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*

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The present experiments investigated the effect of some of the toxic components present in the venom of the spider *Phoneutria nigriventer* on the release of neurotransmitter. The toxic fraction, *Phoneutria nigriventer* toxin-3 (PhTx3), abolished Ca^{2+} -dependent glutamate release, but did not alter Ca^{2+} -independent secretion of glutamate when rat brain cortical synaptosomes were depolarized with 33 mM KCl. This effect was most likely due to interference with the entry of calcium through voltage-gated calcium channels, because PhTx3 reduced by 50% the increase in intrasynaptosomal free calcium induced by membrane depolarization, and did not affect the release of glutamate evoked

by a calcium ionophore (ionomycin). A polypeptide (Tx3-3) present in the PhTx3 fraction reproduced the effects of the PhTx3 fraction on transmitter release and intrasynaptosomal free calcium in the low nanomolar range. We compared the alterations produced by the Tx3-3 with the actions of toxins known to block calcium channels coupled to exocytosis: the results indicated that the Tx3-3 inhibition of glutamate release and intrasynaptosomal calcium resemble that observed with ω -conotoxin MVIIC. We suggest that the Tx3-3 is a calcium-channel antagonist that blocked glutamate exocytosis.

INTRODUCTION

Calcium permeates nerve endings through voltage-gated channels and triggers the exocytosis of transmitter-filled synaptic vesicles docked at the active zones. The unique role of calcium in controlling neurotransmitter release allows calcium channels to play a major role in controlling synaptic transmission. Toxins and drugs that interfere with such channels can, therefore, be used to understand the coupling of calcium and transmitter release.

The venom of the Brazilian spider *Phoneutria nigriventer* possesses several toxic polypeptide fractions, some of which have been purified recently and shown to be neurotoxic [1]. The action of some of these toxins have been studied in intact animals [1] and in preparations *in vitro* [2,3]. One of the toxic fractions, designated *Phoneutria nigriventer* toxin 3 (PhTx3), has been shown to induce a progressive flaccid paralysis in mice when injected intracerebroventricularly. Recently, six neurotoxic peptides (Tx3-1 to Tx3-6) with relative molecular masses ranging from 3500 to 8500 were purified to homogeneity from the fraction PhTx3 [4]. The complete amino acid sequences of Tx3-1, Tx3-2 and Tx3-6 were established, as were the N-terminal sequences of Tx3-3, Tx3-4 and Tx3-5 [4]. Comparison of the obtained sequences with those of other toxins showed weak sequence similarity with other proteins; however, there was a small degree of similarity in the location of cysteine residues when these toxins were compared with other toxins active on

Ca^{2+} channels, i.e. *Hololena curta* toxins and ω -agatoxin IVA (ω -Aga IVA) [4].

The ω -toxins interact specifically with calcium channels and have been found in the venom of marine snails from the genus *Conus* and in the venom of spiders. In particular, two toxins, ω -conotoxin GVIA (ω -CgTX GVIA) from *Conus geographus*, and ω -Aga IVA from the venom of the spider *Agelenopsis aperta*, have been invaluable in identifying pharmacologically active calcium channels involved in cellular processes, such as transmitter release [5].

Purified neuronal calcium channels are multisubunit complexes containing at least four subunits [6,7]. The pore-forming subunit, α_1 , is heterogeneous among calcium channels [8], and it confers upon each functional calcium-channel type distinct biophysical and pharmacological properties. Voltage-activated calcium channels can be classified according to their molecular heterogeneity, voltage dependence for activation and inactivation, unitary conductance, and sensitivity to diverse ligands [5]. The picture emerging is that most of the neuronal calcium channels coupled to transmitter release are high-voltage activated and likely to be products of α_{1A} , α_{1B} and α_{1E} genes (see [5,9]), each of which has characteristic affinities for different ω -toxins. The sensitivity to low concentrations of ω -CgTX GVIA defines pharmacologically the product of the α_{1B} gene, which has electrophysiological properties of the N-type calcium channel [10–12], while ω -Aga IVA defines the so-called P-type channel [14]. Other toxins, such as ω -conotoxin MVIIC (ω -CmTX

Abbreviations used: AM, acetoxymethyl ester; CNS, central nervous system; ω -CgTX GVIA, ω -conotoxin GVIA; ω -CmTX MVIIC, ω -conotoxin MVIIC; ω -Aga IVA, ω -agatoxin IVA; Aga-GI, agatoxin glutamate release inhibitor; KRH, Krebs-Ringers-Hepes; PhTx3, *Phoneutria nigriventer* toxin-3; $[\text{Ca}^{2+}]_i$, intrasynaptosomal free calcium concentration; VSCC, voltage-sensitive calcium channel.

