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Leftward Shift in the Voltage-Dependence for Ca^{2+} Currents Activation Induced by a New Toxin from *Phoneutria reidyi* (Aranae, Ctenidae) Venom

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SUMMARY

Various neurotoxins have been described from the venom of the Brazilian spider *Phoneutria nigriventer*, but little is known about the venoms of the other species of this genus. In the present work, we describe the purification and some structural and pharmacological features of a new toxin (PRTx3-7) from *Phoneutria reidyi* that causes flaccid paralysis in mice. The observed molecular mass (4627.26 Da) was in accordance with the calculated mass for the amidated form of the amino acid sequence (4627.08 Da). The presence of an α-amidated C-terminus was confirmed by MS/MS analysis of the C-terminal peptide, isolated after enzymatic digestion of the native protein with Glu-C endoproteinase. The purified protein was injected (intracerebro-ventricular) into mice at dose levels of 5 μg/mouse causing immediate agitation and clockwise gyration, followed by the gradual development of general flaccid paralysis. PRTx3-7 at 1 μM inhibited by 20% the KCl-induced increase on $[Ca^{2+}]_i$ in rat brain synaptosomes. The HEK cells permanently expressing L, N, P/Q and R HVA Ca^{2+} channels were also used to better characterize the pharmacological features of PRTx3-7. To our surprise, PRTx3-7 shifted the voltage-dependence for activation towards hyperpolarized membrane potentials for L (-4 mV), P/Q (-8 mV) and R (-5 mV) type Ca^{2+} currents. In addition, the new toxin also affected the steady state of inactivation of L-, N- and P/Q-type Ca^{2+} currents.

KEY WORDS: *Phoneutria reidyi*; spider venom; neurotoxin; amidated C-terminus; Ca^{2+}-channel toxin.

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INTRODUCTION

Spider venoms are an important part of biodiversity. They may be regarded as a "treasure chest" of potential future discoveries, because they are a valuable source of a range of novel chemicals with different biological activities. In recent years, there has been an explosion of interest in the small (1.5–9.5 kDa) polypeptides/proteins in venoms that exhibit varying toxicities towards the nervous systems of mammals and/or insects. This is because their mode of action in the prey targets neuronal ion channels (Ca^{2+}, Na^{+}, K^{+} or Cl^{-}), various ionotropic (NMDA, AMPA or kainate) and metabotropic glutamate receptors, pre-synaptic membrane proteins involved in neurotransmitter release, and/or post-synaptic cholinergic receptors (Rash and Hodgson, 2002 (2002). As a result, they have potential use: (i**)** as laboratory tools in gaining a better understanding of the structure and function of neuronal ion channels; (ii**)** in medicine as lead compounds for the development of new drug therapies and (iii**)** in agriculture for the design of new, highly selective and environmentally friendly insecticides (Corzo and Escoubas, 2003; Rash and Hodgson, 2002; Escoubas *et al.*, 2000; Pimenta and De Lima, 2005).

The extremely aggressive Brazilian spider *Phoneutria nigriventer*, responsible for many accidents involving envenomation of humans, is a wandering spider, which does not utilize a web, and therefore produces particularly neurotoxic venom to rapidly subdue its prey. Various neurotoxins active on Na^{+} (Cordeiro *et al.*, 1992; Matavel *et al.*, 2002), K^{+} (Cordeiro *et al.*, 1993; Carneiro *et al.*, 2003) and Ca^{2+} (Diniz *et al.*, 1990, 1993; Santos *et al.*, 2002, Gomez *et al.*, 2002) ion channels, and other highly insecticidal proteins (Figueiredo *et al.*, 1995, 2001; Mafra *et al.*, 1999; De Lima *et al.*, 2002; Oliveira *et al.*, 2003) have been described from the venom of this spider, which is commonly found in Central and South-East Brazil, but very little is known about the venoms of the other species of this genus. Recently, our group has published the comparison of the partial proteomes of venoms from different species of the genus Phoneutria (Richardson *et al.*, 2006). Using different separation methodologies, including 2D-gel and multi-dimensional chromatographic procedures, we were able to find about 400 peptides and proteins of which, nearly 120 have their partial/complete primary structure determined. We now describe the purification, primary structure and identify the molecular target of a new toxin from the venom of *Phoneutria reidyi*, a spider from the northern Brazilian states Amazonas, Pará and Roraima, which causes flaccid paralysis in mice at dose levels of 5 μg/mouse. This toxin, named PRTx3-7, has its C-terminal residue amidated and was found to be homologous to PNTx3-2, a Ca^{2+}-type toxin isolated from *Phoneutria nigriventer* venom. It has been also reported that some toxins from the venom of *P. nigriventer*, like PRTx3-7, cause flaccid paralysis when injected intracerebroventricular (i.c.v.) into mice (Cordeiro *et al.*, 1993). PNTx3-2 is known to partially block L-type Ca^{2+} channels in GH3 cells (Kalapothakis *et al.*, 1998; Gomez *et al.*, 2002), whereas PNTx3-1 is a potent and selective blocker of A-type K^{+} channels (Kushmerick *et al.*, 1999) and PNTx3-3 inhibits all known HVA Ca^{2+} channels, and most effectively the P/Q and R-type currents (Leão *et al.*, 2000).

Electrophysiogical data in HEK cells expressing high-voltage-activated (HVA) Ca^{2+} channels suggest that PRTx3-7 inhibits these channels, change their

voltage-dependent properties and has more affinity for N-type Ca^{2+} channels. The investigation of these natural mutants, along with the related biochemical and biological data will contribute to the global understanding of the mechanisms involving spider toxins and their related targets.

MATERIALS AND METHODS

Toxin Purification

Venom collected from spiders captured near the Usina Tucurui in the state of Para in Northern Brazil was provided by the Butantan Institute (São Paulo, Brazil). Aliquots (25 mg) of lyophilised venom were dissolved in 2 mL of 0.1% (v/v) trifluoroacetic acid (TFA) in water and insoluble materials removed by centrifugation at 10,000 $\times$ *g* for 10 min. Each aliquot of venom was initially fractionated by reverse phase HPLC on a Vydac C4 (214TP1022) preparative column (2.2 cm $\times$ 25 cm), equilibrated with 0.1% TFA in water, and eluted at a flow rate of 5 mL/min with the following solutions: 0–20 min, 0.1% TFA in water; 20–30 min, a gradient of 0–20% acetonitrile in 0.1% TFA in water; 30–110 min, gradient of 20–40% acetonitrile containing 0.1% TFA in water; 110–130 min, 40–50% acetonitrile containing 0.1% TFA in water; 130–140 min, 50–70% acetonitrile with 0.1% TFA in water. The material in the 17th peak (detected by $A_{214\ nm}$) that eluted after 68 min (at a concentration of 29% acetonitrile) was collected and lyophilised. It was then dissolved in 2 mL of 10 mM sodium phosphate buffer pH 6.1, before being subjected to cation exchange FPLC on a column of Resource™ S 1 mL (Pharmacia Biotech), equilibrated in the same buffer and eluted at a flow rate of 1 mL/min with a linear gradient of 0–0.5 M NaCl. The material in the third major peak ($A_{280\ nm}$) that eluted at 0.32 M NaCl was desalted by a final step of reverse phase FPLC on a column of C2/C18 PepRPC™ 15 μm (HR10/10, Pharmacia), using a linear gradient of acetonitrile in 0.1% aqueous TFA.

