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A toxin from the spider *Phoneutria nigriventer* that blocks calcium channels coupled to exocytosis

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1 The aim of the present experiments was to investigate the pharmacological action of a toxin from the spider *Phoneutria nigriventer*, Tx3-3, on the function of calcium channels that control exocytosis of synaptic vesicles.

2 Tx3-3, in confirmation of previous work, diminished the intracellular calcium increase induced by membrane depolarization with KCl (25 mM) in rat cerebrocortical synaptosomes. The toxin was very potent (IC_{50} 0.9 nM) at inhibiting calcium channels that regulate calcium entry in synaptosomes. In addition, Tx3-3 blocked the exocytosis of synaptic vesicles, as measured with the fluorescent dye FM1-43.

3 Using ω-toxins that interact selectively with distinct neuronal calcium channels, we investigated whether the target of Tx3-3 overlaps with known channels that mediate exocytosis. The results indicate that the main population of voltage-sensitive calcium channels altered by Tx3-3 can also be inhibited by ω-agatoxin IVA, an antagonist of P/Q calcium channels. ω-conotoxin GVIA, which inhibits N type calcium channels did not decrease significantly the entry of calcium or exocytosis of synaptic vesicles in depolarized synaptosomes.

4 It is concluded that Tx3-3 potently inhibits ω-agatoxin IVA-sensitive calcium channels, which are involved in controlling exocytosis in rat brain cortical synaptosomes.

Keywords: Phoneutria toxin; exocytosis; calcium channels; FM1-43; ω-agatoxin IVA; ω-conotoxin GVIA; ω-conotoxin MVIIC; synaptic vesicles; synaptosomes

Introduction

Voltage-sensitive calcium channels have a key role in regulating diverse cellular functions including neuronal communication (reviewed by Dunlap *et al*., 1995; Reuter, 1996). Several classes of neuronal type calcium channels that have distinct molecular, biophysical and pharmacological characteristics have been identified, adding complexity to calcium signalling in the nervous system (Snutch *et al*., 1991; Zhang *et al*., 1993; Stea *et al*., 1994, reviewed by Dunlap *et al*., 1995; Varadi *et al*., 1995).

In relation to neurotransmission, the N and P/Q classes of channels have been shown to interact physically with proteins relevant to synaptic vesicle function (Yoshida *et al*., 1992, Lévêque *et al*., 1994, Martin-Moutot *et al*., 1996), to be geographically located at sites of stimulated exocytosis (Westenbroek *et al*., 1995), and to control the release of several neurotransmitters (Turner *et al*., 1992; 1993; Luebke *et al*., 1993; Wheeler *et al*., 1994). L type channels seem to be important for coupling of stimulus-secretion in retinal bipolar cells, but they appear to contribute little to transmitter release in less specialized neurones. (Dunlap *et al*., 1995).

In the last few years, a combination of protein purification, molecular analysis and electrophysiological experiments has revealed several toxins present in the venom of spiders and marine snails that interact with voltage-gated calcium channels (reviewed by Olivera *et al*., 1994). The ω-agatoxin IVA (ω-AGA IVA), from the spider *A. aperta* and several conotoxins obtained from *Conus* snails have been particularly useful for the investigation of calcium channels that control neurosecretion, as they can selectively interact with different classes of channels and alter their function (Olivera *et al*., 1994).

We have previously shown that a toxin present in the venom of the spider *Phoneutria nigriventer*, named Tx3-3, acts as a calcium channel blocker by decreasing calcium entry that contributes to glutamate release (Prado *et al*., 1996). Calcium-dependent glutamate release is thought to occur by exocytosis of synaptic vesicles, suggesting that the phoneutria toxin interferes with a population of calcium channels that is involved with exocytosis (Dunlap *et al*., 1995). However, there has been no attempt to differentiate between the different class of channels that can be inhibited by Tx3-3 in synaptosomes.

In the present study we have extended our previous work by testing the ability of Tx3-3 to interfere with exocytosis of synaptic vesicles, evoked by depolarization in mammalian nerve terminals. We used the fluorescent dye FM1-43, that has been shown to label synaptic vesicles in an activity-dependent way, and allows the estimation of exocytosis in nerve endings (Betz *et al*., 1992; Meffert *et al*., 1994; Henkel *et al*., 1996). In addition, by taking advantage of toxins known to interact with distinct calcium channels, we have also investigated the main classes of presynaptic voltage-gated calcium channels that can control regulated exocytosis in rat brain cortical synaptosomes depolarized with 25 mM KCl.

