Cellular and Molecular Biology [TM] **56**, OL1223-OL1230
DOI 10.1170/139

ISSN 1165-158X
2010 Cell. Mol. Biol.[TM]

EFFECTS OF NEW PHONEUTRIA SPIDER TOXINS ON GLUTAMATE RELEASE AND $[Ca^{2+}]_I$ IN RAT CORTICAL SYNAPTOSOMES

D. S. CARNEIRO[1], L. B. VIEIRA[1,3], M. N. CORDEIRO[2], M. RICHARDSON[2], C. J. CASTRO-JUNIOR[3], M. V. GOMEZ[3] ✉ AND H. J. REIS[1]

1 Laboratório de Neurofarmacologia, Departamento de Farmacologia, ICB-UFMG. Av. Antônio Carlos, 6627. Campus Pampulha. BH-MG, CEP 31270-901, Brazil.
2 Centro de Pesquisas Prof. Carlos Ribeiro Diniz, Fundação Ezequiel Dias, Rua Conde Pereira Carneiro, 80, BH-MG, CEP 30550-010, Brazil.
3 INCT de Medicina Molecular, Faculdade de Medicina – UFMG. Av Alfredo Balena, 190. BH-MG, CEP 30130-100, Brazil
✉ Marcus V. Gomez, PhD; Laboratório de Neurociência, Faculdade de Medicina – UFMG. Av. Alfredo Balena 190, Campus Saúde. 30130-100, Belo Horizonte, MG, Brazil
Tel: +55 31 3248-9650; Fax: +55 31 3409-2685; E-mail: marcusvgomez@gmail.com

Received October 15th, 2009; Accepted December 23th, 2009; Published February 9th, 2010

Abstract – Studies revealed that the venom of the Brazilian "armed" spider *Phoneutria nigriventer* contains potent neurotoxins that caused excitatory symptoms such as salivation, lachrymation, priapism, convulsions, flaccid and spastic paralysis. It was also reported that the main mechanism of action of those neurotoxins are effects on ion channels such as inhibition of the inactivation of Na^+ channels, blockage of K^+ channels and blockage of calcium channels. The venom from *Phoneutria keyserlingi*, as might be expected, contains a series of polypeptides that are very similar, but not identical, to the proteins previously obtained from the venom of *P. nigriventer* in terms of their amino acid sequences and biological activities. We evaluated the effects of some of the toxins of *P. nigriventer* and *P. keyserlingi* on glutamate release and the decrease in $[Ca^{2+}]_i$ by using synaptosomes of rat brain cortices and fluorimetric assays. Sequence comparisons between the Phoneutria toxins of both the species showed great similarity in the location of cysteine residues. However, thus far, no pharmacological assays were performed to evaluate the extension of those biochemical modifications. Our results showed that differences between the amino acid sequences of Phoneutria toxins of both the species lead to the significant changes in the pharmacological properties of these toxins.

Key words: Spider venom, Synaptosomes, Glutamate release, Phoneutria toxins

INTRODUCTION

Several studies have revealed that the venom of the Brazilian "armed" spider *Phoneutria nigriventer* contains potent neurotoxins that can cause excitatory symptoms such as salivation, lachrymation, priapism, convulsions, flaccid and spastic paralysis of the anterior and posterior members, and death following intracerebral injection in mice (4; 22; 6). It was also reported that the main mechanism of action of these neurotoxins are effects on ion channels such as the inhibition of the inactivation of Na^+ channels (1), blockage of K^+ channels (11) and blockage of Ca^{2+} channels (9; 2, 25). Spider neurotoxins have been described as important tools for the future development of new biologically active molecules with potential application in medicine and agriculture (5; 7; 18). In spite of the fact that various neurotoxins from *P. nigriventer* have been described, little is known about the venoms from other species of this genus. Toxins from the venom of *Phoneutria keyserlingi* elicited very similar toxic and lethal effects as those of *P. nigriventer* when tested in mice. However, their effects on ion channels remain unknown. Richardson and colleagues (20) reported that the venom from *P. keyserlingi*, as might be expected, contains a series of polypeptides that are very similar but not identical, to the proteins previously obtained from the venom of *P. nigriventer* in terms of their amino acid sequences and biological activities. Sequence comparisons showed great similarity in the location of cysteine residues when these toxins were compared with other toxins active in the Ca^{2+} channels. We are

currently studying the effects of some of these new toxins from the venom of *P. keyserlingi* and also some toxins from *P. nigriventer,* whose activities are not yet being assessed. Here, we describe the effect of PKTx23C3, PKTx28C4, and PKTx20C4 – neurotoxic peptides purified from the venom of *P. keyserlingi* and PNTx25A1, PNTx19C1, PNTx37C2 and PNTx19C2 obtained from the venom of *P. nigriventer* on glutamate release and the effect of PKTx23C3 and PKTx20C4 on decreasing $[Ca^{2+}]_i$ in the synaptosomes of rat brain cortices.

