**Neurotoxins** can help the understanding of mechanisms involved in neurotransmission. We here report that two neurotoxin isoforms, Tx3-3 and Tx3-4 obtained from the venom of the spider *Phoneutria nigriventer* inhibited the $^{45}$Ca$^{2+}$ influx in rat cortical synaptosomes induced by the scorpion venom tityustoxin. The IC$_{50}$ for Tx3-3 and Tx3-4 were 0.32 and 7.9 nM, respectively. The neurotoxins Tx3-3 and Tx3-4 are very effective in inhibiting $^{45}$Ca$^{2+}$ influx and they should be useful in studies involving Ca$^{2+}$-dependent processes. *NeuroReport* 9: 1371–1373 © 1998 Rapid Science Ltd.

**Key words:** Calcium uptake; *Phoneutria nigriventer*; Synaptosomes; Tityustoxin

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**Introduction**

The venom of the Brazilian spider *Phoneutria nigriventer* possesses several toxic polypeptide fractions, some of which have been purified and shown to be neurotoxic. One of the toxic fractions, designated PhTx-3 has six neurotoxic peptides (Tx3-1 to Tx3-6) with relative molecular masses ranging from 3.5 to 8.5 kDa. We have shown that PhTx-3 and one of the peptides named Tx3-3 act as calcium channel blockers by decreasing the calcium entry that contributes to acetylcholine and glutamate release in rat brain cortical slices and synaptosomes. We have also shown that Tx3-3 blocks calcium channels coupled to exocytosis.

Tityustoxin (TsTX), a neurotoxin purified from the venom of the Brazilian scorpion *Tityus serrulatus*, mimics many effects of electrical stimulation, causing cell depolarization that increases the release of the neurotransmitters acetylcholine and glutamate, as well as the uptake of Na$^+$ and Ca$^{2+}$ in rat brain cortical slices and synaptosomes. These effects of TsTX are sodium and calcium-dependent and are inhibited by tetrodotoxin. Thus, TsTX is a useful tool to study the effects of Ca$^{2+}$ ions on synaptic transmission. The present study was undertaken to assess the effect of the PhTx3 isoforms Tx3-3 and Tx4-4, on the TsTX-induced increase in $^{45}$Ca$^{2+}$ influx in rat brain cortical synaptosomes.

**Materials and Methods**

Tityustoxin was purified from the venom of *T. serrulatus* as described previously. The PhTx3 isoforms, Tx3-3 and Tx3-4, were purified from the venom of *P. nigriventer* by a combination of chromatographic steps as described by Cardeiro et al. Wistar rats weighing 150–180 g were killed by cervical dislocation followed by decapitation. The brain was dissected out of the cranial cavity the cortices transferred to a Potter flask and homogenized in 10 ml of...
a solution containing sucrose 0.32 M, DTT 0.25 mM and EDTA 1.0 mM. Synaptosomes were prepared by Percoll density gradient centrifugation. Briefly, the cortices were homogenized with a teflon pestle attached to a motor (14 up and down strokes). The homogenate was layered on top of a discontinuous Percoll gradient and centrifuged Phases 3 and 4 of the gradient were collected, combined, resuspended in Krebs–Ringer–HEPES buffer (KRH) containing (in mM) NaCl 124, KCl 4.7, CaCl2 1.4, NaH2PO4 1.3, MgSO4 1.2, HEPES 20, glucose 11, pH 7.4. Synaptosomes were stored on ice for no more than 30 min prior to the beginning of the experiments. The isoforms, Tx3-3 or Tx3-4, were preincubated with 500 µl of the resuspended synaptosomes (0.5 mg protein) in KRH for 10 min at 30°C. Influx of 45Ca2+ was initiated by rapid addition of 500 µl KRH containing ~2 µCi/ml 45Ca2+ in the absence (control) or in the presence of TsTX 2.5 µM (test). After 30 s incubation at 30°C, 45Ca2+ influx was stopped by the addition of 5 ml KRH containing 4.0 mM EGTA at 4°C. The samples were immediately filtered through glass fiber filters using a Sartorius manifold system. The filters were rapidly washed with 2× 5 ml KRH containing Ca2+ 4.0 mM at 4°C, dried and the radioactivity in the filters counted in 5 ml of scintillation fluid (ethanol 30% v/v, dioxane 30% v/v, toluene 30% v/v, triton 1% v/v, naftalene 7 g% w/v, POPOP 0.02 g and PPO 0.5% w/v) in a liquid scintillation spectrophotometer. Experiments were performed in duplicate and the net 45Ca2+ uptake induced by TsTX was determined by the difference between the uptake in the presence and in the absence of Tx3-3 or Tx3-4. Protein concentration was determined by the method of Lowry et al.

