLA_2 (E.C. 3.1.14) are Ca^{2+}-dependent enzymes that catalyze the hydrolysis of phospholipids at position sn-2 (51). Snake venom PLA_2 occurs in groups I (Elapidae and Hydrophidae) and II (Viperidae). Group II contains the catalytically-active Asp49

PLA_2, as well as the Lys49 isoforms. The latter type of proteins are considered PLA_2 homologues since they are probably devoid of (or may have very low) catalytic activity. In addition to their enzymatic activity, venom PLA_2 may have other actions, such as neurotoxicity and myotoxicity (25).

In the present work, two presynaptic neurotoxic PLA_2, named neuwieditoxin-I (NeuTX-I) and neuwieditoxin-II (NeuTX-II), were isolated from BNP venom and partially sequenced. This is the first time to our knowledge that a pure presynaptically active neurotoxin such as NeuTX-I has been isolated from botropic venom and partially sequenced.

MATERIALS AND METHODS

Reagents and venom

Acetylcholine chloride, solvents (HPLC grade) and other reagents were obtained from Sigma and Aldrich Chemical Company (St Louis, MO, USA). *Bothrops neuwiedi pauloensis* venom, collected in the state of São Paulo, was provided by Butantan Institute (São Paulo, São Paulo State, Brazil). The venom was obtained from adult specimens, and was desiccated and stored at 2-4°C.

Purification of Neuwieditoxins I and II

Molecular exclusion HPLC: *Bothrops neuwiedi pauloensis* venom (25mg) was loaded onto a Protein-Pak 300SW (Waters, Milford, MA, USA) column (0.78cm X 30cm) and eluted with 0.25M ammonium bicarbonate, pH 7.9, at a flow rate of 0.3ml/min. The elution profile was monitored at 280nm using a Waters 490 programmable multiwavelength detector (Waters, Milford, MA, USA); fractions were collected using a Foxy 200 automatic fraction collector (Isco, Inc., Lincoln, NE, USA). The chromatographic runs were recorded on a 746 data module dual channel recorder (Waters, Milford, MA, USA).

Reverse-phase HPLC: Lyophilized fractions from molecular exclusion HPLC were screened for neuromuscular activities and those of interest were then fractionated using a μBondapak C18 (Waters, MA, USA) column (0.78cm X 30cm). The column was equilibrated with buffer A (0.1% trifluoroacetic acid – TFA, in water) and the proteins were eluted with a linear gradient of buffer B (0.035% TFA in 66% acetonitrile) at a flow rate of 1.0ml/min. The elution profile was monitored at 280nm.

The fractions from reverse-phase HPLC were screened for neuromuscular activities. Those of interest were then pooled, lyophilized and stored at -20°C.

Phospholipase A_2 activity

PLA_2 activity was measured using the assay previously described by Cho and Kezdy (7) and Holzer and Mackessy (20), modified for 96-well plates (3). The enzyme activity, expressed as the reaction initial velocity (Vo), was calculated based on the increase in absorbance after 20 min. Absorbances at 425nm were measured using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA, USA).

Electrophoresis

Tricine-PAGE in a discontinuous gel and buffer system (45) was used to estimate the molecular mass of the proteins, in the presence or absence of dithiothreitol (DTT). Marker proteins were included in the runs and the gels were stained with coomassie blue.

N-terminal sequencing

Direct sequencing of the N-terminal was done with reduced and carboxymethylated protein, using a Procise protein sequencing system (Applied Biosystems, CA, USA). Phenylthiohydantoin amino acids were identified by comparing their retention times with those of 20 phenylthiohydatoin amino acid standards.

Chick biventer cervicis (CBC) nerve-muscle preparation

A protocol (n° 431-1) according to the Ethical Principles for Animal Research, established by the Brazilian College for Animal Experimentation (COBEA) and approved by the Institutional Committee for Ethics in Animal Research (State University of Campinas – UNICAMP), was used. The biventer cervicis was removed from chicks (which had been killed by exposure to halothane) as described by Ginsborg and Warriner (16) and mounted under a tension of 0.5g in a 5ml organ bath containing aerated (95% O_2, 5% CO_2) Krebs solution (pH 7.5, 37°C) of the following composition (in mM): NaCl, 118.6; KCl, 4.69; $CaCl_2$, 1.88; KH_2PO_4, 1.17; $MgSO_4$, 1.17; $NaHCO_3$, 25.0; and glucose, 11.65. Indirect stimulation (0.1Hz, 0.2ms, 6-7V) from a Grass S4 stimulator (Grass Instruments, Quincy, MA, USA) was used; muscle

