Tx3-4 a toxin from the venom of spider *Phoneutria nigriventer* blocks calcium channels associated with exocytosis

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ABSTRACT

The purpose of the present work was to investigate the pharmacological action of a calcium channel-blocking toxin from the venom of the spider *Phonetic nigriventer*, Tx3-4 on calcium channels coupled to exocytosis of synaptic vesicles. Tx3-4 blocked KCl-induced exocytosis of synaptic vesicles with an IC50 of 1.1 nM. To investigate whether the target of Tx3-4 overlaps with known calcium channels that mediate calcium entry and exocytosis, we used ω-toxins that interact selectively with neuronal calcium channels. The results indicate that the main population of voltage-sensitive calcium channels altered by Tx3-4 is P/Q calcium channels. In conclusion, Tx3-4 is a potent inhibitor of calcium channels involved in the KCl-induced exocytosis of synaptic vesicles in brain cortical synaptosomes.

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Voltage-sensitive calcium channels (VSCC) have a key role in regulating diverse cellular functions, including neuronal communication [7,19]. The opening of VSCC Ca^{2+} channels may produce a localized rise in Ca^{2+} and exocytosis of neurotransmitters. Thus, calcium permeates nerve endings through VSCC triggering exocytosis of synaptic vesicles docked at the active zones.

Electrophysiological studies have classified VSCC into T (low-voltage activated) and L-, N-, P-, Q- and R-types (high-voltage activated) [23,24]. Peptides isolated from the venom of the spiders or cone snails have been used to distinguish the different types of calcium channels. These peptides can inhibit current through specific channels with nanomolar potency [1]. The venom of the spider *Phoneutria nigriventer* contains several neurotoxins peptides with actions such as inhibition of the inactivation of Na^+ channels [3], blockage of K^+ channels [10], blockage of Ca^{2+} channels [5] and inhibition of $^{45}Ca^{2+}$ influx in depolarized synaptosomes [15]. Thus, *Phoneutria nigriventer* spider venom contains a cocktail of toxins that affects ionic channels (see review [8]) and most of these

peptide toxins are calcium channel blockers that may are used to capture prey.

The aim of this paper was to investigate, on rat brain cortical synaptosomes, the KCl-induced increase on exocytosis of synaptic vesicles in the presence of Tx3-4. We extend the study comparing the interaction of Tx3-4 with others toxins calcium channel blockers (reviewed by Olivera et al. [17]) on exocytosis. We suggest that Tx3-4 is an antagonist of calcium channels coupled to exocytotic release of neurotransmitters.

Tx3-4 purified according Cordeiro et al., 1993 is a protein of 8449 Da. FM2-10 was obtained from Molecular Probes (Eugene, OR, USA). ω-CgTX MVIIC, ω-CgTX GVIA and ω-AGA IVA, Fura-2 acetoximethyl ester (Fura-2AM) and Percoll were obtained from Sigma Chemical Co. (MO, USA).

All animal procedures were approved by a local Ethics Committee and followed the guidelines for the Use and Care of Animals for Research issued by the NIH. Male wistar rats (180–200 g) were killed by decapitation and their brains rapidly (<1 min) removed and homogenized 1:10 (w/v) in 0.32 m sucrose solution containing 0.25 mM dithiothreitol and 1.0 mM EDTA. The homogenate centrifuged (1000 × g for 10 min) and synaptosomes were purified from the supernatant by discontinuous Percoll-density gradient

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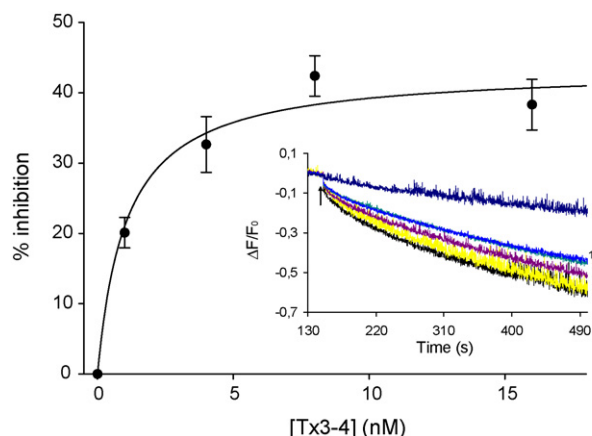*E-mail address:* celiojcjunior@yahoo.com.br (C.J. de Castro Junior).