MATERIALS AND METHODS

Reagents

Glutamate dehydrogenase type II (EC1.4.1.3), $NADP^+$, glutamate, acetoxymethylester sucrose, Percoll®, EGTA and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Fura-2 AM was obtained from Molecular Probes (Eugene, OR, USA). ω-Aga IVA, ω-CgTX GVIA and ω-CmTX MVIIC were purchased from Peptides Inc. (Japan). All other reagents were of analytical grade.

Isolation of neurotoxins

The toxins were purified from the venom samples of *P. nigriventer* and *P. keyserlingi* by a combination of chromatographic steps, as previous described (3).

Purification of synaptosomes

Adult Wistar rats of both sexes (180–200 g) were killed by decapitation. The cortices were removed and homogenized in 1:10 (w/v) 0.32 M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (1 mM). Homogenates were first centrifuged (1000 X g for 10 min) and synaptosomes were isolated by Percoll® gradient centrifugation (21). Synaptosomes were re-suspended in HEPES-buffered salt solution (HBSS: 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, adjusted to pH 7.4 with 5.0 M NaOH) at 10.0 mg/mL, divided into aliquots of 200 µL and kept on ice until it used for the measurement of glutamate release or loaded with Fura-2 AM.

Measurement of continuous glutamate release

Glutamate release was assayed by monitoring the increase in fluorescence due to the production of NADPH in the presence of $NADP^+$ and glutamate dehydrogenase (15). Briefly, synaptosomes (10 mg/mL) were incubated for 60 min in the same way as for measurement of Ca^{2+} (see below), washed with HBSS medium, and transferred to a cuvette (final synaptosomal concentration, 0.5–1.0 mg/mL), which was maintained at 35°C with constant stirring. At the start of each assay, $CaCl_2$ (1.0 mM), $NADP^+$ (1.0 mM) and glutamate dehydrogenase (50 units) were added to the synaptosomes. The excitation wavelength was set at 360nm and the emission wavelength at 450 nm. $CaCl_2$ addition was omitted in the experiment performed in the presence of 1.0 mM EGTA in order to assess calcium-independent glutamate release under different conditions. Toxins were added 60 s before membrane depolarization with 33 mM KCl. After stabilization of the fluorescence signal at a plateau level, 5.0nmol of glutamate was added as an internal standard. Data was expressed as nmol of glutamate per milligram of protein.

Measurements of intrasynaptosomal free Ca^{2+} concentration $[Ca^{2+}]_i$

Fura-2 AM (stock solution 1 mM DMSO) measurements of intrasynaptosomal free calcium concentration $[Ca^{2+}]_i$ in synaptosomes were performed in a PTI spectrofluorimeter according to Grynkiewicz and colleagues (8) with minor modifications described previously (21; 17). Fluorescence emission was recorded at 500nm using 340/380 nm excitation ratio. $CaCl_2$ was added to the synaptosomal suspension at the beginning of each fluorimetric assay (1.0 mM final concentration). Toxins were added to the synaptosomal suspension 60 s before membrane depolarization with KCl (33 mM). The $[Ca^{2+}]_i$ increase induced by membrane depolarization is strictly dependent on the presence of extracellular Ca^{2+} in these conditions (21).

Statistical analysis

Results described are mean±S.E.M of at least three independent experiments. Statistical significance was by analysis of variance (ANOVA). A value of $P < 0.05$ was considered as statistically significant.

RESULTS

Effect of toxins on glutamate release from brain cortical synaptosomes

Table 1 shows the effect of PKTx23C3, PNTx19C1, PKTx28C4, PNTx25A1, PKTx20C4, PNTx37C2 and PNTx19C2 on K^+-induced glutamate release. Only PKTx23C3 and PKTx20C4 decreased the KCl-evoked glutamate release from cerebrocortical synaptosomes (Figure 1). The concentration of PKTx23C3 and PKTx20C4 ranging, respectively, from 8nM to 300nM and 0.3 µM to 3 µM gradually decreased the KCl-evoked release of glutamate. The calculated IC_{50} values for PKTx23C3 and PKTx20C4 inhibition were 116.4 nM and 1.48 µM, respectively. In the absence of Ca^{2+}, in the reaction media (no added Ca^{2+} plus 2mM EGTA), the KCl-induced release of glutamate was 6.4±1.9nmol/mg of protein. This value represents the calcium-independent pool and 52% of the total glutamate release (Table 1) according to previous experiments (19) in the central nervous system.