The homogeneity of the isolated protein was examined by high-resolution propionic acid/urea PAGE (Chettibi and Lawrence, 1989). The toxicity of the material was assayed by intracerebro-ventricular injection in mice as described previously (Rezende *et al.*, 1991).

Mass Spectrometry Analyses

The molecular mass of the protein was determined by electrospray mass spectrometry (Q-TOF Micro®, Micromass, UK) in positive ion mode as previously described (Pimenta *et al.*, 2005). The protein (20 μg) was solubilised in 100 μL of 50% acetonitrile in aqueous 0.1% TFA and applied to mass spectrometer by a syringe pump system at a flow rate of 5–10 μL/min. Alternatively, a nanospray interface was used to increase sensitivity (flow rate of 0.5–1.0 μL/min). The capillary voltages were 3–3.5 kV and cone voltages were 40–60 V. The spectrum used was the result from 20 scans (2.4 s) combined. Original data (m/z) were treated (base line subtraction, smoothing and centring) and transformed into a mass (Da) spectrum.

Sequencing by tandem mass spectrometry (MS/MS) was performed using collision-induced dissociation (CID) technique, carried out using argon and collision energies in the range 30–45 eV. Data were analysed by MassLynx® 3.5 software.

Edman Degradation Sequencing

The protein was S-reduced and alkylated using 4-vinylpyridine as described (Wilson and Yuan, 1988). Samples of the native and alkylated protein (3–4 nmol) were sequenced by Edman degradation using an automated Shimadzu PPSQ-21A protein sequencer coupled to reversed phase separation of PTH-amino acids on a WAKOSIL-PTH (4.6 mm × 250 mm) column.

Enzymatic Hydrolysis of PRTx3-7

A sample (20 nmol) of the native protein was digested with 5 μg of the Glu-C endoproteinase from *S. aureus* V8 strain (Sigma) in 0.1 M Na phosphate buffer, pH 7.8, for 24 h at 37°C. The resulting peptides were separated by reverse phase HPLC on an analytical column (0.46 cm × 25 cm) of Vydac C18 small pore (201SP54), using a gradient of 0–50% acetonitrile in 0.1% (v/v) aqueous TFA (with 1.0 mL/min flow and detection at 214 nm) and the C-terminal peptide was submitted to MS/MS sequencing.

Solutions and Drugs

Stock solutions of PRTx3-7 were prepared in distilled water and stored at −20°C. Toxin was thawed shortly before the experiments and diluted into the bath solution at the appropriate concentration. BSA (0.025%, Jackson Immuno Res., West Grove, PA, USA) was included in external solutions to avoid non-specific binding. For experiments with permanently expressed HVA Ca^{2+} channels, the perfusion system consisted of a custom-made multiple solution perfusion manifolds with four input and four output capillary tubes (custom microfil, 28 gauge, 250 μm inner diameter and 350 μm outer diameter; World Precision Instruments, Sarasota, FL) sheathed in a glass pipette. High chemical-resistant Tygon Chemfluor FEP (Norton Performance Plastics, Akron, OH) and Silastic (Fisher Scientific, Nepean, ON) tubing was used to connect the perfusion manifold to the syringe valve. Gravity-driven perfusion occurred at a rate of approximately 400 μL/min. The outputs of the manifold were placed within close proximity of the cell, resulting in the cell being bathed in new solutions with minimal delay (within 1 s).

Preparation of Synaptosomes

Adult Wistar rats of both sexes (180–200 g) were decapitated and had their cortices removed and homogenized 1:10 (w/v) in 0.32 M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (1 mM). Homogenates were then submitted to low-speed centrifugation (1000 × *g* for 10 min) and synaptosomes were purified from the supernatant by discontinuous Percoll™-density-gradient

centrifugation (Dunkley *et al.*, 1988; essentially as described by Romano-Silva *et al.*, 1993). The isolated nerve terminals were re-suspended in Krebs–Ringer–Hepes solution (124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM Hepes, pH 7.4) with no added $CaCl_2$, to a concentration of approximately 10 mg/mL, divided into aliquots of 200 μL and kept on ice until loaded with fura-2AM^{TM}.

Measurements of Intracellular Calcium Concentration $[Ca^{2+}]_i$

Fura-2AM^{TM} (stock solution 1 mM in DMSO) was added to the synaptosomal suspensions to give a final concentration of 5 μM and the mixture incubated for 30 min (35°C) and then diluted with medium to a concentration of 1.2 mg/mL followed by a further 30 min incubation period. Fura-2-loaded isolated nerve terminals were washed, re-suspended with medium (1 mg/mL) and immediately used for ratiometric quantification of intracellular free calcium (Grynkiewicz *et al.*, 1985) in a PTI^{TM} spectrofluorimeter. Fluorescence emission ratio was calculated for emission at 500 nm using excitation at 330/370 (FR = I330/I370, where I330 and I370 are the emission intensities for excitation at corresponding wavelengths). Calibration of fura-2 signals and estimation of synaptosomal background fluorescence were performed as described elsewhere (Romano-Silva *et al.*, 1993). $CaCl_2$ was added to the synaptosomal suspension at the beginning of each fluorimetric assay (for 1.0 mM final concentration). Synaptosomes were stirred throughout the experiment in a cuvette maintained at 35°C. Toxin was added to synaptosomal suspension 60 s prior to membrane depolarisation with 33 mM KCl. The $[Ca^{2+}]_i$ increase induced by membrane depolarisation is strictly dependent on the presence of extracellular Ca^{2+}.

Cell Culture

Human embryonic kidney cells (HEK 293) expressing a simian virus 40T-antigen (tsA-201), were grown in standard DMEM (10% fetal bovine serum and 50 U/mL penicillin–50 μg/mL streptomycin). HEK cells were split at 80% confluence using trypsin–EDTA, plated on 35-mm culture dishes at 5–10% confluence and maintained at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. The cells were allowed to recover for 12 h before transfection.