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MVIIC), seem to be much less selective in discriminating between the diverse voltage-gated Ca^{2+} channels (see [5]).

The use of the pharmacological tools described above has helped in the investigation of calcium channels coupled to the release of glutamate, the major excitatory transmitter in the brain. Release of glutamate from brain synaptosomes is at least partially dependent on calcium penetration through ω -Aga IVA-sensitive channels [15], and apparently totally abolished by another component of the venom of *Agelenopsis aperta*, Aga-GI (agatoxin glutamate release inhibitor) [16]. Glutamatergic synaptic transmission can also be affected by ω -CgTX GVIA and ω -CmTX MVIIC in the hippocampus [17].

The effect of PhTx3 *in vivo* suggests that it inhibits synaptic transmission; however, there is little direct functional data on the effect of the PhTx3 fraction or the Tx3 isotoxins on transmitter release or ion fluxes in nerve terminals. In this study we tested the hypothesis that PhTx3 and its toxic components alter transmitter release. We suggest here that Tx3-3, one of the isotoxins present in the PhTx3 fraction, is an antagonist of calcium channels coupled to exocytotic glutamate release.

EXPERIMENTAL

Materials

Glutamate dehydrogenase type II (EC 1.4.1.3), NADP⁺, glutamate, fura-2 acetoxymethyl ester (AM), sucrose, Percoll, ionomycin, SDS, EGTA and Hepes were obtained from Sigma Chemical Co., MO, U.S.A. ω -CmTX MVIIC and ω -Aga IVA were purchased from Peptides, Japan. All other reagents were of analytical grade.

Purification of synaptosomes

Adult Wistar rats of both sexes (180 to 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 [18], essentially as described by Romano-Silva et al. [3]. The isolated nerve terminals were resuspended in Krebs-Ringer-Hepes solution (KRH; 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 approx. 10 mg/ml, divided into aliquots of 200 μl and kept on ice until loaded with fura-2 AM or used for measurement of glutamate release.

Measurements of intrasynaptosomal free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$)

Fura-2 AM (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 approx. 1.2 mg/ml followed by a further 30 min incubation period. Fura-2-loaded isolated nerve terminals were washed, resuspended with medium (1 mg/ml) and immediately used for ratiometric quantification of intracellular free calcium [19] in a PTI spectrofluorimeter. Fluorescence emission was recorded at 500 nm using 340/380 excitation ratio. Calibration of fura-2 signals and estimation of synaptosomal autofluorescence were performed as described elsewhere [3]. CaCl_2 was added to the synaptosomal suspension at the beginning of each fluorimetric assay (1.0 mM final concentration). Synaptosomes were stirred throughout the experiment in a cuvette maintained at 35 °C. Toxins were added to

synaptosomal suspension 60 s prior to membrane depolarization with 33 mM KCl. The $[\text{Ca}^{2+}]_i$ increase induced by membrane depolarization is strictly dependent on the presence of extracellular Ca^{2+} in these conditions [3].

Measurement of continuous glutamate release

The glutamate release assay was performed by following the increase in fluorescence due to the production of NADPH in the presence of glutamate dehydrogenase and NADP⁺ [3,20]. Briefly, synaptosomes (10 mg/ml) were incubated for 60 min in the same way as for the measurement of calcium, washed with KRH medium, and transferred to a cuvette (final synaptosomal concentration 0.5 mg/ml to 1 mg/ml) which was maintained at 35 °C with constant stirring. At the start of each assay CaCl_2 (1 mM), NADP⁺ (1 mM) and glutamate dehydrogenase (50 units) were added to synaptosomes. The excitation wavelength was set at 360 nm and the emission wavelength to 450 nm. CaCl_2 addition was omitted and the experiment performed in the presence of 1.0 mM EGTA in order to assess the Ca^{2+} -independent glutamate release under different conditions. Toxins were added 60 s before membrane depolarization with 33 mM KCl.