Effect of PKTx23C3 and PKTx20C4 on calcium-independent release of glutamate

The KCl-induced release of glutamate is the sum of two components distinguished by their dependence, or independence, of extracellular Ca^{2+} (for a review, see (16)). The calcium-independent release was assayed in a medium lacking $CaCl_2$ and by adding 2.0mM EGTA 2

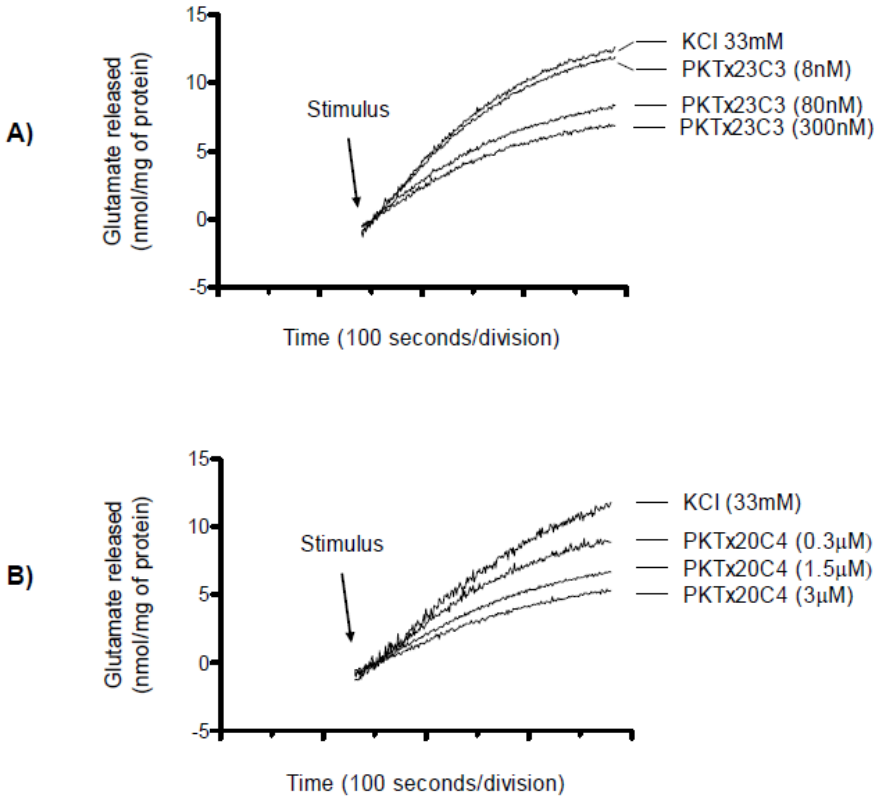

Figure 1. Effect of PKTx23C3 and PKTx20C4 isotoxins on glutamate release.
Synaptosomes, which are prepared as described in the Materials and Methods section, are depolarized with 33 mM KCl, and this is followed by the continuous release of glutamate. **(A)** Total release of glutamate from control and toxin-treated synaptosomes of PKTx23C3 (8.0, 80.0 and 300 nM). **(B)** Total release of glutamate from control and toxin-treated synaptosomes of PKTx20C4 (0.3, 1.5 and 3.0 µM). All toxins are added 60 s before the addition of KCl. The figures show representatives curves from three independent experiments.

Table 1. Effect of *Phoneutria keyserlingi* and *Phoneutria nigriventer* isotoxins on glutamate release from depolarized cerebrocortical synaptosomes.

Toxin	Glutamate released (nmol/mg of protein)	Spider
PKTx23C3 (300nM)	6.9±2.7*	*Phoneutria keyserlingi*
PNTx19C1(12µM)	10.2±2.4	*Phoneutria nigriventer*
PKTx28C4 (3µM)	10.6±0.7	*Phoneutria keyserlingi*
PNTx25A1 (19µM)	10.3±0.8	*Phoneutria nigriventer*
PKTx20C4 (3µM)	5.9±0.3*	*Phoneutria keyserlingi*
PNTx37C2 (100nM)	12.8±1.3	*Phoneutria nigriventer*
PNTx19C2 (2µM)	10.3±1.0	*Phoneutria nigriventer*
EGTA (2mM)	6.4±1.9*	-
KCl (33mM)	12.4±0.5	-

*P<0.05 compared with control (33mM KCl)

min before depolarization with 33mM KCl. The resulting release in those conditions was around 52% of regular medium containing calcium. In the presence of PKTx23C3 (300nM) and PKTx20C4 (3µM), we did not observed any significant effect on calcium-independent glutamate release (Figure 2). These results indicated that the effects of PKTx23C3 and PKTx20C4 were restricted to the calcium-dependent (exocytotic) glutamate pool. After demonstrating that PKTx23C3 and PKTx20C4 possibly blocked the calcium-dependent glutamate release, we tested the effect of these toxins on the KCl-induced increase of synaptosomal $[Ca^{2+}]_i$.