contractions and contractures were recorded via a force-displacement transducer (BG 25 GM, Kulite Semiconductor Products, Inc., Leonia, NJ, USA) coupled to a Gould RS 3400 recorder (Gould Inc., Cleveland, OH, USA). The preparations were allowed to stabilize for at least 15 min before the addition of fractions (10μg/ml). Contractures to exogenously applied submaximal concentrations of acetylcholine (110μM) and KCl (13.4mM) were obtained in the absence of nerve stimulation prior to the addition of toxins and at the end of the experiment in order to test for the presence of neurotoxic and myotoxic activities (18).

Electrophysiological procedure

Preparation: Left hemidiaphragm of mouse was prepared as previously described by Re *et al*. (37, 38). Briefly, Charles River male mice, 30-40 days old, were killed and the left hemidiaphragm was dissected together with a short length of phrenic nerve. The preparation was bathed in Krebs' solution of the following composition (mM): NaCl (133), KCl (4.7), $MgCl_2$ (1.2), $CaCl_2$ (7.2), NaH_2PO_4 (1.3), $NaHCO_3$ (16.3), Glucose (7.8), pH 7.4, gassed with 95% O_2-5% CO_2 and kept at room temperature (18-22°C). Concentrations of $MgCl_2$ (5 to 15mM) and $CaCl_2$ (0.9 to 2mM) were adjusted in order to abolish the twitch of the muscle fiber.

The muscle was pinned on Sylgard resin, and placed on the stage of a Leitz inverted microscope. End-plates were visible by transillumination of the preparation using an optic fiber system. The preparation was equilibrated in saline for 30 minutes before starting the experiments.

End-plate signals: Spontaneous and evoked end-plate currents were recorded using a focal extracellular pipette pressed against the edge of an end-plate. Drummond 100µl measuring pipettes of soft glass (1.4mm) were pulled with a Kopf 700C Puller, which had a final tip diameter ranging from 3 to 15µm and was fire polished with a Narishighe MF83 Microforge. After filling with physiological solution, the electrode was connected to a List LM EPC7 current to voltage converter. Pipette resistances were of 100-300KΩ, and seal resistances, measured after pressing the pipette against the sarcolemma, ranged between 300-600KΩ. The loose patch clamp method (47) enables a good control of the series resistance all over the experiment (40). Furthermore, the voltage of the muscle fiber is well monitored using the same technique and virtually clamped at the resting value. Indirect stimulation of the

muscle was achieved by means of a suction electrode. Supramaximal square wave pulses of 0.1ms duration at 2Hz were applied via a DA analogic output of a PCL 818 card (Advantech). The signals were visualized on a Tektronix 5113 dual-beam storage oscilloscope and fed to the input stage of the PCL 818 analogue-to-digital converter mounted on a computer system (PC IBM) enabling a fully automated analysis of the data (39). The decay phase of the miniature end-plate currents (mepc's) was analyzed to calculate the decay time constant. The elaboration was carried out on the mepc decay part that fell within 10-90% of its peak amplitude. The function used was:

$$I_t = I_o \exp^{-t/\tau}$$

where I_t is the current at time t; I_o is the current at time zero, i.e. the peak current; and τ is the decay time constant. Marquardt's least-squares method was used for the fitting.

The parameters were obtained using the protocol previously described by Re *et al*. (38). The technique led to automatic evaluation of the mean τ value of the mepc's decay, the mean peak value of the miniature events (mepc), the spontaneous release frequency (f), and the mean epc's peak value (epc). The resting membrane potential (RMP) was measured using conventional intracellular microelectrodes (3M KCl) connected to a P16 differential amplifier (GRASS).

Statistical analysis

Each experiment was repeated at least three times. Results were expressed as mean $\pm$ standard error of mean (S.E.M.). Student's t-test was used for statistical analysis of the data, and values of $p<0.05$ indicated significance.

RESULTS

Bothrops neuwiedi pauloensis (BNP) venom was initially separated into 8 fractions by molecular exclusion (Figure 1A). Since the venom contains PLA_2 activity (12.7nmol/min/mg), this enzyme was assayed along the venom elution profile. Only fractions 2 and 3, corresponding to the second and third peaks, had PLA_2 activity: 25.8 and 48.2nmol/min/mg, respectively.