Methods

Purification of synaptosomes

Adult Wistar rats of either sex (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 first centrifuged ($1000 \times g$ for 10 min) and synaptosomes were isolated from supernatant by discontinuous Percoll-density-gradient centrifugation (Dunkley *et al*., 1988). Isolated nerve terminals were

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resuspended in HEPES-buffered salt solution (HBSS: 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, pH 7.4), to a concentration of 10 mg ml^{-1}, divided into aliquots of 200 μl and kept on ice until loaded with fura-2 AM. Synaptosomes were diluted to 0.3 mg ml^{-1} and divided into aliquots of 2 ml and kept on ice for FM1-43 experiments (see below).

Measurements of intrasynaptosomal free Ca^{2+} concentrations

Synaptosomal suspensions (10 mg ml^{-1}) were incubated for 30 min (35°C) with fura-2 AM (stock solution 1 mM in DMSO), to a final concentration of 5 μM and then diluted with medium to a concentration of 1.2 mg ml^{-1} protein. After a further 30 min incubation period, fura-2 labelled synaptosomes were washed, resuspended in medium (1 mg ml^{-1}) and immediately used for ratiometric quantification of intracellular free calcium ($[Ca^{2+}]_i$) (Grykiewicz *et al*., 1985) in a PTI spectrofluorimeter. Fluorescence emission was recorded at 500 nm at an excitation ratio of 330/370. Calibration of fura-2 signals and estimation of synaptosomal autofluorescence were performed as described by Prado *et al*. (1996). Calcium (2 mM, final concentration) was added to the synaptosomal suspension 30 s after the start of each assay. Isolated nerve terminals were stirred throughout the experiment in a cuvette maintained at 35°C. According to the experiment, the toxins were added to the synaptosomal suspension 30 min before depolarization with 25 mM KCl. Maximum concentrations of toxin were also effective when incubated for 5 min (not shown). The IC_{50} value for the inhibition by Tx3-3 of intracellular calcium changes evoked by KCl was calculated, by use of the logistic function on the dose-response curve of Figure 1 with SigmaPlot software (JandelScientific).

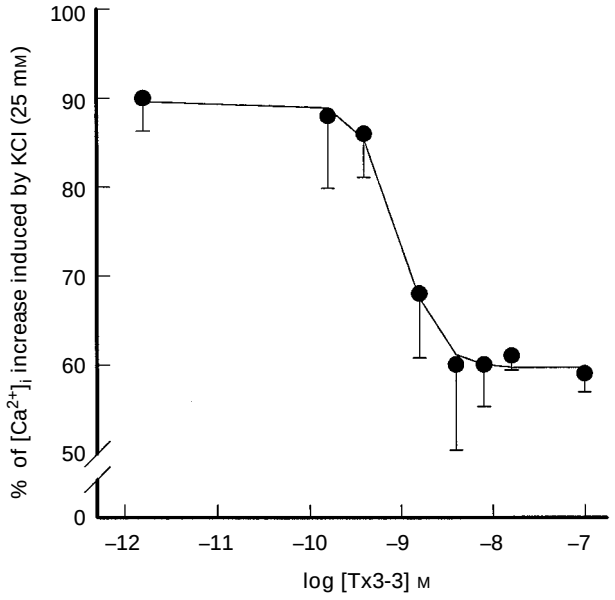

Figure 1 Effect of Tx3-3 on $[Ca^{2+}]_i$ increase induced by KCl. Rat cerebrocortical synaptosomes were loaded with fura-2 as described in Methods and then incubated in the presence or absence of Tx3-3, at various concentrations, for 30 min. Synaptosomal suspensions were transferred to a fluorimeter and then depolarized with 25 mM KCl. The variation of $[Ca^{2+}]_i$ (stimulated-basal) in the presence of toxin is plotted as a percentage of that induced by KCl. The symbols are the mean for five (16 nM), four (100 nM) and three (other concentrations) experiments; vertical lines show s.e.mean. The line represents the curve-fitting generated with the logistic function. The average increase of $[Ca^{2+}]_i$ by KCl in control conditions was 183 ± 10 nM.