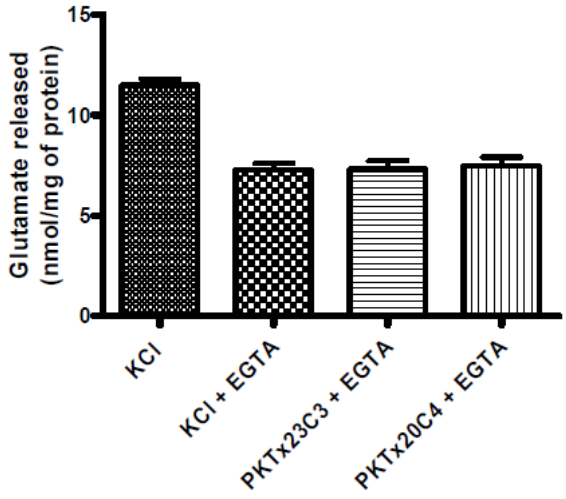

Figure 2. Effect of PKTx23C3 and PKTx20C4 on Ca^{2+}-independent release of glutamate.
The release of glutamate is performed in HBSS medium lacking calcium (1.0mM EGTA) and control or PKTx23C3 (300 nM) and PKTx20C4 (3 µM) – exposed synaptosomes are depolarized by using 33 mM KCl. The amount of glutamate released under calcium-deprived conditions is similar to that shown in Table 1 for maximal concentration of those toxins. The mean values from five independent experiments are shown.

Effect of PKTx23C3 and PKTx20C4 on $[Ca^{2+}]_i$.

Depolarization with KCl (33mM) induced an increase of 250nM (from 250 to 500nM) on average $[Ca^{2+}]_i$ measured using Fura-2-AM in brain cortical synaptosomes. The toxins PKTx23C3 (300nM) or PKTx20C4 (3 µM) were separately added 60 s before membrane depolarization with the synaptosomal suspension. The resulting release was approximately 79% (PKTx23C3) and 75% (PKTx20C4) of that measured in medium lacking toxins (Figure 3). In order to test whether the PKTx23C3 and PKTx20C4 targeted channels overlap with known neuronal subtypes of calcium channels, such as those blocked by ω-Aga IVA, ω-CgTX GVIA and ω-CmTX MVIIC, we measured intracellular calcium changes induced by KCl (33mM) in the presence of different toxins. Figure 3 shows that the new Phoneutria toxins PKTx23C3 (300 nM) and PKTx20C4 (3 µM) decreased the changes in $[Ca^{2+}]_i$ by about 20%, which was induced by 33 mM KCl when exposed to the synaptosomes 60 s before depolarization. To test for the overlapping of calcium channels inhibited by ω-Aga IVA (100nM), ω-CgTX GVIA (1 µM) and PKTx23C3 (300nM), these toxins were incubated together (ω-Aga IVA + PKTx23C3 and ω-CgTX GVIA + PKTx23C3) 60 s before KCl (33 mM) depolarization. ω-Aga IVA and ω-CgTX GVIA, blockers of P- and N-type Ca^{2+} channels, respectively, caused a small inhibition of the KCl-induced increase in $[Ca^{2+}]_I$ (data not shown). The results caused by PKTx23C3 in association with either ω-Aga IVA or ω-CgTX GVIA was similar to that obtained with PKTx23C3 alone (Figure 3). Addition of PKTx23C3 (300 nM) with either ω-Aga IVA or ω-CgTX GVIA caused an inhibition that was not significantly different from that observed in the presence of PKTx23C3 (300 nM). These results might indicate that PKTx23C3 (300 nM) binds in the outer vestibule of the pore that is allosterically coupled to ω-Aga IVA interfering with the interaction between ω-Aga-IVA and P/Q calcium channel, in a similar way as describe to calcicludine, a toxin extracted from green mamba snake (26). Furthermore, the association of PKTx23C3 (300 nM) with ω-CmTX MVIIC caused an inhibition that was not different from that induced by ω-CmTX MVIIC alone (Figure 3 and data not shown).

The inhibition caused by PKTx20C4 in association with either ω-Aga IVA and ω-CgTX GVIA was similar to that obtained with PKTx20C4 alone (Figure 3). ω-Aga IVA (100nM), ω-CgTX GVIA (1 µM) and PKTx20C4 (3 µM) were incubated together (ω -Aga IVA + PKTx20C4 and ω-CgTX GVIA + PKTx20C4) 60 s before KCl (33mM) depolarization (Figure 3). The combined use of PKTx20C4 (3 µM) with ω-Aga IVA (100nM) caused an inhibition that was significantly different from that observed in the presence of PKTx20C4 (3 µM) alone. However, the association with either ω-CgTX GVIA or ω-CmTX MVIIC (1 µM) caused an inhibition that was not significantly different from GVIA (1µM) or Aga IVA (100 nM) alone (data not shown).

Although the joint application of toxins (ω-Aga IVA (100 nM), ω-CgTX GVIA (1 μM) ω-CmTX MVIIC (1 μM) and PKTx23C3 (300 nM)) appears to cause an inhibition $[Ca^{2+}]_I$, the difference between this inhibition and that induced by PKTx23C3 alone failed to achieve statistical significance. On the other hand, the association between ω-Aga IVA with PKTx20C4 caused significant inhibition on $[Ca^{2+}]_I$, thus indicating that there was no overlap between both toxins.