In indirectly stimulated CBC preparations, only fraction 3, corresponding to the third peak, produced complete blockade without significantly inhibiting the responses to ACh and KCl. At 10μg/ml, fraction 3 produced complete and irreversible neuromuscular blockade in 70±6 min at 37°C (*n*=4) (not shown). Low temperature (20-24°C) abolished the neuromuscular action of this fraction (*n* = 3) (not shown).

After repurification of fraction 3 (Figure 1B), only fractions corresponding to peak c (5-7% of venom protein), namely NeuTX-I, and peak b (3.5% of venom protein), namely NeuTX-II, induced neuromuscular blockade in CBC preparation. Tricine SDS-PAGE in the presence or absence of dithiothreitol showed that NeuTX-I and NeuTX-II had a molecular mass of ~14 and ~28kDa, respectively (Figure 1B, inset). NeuTX-I (10μg/ml) produced complete neuromuscular blockade up to 80 min (n=3), without inhibiting the responses to ACh and KCl (Figures 2A, B and D), whereas the response to KCl were partially inhibited after incubation with NeuTX-II (10 μg/ml) (Figures 2A, C and D).

The N-terminal sequences were determined up to the 36th for NeuTX-I, and up to the 41st for NeuTX-II amino acid residues (Figures 3 and 4).

To better clarify the possible molecular event involved in the NeuTX-I (the only toxin that induced complete neuromuscular blockade without inhibiting ACh and KCl-induced contracture) action, we studied the effects of the toxin (5, 10 and 50μg/ml) on mouse neuromuscular junction using the loose patch clamp technique (LPC). The results obtained were summarized in Table 1. The toxin induced an increase in the amplitude and frequency of the spontaneous release. Giant mepc's were frequently observed at NeuTX-I concentration of 10μg/ml (Figure 5). After this apparent facilitation, a complete block of the evoked release was always observed (Figures 6 a, b, c). Usually, the recovery of the evoked signals was incomplete after the final wash out. However, the block was overcome by adding calcium to the physiological solution (Figures 6 d, e).

During treatments with NeuTX-I, resting membrane potential values were not different from those obtained during control (toxin-free medium) experiments.

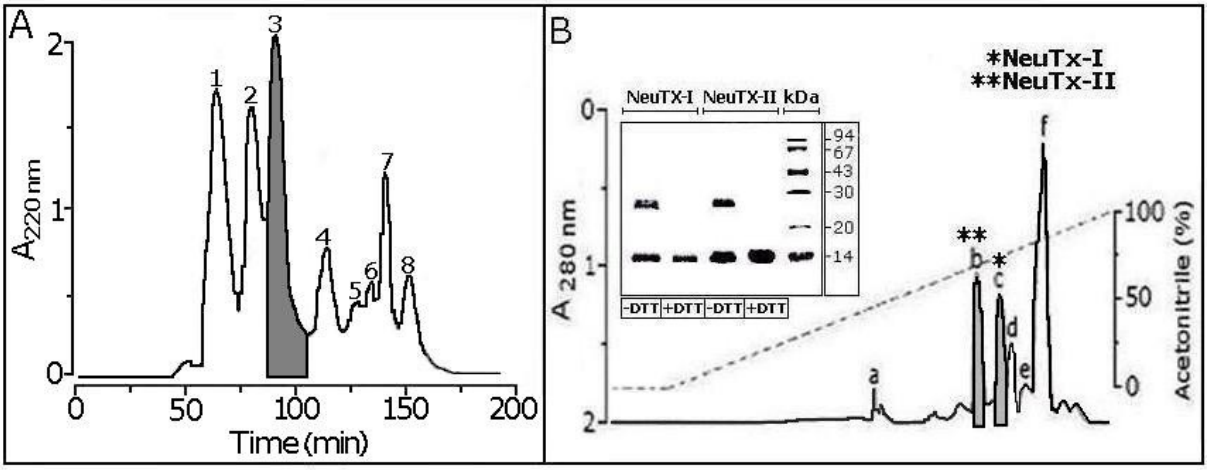

Figure 1. Purification of NeuTXs-I and II. A: Molecular exclusion HPLC of *Bothrops neuwiedi pauloensis* venom (25mg). Fractions were collected and the elution profile was monitored at 280nm. B: Repurification of fraction 3 by reverse phase HPLC. NeuTX-I (*) was recovered in peak c and NeuTX-II (**) was recovered in peak b. The inset shows a Tricine-SDS-PAGE gel of the toxins in the absence and presence of dithiothreitol (DTT).