Fig. 1. Effect of Tx3-4 on KCl-induced exocytosis. Rat cerebrocortical synaptosomes were incubated in KRH in the presence of FM2-10 (50 μM). Tx3-4 (1.0, 4.0, 8.0 and 16 nM) was added 5 min before depolarization by 30 mM KCl. The plot shows the mean ± S.E.M. for the percent of fluorescence inhibition induced by Tx3-4. The line represents the curve fitting generated by non-linear regression, $f = ax/(b+x)$. The insert shows the fluorescence traces in the absence/presence of 30 mM KCl and Tx3-4 at the indicated concentrations. The arrow indicates the addition of 30 mM KCl.

centrifugation [6], essentially as described [20]. The isolated nerve terminals were resuspended in Krebs–Ringer–Hepes medium (KRH): (124 mM NaCl; 1.3 mM CaCl₂, 4.0 mM KCl; 1.2 mM MgSO₄ 10 mM glucose, 25 mM Hepes, pH 7.4), to a protein concentration of 10 mg/ml, divided into aliquots of 1 ml kept on ice for the experiments.

The exocytosis measurements were performed in synaptosomes (500 μl) diluted to 1.0 ml with KRH medium in a stirred cuvette and incubated with FM2-10 (50 μM) and 1.3 mM calcium for 3 min at 37 °C as previously done for FM1-43 [9,11]. Vesicle cycling was stimulated with 30 mM KCl and otherwise stated for 45 s. The sample was washed with two short 10 s spins to remove externally bound dye, and resuspended in 2 ml of fresh KRH. The synaptosomes were stirred at 37 °C for approximately 5 min to further remove dye from the plasma membrane. Labelled vesicles were released during a second round of vesicle cycling, stimulated with KCl in KRH medium. We measured the decrease in fluorescence $((F - F_0)/F_0)$ when the dye was exocytosed in a Shimadzu Spectrophotometer model RF-5301 PC, exciting at 488 nm and collecting at 570 nm. The fluorescence decay was analyzed at an average of 480–490 s. The dye FM1-43 has been used previously to measure exocytosis in synaptosomes [9,13], but FM2-10 is more easily washed from external membranes giving a lower background signal, which it was used this study.

Analysis for the significance of the differences between the toxins treatment were performed by one-way analysis of variance.

Fig. 1 and its insert shows the concentration curve for the inhibitory effect of Tx3-4 on the exocytosis of synaptic vesicles measured with FM2-10. Tx3-4 at the concentrations of 1.0, 4.0, 8.0 and 16.0 nM inhibited the KCl-induced exocytosis of synaptic vesicles by 20 ± 2.1, 33 ± 3.9, 42 ± 2.9 and 38 ± 3.6%, respectively, $P < 0.05$. Thus the inhibitory effect of Tx3-4 on the KCl-induced exocytosis of synaptic vesicles reached a plateau at 16.0 nM with an IC₅₀ of 1.1 nM.

Next we tested for the overlapping between Tx3-4 and other toxins, calcium channel blockers, on the exocytosis of synaptic vesicles (Fig. 2). Tx3-4 (10 nM) reduced by 37 ± 2.8% the 30 mM KCl-induced exocytosis of synaptic vesicle, whereas the reduction evoked by ω-CgTX-MVIIC (2.5 μM) and ω-Aga-IVA (30 nM) were lower 21 ± 3.5 and 10 ± 5.0%, respectively, $P < 0.05$. The ω-CgTX GVIA (1.0 μM), blocker of N-type calcium channel, caused a small and no significant

effect on the KCl-induced exocytosis ($P > 0.05$). The joint application of Tx3-4 and ω-CgTX MVIIC did not induce higher inhibition on exocytosis than that induced by Tx3-4 alone, $P > 0.05$. In contrast, the joint use of Tx3-4 (10 nM) and Aga-IVA (30 nM) caused an additive inhibitory effect of 53 ± 3.9% on the 30 mM KCl-induced exocytosis of synaptic vesicles ($P < 0.05$). Otherwise the association of Tx3-4 (10 nM) with ω-CgTX GVIA (1.0 μM) caused inhibition on the exocytosis that was, however, not statistically different from the inhibition induced by Tx3-4 alone ($P > 0.05$). Cd²⁺ (100 μM), an inespecific blocker of calcium channels, inhibited by 54 ± 3.8% the KCl-induced exocytosis, $P < 0.05$.