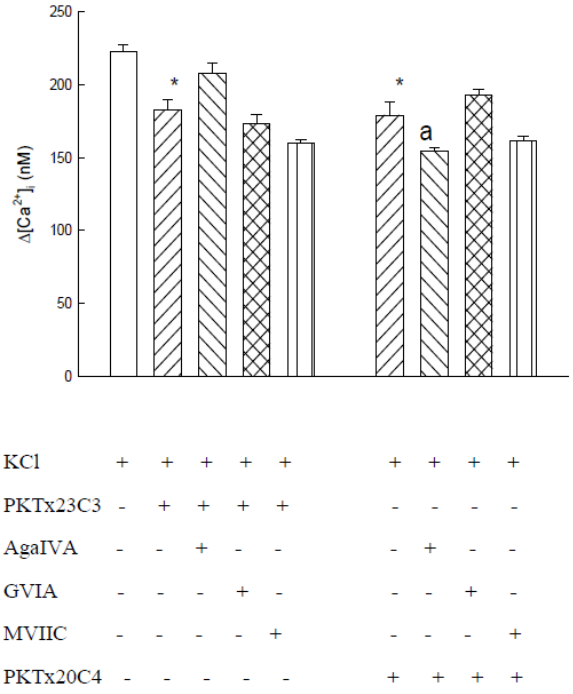

Figure 3. Effect of calcium channel blockers, PKTx23C3 and PKTx20C4 on the increase in $[Ca^{2+}]_I$ in rat cortical synaptosomes.

Synaptosomes were obtained and loaded with fura-2 as described in the Materials and Methods section. They were incubated in HBSS medium containing 1.0 mM $CaCl_2$ for 60s in the presence of the toxins, alone or in combination (100 nM ω-Aga IVA; 1.0μM ω-CgTX GVIA; 1.0 μM ω-CmTX MVIIC; 300 nM PKTx23C3; 3 μM PKTx20C4) followed by depolarization with 33 mM KCl during 2.5 min. Results are expressed as mean±S.E.M. from five independent experiments. ***** means statistical difference compared with 33mM KCl ($P<0,001$), and ***a*** means statistical difference compared with PKTx20C4 alone ($P<0,05$).

DISCUSSION

Sequence of PNTx19C1 and its effect on glutamate release

In our study, we observed that the toxin PNTx19C1 had no significant effect in inhibiting the glutamate release evoked by KCl when used doses up to 12 μM. The sequence of toxin PNTx19C1 is not completely determined; however, we can see that it shows great similarity when compared to the sequences of toxins belonging to the family Tx3-6 which, as previously mentioned, have a great effect on the inhibition of calcium currents (20). We also noticed a chemical variation in the properties of amino acids existing on the PNTX3-6 and PNTx19C1 chain structure. The main changes occurred between hydrophobic amino acids and amino acids charged polarized (Figure 4C/D). One important modification in the amino acid positions is the alanine residue (position 1 in PNTx3-6 sequence) by glycine (in PNTx19C1). Such change might cause major effects on its biochemical and pharmacological properties.

Thus, we may suggest that no significant effect on the release of glutamate at the concentration of PNTx19C1 (12 μM) might be explained by the changes in the polarization of the chain, which leads to a decrease in affinity between the channel and the toxin.

Sequences of PKTx28C4 and PNTx25A1 and their effects on glutamate release.

Recent studies have shown that these two new peptides extracted from spiders and *P. keyserlingi and P. nigriventer* have very toxic effects when injected into the thoracic cavity of rats and houseflies (20). However, in the present study, these peptides did not show significant effects in the inhibition of glutamate release evoked by KCl in rat brain synaptosomes. These two peptides have 43%–51% of sequence homology when compared to the toxins of the family Tx2 (20) (Figure 4E). It is known that the toxins of the family PNTx2 act by increasing the activation of sodium channels (1; 27; 20) and increasing the inflow of sodium ions in synaptosomes, thereby inducing depolarization of the membrane, calcium ions influx and release of glutamate in relation to TTX (21). The main amino acid changes observed when we compared both toxins with PnTx2-1 are the presence of a basic amino acid (position 1 in PKTx28C4 and PNTx25A1) and the alterations of several amino acids in the sequence of the remaining toxins. Investigations of the effects of both toxins on Na^+ influx must be performed to clarify if such changes are crucial to the structures and functions of those toxins.

Sequence of PNTx37C2 and its effect on the glutamate release.