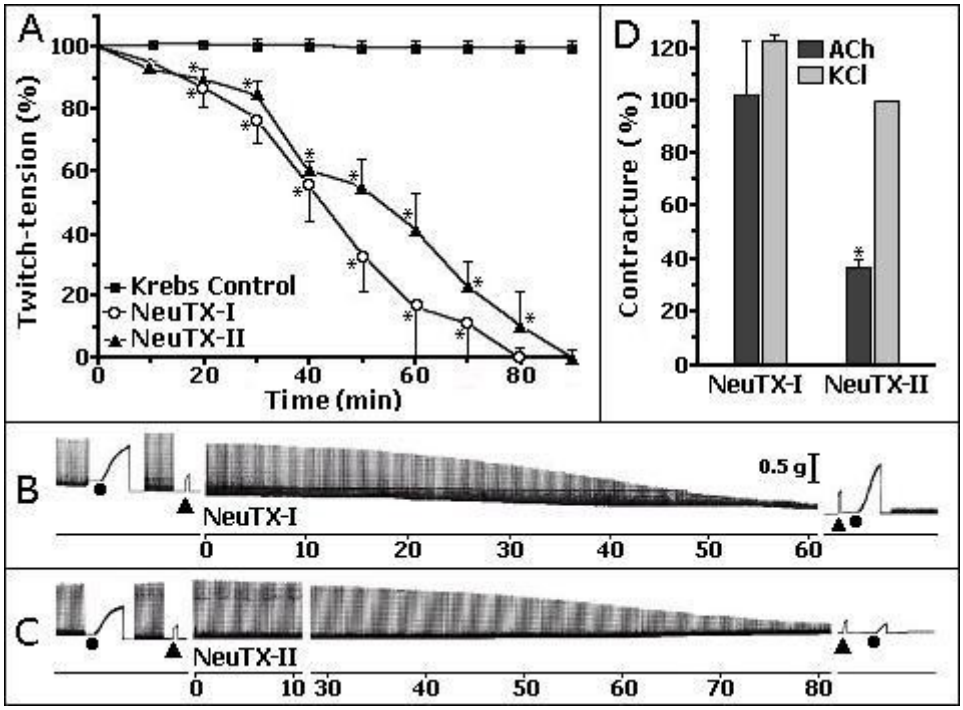

Figure 2. Neuromuscular blockade of chick biventer cervicis preparations at 37°C by NeuTXs-I and II. A: Time-dependent blockade produced by each toxin (10μg/ml), on indirectly stimulated preparations, compared to the Krebs control (*p<0.05). Each point represents the mean ± S.E.M. of 3-5 experiments. B and C: Representative recordings of the responses of indirectly stimulated chick preparation to NeuTX-I and NeuTX-II (10 μg/ml), respectively. Responses to exogenous acetylcholine (▲, 110μM) and KCl (●, 13.4mM) were obtained before and after the addition of toxins. Note that, in contrast to the response to KCl, response to acetylcholine after the blockade by NeuTX-II was still identical to the control. This result is representative of 3 experiments. D: Muscle contractures to KCl (13.4mM) and acetylcholine (ACh, 110μM) after 120min incubation with each toxin (10μg/ml) (mean ± S.E.M. of 3-5 experiments), expressed as a percentage of the pre-toxin values (*p<0.05).