Exocytosis measurements

Rat cerebrocortical synaptosomes (usually obtained from one rat), were incubated for 45 min at 35°C, and then exposed to FM1-43 (4 μM) in the presence of calcium (2 mM) for 10 min. Synaptosomes were labelled with FM1-43 according to the protocol of Meffert *et al*. (1994) with minor modifications. Synaptosomes were depolarized with KCl (40 mM) for 1 min, washed three times with HBSS containing BSA (1 mg ml^{-1}) and transferred to a cuvette. Depending on the experiment, samples were incubated for 300 s in the presence or absence of calcium-channel blockers in HBSS with calcium (2 mM) before the fluorescence was recorded. After this incubation period, we monitored FM1-43 fluorescence for variable times (typically 400 s) and KCl (25 mM or 60 mM) or TsTX were added with the help of an automatic dispenser 40 s after the start of the fluorimetric assay. Samples were excited at 488 nm and fluorescence emission (560 nm with 10 nm bandwidth filter) was recorded every second.

Synaptosomes loaded with FM1-43 showed a time-dependent decrease in fluorescence even in the absence of depolarization. We assumed that this corresponded to spontaneous exocytosis that occurs in our basal conditions, thus for each of the experiments this fluorescence decrease was also recorded. The experiments were performed with continuous stirring at room temperature (approximately 28°C), because higher temperatures lead to an excessive increase in the basal output of FM1-43. In addition, control experiments performed in the absence of calcium indicate that close to 20% of the basal release depended on calcium entry into synaptosomes (not shown). To estimate the amount of exocytosis induced by depolarization during exposure to different pharmacological agents, the magnitude of the fluorescence decrease after KCl addition was calculated in the presence of channel blockers and normalized to that induced by KCl in the absence of drugs.

Chemicals

FM1-43 was obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). ω-Conotoxin MVIIC (ω-CmTX MVIIC) and ω-AGA IVA were purchased from Peptides (Japan); ω-conotoxin GVIA (ω-CgTX GVIA), Percoll, fura-2 acetoxymethyl ester (AM), bovine serum albumin (BSA), HEPES and cadmium chloride were obtained from Sigma Chemical Co. (MO, U.S.A.) The Tx3-3 was purified according to Cordeiro *et al*. (1993) and tityustoxin (TsTX) was obtained according to Gomez and Diniz (1966). All other reagents were of analytical grade obtained from commercial sources.

Statistical analysis

Analysis for significance of differences between the pharmacological treatments was done by one-way analysis of variance followed by the least significant difference test.

Results

Effect of Tx3-3 on $[Ca^{2+}]_i$

Depolarization with KCl (25 mM) induced an increase of 200 nM (from 200 to 400 nM) on average $[Ca^{2+}]_i$ measured with fura-2 in cortical synaptosomes. The Tx3-3, incubated for 30 min with the synaptosomal suspension, decreased this effect evoked by depolarization in a dose-dependent way (Figure 1). The IC_{50} for the inhibition by the phoneutria toxin of KCl-induced changes in intracellular calcium was around 0.9 nM. There was no statistical difference between the effect of the toxin at 8, 16 or 100 nM, thus the concentration of 8 nM was chosen to perform most of the subsequent experiments.

In order to test whether the Tx3-3 targeted channels overlap with known neuronal classes of calcium channels, such as those blocked by ω-AGA IVA and ω-CgTX GVIA, we mea-

sured intracellular calcium changes induced by KCl (25 mM) in the presence of different toxins. Figure 2 shows that ω-AGA IVA (100 nM) and Tx3-3 (8 nM) decreased by about 50% the changes in $[Ca^{2+}]_i$ induced by 25 mM KCl when exposed to synaptosomes 30 min before depolarization. In contrast, ω-CgTX GVIA under the same conditions had little interference (12%) with the KCl-induced calcium influx measured by the fura-2 signal. The effect of ω-AGA IVA (100 nM) was saturating, as higher concentrations of the toxin did not produce larger effects (mean $\pm$ s.d.: 116 ± 8 and 113 ± 10 nM for the increase in $[Ca^{2+}]_i$ induced by 25 mM KCl in the presence of 1 μM and 100 nM ω-AGA IVA, respectively). To test for overlapping of calcium channels inhibited by ω-AGA IVA (100 nM) and Tx3-3 (8 nM), these toxins were incubated together for 30 min with the synaptosomal suspension before KCl (25 mM) depolarization. Although the joint application of both toxins appears to cause a larger inhibition, the difference between this inhibition and that induced by Tx3-3 failed to achieve statistical significance.