Transient Transfection with L-, P/Q- and R-type Ca Channels

HEK cells were grown to approximately 80% confluency, enzymatically dissociated using 0.25% trypsin in DMEM containing 1 mM EDTA (trypsin/EDTA), plated on 35-mm culture dishes at 5–10% confluency, and allowed to recover for 12 h. The medium was then replaced, and the cells were transiently transfected with cDNA plasmids encoding one type of Ca^{2+} channel α subunit [α_{1A} (Starr *et al.*, 1991), α_{1C} (Bourinet *et al.*, 1994), or α_{1E} (Soong *et al.*, 1993)] together with auxiliary subunits β_{1b} and $\alpha_2\delta$ (Zamponi *et al.*, 1997) and CD8 marker plasmid at a molar ratio 1:1:1:0.25 using Lipofectamine (Invitrogen) following the manufacturer's instructions. After 12 h, the cells were washed with fresh medium, stored at 37°C and

allowed to recover for an additional 36–72 h before electrophysiology recordings. Transiently transfected cells were selected for expression of CD8 by adherence of Dynabeads (Dynal, Great Neck, NY).

Generation of Stable Cell Lines Expressing N- and T-type Ca Channels

HEK cells were transfected using linearized cDNA encoding either α_{1G} (McRory *et al.*, 2001) alone or α_{1B} (Dubel *et al.*, 1992 together with β_{1b} and $\alpha_2\delta$ using standard Ca-phosphate precipitation method, The stable cell lines were enzymatically dissociated using 0.25% trypsin in DMEM containing 1 mM EDTA (trypsin/EDTA), plated on 35-mm culture dishes, and allowed to recover 24–36 h before recordings.

Electrophysiological Recordings

Macroscopic currents on the HEK cells were recorded using the whole-cell patch-clamp technique. The external recording solution contained (in mM): 2 $BaCl_2$, 1 $MgCl_2$, 10 HEPES, 40 TEACl, 92 CsCl, and 10 glucose, pH = 7.4. The internal pipette solution contained (in mM): 120 CsCl, 1 $CaCl_2$, 11 EGTA, 10 HEPES, 2 Mg-ATP, pH = 7.2. Whole-cell currents were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), controlled and monitored with a personal computer running pClamp software version 6.03 (Axon Instruments). Patch pipettes (borosilicate glass BF150-86-10; Sutter Instruments, Novato, CA), were pulled using a Narishige (Tokyo, Japan) puller, with typical resistances of 3–5 MΩ (when filled with internal solution). The bath was connected to the ground via a 3 M KCl Agar-bridge. Voltage errors due to series resistance were kept to <3 mV by selecting cells whose peak whole-cell currents were <2 nA. All recordings were performed at room temperature (20–24°C). Data were sampled at 10 kHz and low-pass filtered at 2 kHz using the built-in Bessel filter of the amplifier, and the amplifier was also used for whole-cell capacitance compensation on every cell. In some cases, capacitance and leakage subtraction were performed online using P/P4 protocol. Current–voltage relationships were fitted to the equation: $I_m = G_{max} \times (V_m - E_{rev})/\{1 + \exp((V_m - V_{50a})/k_a)\}$, where V_m is the test potential, V_{50a} is the half-activation potential, E_{rev} is the extrapolated reversal potential, G_{max} is the maximum slope conductance, and k_a reflects the slope of the activation curve. The steady-state inactivation curves were obtained by applying test depolarisation to peak potentials at the end of 10 s pre-pulses ranging in voltages from -110 to $+20$ mV in 10 mV increments. The data were fitted with a Boltzmann equation: $I/I_{max} = [1 + \exp(\{V - V_{50i}\}/k_i)]^{-1}$, where I is the peak current, I_{max} is the peak current when the conditioning pulse was -110 mV, V and V_{50i} are the conditioning potential and the half-inactivation potential respectively, and k_i is the inactivation slope factor. Time courses of channel's blockade, activation and inactivation rates during steps to peak potential and deactivation of currents following brief test pulses were well described by single exponential and the calculated time constants were cited in the text as: τ_{on}, τ_{act}, τ_{inac}, τ_{deact}, respectively.

Recordings were analysed using Clampfit 6.03 (Axon Instruments) and leak subtraction was applied on cells that were not subtracted on-line and low-pass filtering at 1000 Hz. Figures and fittings were obtained by using the software program Microcal Origin (Version 6.0). Statistical significance was determined by Student's *t*-test, and significant values were set as $p < 0.05$ or as indicated in the text and figure legends.

RESULTS

Purification, Sequencing and Mass Spectral Analysis of PrTx3-7

The new neurotoxin PRTx3-7 was isolated from the venom of the Brazilian spider *Phoneutria reidyi* using only three steps of purification: preparative reverse phase HPLC on Vydac C4, cationic exchange chromatography on Resource S at pH 6.1 and a final step of reverse phase FPLC on an analytical column of C2/C18 PEP-RPC. The isolated protein yielded a single narrow band when examined by high resolution propionic acid/urea PAGE and ESI-Q-TOF/MS analysis indicated a molecular mass of 4627.26 ± 0.03 Da. The complete amino acid sequence of the protein was determined without ambiguity by automated Edman sequencing (Fig. 1). PRTx3-7 also shows approximately 55% sequence identity with PNTx3-1, and 38% with PNTx3-3.

The molecular mass calculated for PRTx3-7 from the amino acid sequence was 4628.07 Da, taking into account a free carboxyl group at the C-terminal and that all Cys residues involved in disulfide bonds formation. The mass difference, i.e. 0.9 a.m.u., could indicate a presence of a α-amide C-terminal (calculated mass 4627.08 Da) and, in that case, the calculated mass difference would be only −0.09 a.m.u. To investigate the possible presence of an α-amide derivative at the

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PRTx3-7    ACAGL YKKCGKGVNTCCENRP      CKCDLAM G NCICKKKFVEFFGG
PNTx3-2    ACAGL YKKCGKGASPCCEDRP      CKCDLAM G NCICK
PNTx3-2*   ACAGL YKKCGKGASPCCEDRP      CKCDLAM G NCICKKKFIEFFGGGK
PNTx3-1   AECAAV YERCGKGYKRCCEERP      CKCNIVMD  NCTCK KFISE
Pn3a*      ACADV YKECWYPEKPCCKDRA      CQCTLGM  T CKCKATLGDLFGR
PNTx3-3    GCANA YKSC NGPHTCCWGYNGYKKACICSGSNWK NCKCK
ωAGAIVA KKKCIAKDYGRCKWGGTPCCRGRG      CICSI M GTNCECKPRLIMEGLG
ωAGAIVB     CIAEDYGKCTWGGTKCCRGRP      CRCS  MIGTNCECKK LISELFG
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Fig. 1. Amino acid sequence alignment between PRTx3-7 and other spider toxins. Comparison of the amino acid sequence of the toxin PRTx3-7 from the venom of the spider *Phoneutria reidyi* with the complete sequences of the toxins PNTx3-1, and PNTx3-2 and the N-terminal sequence of PNTx3-3 from *Phoneutria nigriventer* (Cordeiro *et al.*, 1993). Also shown (PNTx3-2* and Pn3a*) are the amino acid sequences deduced from the analyses of clones from a cDNA library constructed using the venom gland of *P. nigriventer* (Kalapothakis *et al.*, 1998), and ω-AGAIVA and ω-AGAIVB are toxins from the venom of the funnel-web spider *Agelenopsis aperta* (Adams *et al.*, 1993; Mintz *et al.*, 1992). Gaps were introduced for better alignment. The amino acid residues highlighted in bold are those which are identical with the residues in PRTx3-7.