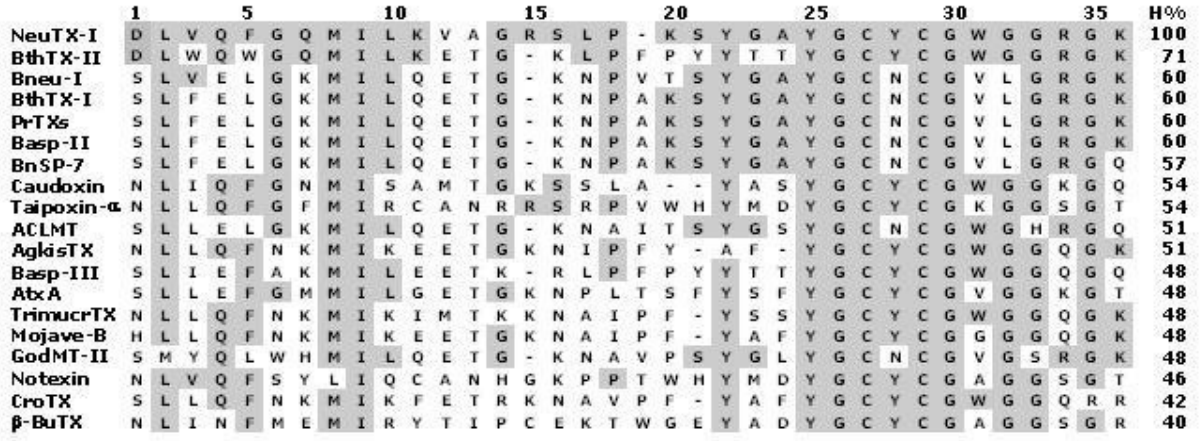

Figure 3. Amino acid sequence alignment of NeuTX-I with myotoxic and presynaptic neurotoxic PLA_2 toxins. Bothropstoxin-II (BthTX-II), from *B. jararacussu* (36); Bneu-I, from *B. neuwiedi* (15); bothropstoxin-I (BthTX-I), from *B. jararacussu* venom (8); piratoxins (PrTXs), from *B. pirajai* venom (48); Basp-II, from *Bothrops asper* venom (14); BnSP7, from *B. neuwiedi* (46); caudoxin, a presynaptic neurotoxin from *Bitis caudalis* (52); taipoxin-α, a presynaptic neurotoxin from *Oxyuranus s. scutellatus* (31); ACLMT, a myotoxin from *Agkistrodon contortrix laticinctus* (22); agkistrodotoxin (AgkisTX), a presynaptic neurotoxin from *Agkistrodon halys pallas* (28); Basp-III, from *Bothrops asper* venom (23); ammodytoxin A (AtxA), from *Vipera ammodytes ammodytes* (42); trimucrotoxin (TrimucrTX), from *Trimeresurus mucrosquamatus* (49); basic component of mojavetoxin (Mojave-B), from *Crotalus scutulatus scutulatus* (2); GodMT-II, myotoxic PLA_2, from *Cerrophidion (Bothrops) godmani* (11); notexin, from *Notechis scutatus scutatus* (17); basic component of crotoxin (CroTX), from *Crotalus durissus terrificus* (1); β-bungarotoxin (β-buTX), from *Bungarus multicinctus* (26, 27). Conserved residues are in gray. The percentage homology (H%) is also shown.