Isolation of *Phoneutria nigriventer* toxins

The PhTx3 and isotoxins therein were purified from the venom of *Phoneutria nigriventer* by a combination of chromatographic steps according to the method of Cordeiro et al. [4].

Reproducibility and statistical analysis

Results shown here are means of at least three experiments. When necessary statistical significance was assessed by analysis of variance (ANOVA). A value of $P < 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

Rat cortical synaptosomes are a well-recognized model for studying neurotransmitter release and other nerve-terminal-related mechanisms. Thus, synaptosomes are good agents for the screening of substances with potential activity on the central nervous system (CNS). In this study, synaptosomes were stimulated with a high concentration of KCl (33 mM) to depolarize their membranes and induce neurotransmitter release. KCl depolarization has been widely used and the amount of data available in the literature accounts for its choice here in order to simplify the comparison of results.

The protocol used to perform the experiments in synaptosomes was guided, initially, by data obtained following intracerebroventricular injections of PhTx3 [4]. The paralysis provoked by this fraction of the *P. nigriventer* venom suggested strongly that it was inhibiting CNS neurotransmitter release. In order to test this possibility we decided to initiate our investigation by examining the effect of PhTx3 on the release of the major excitatory neurotransmitter in the CNS, the amino acid L-glutamate.

When synaptosomes are depolarized with KCl the release of glutamate occurs in a biphasic pattern: a short rapid phase followed by a long-lasting slow release. Figure 1(A) shows the effect of three different concentrations of PhTx3 (0.1, 1.0 and 10 $\mu\text{g}/\text{ml}$) added 1 min before depolarization of synaptosomes with 33 mM KCl. The depolarization-induced glutamate release was inhibited by 25, 50 and 50 % respectively. It was clear that

Figure 1 PhTx3 abolishes the Ca^{2+} -dependent release of glutamate from brain synaptosomes

Synaptosomes prepared as described in the Experimental section were depolarized with 33 mM KCl and the continuous release of glutamate was followed. **(A)** Total release of glutamate from control and toxin-treated synaptosomes. PhTx3 (0.1, 1.0 and 10 µg/ml) was added 60 s before addition of KCl. **(B)** Ca^{2+} -independent release of glutamate. The release of glutamate was performed in medium lacking calcium (1 mM EGTA), and control or PhTx3-exposed synaptosomes (1 µg/ml) were depolarized by 33 mM KCl. The amount of glutamate released under calcium-deprived conditions was similar to that shown in **(A)** for maximal concentrations of PhTx3 ($P > 0.05$). The results represent mean values of four independent experiments.

there was no further reduction of glutamate release in response to a further increase in the PhTx3 concentration (from 1.0 to 10 µg/ml).

The KCl-evoked release of glutamate is the sum of two components distinguished by their dependence on, or independence of, extracellular Ca^{2+} (for a review see [21]). The Ca^{2+} -independent release was assayed in a medium lacking CaCl_2 and by adding 1.0 mM EGTA 2 min before depolarization with KCl. The resulting release in these conditions was approx. 50 % of that measured in normal medium containing calcium (Figures 1A and 1B), and had the same magnitude as that obtained in the presence of 1.0 or 10 µg/ml PhTx3 (Figure 1A). In contrast, we found that PhTx3 had no effect on the Ca^{2+} -independent release (Figure 1B). These results indicated that the PhTx3 effect was restricted to the Ca^{2+} -dependent (exocytotic) glutamate release.

Figure 2 Effect of PhTx3 on KCl-induced increase in $[\text{Ca}^{2+}]_i$

Synaptosomes loaded with fura-2 as described in the Experimental section were incubated, when necessary, for 60 s with PhTx3 (1.0 and 10 µg/ml) before the addition of 33 mM KCl. Isolated nerve terminals treated with PhTx3 showed a significant reduction of the $[\text{Ca}^{2+}]_i$ increase induced by 33 mM KCl ($P < 0.05$). Results shown are mean values from three [KCl and PhTx3 (1 µg/ml)] or two [PhTx3 (10 µg/ml)] independent experiments.