C-terminal residue, the native protein was digested for 24 h with the Glu-C specific endoproteinase V8 from the *S. aureus* strain, and the products of the digestion were separated by reverse phase HPLC on an analytical column of Vydac C18 small pore (not shown). The C-terminal peptide Phe-Phe-Gly-Gly, which resulted from the hydrolysis of the peptide bond Glu_{39}-Phe_{40} was eluted at an acetonitrile concentration of 20%. Its molecular mass was measured as 425.35 Da, which is in high accordance with a calculated monoisotopic mass for the amide derivative form (425.21 Da). This peptide was subjected to collision-induced dissociation (MS/MS) to confirm that the C-terminal residue Gly_{43} was indeed an amide (Fig. 2).

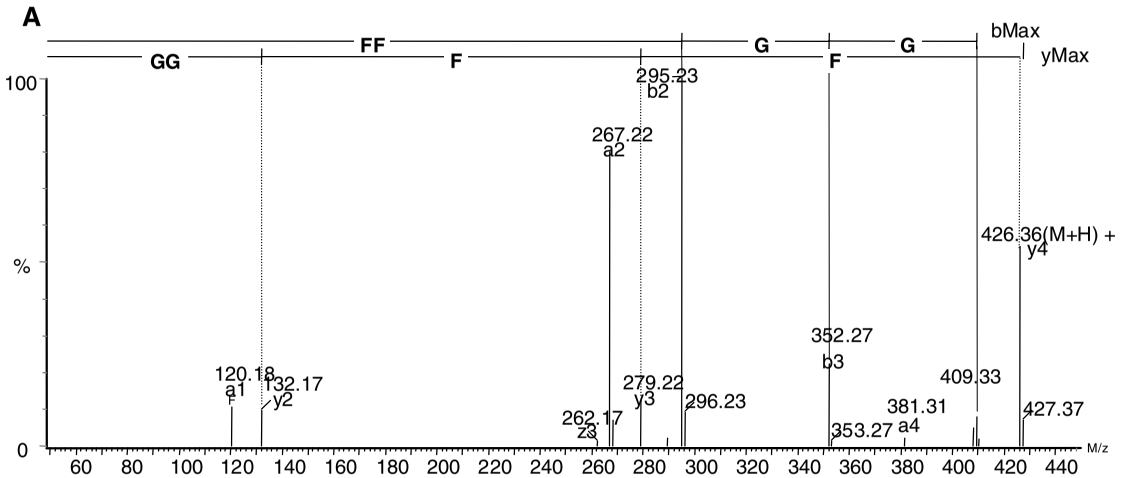
PrTx3-7 Inhibits Partially KCl-Induced Increase on $[Ca^{2+}]_i$ in Synaptosomes

To verify whether PRTx3-7 targets Ca^{2+} channels as has been described for other *Phoneutria* toxins, we tested the effects of 0.4 μM and 1 μM of PRTx3-7 on KCl-induced increase in $[Ca^{2+}]_i$ in rat brain cortical synaptosomes. Synaptosomes were stirred throughout the experiment in a cuvette maintained at 35°C. Toxin was added to the synaptosomal suspension 60 s prior to membrane depolarization with KCl (33 mM). The $[Ca^{2+}]_i$ increase by membrane depolarization is strictly dependent on the presence of extracellular Ca^{2+} in these conditions (Romano-Silva *et al.*, 1993). Supplementary Fig. 1 shows that 0.4 μM of PRTx3-7 has no significant effect in the increase in synaptosomal $[Ca^{2+}]_i$. However, 1 μM of PRTx3-7 inhibited by 20% the KCl-induced increase in $[Ca^{2+}]_i$ in synaptosomes.

Electrophysiological Analysis

PRTx3-7 Blocks High Voltage-Activated Ca^{2+} Channels

The electrophysiological action of PRTx3-7 was investigated using HEK (tsA-201) cells expressing L-, N-, P/Q- and R-type Ca^{2+} channels (α_1 subunits plus auxiliary subunits ($\beta_{1b} + \alpha_2\delta$) and stable HEK cells line expressing the T-type channel. As shown in Fig. 3, representative current traces show that the application of 1 μM PRTx3-7 (filled circles) has different effects on high voltage-activated Ca^{2+} currents and no significant effect on low-voltage-activated Ca^{2+} currents. To better evaluate the blocking efficiency of PRTx3-7, we chose one type of cloned Ca^{2+} channels to be exposed to a range of concentrations of the toxin (Fig. 3F). The peak Ba^{2+} currents were blocked in an incomplete concentration-dependent manner (2 μM PRTx3-7 was the maximal concentration tested) and the maximal blockade obtained was close to 40% (IC_{50} = 436 nM and Hill coefficient = 0.8). Moreover, other experiments showed that the application of 1 μM of PRTx3-7 with a fast perfusion system does not completely block current activity of L-type (time constant of channel's blockade, $\tau_{on} = 37 \pm 13$ s), N- ($\tau_{on} = 18.2 \pm 1.5$ s), P/Q- ($\tau_{on} = 10.7 \pm 1.7$ s) and R- ($\tau_{on} = 19.9 \pm 2.7$ s) Ca^{2+} currents. Following the washout, the currents did not fully recovered (Fig. 4A–D).