The sequence of the toxin PNTx37C2 (unpublished data) showed great similarity to the

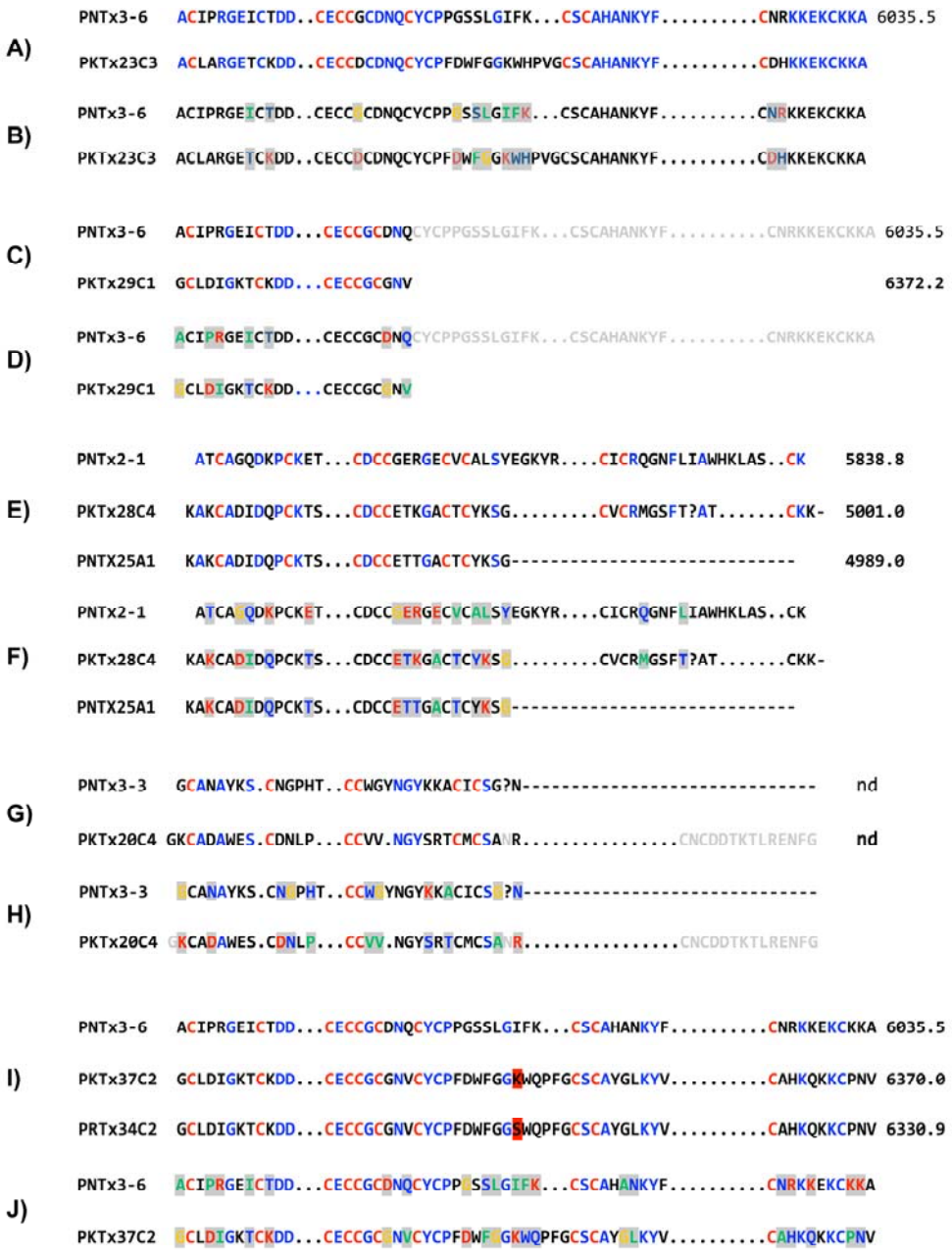

Figure 4. Chart of amino acid sequences alignments of Phoneutria toxins.
The sequences are aligned according to the cysteine residues represented in red. Hydrophobic amino acids are shown in green, charged amino acids in red, polar residues in blue, and glycine in yellow. To further discussion see text.

toxins of the family Tx3, particularly PnTx3-6 (Figure 4I). As previously mentioned, the toxin PnTx3-6 operates mainly in N type channels (25). In our study, there was no inhibition of glutamate release by PNTx37C2. On comparing the amino acid sequences of PnTx3-6 and PnTx37C2, we found that both the toxins have the same number of cysteine residues and a homology of approximately 45% (Figure 6I and J). We also observed that the toxin PRTx34C2 extracted from the venom of the spider *P. reidyi* (20) shows great similarity to PNTx37C2, differing with regard to only one residue (Figure 4I).

Sequence analysis showed that alanine at position 1 (PNTx3-6) was replaced by glycine (PNTx37C2) and the last amino acid (an alanine-PNTx3-6 by valine – PNTx37C2), which may have caused major changes in its biochemical and pharmacological properties. Such changes could vary the polarization or toxin linkage, which may reduce its affinity for N type calcium channels, thereby justifying the effects observed in our experiments.