Effect of the Tx3-3 on the exocytosis of synaptic vesicles measured with FM1-43

In order to investigate the action of the Tx3-3 on the exocytosis of synaptic vesicles, we first studied the characteristics of FM1-43 release from synaptosomes under our experimental conditions. This dye is much more fluorescent when partitioned in membranes, therefore after staining, dye release from synaptic vesicles can be followed as a decrease in fluorescence (Betz *et al*., 1992; Meffert *et al*., 1994). In synaptosomes loaded with FM1-43 (see Methods), addition of KCl (25 mM or 60 mM) induced a significant drop in fluorescence (Figure 3), most likely reflecting dye release from exocytotic vesicles. Synaptosomes depolarized with 60 mM KCl showed a somewhat larger degree of dye release in the initial moments of depolarization when compared to that evoked by 25 mM KCl.

Depolarization with KCl is believed to open calcium channels directly, and in agreement with this premise tetrodotoxin did not interfere with KCl-induced release of FM1-43 (not shown). In contrast, when sodium channels were main-

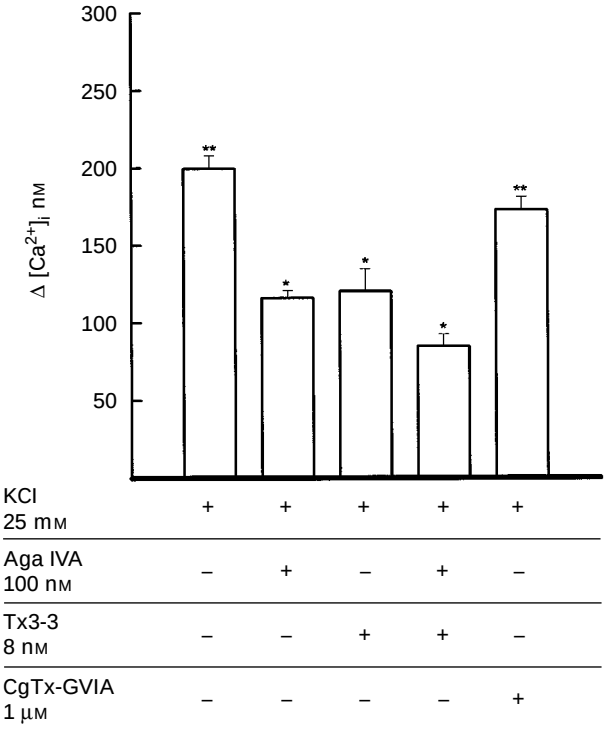

Figure 2 Effect of Tx3-3, ω-AGA IVA and ω-CgTx GVIA on $[Ca^{2+}]_i$ increase induced by KCl. Synaptosomes were loaded with fura-2 as described in Methods. Toxins were preincubated with the synaptosomal suspension for 30 min before fura-2 fluorescence was followed. After recording basal $[Ca^{2+}]_i$ for 6 min, the suspension was depolarized with 25 mM KCl. $[Ca^{2+}]_i$ variation represents the levels of calcium after KCl addition subtracted from the basal $[Ca^{2+}]_i$. Columns are the means $\pm$ s.e.mean for three experiments. $^*P<0.05$ compared to KCl; $^{**}P<0.05$ compared to ω-Aga IVA + Tx3-3.