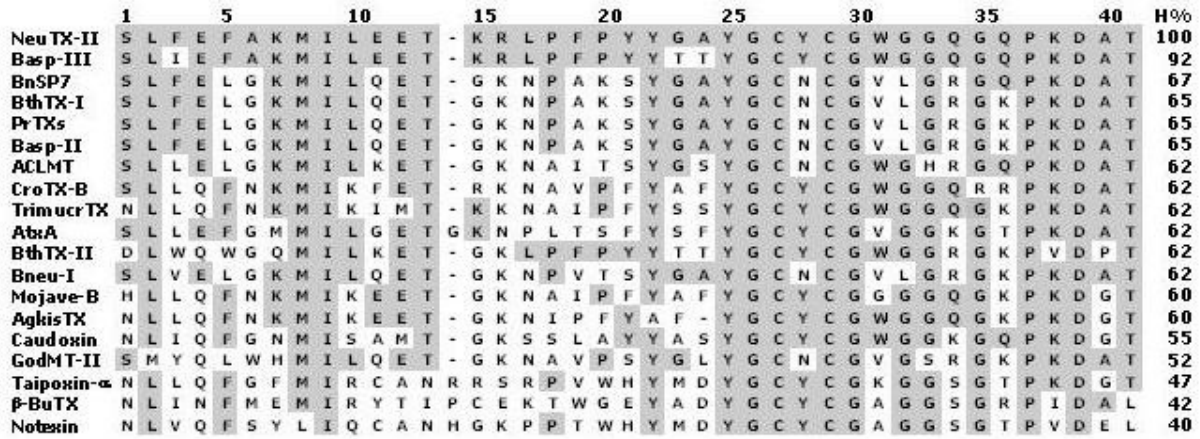

Figure 4. N-terminal amino acid sequence alignment of NeuTX-II with myotoxic and presynaptic neurotoxic PLA_2 toxins. Basp-III, from *Bothrops asper* venom (23); BnSP7, from *B. neuwiedi* (46); bothropstoxin-I (BthTX-I), from *B. jararacussu* venom (8); piratoxins (PrTXs), from *B. pirajai* venom (48); Basp-II, from *Bothrops asper* venom (14); ACLMT, a myotoxin from *Agkistrodon contortrix laticinctus* (22); basic component of crotoxin (CroTX), from *Crotalus durissus terrificus* (1); trimucrotoxin (TrimucrTX), from *Trimeresurus mucrosquamatus* (49); ammodytoxin A (AtxA), from *Vipera ammodytes ammodytes* (42); bothropstoxin-II (BthTX-II), from *B. jararacussu* (36); Bneu-I, from *B. neuwiedi* (15); basic component of mojavetoxin (Mojave-B), from *Crotalus scutulatus scutulatus* (2); agkistrodotoxin (AgkisTX), from *Agkistrodon halys pallas* (28); caudoxin, from *Bitis caudalis* (52); GodMT-II, a myotoxic PLA_2 from *Cerrophidion* (*Bothrops*) *godmani* (11); taipoxin-α, a presynaptic neurotoxin from *Oxyuranus s. scutellatus* (31); β-bungarotoxin (β-buTX), from *Bungarus multicinctus* (26, 27); notexin, from *Notechis scutatus scutatus* (17). Conserved residues are in gray. The percentage homology (H%) is also shown.

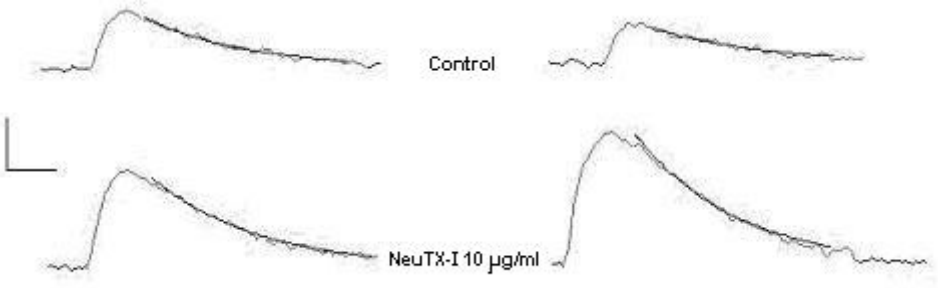

Figure 5. Spontaneous miniature end-plate currents recorded at mouse neuromuscular junction. The figure shows digitized raw data related to control and treatment with NeuTX-I concentration of 10μg/ml. Note the presence of giant mepc's. Calibration bars, vertical: 250pA, horizontal: 1ms.

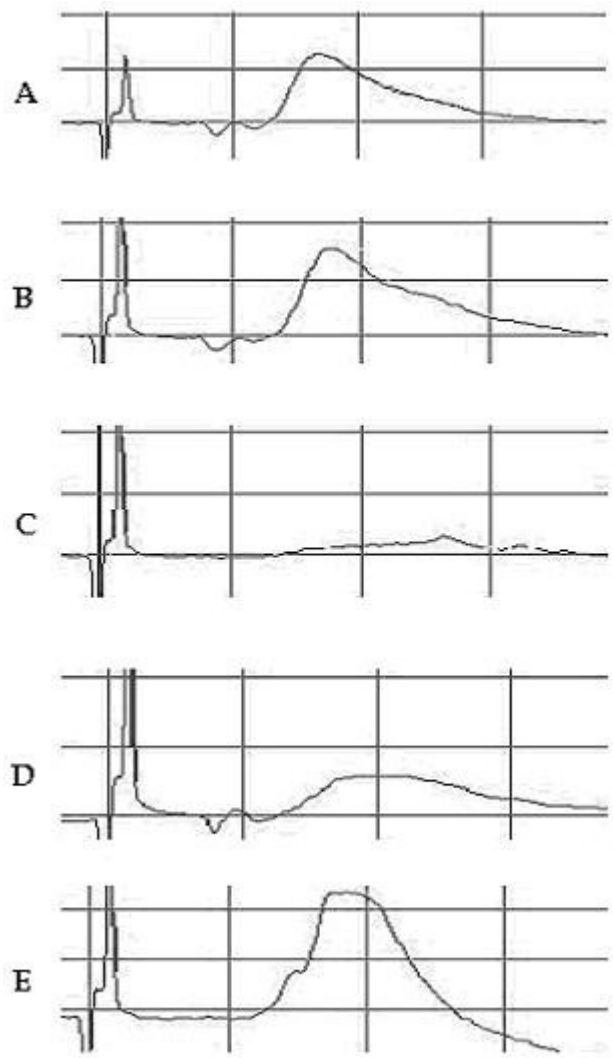

Figure 6. Averaged signals obtained by 200 evoked end-plate currents recorded at mouse neuromuscular junction. The figure shows digitized raw data related to control (A), treatment with NeuTX-I concentration of 5μg/ml (B) and 10μg/ml (C); the effects obtained after a wash out (D) and a subsequent flow of a physiological solution with calcium concentration increased from 1 to 1.5mM (E). Calibration bars, vertical: 2nA, horizontal: 2ms.