B

Amidated

a	**120.08**	**267.15**	324.17	**381.19**
	- 0.10	**- 0.07**	---	**- 0.12**
b	148.08	**295.14**	**352.17**	**409.19**
	---	**- 0.08**	**- 0.10**	**- 0.14**
	Phe	**Phe**	**Gly**	**Gly**
y	**426.21**	**279.15**	**132.08**	75.06
	- 0.15	**- 0.08**	**- 0.09**	---
z	**409.18**	**262.12**	115.05	58.03
	- 0.15	**- 0.05**	---	---

C

Non-amidated

a	**120.08**	**267.15**	324.17	**381.19**
	- 0.10	**- 0.07**	---	**- 0.12**
b	148.08	**295.14**	**352.17**	**409.19**
	---	**- 0.08**	**- 0.10**	**- 0.14**
	Phe	**Phe**	**Gly**	**Gly**
y	427.20	280.13	133.06	76.04
	---	---	---	---
z	410.17	263.10	116.03	59.01
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Fig. 2. Collision-induced dissociation (CID) spectrum and sequencing data of the C-terminal peptide (FFGG) from PRTx3-7. The peptide was obtained by enzymatic digestion using the Glu-specific endoproteinase from *S. aureus* V8. (A) Collision-induced dissociation spectrum obtained by fragmenting the peptide FFGG using argon (collision energy 30–45 eV). (B) and (C) show the theoretical fragmentation ion (a, b, y and z) maps of the peptide with or without C-terminal amidation, respectively, and indicates the matched observed ions (bold) and their respective differences.

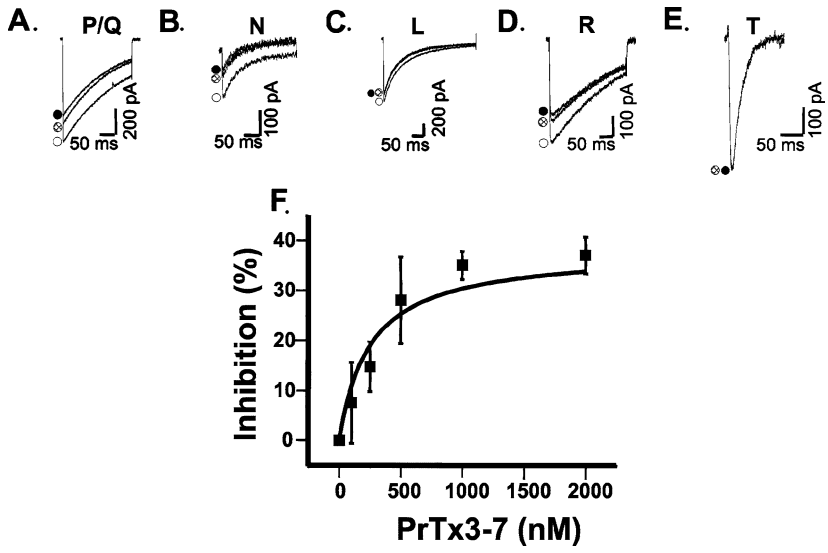


Fig. 3. PrTx3-7 blocks partially high voltage-activated Ca^{2+} channels. (A–E) Representative Ba^{2+} current traces (for P/Q-(A), N-(B), L-(C), R-(D) and T-(E) type Ca^{2+} channels) recorded in control (open circles), during the application of 1 μM PRTx3-7 (filled circles), and after washout of the toxin (semi-filled circles). Currents were elicited by stepping from a holding potential of -100 mV to peak current potential (P/Q, -10 mV; N, $+10$ mV; L, -5 mV; R, -20 mV and T, -30 mV). (F) Concentration–response curve shows that PRTx3-7 blocks partially the N-type Ca^{2+} channel. Line represents the best fit of the Hill equation: $I = I_{max}/\{1 + (IC_{50}/[PrTx3\text{-}7])^n\}$, where I is the observed percentage of inhibition at a given concentration of the toxin, I_{max} is the predicted maximal inhibition, and the Hill coefficient (n) gives a measure of the steepness of the curve. Data points represent mean $\pm$ SEM of four to seven independent measurements.

PrTx3-7 Alters Voltage-Dependent Properties of High-Voltage Ca^{2+} Channels But Not the Kinetics

We tested the effects of 1 μM PRTx3-7 on the high-voltage Ca^{2+} channels. In Fig. 5, control cells were half-maximal activation at membrane potential of -9.1 ± 0.6 mV (P/Q-), $+2.7 \pm 0.9$ mV (N-), -6.0 ± 0.4 mV (L-) and -19 ± 0.6 mV (R-). After application of the toxin, these parameters were changed: $-18 \pm 0.8-$mV (P/Q-), -10.3 ± 1.2 mV (L-), -24 ± 0.4 mV (R-) and not changed for $+2.0 \pm 1.6$ mV (N-). As shown in Fig. 5, the treatment of the cells with 1 μM of PRTx3-7 caused a hyperpolarized shift in the voltage-dependent activation of the L- (-4 mV), P/Q- (-8 mV) and R- (-5 mV) type currents. In addition to the shift in the voltage-dependence of activation, the toxin also affected the steady state of inactivation of N- ($+7$ mV), L- (-5 mV) and P/Q- (-4 mV) currents (Fig. 6). Some time control experiments were performed to ensure that the shift in the activation and inactivation curves did not occur spontaneously (data not shown). It was also shown that 1 μM of PRTx3-7 caused neither a significant shift in the voltage-dependence of activation of N-type Ca^{2+} channels nor a variation in the steady state of inactivation of R-type channels (Figs. 5B and 6D). Analysing the