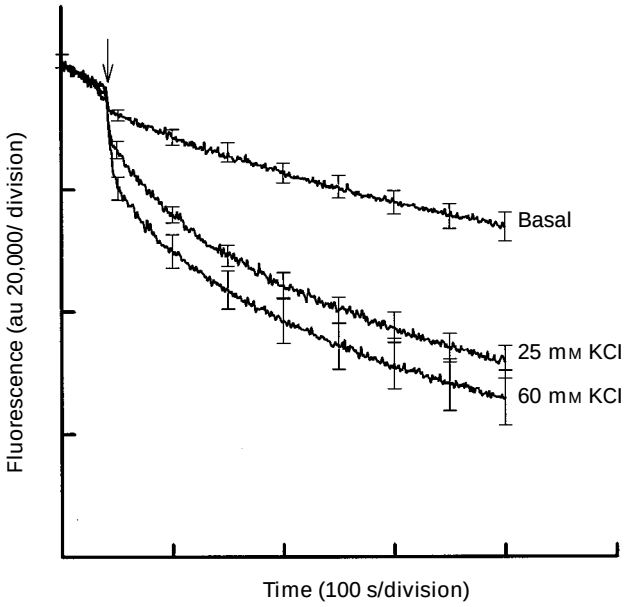

Figure 3 FM1-43 destaining evoked by membrane depolarization. Rat cerebrocortical synaptosomes were incubated in presence of FM1-43 (4 μM) and calcium (2 mM), depolarized with KCl (40 mM), washed in dye-free medium and transferred to a fluorimetre cuvette for fluorescence measurements (see Methods). FM1-43 release was monitored after addition of calcium (2 mM) to the synaptosomal suspension. The plot represents mean of thirty-two (pooled basal), twelve (25 mM KCl) and twenty-three (60 mM KCl) normalized fluorescence records; vertical lines show s.e.mean. Arrow indicates addition of KCl or medium (basal traces). au – arbitrary units.

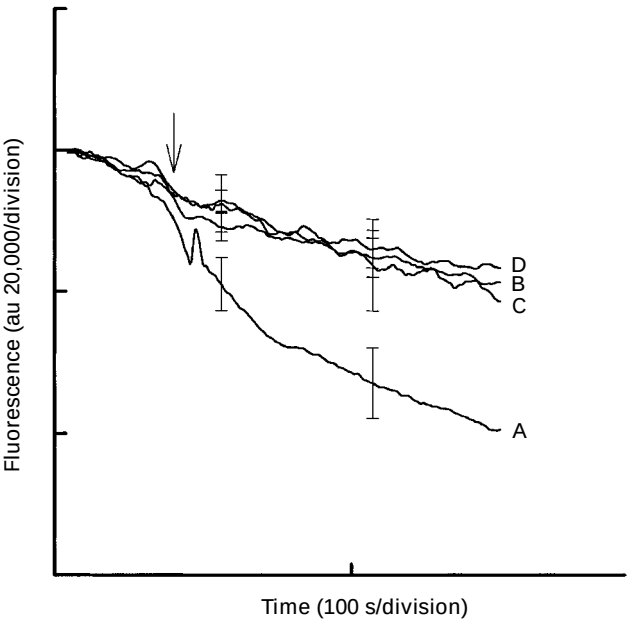

Figure 4 FM1-43 destaining induced by opening of voltage-sensitive sodium channels. Synaptosomes loaded with FM1-43 as described in Figure 3 and Methods were preincubated for five minutes (Ca^{2+} 2 mM) in the absence (A) or presence of TTX (5 μM, C) or cadmium (100 μM, D). At the arrow, TsTX (0.5 μM) (A, C and D) or control medium (B) were added to the synaptosomal suspension. The traces are mean for three experiments, vertical lines show s.e.mean. au – arbitrary units.

tained in an open state with a scorpion toxin, TsTX (0.5 μM, Gomez *et al*., 1995), the release of FM1-43 was diminished both by Cd^{2+} (100 μM) and tetrodotoxin (5 μM, Figure 4).

It is generally accepted that FM1-43 labelling of the synaptic vesicles depends on at least one cycle of exo-endocytosis (Betz *et al*., 1996). Thus, we tested whether suppression of exocytosis during FM1-43 loading, by omitting calcium from the incubation medium, altered the labelling of synaptosomes with dye. Figure 5a shows that depolarized (60 mM KCl) synaptosomes labelled in the absence of calcium release much less FM1-43 than those labelled when calcium was present. In fact, synaptosomes labelled in the absence of calcium had a decrease of fluorescence similar to basal samples.