Comparative analysis of the sequence of PKTx23C3 and its effects on calcium dependent glutamate release.

Richardson and colleagues (20) recently reported a comparison of the partial proteomes of the venoms of the following spiders: *P. keyserlingi, P. nigriventer and Phoneutria reidyi*. It was shown that the toxins of the family Tx3, which were purified from the venom of the spider *P. nigriventer*, had great similarity (40%–50%) in their amino acid sequences when compared with toxins Agatoxins III and AgorTxB7a, which are toxins from the venom of the spider *Agelena orientalis* (10). Members of the family of Tx3-6 toxins are characterized by being the only ones to have cysteine residues in the sequence arranged CxCCxC (Figure 4A). It is known that these toxins have low toxicity when injected into mice but have great effect on the inactivation of calcium currents and release of glutamate-dependent calcium channels, acting mainly on the P/Q- type (25; 23).

We verified the inhibition of calcium-dependent glutamate release from rat cortical synaptosomes in the presence of PKTx23C3. The amino acid sequence of this toxin clearly indicates that it belongs to the family Tx3-6. It has the same number of cysteine residues positioned in similar locations in the chain of amino acids and a homology of approximately 70% when compared to the sequences of the other members of the Tx3-6 family (Figure 4A/B). We also noticed that the amino acids that differ between the two chains have different chemical properties. Exchanges occur between amino acids of different groups, namely, hydrophobic amino acids with chains, chains of amino acids and amino acids charged polarized (Figure 4B). Thus, we may suggest that the inhibition of lower intensity shown by PKTx23C3 when used in doses of 300nM when compared to PNTx3-6 can be explained by the presence of different amino acids between the two chains. Such changes could modify the polarization of the chain of this toxin by reducing its affinity for calcium channels, thus explaining the difference between the effects shown in both the toxins.

Sequence of PKTx20C4 and its effect on calcium dependent glutamate release.

The sequence of the toxin PKTx20C4 showed that it has a great similarity with toxins from the family Tx3, particularly with the toxin Tx3-3 (1; 27; 20). Tx3-3 when injected into the brains of mice at a dose of 5µg causes flaccid paralysis and rapid death between 10 and 30 min. Several studies have shown that the toxin PNTx3-3 is a potent inhibitor of calcium channels (17; 9; 13) blocking calcium current P/Q and R (12; 14).

In our study, PKTx20C4 (3 µM) showed inhibition of 52.4% in the release of glutamate after depolarization with 33 mM KCl. As previously reported (24), preparation of brain cortical synaptosomes is ineffective as a tool for studying the effect of toxins directly on ion channels. However, we associated PKTx20C4 with classic calcium channel blockers in order to find out some interaction between the toxin and calcium channels. Our results showed more affinity by N type calcium channels since no addictive effect was observed in the association of PKTx20C4 and ω-CgTX GVIA.

Comparison of their sequences revealed an important change between the PNTx3-3 chain and PKTx20C4. A glycine was replaced at position 1 in PNTx3 and PKTx20C4 gained extra two amino acids – glycine and lysine – plus other changes elsewhere in its chain. These modifications may affect the polarization of the structure of this toxin or may cause adjustment to the channel link by reducing its affinity to P/Q and R-type calcium channels or also by increasing its affinity for N type channel.

Acknowledgements –This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq/MCT (Instituto do Milênio), FAPEMIG and CAPES.