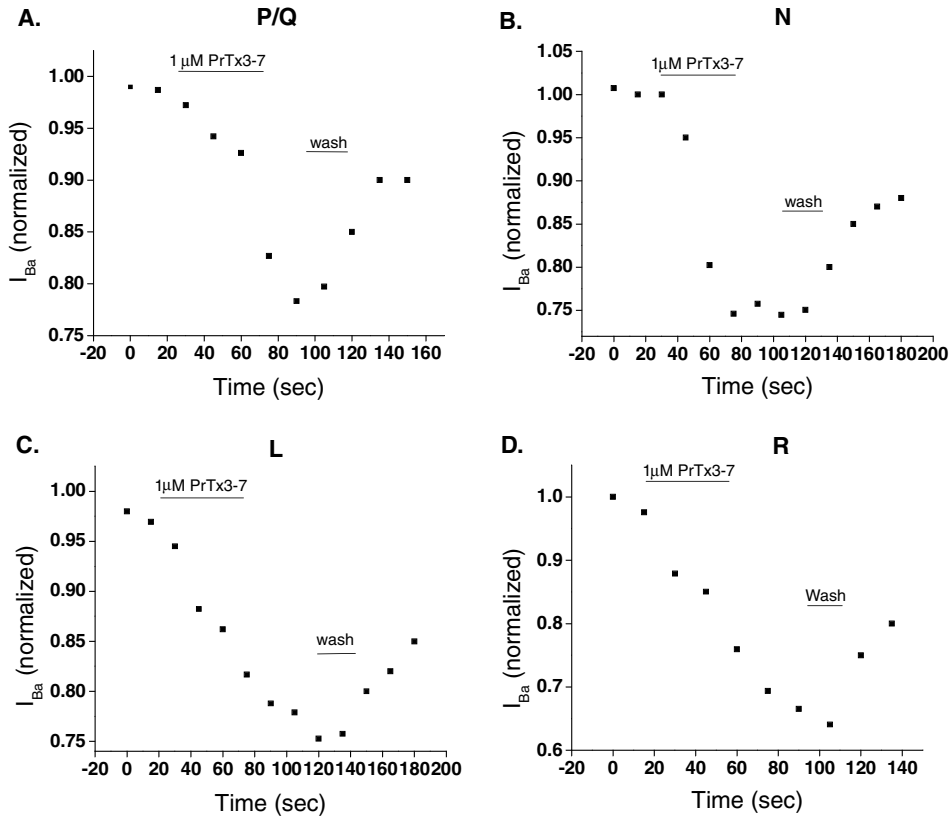


Fig. 4. Blockade of high voltage-activated Ca^{2+} channels by PRTx3-7 is incomplete and not fully reversible. (A–D) Application of 1 μM PRTx3-7 (no significant run-down was observed after 2–4 min prior the addition of PRTx3-7) blocks partially high voltage-activated Ca^{2+} channels (A: P/Q, B: N, C: L, D: R) and even after washout the recovery of all four types of currents were not complete. The time course of PRTx3-7 effects were investigated using 80–400 ms steps to peak current potential every 10 s from a holding potential of −100 mV. Note that PRTx3-7 was added corresponding to the length of time indicated by the solid bar above the graphs. The whole-cell current values for each point were divided by the maximal current to give normalized current.

effect of PRTx3-7 on the kinetics of the HVA Ca^{2+} channels, we observed that after treatment with 1 μM of PRTx3-7 for all HVA currents there was no significant alteration in the rates of activation, inactivation or deactivation (Table I).

DISCUSSION

It has been hypothesised that the key role of α-amide modification is to prevent the ionisation of the COOH-terminus of the peptide, rendering it more hydrophobic and, therefore, increasing its ability to bind to a receptor (Eipper *et al.*, 1993). In some instances, the charge elimination promoted by the amide

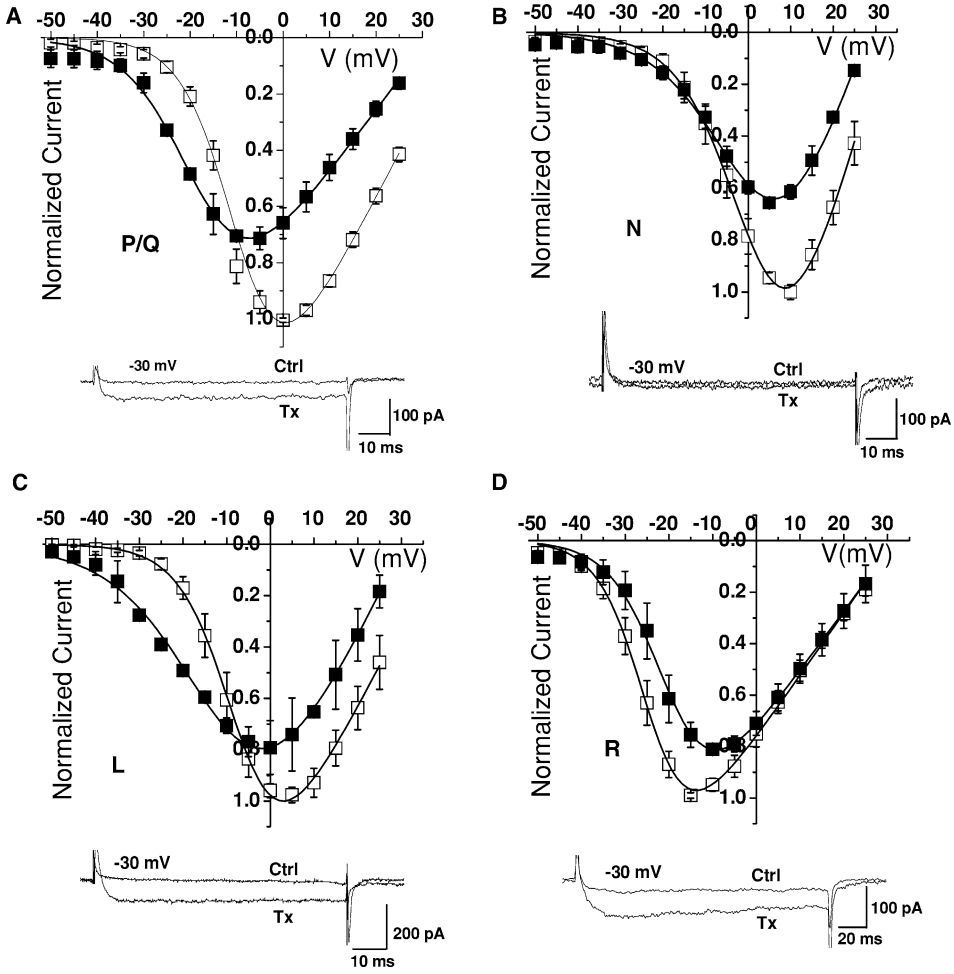


Fig. 5. Current–voltage relationships for PRTx3-7 inhibition. Normalized current–voltage relationships are shown before (open circles) and after treatment (filled circles) with the toxin. Depolarizing pulses were applied from a holding potential of −100 mV to membrane potentials increasing in 10 mV steps. Current–voltage relationships were fitted as described in Materials and Methods and used for calculate V_{50a}, k_a, E_{rev} and G_{max}. The voltage for half activation (V_{50a}) was hyperpolarized shifted after application of 1 μM PRTx3-7 for P/Q (A) currents (control $V_{50a} = -9.1 \pm 0.6$ mV vs. treated $V_{50a} = -18 \pm 0.8$ mV, $n = 4$); for L (C) currents (control $V_{50a} = -6.7 \pm 0.4$ mV vs. treated $V_{50a} = -10.3 \pm 1.2$ mV, $n = 3$); for R (D) currents (control $V_{50a} = -24 \pm 0.4$ mV vs. treated $V_{50a} = -19 \pm 0.6$ mV, $n = 5$). No significant change was observed for N (B) currents when PRTx3-7 (1 μM) was applied (control $V_{50a} = +2.7 \pm 0.9$ mV vs. treated $V_{50a} = +2.0 \pm 1.6$ mV, $n = 5$). Insets, representative current traces at −30 mV showing the increase of conductance for P/Q-, L- and R-currents in the presence of toxin.

modification can significantly affect the three-dimensional structure and the activity of a toxin as NMR and molecular modelling data have showed (Sforça *et al.*, 2004). Furthermore, the modification of the C-terminus into NH_2-terminus could act as a protective shield, preventing the cleavage of the peptide by carboxypeptidases and increasing the half-life of the toxin inside the victim's tissue. To our knowledge, this