Figure 3 PhTx3 does not alter ionomycin-induced release of glutamate

Synaptosomes prepared as described in Figure 1 were stimulated by ionomycin (2 µM) in the presence or absence of PhTx3 (1 µg/ml). There was no significant difference between the response of control and PhTx3-exposed synaptosomes to the increase of calcium induced by the ionophore ($P > 0.05$).

Exocytosis is triggered by calcium influx through voltage-sensitive calcium channels (VSCCs) located close to vesicle docking sites. It has been proposed that the Ca^{2+} -channels involved in glutamate release from cerebrocortical synaptosomes were opened by depolarizing stimuli and are maintained in a long-lasting non-inactivating state [21]. Changes in cytosolic Ca^{2+} can be monitored continuously in fura-2-loaded synaptosomes (see the Experimental section); when synaptosomes are depolarized upon addition of KCl (33 mM) the response pattern observed is a fast rising spike followed by a lower steady plateau (Figure 2). Addition of PhTx3 (1.0 or 10 µg/ml) 1 min before KCl depolarization inhibited the

Table 1 Effect of the Tx3 isotoxins on $[Ca^{2+}]_i$ and glutamate release from depolarized synaptosomes

Glutamate release and $[Ca^{2+}]_i$ were measured as described in the Experimental section and Figures 1 and 2. Results show the means $\pm$ S.E.M. for three experiments where the intracellular increase of Ca^{2+} induced by KCl in the presence or absence of toxins was calculated, or the cumulative release of glutamate for 10 min was measured in at least three separate experiments. The concentrations used were 1 μ g/ml for Tx3-1, Tx3-2, Tx3-4, Tx3-5 and 0.1 μ g/ml for Tx3-3 and Tx3-4. Abbreviation: n.a., not available.

	$[Ca^{2+}]_i$ (nM)	Glutamate released (nmol/mg of protein)
33 mM KCl (control)	238 $\pm$ 16	11.2 $\pm$ 0.5
Tx3-1 (220 nM)	222 $\pm$ 9	10.8 $\pm$ 1.0
Tx3-2 (280 nM)	217 $\pm$ 49	9.7 $\pm$ 0.3
Tx3-3 (16 nM)	165 $\pm$ 24*	5.7 $\pm$ 0.5*
Tx3-4 (12 nM)	235 $\pm$ 24	n.a.
Tx3-4 (120 nM)	211 $\pm$ 28	7.8 $\pm$ 0.7*
Tx3-5 (200 nM)	260 $\pm$ 2	9.7 $\pm$ 0.5
Tx3-6	n.a.	n.a.
PhTx3 (1.0 μ g/ml)	112 $\pm$ 8*	6.6 $\pm$ 1.1*
EGTA (1.0 mM)	n.a.	6.1 $\pm$ 0.4*

* $P < 0.05$ compared with control.

'plateau' increase of Ca^{2+} by 53 %. This result is similar to a report by Pocock and Nicholls [16] of a toxin (Aga-GI) from the venom of the spider *Agelenopsis aperta*. These authors showed that Aga-GI blocked Ca^{2+} -dependent glutamate release completely and inhibited the depolarization-induced increase in $[Ca^{2+}]_i$ by approx. 40 %. Moreover, the protocol used opens calcium channels without need of sodium influx, therefore interference with sodium channels could not explain the above results.

The supposition that PhTx3 acts on VSCCs was supported by the results obtained with the calcium ionophore ionomycin (Figure 3). Ionomycin increases $[Ca^{2+}]_i$ without the participation of calcium channels and induces neurotransmitter release by raising cytosolic Ca^{2+} to the levels necessary to trigger exocytosis [16]. We have found that PhTx3 (1.0 μ g/ml) was ineffective against ionomycin-induced glutamate release from synaptosomes. This indicated that in the presence of PhTx3 the exocytotic machinery remained functional, reinforcing the notion that the PhTx3 effect must be related to an interference with the VSCC step.