Table 1. Effects of NeuTX-I on mouse neuromuscular junction.

	Control Values	NeuTX-I 5μg/ml	NeuTX-I 10μg/ml	NeuTX-I 50μg/ml	Wash Out
epc (nA)	**1.57 (±0.58; 5)**	+16.9 (±7.62; 5)*	**-65.2 (±20.0; 5)**	**-84.0 (±8.61; 5)**	**-69.8 (±16.8; 5)**
mepc (nA)	**0.20 (±0.08; 5)**	+19.7 (±19.8; 5)*	+30.6 (±40.9; 5)*	+47.1 (±57.3; 5)*	+63.5 (±92.6; 5)*
f (Hz)	**2.11 (±0.81; 5)**	+14.2 (±11.4; 5)*	+8.9 (±20.8; 5)*	+18.4(±52.0; 5)*	-2.52 (±28.9; 5)*
τ (ms)	**1.16 (±0.13; 5)**	+1.36 (±7.75; 5)*	+0.64 (±15.1; 5)*	+4.7 (±17.5; 5)*	-1.84 (±9.42; 5)*

Values show the effects of the indicated NeuTX-I concentrations on four parameters related to the function of mouse neuromuscular junction compared with control and final wash out. The amplitudes of the evoked (**epc**) and spontaneous (**mepc**) release, the frequency of the quantal release (**f**) and the mepc's decay time constant (τ) were analyzed. Data were expressed as mean percentage variations in respect to mean control values. Standard deviations and number of experiments are indicated between parentheses.

* Indicates non-significant differences. Values in bold indicate significant mean with $p<0.05$.

DISCUSSION

Although neurotoxicity has been demonstrated *in vitro* (4, 5, 9, 10, 12, 44, 46, 53) and *in vivo* (9, 53) for various *Bothrops* venoms, there is no conclusive evidence for such an effect after snakebite in humans. *In vitro*, the neurotoxicity of BNP venom is mainly presynaptic (4, 5, 12, 44, 53), although its potency varies from sample to sample (5).

Presynaptic inhibition is conveniently studied using dissected BCP preparation. A pure presynaptically active neurotoxin would abolish nerve-evoked twitches, without affecting responses to cholinoceptor agonists or the responses to direct muscle stimulation, but would not affect the responses to elevated K^+ concentration (18). As a pure presynaptically neurotoxin, NeuTX-I induced complete neuromuscular blockade without inhibiting the responses to ACh and KCl. Consequently, its effects were studied using loose patch clamp technique on a mammalian neuromuscular junction. The electrophysiological data were indicative of a pure presynaptic effect.

After an initial facilitation leading to depletion of ACh release, a complete calcium-dependent blockade was observed. The presynaptic action is confirmed by the constancy of the mepc's decay time, i.e. the rate constant of the receptor-ion channel complex conformational change, reflecting the closing of the ACh-sensitive channels (24, 29) at the postsynaptic sites.

In general, snake venoms contain a mixture of toxins active at prejunctional and postjunctional sites, as well as myotoxic components. Therefore, probably more than one component is capable of contributing to the neuromuscular action produced by the venom. Myotoxic components reduce the response of biventer cervicis muscle to exposure to elevated K^+ concentration and/or initiate contractures in the muscle (18). These effects were produced by BNP venom (4) and NeuTX-II.

In previous reports, the neuromuscular blocking effect of BNP venom in BCP and MPND was temperature and Ca^{2+}-dependent and this fact also indicated that enzymatic activity is necessary for neuromuscular action (4, 12). The neuromuscular blocking effect of the fraction corresponding to peak 3, from the molecular exclusion step, which showed PLA_2 activity and revealed NeuTX-I and NeuTX-II after reverse-phase HPLC, was temperature-dependent, indicating that its neurotoxicity may require enzymatic activity, as suggested for the venom (4).