Results

Figure 1 shows the time course effect of TsTX-induced 45Ca2+ influx in rat brain cortical synaptosomes. TsTX (2.5 µM)-stimulated calcium uptake in synaptosomes was linear up to 30 s incubation period. Thereafter calcium influx reached a plateau. At 30 s TsTX-induced 45Ca2+ influx was 2.75 ± 0.11 times (n = 22) greater than the control level. Figure 2 shows the effect of toxins Tx3-3 and Tx3-4 on TsTX-stimulated 45Ca2+ influx in rat brain cortical synaptosomes. Both TX3-3 and TX3-4 inhibited the TsTX-induced increase in 45Ca2+ influx; however, the inhibitory effect of TX3-3 was greater than that caused by Tx3-4. The IC50 for the neurotoxins Tx3-3 and Tx3-4 were 0.32 nM and 7.9 nM, respectively. Basal Ca2+ uptake by rat synaptosomes was not affected by Tx3-3 and Tx3-4 at any of the concentrations tested (data not shown).

![FIG. 1. Time course effect of tityustoxin on the uptake of 45Ca2+ in synaptosomes. Rat brain cortical synaptosomes were incubated in KRH buffer at 30°C at the indicated times in the absence (control) or in the presence of tityustoxin 2.5 µM. Results are means ± s.e.m. of duplicates of at least three experiments. For other details see Material and Methods.](image1)

![FIG. 2. Effects of spider neurotoxins Tx3-3 and Tx3-4 on the tityustoxin-induced influx of 45Ca2+ in synaptosomes. Rat brain cortical synaptosomes (0.5 mg protein) were preincubated for 10 min in KRH buffer and then for 10 min at 30°C in the presence of Tx3-3 (●) or Tx3-4 (○) at the indicated concentrations. They were then incubated for 30 s with or without 2.5 µM tityustoxin. Results are means ± s.e.m. of duplicates of three experiments. For other details see Material and Methods.](image2)
Discussion

Synaptosomes are useful preparations to screen substances with potential activity on the central nervous system. We studied the $^{45}$Ca$^{2+}$ influx in synaptosomes using TsTX that depolarizes their membranes and induces neurotransmitter release. TsTX is a $\alpha$-scorpion toxin that causes cell depolarization by delaying the inactivation of Na$^+$ channels.\(^{14}\) Although neurotransmitter release induced by TsTX is a calcium-dependent process,\(^{2,3,8}\) the mechanisms by which this toxin affects calcium entry into the cells is still unknown. Our previous results indicated that the effect of the Phoneutria spider toxin, fraction PhTx3, was restricted to the Ca$^{2+}$-dependent process of acetylcholine\(^2\) and glutamate\(^3\) release. The Phoneutria neurotoxins isoforms Tx3-1 to Tx3-6 had pharmacological effects in vivo, but only Tx3-3 and Tx3-4 produce death.\(^{11}\) In vitro experiments showed that only these isoforms were effective against the calcium-dependent KCl-evoked glutamate release.\(^3\) Here we report the inhibitory effect of the P. nigriventer spider toxins, Tx3-3 and Tx3-4, in the TsTX-induced increase in $^{45}$Ca$^{2+}$ influx in synaptosomes. The inhibitory effect of Tx3-3 on $^{45}$Ca$^{2+}$ influx in synaptosomes was greater than that caused by Tx3-4. These results agree with the inhibition caused by both toxins in KCl-induced increase on [Ca$^{2+}$], and glutamate release.\(^3\) The inhibition of the TsTX-induced increase in the $^{45}$Ca$^{2+}$ influx by Tx3-3 and Tx3-4 is also in agreement with previous data showing the calcium dependency for the TsTX evoked release of neurotransmitters.\(^{2,6}\)

Previous data suggest that the inhibitory effect of Tx3-3 and Tx3-4 on KCl-evoked glutamate release\(^3\) resembles that observed with $\omega$-Conotoxin MVIIIC, a blocker of P/Q calcium channels. This indicates that both toxins interact with the same channel. Further experiment are necessary, however, to clarify the electrophysiological characterization of the channels blocked by Tx3-3 and Tx3-4. Tx3-3 is a protein of 6.3 kDa whose partial amino acid sequence shows weak homology with other toxins known to block calcium channel\(^{11}\) and which might be of importance in spatial modeling of peptide Ca$^{2+}$ channels antagonists. Finally, owing to its ability to abolish Ca$^{2+}$-dependent processes, Tx3-3 may be a useful tool to study calcium channels involved in neurotransmitter release.

Conclusion

The present study is the first to show that P. nigriventer neurotoxins Tx3-3 and Tx3-4 block the TsTX-induced $^{45}$Ca$^{2+}$ influx in synaptosomes. The Phoneutria toxins have weak homology with other toxins known to act on calcium channels and they may interact with different molecular variants of calcium channels.

References


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