Further purification of PhTx3 yielded six homogenous isotoxins, Tx3-1 to Tx3-6 [4]. All Tx3s manifested toxic effects when injected intracerebroventricularly, varying from general flaccid paralysis followed by death in 30 min (Tx3-3 and Tx3-4) to paralysis of the hindlimbs and gradual reduction of movement and aggression over a period of 24 h [4]. Thus, we have characterized the effect of the isotoxins on $[Ca^{2+}]_i$ and glutamate release in the same system used to investigate PhTx3. The choice of the doses used was based on the potency of PhTx3 (maximal effect at 1 μ g/ml), thus all the isotoxins were tested in concentrations up to 1 μ g/ml. Although all the isotoxins had pharmacological effects *in vivo*, we have identified two (Tx3-3 and Tx3-4) that were effective against KCl-evoked glutamate release, at concentrations up to 1.0 μ g/ml (Table 1). Coincidentally, these isotoxins are the only ones to produce flaccid paralysis *in vivo* followed by death [4]. However, the toxin Tx3-3 was much more potent than Tx3-4, completely inhibiting the calcium-dependent glutamate release at a concentration of 0.05 μ g/ml (8 nM, Figures 4A and 4B). Tx3-4 significantly reduced the KCl-induced release

Figure 4 Effect of Tx3-3 on glutamate release

(A) Synaptosomes obtained as described in the text were depolarized with 33 mM KCl in the presence or absence of Ca^{2+} (1 mM EGTA). (B) The same population of synaptosomes presented in (A) were exposed to different concentrations of Tx3-3 1 min before depolarization with 33 mM KCl. The Tx3-3 in maximum concentrations reduced the glutamate release to the same extent as medium deprived of calcium ($P > 0.05$).

(by 30 %; Table 1) only at a concentration of 1.0 μ g/ml (120 nM), and was ineffective at lower concentrations (results not shown). Therefore, Tx3-3 was by far the most active toxin against KCl-induced glutamate release in the PhTx3 fraction.

The above results on glutamate release were in agreement with measurements of $[Ca^{2+}]_i$: Tx3-1, -2, -4 and -5 (1.0 μ g/ml) did not have any significant effects, whereas Tx3-3 (0.1 μ g/ml; 16 nM) reduced the KCl-induced response by 30 % (Table 1 and Figure 5). It is important to notice that the measurements of $[Ca^{2+}]_i$ were done in the population of synaptomes, while glutamate release is indicative of transmitter release from only part of this population. In fact, some treatments can alter calcium influx without altering

Figure 5 Tx3-3 decreases calcium influx

Synaptosomes loaded with fura-2 as described in the Experimental section were depolarized in the presence or absence of 16 nM of Tx3-3. The *Phoneutria* toxin decreased significantly ($P < 0.05$) the increase of intracellular calcium due to the opening of calcium channels.

Figure 6 Comparison of the effects of Tx3-3 with other calcium-channel antagonists on $[Ca^{2+}]_i$ and glutamate release from synaptosomes stimulated with 33 mM KCl

(A) Variations of $[Ca^{2+}]_i$ in response to 33 mM KCl in the presence or absence of toxins that block distinct calcium channels. $[Ca^{2+}]_i$ was measured as described in the Experimental section and Figure 2. (B) Cumulative glutamate release in the presence or absence of toxins that interfere with voltage-gated calcium channels. Synaptosomes were obtained and experiments performed as in Figure 1 and Table 1. Concentrations used were: KCl (33 mM), PhTx3 (1 μ g/ml), Tx3-3 (16 nM), ω -CmTX MVIIC (1 μ M) and ω -AGA IVA (100 nM). Results represent means $\pm$ S.E.M. of at least three experiments. Asterisks indicate results significantly different from those obtained with synaptosomes exposed to 33 mM KCl ($P < 0.05$).

glutamate release and vice versa ([22] and Figures 6A and 6B). Thus, it is conceivable that Tx3-4 could also alter calcium influx through a channel that has a minor contribution to the overall calcium response, but partially contributes to glutamate release.