This is the first report of purification and N-terminal sequence of presynaptic PLA_2 toxins from *Bothrops* venom. Several snake presynaptic PLA_2, which produce the same pharmacological action of NeuTX-I in CBC at low concentrations ($\leq$10μg of toxin/ml), have been described, such as the presynaptic PLA_2 caudoxin (30) from the venom of *Bitis caudalis*, which shares 54% homology with NeuTX-I. On the other hand, NeuTX-II shares 62% homology with the basic component of crotoxin, a potent neurotoxin from the venom of the South American rattlesnake *Crotalus durissus terrificus*. Crotoxin is a β-neurotoxin consisting of a heterodimer of a non-toxic, non-enzymatic acidic protein, named crotapotin, and a basic Asp-49 protein with PLA_2 activity. The PLA_2 is a single polypeptide chain of 123 amino acids with considerable similarity to other PLA_2, while crotapotin consists of three polypeptides linked by several disulfide bridges (1).

A recent comparative study of the pharmacological activities of *B. insularis*, *B. neuwiedi* and *C. d. terrificus* in chick neuromuscular preparations (44) indicated that *B. neuwiedi* and *B. insularis* venoms may contain components that act

presynaptically at the skeletal neuromuscular junction in a manner similar to *C. d. terrificus* venom and its main toxin, crotoxin. These conclusions were based on several evidences, such as the blockade of twitch-tension by *B. neuwiedi* and *B. insularis* venoms without affecting the response to the cholinoceptor agonist, thus suggesting a predominant presynaptic action. These authors also pointed out the persistence of KCl-induced contractures after neuromuscular blockade and the fact that at low concentrations both venoms were incapable of increasing creatine kinase activity.

The initial work on the neuromuscular blocking action of bothropic venoms by Rodrigues-Simioni *et al*. (43) led to the discovery of bothropstoxin-I (21), a myotoxin that has since been widely studied (19, 34, 35). The presence of a presynaptic PLA_2 in *B. n. pauloensis* venom was expected, since several reports had already indicated that this venom had presynaptic neurotoxicity (4, 5, 12, 44, 53).

The fact that NeuTX-I has Gln-4 (Q-4), Phe-5 (F-5) and Tyr-28 (Y-28), and that NeuTX-II has Phe-5 (F-5) and Tyr-28 (Y-28), amino acid residues conserved in all Asp-49 (D-49) PLA_2 variants purified so far, strongly suggests that both toxins are Asp-49 PLA_2. The inactive or little catalytically active Lys-49 (K-49) PLA_2 toxins have Leu-5 (L-5) for Phe-5 (F-5) and Asn-28 (N-28) for Tyr-28 (Y-28). The presence of Asp-49 is essential for effective hydrolysis of phospholipids. Besides Asp-49, the presence of Tyr-28, Gly-30 (G-30) and Gly-32 (G-32) is involved in the catalysis (50). NeuTX-I and NeuTX-II have Gly-30 and Gly-32, but Lys-49 toxins such as BnSP-7, a myotoxic PLA_2 from *B. n. pauloensis* venom (13, 32, 46), besides bothropstoxin-I, piratoxins, Basp-II and Bneu, have Leu-32 (L-32) for Gly-32. In addition, the fraction that originated NeuTX-I and NeuTX-II, third peak from HPLC molecular exclusion elution profile, had PLA_2 activity and its blocking action was temperature-dependent. Loose patch clamp confirmed the calcium-dependent blockade produced by NeuTX-I. All these findings together suggest that enzymatic activity is required for the pharmacological effects observed. Moreover, NeuTX-I and NeuTX-II may be the main components responsible for the neuromuscular blocking action of BNP venom. Based on our findings, the new toxins, NeuTX-I and NeuTX-II probably belong to two classes of PLA_2 toxins: (1) group II, which include those toxins isolated from Viperinae and Crotalinae snake venoms; (2) β-neurotoxins group, which include presynaptic neurotoxic PLA_2 toxins.

Although the neurotoxic action of *Bothrops* venoms is not a clinical problem, an understanding of the effects of other species may provide insights into a variety of physiological processes. Snake presynaptic neurotoxins with PLA_2 activity affect the release of ACh and block the neurotransmission in an unknown way. Once their mechanism of action is firmly established, presynaptic PLA_2 neurotoxins could be employed as tools to investigate specific aspects of neuroexocytosis. In addition, the isolation, identification, and pharmacological and biochemical characterization of PLA_2 enzymes help to understand their structure-function relationships, which can be useful in developing prototypes of novel tools and pharmaceutical drugs (33).

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