Measurements of FMs dyes decay [4,9,13] provide a good indication of transmitter release of synaptosomes. KCl-induced decrease in fluorescence in the calcium absence (EGTA 8 mM) was greatly diminished by about 25% of control conditions using 2 mM calcium (data not shown). In the present paper, the FM labeling decay induced by KCl depolarization was also greatly diminished in the presence of Tx3-4 and conotoxins and thus, dependent of active exocytosis. We take advantage of this technique to show that Tx3-4 caused a dose-dependent inhibition of exocytosis. Exocytosis of synaptic vesicles is triggered by a raise in local calcium concentration and is tightly coupled to a selective calcium channel subtype. Tx3-4, ω-Aga-IVA and ω-conotoxin MVIIC inhibited the exocytotic responses induced by KCl depolarization of nerve terminals. Tx3-4 caused a dose-dependent inhibition of exocytosis with an IC₅₀ of 1.1 nM. The small and not significant effect on exocytosis induced by ω-CgTX GVIA suggests that N-type calcium channel does not contribute much to exocytosis of central nerve terminals [21]. N-type calcium channels are more prevalent in peripheral nerve terminals and are largely responsible for synaptic transmission in autonomic and sensory terminals [16]. Considerable research in the last decade has focused on potential role of N-type calcium channel inhibitors for development of novel analgesic drugs [2]. The various VSCC-regulated components of
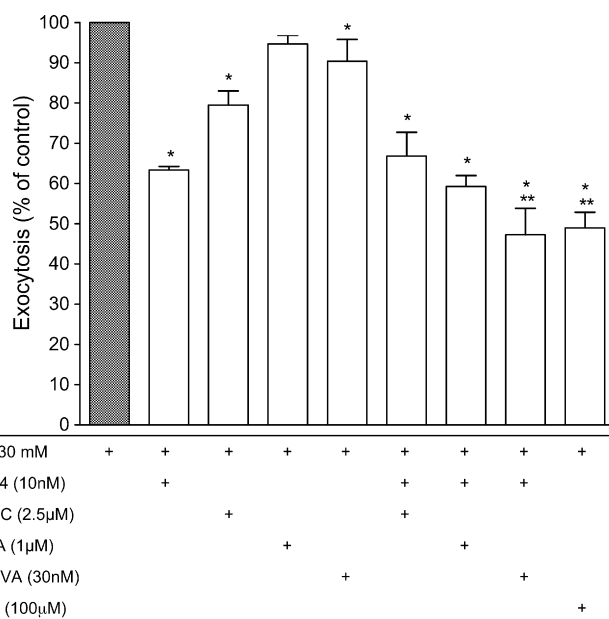

Fig. 2. Effect of Tx3-4, ω-conotoxin MVIIC, ω-CgTx GVIA, ω-Aga IVA and cadmium on exocytosis induced by 30 mM KCl. Rat cerebrocortical synaptosomes were incubated in KRH in the presence of FM2-10 (50 μM). The toxins and cadmium were added 5 min before depolarization by 30 mM KCl. Columns show the percent of exocytosis inhibition induced by 10 nM Tx3-4, 2.5 μM ω-conotoxin MVIIC, 1.0 μM ω-CgTx GVIA, 30 nM ω-Aga IVA or 100 μM cadmium on the fluorescence induced by 30 mM KCl (control). Columns show the mean ± S.E.M. for at least three experiments. *$P < 0.05$ compared to the control 30 mM KCl and **$P > 0.05$ compared to the Tx3-4 value.

transmitter release and electrical signaling contribute to numerous physiological processes that are potential targets for therapeutic intervention.

At the concentration of 30 nM ω-AgaIVA blocks P-type calcium channels [18] without interfering with the Q-type channel. The P-type [14] and Q-type calcium currents are quite similar and both currents are completely blocked by saturating concentration of ω-conotoxin MVIIC. The data showing the lack of an additive inhibitory effect between the Tx3-4 plus saturating concentrations of ω-conotoxin MVIIC and also the observed additive inhibitory effect on exocytosis induced by the joint use of 10 nM Tx3-4 and 30 nM ω-Aga-IVA suggests that Tx3-4 may acts on P/Q type calcium channels in nerve terminals. P/Q-type calcium channels are most important for transmitter release at central terminals [9,22]. However, considering that ω-conotoxin MVIIC and ω-CgTX GVIA compete for the same binding site on the N-type calcium channel [12], Tx3-4 may also affects N-type calcium channel. Tx3-4 has homology structure with Phoneutria ω-Ptx-IIA that was shown blocks N-type calcium channels of rat dorsal root ganglion [5].

Multiple types of calcium channels coexist in nerve terminals, and they regulate jointly calcium entry and synaptic vesicles exocytosis. Tx3-4 may binds to brain synaptosomes in multiple binding sites and with the present experiments is no possible to exclude the participation of more than one calcium channel involved on the Tx3-4 inhibition of the KCl-induced increase of exocytosis of nerve terminals. The joint use of Tx3-4 and conotoxins do not completely block the vesicle exocytosis suggesting the presence of additional resistant exocytotic channel.

The control of the calcium-dependent secretion of neurotransmitters from presynaptic nerve terminal is one the most important function to initiate the synaptic transmission. Tx3-4 shows a very potent inhibition of exocytosis with an IC_{50} of 1.1 nM. Conotoxins, well-known calcium channels blockers, also inhibited KCl-induced exocytosis but with higher concentrations than that used by Tx3-4. Thus, Tx3-4 may represents a potent tool to study the participation of calcium channels on neurotransmitter release from nerve terminals.

Acknowledgements

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