The identification of Tx3-3 as an active peptide with effectiveness similar to that of the PhTx3 fraction led us to study its effects in comparison with those of other known peptide Ca^{2+} -channel blockers (ω -CmTX MVIIC and ω -Aga IVA). These toxins were chosen for comparison because of their ability to inhibit glutamatergic transmission [15,17,23]. The N-terminal sequence of Tx3-3 (GCANAYKSCNGPHTCCWGYNGYKK-ACICSG?NWK, approx. M_r 6300) has been compared with several other toxins, but only weak sequence similarities were observed [4]. Figure 6(A) shows that the various toxins were active at lowering the KCl-induced plateau of $[Ca^{2+}]_i$ by approximately the same amount ($\sim 50\%$) at the following concentrations: PhTx3, 1.0 μ g/ml; Tx3-3, 16 nM; ω -CmTX MVIIC,

1 μ M; ω -Aga IVA, 100 nM. ω -CmTX MVIIC and ω -Aga IVA are known to block the N- and Q-, and P-type calcium channels respectively at these concentrations [14,17,24]. All values were statistically different from the control ($P < 0.05$) but not from each other. However, Figure 4(B) revealed differences in the actions of the toxins on glutamate release; PhTx3, Tx3-3 and ω -CmTX MVIIC were able to inhibit glutamate release but ω -Aga IVA had no effect. At first glance this seems to contradict earlier reports on the effect of ω -Aga IVA on glutamate release from CNS preparations [15,23]. However, some experimental details are relevant to the discussion; the ω -Aga IVA effect on glutamate release has been shown to be inversely proportional to the strength and duration of the depolarizing stimulus [23], therefore this toxin was more effective at concentrations of KCl below 30 mM and during short-duration pulse depolarizations. More prolonged and/or stronger stimuli would be capable of activating a secondary Ca^{2+} -channel insensitive to ω -Aga IVA and stimulating transmitter release [15,23]. These facts could explain our finding that ω -Aga IVA reduces $[Ca^{2+}]_i$ (demonstrating that the toxin was active) but did not diminish glutamate release (probably due to the sustained KCl depolarization). These results support the notion that Tx3-3 is probably able to block all Ca^{2+} channels coupled to glutamate release in a non-selective manner at the concentration used. The effects of Tx3-3 resemble those of ω -CmTX MVIIC, both on KCl-induced glutamate release and on changes in $[Ca^{2+}]_i$ (Figures 6A and 6B).

The ω -CmTX MVIIC, targets N-, P- and Q-type Ca^{2+} channels at the concentration used in our experiments [17,24,25]. An earlier report described inhibition of excitatory post-synaptic membrane potentials, due to decreased glutamate release, in hippocampal preparations by ω -CmTX MVIIC [17]. As mentioned above, the effect of Tx3-3 was very similar to that of ω -CmTX MVIIC, which could mean that both toxins interact with the same channels. Further experimentation will be necessary to shed more light to this topic, especially on the electrophysiological characterization of the channels blocked by the Tx3-3 and the effect of this toxin on the release of other transmitters.

The types of Ca^{2+} channels coupled to glutamate release have been a source of discussion for some time. The difficulty of assigning a definitive calcium-channel subtype to exocytotic glutamate release probably reflects the heterogeneity of calcium channels that can be activated by different stimuli [23]. The participation of more than one sort of channel in transmitter release at the same nerve terminal has been suggested by several authors [15,17,23] and variations were shown between different areas of the nervous system in the combinations of channel types involved in the release of particular transmitters (see [5]); these facts underlie the difficulties of analysing Ca^{2+} -channel-glutamate release coupling. To this extent the discussion on the possible channels inhibited by Tx3-3 is speculative.

In conclusion, Tx3-3 was able to block Ca^{2+} -dependent KCl-evoked glutamate release at low concentration (8 nM), probably by acting with high affinity on Ca^{2+} channel(s) coupled to exocytosis. This toxin does not have significant amino acid sequence similarity with any other known toxin able to block Ca^{2+} channels [4], a point that might be of importance in spatial modelling of peptide Ca^{2+} -channel antagonists, since non-selective toxins may provide information on peptide domains shared by different classes of calcium channel. Finally, owing to its ability to abolish the Ca^{2+} -dependent step of glutamate release, Tx3-3 is a potential tool to discriminate between the roles of the Ca^{2+} -independent and -dependent components on *in vivo* glutamate release in distinct pathological situations, such as epilepsy and ischaemia/anoxia [26,27], where reversal of

glutamate transporter action might be a key factor in causing lesions.