REFERENCES

1. Araujo, D. A., M. N. Cordeiro, et al., 1993. Effects of a toxic fraction, PhTx2, from the spider Phoneutria nigriventer on the sodium current. *Naunyn Schmiedebergs Arch Pharmacol* 347(2), 205-8.
1. Cassola, A. C., H. Jaffe, et al., 1998. Omega-phonetoxin-IIA: a calcium channel blocker from the spider phoneutria nigriventer. *Pflugers Arch* 436(4), 545-52.
2. Cordeiro MN, Figueiredo SG, Valentim AC, Diniz CR, Eicksted RD, Goroy J, and Richardson M., 1993. Purification and amino acid sequences of six TX3 type neurotoxins from the venom of the Brazilian "armed" spider Phoneutria nigriventer. *Toxicon* 31, 35-42.
3. Diniz, M. R. V., Paine, M. J. I., Diniz, C. R., Theakston, R. D. G., and Crampton, J. M., 1993. Sequence of the DNA coding for the lethal neurotoxin Tx1 from the Brazilian ldquoarmedrdquo spider Phoneutria nigriventer predicts the synthesis and processing of a preprotoxin. *J. Biol. Chem.* 268,15340–15342.
4. Escoubas, P., S. Diochot, et al., 2000. Structure and pharmacology of spider venom neurotoxins. *Biochimie* 82(9-10), 893-907.
5. Entwistle, I. D., R. A. Johnstone, et al., 1982. Isolation of a pure toxic polypeptide from the venom of the spider Phoneutria nigriventer and its neurophysiological activity on an insect femur preparation. *Toxicon* 20(6), 1059-67.
6. Gomez, M. V., E. Kalapothakis, et al., 2002. Phoneutria nigriventer venom: a cocktail of toxins that affect ion channels. *Cell Mol Neurobiol* 22(5-6), 579-88.
7. Grynkiewicz, G., M. Poenie, et al., 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* 260(6), 3440-50.
8. Guatimosim, C., M. A. Romano-Silva, et al., 1997. A toxin from the spider Phoneutria nigriventer that blocks calcium channels coupled to exocytosis. *Br J Pharmacol* 122(3), 591-7.
9. Kozlov, S., A. Malyavka, et al., 2005. A novel strategy for the identification of toxin like structures in spider venom. *Proteins* 59(1), 131-40.
10. Kushmerick, C.; Kalapothakis E; Beirao PS; Penaforte CL; Prado VF; Cruz JS; Diniz CR; Cordeiro MN; Gomez MV; Romano-Silva MA; Prado MA., 1999. Phoneutria nigriventer toxin Tx3-1 blocks A-type K+ currents controlling Ca2+ oscillation frequency in GH3 cells. *Journal of Neurochemistry* 72(4),1472-81.
11. Leao, R. M., J. S. Cruz, et al., 2000. Inhibition of neuronal high-voltage activated calcium channels by the omega-phoneutria nigriventer Tx3-3 peptide toxin. *Neuropharmacology* 39(10), 1756-67.
12. Miranda, D. M., M. A. Romano-Silva, et al., 1998. Phoneutria nigriventer toxins block tityustoxin-induced calcium influx in synaptosomes. *Neuroreport* 9(7), 1371-3.
13. Miranda, D. M., M. A. Romano-Silva, et al., 2001. Spider neurotoxins block the beta scorpion toxin-induced calcium uptake in rat brain cortical synaptosomes. *Brain Res Bull* 54(5), 533-6.
14. Nicholls, D. G., T. S. Sihra, Sanchez-Prieto, J. 1987. Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J Neurochem* 49(1), 50-7.
15. Nicholls DG., 1993. Ion channels and the regulation of neurotransmitter glutamate release. *Biochem Soc Trans* 21(1), 53-8.
16. Prado, M. A., C. Guatimosim, et al., 1996. A novel tool for the investigation of glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider Phoneutria nigriventer. *Biochem J* 314 (Pt 1), 145-50.
17. Rash, L. D. and Hodgson, W. C., 2002. Pharmacology and biochemistry of spider venoms. *Toxicon* 40(3), 225-54.
18. Reis HJ, Prado MA, Kalapothakis E, Cordeiro MN, Diniz CR, De Marco LA, Gomez MV, Romano-Silva MA., 1999. Inhibition of glutamate uptake by a polypeptide toxin (phoneutriatoxin 3-4) from the spider Phoneutria nigriventer. *Biochem J* 343 (Pt 2),413-8.
19. Richardson, M., A. M. Pimenta, et al., 2006. Comparison of the partial proteomes of the venoms of Brazilian spiders of the genus Phoneutria. *Comp Biochem Physiol C Toxicol Pharmacol* 142(3-4), 173-87.
20. Romano-Silva, M. A., R. Ribeiro-Santos, et al., 1993. Rat cortical synaptosomes have more than one mechanism for Ca2+ entry linked to rapid glutamate release: studies using the Phoneutria nigriventer toxin PhTX2 and potassium depolarization. *Biochem J* 296(Pt 2), 313-9.
21. Schenberg, S., and Lima, F. A., 1966. Pharmacology of the polypeptides from the venom of the spider Phoneutria fera. *Mem. Inst. Butantan.* 33, 627–638.
22. Souza, A. H., J. Ferreira, et al., 2008. Analgesic effect in rodents of native and recombinant Ph alpha 1beta toxin, a high-voltage-activated calcium channel blocker isolated from armed spider venom. *Pain* 140(1), 115-26.
23. Vieira, L. B., C. Kushmerick, et al., 2003. PnTx3-6 a spider neurotoxin inhibits K+-evoked increase in [Ca2+](i) and Ca2+-dependent glutamate release in synaptosomes. *Neurochem Int* 42(4), 277-82.
24. Vieira, L. B., C. Kushmerick, et al., 2005. Inhibition of high voltage-activated calcium channels by spider toxin PnTx3-6. *J Pharmacol Exp Ther* 314(3), 1370-7.
25. Wang, X., Du, L., and Peterson, B. Z. 2007. Calcicludine binding to the outer pore of L-type calcium channels is allosterically coupled to dihydropyridine binding. *Biochemistry* 46(25): 7590-98.
26. Yonamine, C. M., L. R. Troncone, et al., 2004. Blockade of neuronal nitric oxide synthase abolishes the toxic effects of Tx2-5, a lethal Phoneutria nigriventer spider toxin. *Toxicon* 44(2), 169-72.