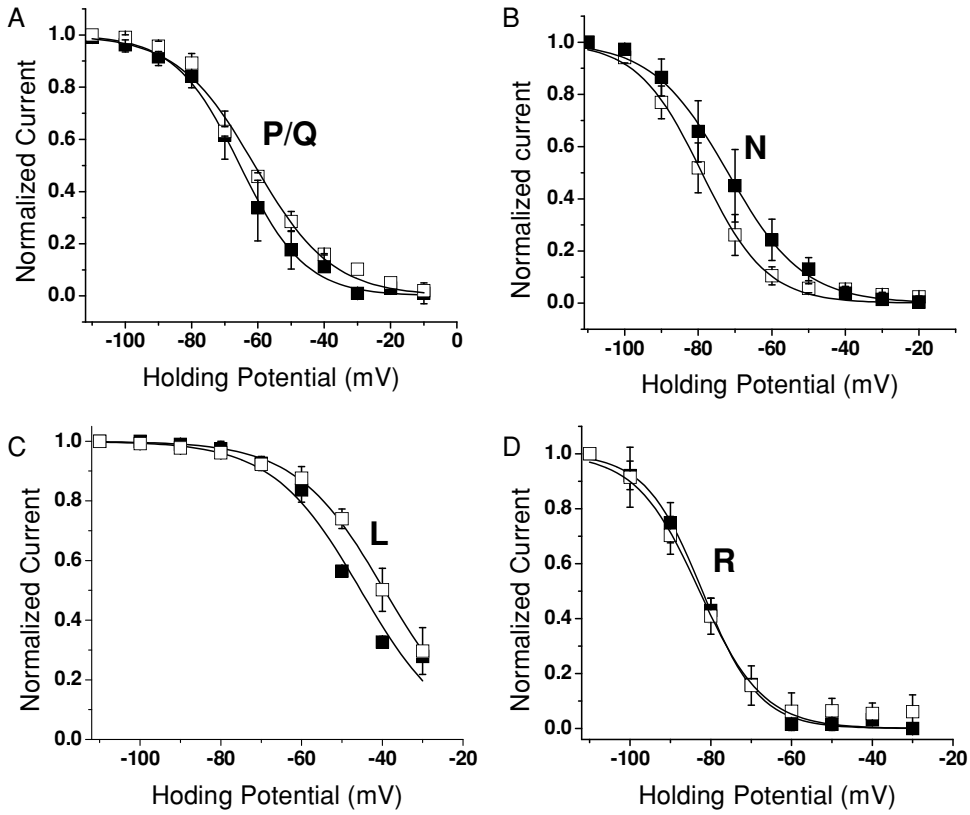


Fig. 6. The voltage-dependence of steady-state inactivation of Ca^{2+} channels is affected by PRTx3-7. Cells were voltage clamped at the indicated prepulse potential for 10 s and then stepped to the peak current potential for each channel type (P/Q, −10 mV; N, +10 mV; L, −5 mV; R, −20 mV). Currents during the test pulse were normalized to the current obtained after a prepulse to −120 mV and fit with a Boltzmann equation: $I/I_{max} = [1 + \exp(\{V - V_{50i}\}/k_i)]^{-1}$, where I is the peak current, I_{max} is the peak current when the conditioning pulse was −110 mV, V and V_{50i} are the conditioning potential and the half-inactivation potential respectively, and k_i is the inactivation slope factor. PRTx3-7 (1 μM) caused significant shift in the half-inactivation potential (V_{50i}) for P/Q (A) currents (control $V_{50i} = -61 \pm 0.9$ mV, $k_i = -7.1$; treated $V_{50i} = -65 \pm 0.5$ mV, $k_i = 0.5$, $n = 5$); N (B) currents (control $V_{50i} = -79 \pm 0.6$ mV, $k_i = -8.8$; treated $V_{50i} = -72 \pm 0.5$ mV, $k_i = 10.3$, $n = 6$); L (C) currents (control $V_{50i} = -39.5 \pm 0.4$ mV, $k_i = 11$; treated $V_{50i} = -45 \pm 1.2$ mV, $k_i = 10.9$, $n = 5$). No significant change was observed in R (D) currents (control $V_{50i} = -83 \pm 0.4$ mV, $k_i = 7.9$; treated $V_{50i} = -82 \pm 0.4$ mV, $k_i = 7$, $n = 7$). Note that in all graphs showed above, (PRTx3-7)-treated cells are shown with filled symbols whereas control cells are shown with open symbols.

is the first report providing direct evidence for the occurrence of a polypeptide with an amidated C-terminal amino acid from the venom of a spider from the *Phoneutria* genus, although it has been reported for a number of different venom toxins from other spiders (Bernard *et al.*, 2000; Stapleton *et al.*, 1990), scorpions (Pimenta *et al.*, 2003; Becerril *et al.*, 1997), cone snails (McIntosh *et al.*, 1999) and wasps (Sforça *et al.*, 2004).

Table I. Effect of PRTX3-7 on the Kinetics of HVA Ca^{2+} Channels

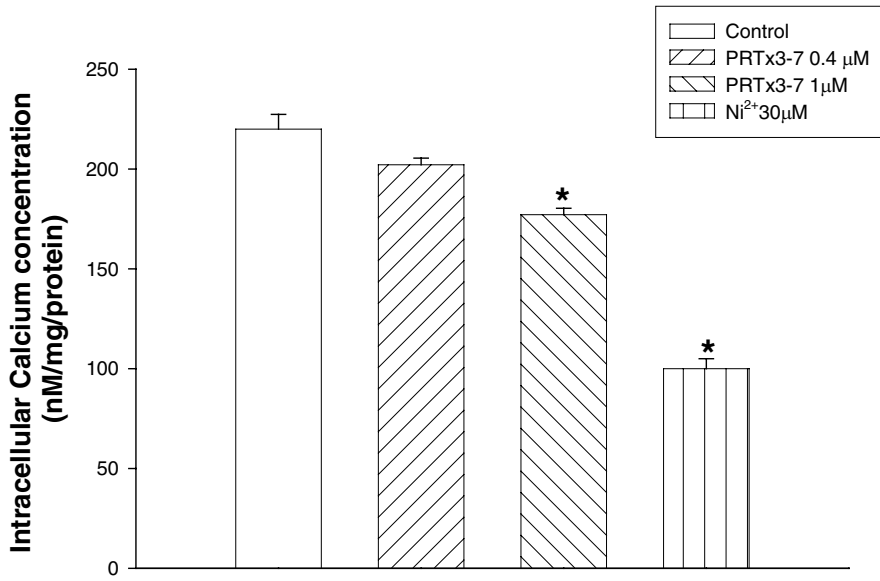
Ca current	Treatment	τ_{act} (ms)	τ_{inact} (ms)	τ_{deact} (ms)
P/Q	Control	1.3 ± 0.3 (9)	100 ± 14.7 (9)	0.2 ± 0.01 (9)
	1 μM PRTx3-7	1.1 ± 0.2 (9)	90 ± 10.7 (9)	0.22 ± 0.05 (9)
N	Control	2.17 ± 0.3(6)	191 ± 60 (6)	0.7 ± 0.2 (6)
	1 μM PRTx3-7	2.2 ± 0.26 (6)	164 ± 34 (6)	0.9 ± 0.3 (6)
L	Control	2.8 ± 2.4 (9)	166 ± 28.9 (9)	0.9 ± 0.2 (9)
	1 μM PRTx3-7	2.7 ± 1.3 (9)	149 ± 24 (9)	1.1 ± 0.2 (9)
R	Control	1.76 ± 0.22 (7)	155 ± 39 (5)	0.6 ± 0.1 (5)
	1 μM PRTx3-7	1.81 ± 0.26 (7)	156 ± 39 (5)	0.4 ± 0.1 (5)