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REFERENCES

- Rezende Jr., L., Cordeiro, M. N., Oliveira, E. B. and Diniz, C. R. (1991) *Toxicon* **29**, 1225–1233
- Araújo, D. A., Cordeiro, M. N., Diniz, C. R. and Beirão, P. S. (1993) *Naunyn Schmiedeberg's Arch. Pharmacol.* **347**, 205–208
- Romano-Silva, M. A., Ribeiro-Santos, R., Ribeiro, A. M., Gomez, M. V., Diniz, C. R., Cordeiro, M. N. and Brammer, M. J. (1993) *Biochem. J.* **296**, 313–319
- Cordeiro, M. N., Figueiredo, S. G., Valentim, A. C., Diniz, C. R., von Eickstedt, V. R. D., Gilroy, J. and Richardson, M. (1993) *Toxicon* **31**, 35–42
- Olivera, B. M., Miljanich, G. P., Ramachandran, J. and Adams, M. E. (1994) *Annu. Rev. Biochem.* **63**, 823–867
- McEnery, M. W., Snowman, A. M., Sharp, A. H., Adams, M. E. and Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 11095–11099
- Witcher, D. R., De Waard, W. M., Sakamoto, J., Franzini-Armstrong, C., Pragnell, M., Kahl, S. D. and Campbell, K. P. (1993) *Science* **261**, 486–489
- Snutch, T. P., Leonard, J. P., Gilbert, M. M., Lester, H. A. and Davidson, N. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3391–3395
- Zhang, J.-F., Randall, A. D., Ellinor, P. T., Horne, W. A., Sather, W. A., Tanabe, T., Schwarz, T. L. and Tsien, R. W. (1993) *Neuropharmacology* **32**, 1075–1088
- Fox, A. P., Nowicky, M. C. and Tsien, R. W. (1987) *J. Physiol. (London)* **394**, 149–172
- McCleskey, E. W., Fox, A. P., Feldman, D. H., Cruz, L. J., Olivera, B. M. and Tsien, R. W. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4327–4331
- Dubel, S. J., Starr, T. V. B., Hell, J., Ahljianian, M. K., Enyeart, J. J., Caterall, W. A. and Snutch, T. P. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5058–5062
- Reference deleted
- Mintz, I. M., Venema, V. J., Swiderek, K. M., Lee, T. D., Bean, B. P. and Adams, M. E. (1992) *Nature (London)* **355**, 827–829
- Turner, T. J., Adams, M. E. and Dunlap, K. (1992) *Science* **258**, 310–313
- Pocock, J. M. and Nicholls, D. G. (1992) *Eur. J. Pharmacol.* **226**, 343–350
- Wheeler, D. B., Randall, A. and Tsien, R. W. (1994) *Science* **264**, 107–111
- Dunkley, P. R., Heath, J. W., Harrison, S. M., Jarvie, P. E., Glenfield, P. J. and Rostas, J. A. P. (1988) *Brain Res.* **441**, 59–71
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Nicholls, D. G., Sihra, T. S. and Sanchez Prieto, J. (1987) *J. Neurochem.* **49**, 50–57
- Nicholls, D. G. (1993) *Eur. J. Biochem.* **212**, 613–631
- Romano-Silva, M. A., Gomez, M. V. and Brammer, M. J. (1994) *Neurosci. Lett.* **178**, 155–158
- Luebke, J. I., Dunlap, K. and Turner, T. J. (1993) *Neuron* **11**, 895–902
- Sather, W. A., Tanabe, T., Zhang, J.-F., Mori, Y., Adams, M. E. and Tsien, R. W. (1993) *Neuron* **11**, 291–303
- Grantham, C. G., Bowman, D., Bath, C. P., Bell, D. C. and Bleakman, D. (1994) *Neuropharmacology* **33**, 255–258
- Rothman, S. M. and Olney, J. W. (1986) *Ann. Neurol.* **19**, 105–111
- Meldrum, B. and Garthwaite, J. (1990) *Trends Pharmacol. Sci.* **11**, 379–387

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