FM1-43 release from nerve endings has been shown to be dependent on calcium entry in a variety of preparations (Betz *et al*., 1992; 1996; Meffert *et al*., 1994) and the above experiments confirmed that for loading synaptic vesicles with the dye. Figure 5b shows that KCl (60 mM) also induces a large calcium-dependent release of FM1-43. In this experiment, the traces obtained for KCl depolarization in normal conditions were subtracted from those obtained in the presence of 100 μM Cd^{2+}. It is clear that most of the decrease in fluorescence from depolarized synaptosomes occurs due to calcium entry.

After confirming that FM1-43 release can be used to follow exocytosis of synaptic vesicles in synaptosomal preparations, we tested the effect of Tx3-3 on exocytosis. Figure 6 (inset) shows the fluorescence change induced by KCl (25 mM) in the presence and absence of Tx3-3 (8 nM) incubated for 5 min before the measurements were taken. The results (a representative experiment out of the three performed) show that Tx3-3 was able to decrease almost totally the depolarization-evoked exocytosis of synaptic vesicles. Because the channels that are inhibited by Tx3-3 can have a major influence on the exocytosis of synaptic vesicles, it seemed of importance to investigate the sensitivity of FM1-43 release to blockers of voltage-gated calcium channels with known selectivity. Thus, we evoked FM1-43 release in the presence or absence of several pharmacological manipulations known to interfere with the function of neuronal type calcium channels. In these experiments, the magnitude of the fluorescence drop, evaluated as a variation of the fluorescence before and after addition of KCl, is expressed as a percentage of KCl-induced exocytosis, to account for any variations between experiments performed in different trials. The spontaneous fall in fluorescence observed