Note. Averages were compiled from different cells, where in parenthesis are shown the number of cells. Mean values (± standard error). There were not significant differences between the cells and treated ($p < 0.5$) in all cases above.

Comparison of the amino acid sequence of PRTx3-7 with the sequences of toxins previously isolated from the venoms of spiders of other genera reveals that it has low levels (28–40%) of sequence identity (Fig. 1) with the omega (ω)-agatoxins IVA and IVB from the venom of the funnel-web spider *Agelenopsis aperta* (Adams *et al.*, 1993; Mintz *et al.*, 1992). When compared with the toxins previously isolated from *Phoneutria nigriventer*, it can be seen that it is very similar (88% identity) to PNTx3-2 (Cordeiro *et al.*, 1993), differing in only four positions (Fig. 1). However, it is clear that PRTx3-7 has not suffered the post-translational removal of the C-terminal region as was indicated for PNTx3-2 by the sequencing of cDNA clones (Kalapothakis *et al.*, 1998). Furthermore, the PNTx3-2 sequence predicted from cDNA shows the presence of two additional residues after the C-terminus of the native toxin, which are Gly_{44} and Lys_{45}, also indicating a possible C-terminal amide formed from Gly_{44}.

When the purified PNTx3-7 was injected (intracerebro-ventricular) into mice at dose levels of 5 μg/mouse, it caused immediate agitation and clockwise gyration, followed by the gradual development of general flaccid paralysis. These symptoms were very similar to those previously described when various Tx3-type toxins from *P. nigriventer* were injected into mice (Cordeiro *et al.*, 1993; Rezende *et al.*, 1991).

As shown in supplementary Fig. 1, PRTx3-7 affects the KCl-induced increase in $[Ca^{2+}]_i$ in rat brain cortical synaptosomes suggesting the involvement of this toxin with Ca^{2+} channels. The synaptosomal preparation is definitely not the most adequate preparation to identify which channels were involved in the inhibition of Ca^{2+} release upon toxin exposure. Another important drawback is the fact that we need to depolarise the synaptosomal membrane to provoke intracellular Ca^{2+} variations and by doing that we could miss valuable information at more negative membrane potentials. The involvement of P/Q-type Ca^{2+} channels in the PRTx3-7 action was also confirmed in heterologous system, in which PRTx3-7 inhibited by 30% the P/Q-type Ca^{2+} current (Fig. 3). Thus, PRTx3-7 affects ion channels as was shown for other *Phoneutria* toxins (Leão *et al.*, 2000; Kushmerick *et al.*, 1999; Cassola *et al.*, 1998; Miranda *et al.*, 1998; Guatimosim *et al.*, 1997). However, according to our data, PRTx3-7 has higher affinity for N-type channels than for the other Ca^{2+}-channel subtypes (1 μM of PRTx3-7 blocks almost 40% of the N-type currents). The poor sensitivity of N-type Ca^{2+} channels in brain cortical slices and



Supl Fig. 1. Effect of PRTx3-7 in KCl-induced increase in $[Ca^{2+}]_i$ in synaptosomes. Synaptosomes loaded with fura-2 as described in Materials and Methods were incubated in KRH medium containing 1.0 mM $CaCl_2$ for 60 s in the presence of 400 nM or 1 μM of PRTx3-7 then, they were stimulated by KCl 33 mM during 2.5 min. The measured fura-2 fluorescence (Exc = 330/370 nm and Em = 510 nm) was used to calculate $[Ca^{2+}]_i$ as described by Grynkiewicz *et al.* (1985). Calibration was performed with sequential addition of SDS and EGTA at the end of each experiment. Data are mean values of at least three separate experiments.

synaptosomes is well known (Lundy *et al.*, 1991; Suszkiw *et al.*, 1986) and we have shown that a specific blocker of N-type Ca^{2+} channels, ω-CgTX-GVIA, had no effect on the tityustoxin-stimulated release of acetycholine in rat brain cortical slices (Casali *et al.*, 1995).

Our electrophysiological data obtained using a heterologous expression system demonstrates that the new polypeptide toxin PRTx3-7 produces a significant leftward shift in the voltage-dependence for activation of all Ca^{2+} channels studied except for the N-type Ca^{2+} channel. This effect is interesting since the majority of toxins that target Ca^{2+} channels (e.g., ω-Aga-IVA, kurtoxin and ω-grammotoxin) shift activation curves towards depolarised values (McDonough *et al.*, 1997a,b; Chuang *et al.*, 1998). Also, Meunier *et al.* (2002) described a toxin from *Glycera convoluta*, Glycerotoxin (GLTx) that also causes a leftward shift of the activation curve in HEK cells expressing $Ca_v2.2$ channels promoting an up-regulation of current activity. Another toxin that has a similar effect to PRTx3-7 is the β-scorpion toxins which shift the voltage-dependence of activation of Na^+ channels to a more negative potential (for a review, see Zuo and Ji (2004)). It also seems that the voltage shift and the maximal current blocking are separable effects since the dose–response curve for N-type currents has a tendency to reach a plateau around 40% of blockade. To understand how PRTx3-7 is changing the voltage-dependent properties and not modifying the

kinetics of Ca^{2+} channels, more experiments need to be performed. Our next step will be to investigate the binding site of the toxin on the HVA Ca^{2+} channels.

Most Ca^{2+} channels contain multiple target sites for antagonist-binding, including binding sites for natural toxins. Although PRTx3-7 is not selective as other toxin blockers; its ability to modify some properties of HVA Ca^{2+} channels may be useful in understanding synaptic transmission.

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