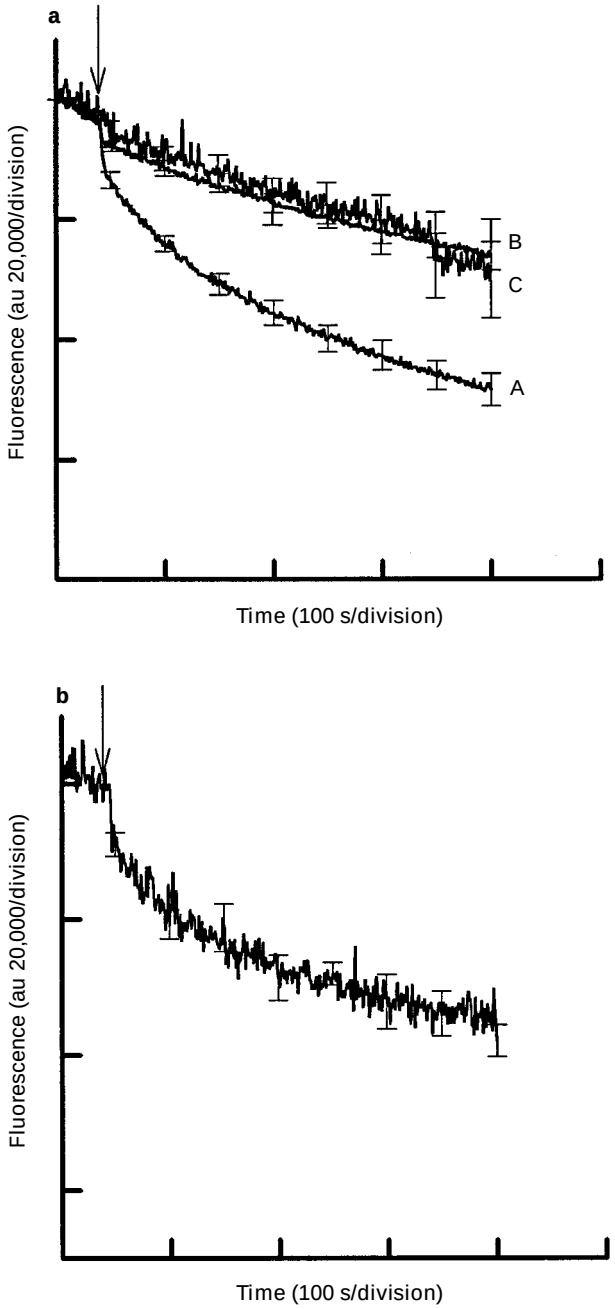

Figure 5 FM1-43 labelling and release are calcium-dependent. (a) Synaptosomes were labelled as described in Methods in the presence (A,B) or absence of 2 mM Ca^{2+} (C). After washing the excess of dye, FM1-43 release was monitored. Arrow indicates addition of 60 mM KCl (A and C) or control medium (B). The traces represent mean for three experiments; vertical lines show s.e.mean. au-arbitrary units. (b) Synaptosomal suspensions loaded with the styryl dye FM1-43 (see Methods) were incubated for five minutes in the presence or absence of cadmium (100 μM) in HBSS with Ca^{2+} (2 mM) before the beginning of the experiment. After that, the record of FM1-43 fluorescence was started and 60 mM KCl was added to the synaptosomes at 40 s (arrow). The traces in the absence of cadmium were normalized and subtracted from those obtained from cadmium-treated synaptosomes. The plot shows the mean of three experiments; vertical lines show s.e.mean. au-arbitrary units.

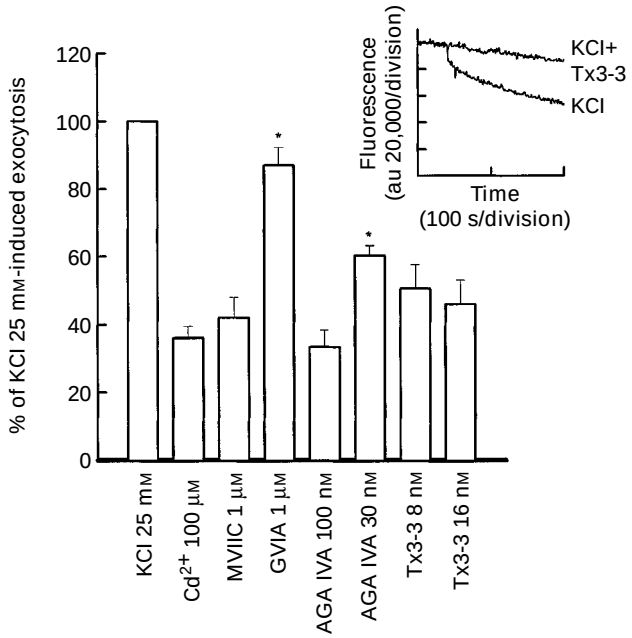

Figure 6 Effect of Tx3-3, ω-AGA IVA and ω-CgTx GVIA on exocytosis. Synaptosomal suspensions loaded with FM1-43 were exposed to distinct calcium channels antagonists for 340 s before depolarization with KCl (25 mM). Results are plotted as the percentage of the KCl-induced exocytosis in control conditions (no antagonists). Columns show the mean $\pm$ s.e.mean for three experiments in each condition. The inset shows the traces for KCl (25 mM)-induced FM1-43 release in the presence and absence of Tx3-3 (8 nM). $^*P<0.05$ in comparison to cadmium.

in control experiments amounted to 40% of the KCl (25 mM)-induced exocytosis (not shown). Depolarization by KCl 25 mM induced a decrease in fluorescence that was sensitive to the non-specific calcium channel blocker ω-CmTX MVIIC (1 μM) in a way similar to Cd^{2+} (Figure 6). To test for the involvement of specific calcium channels to trigger exocytosis in mammalian nerve terminals, the action of more selective antagonists on FM1-43 release evoked by 25 mM KCl stimulation was investigated (Figure 6). The results indicate that suppression of calcium entry through ω-CgTx GVIA-sensitive calcium channels has little effect on FM1-43 destaining (13%), whilst blockade of calcium channels by ω-AGA IVA (100 nM) abolished synaptic vesicle exocytosis induced by 25 mM KCl. This effect of ω-AGA IVA was dose-dependent, and a concentration that is believed to block P-type channels (30 nM, Randall & Tsien, 1995), without interfering with the Q type, had a somewhat smaller effect on exocytosis ($P<0.05$ compared to inhibition by Cd^{2+}). The inhibition obtained for Tx3-3 (8 and 16 nM) was not statistically different from that seen in cadmium-treated nerve terminals ($P>0.05$).

FM1-43 release due to depolarization by 60 mM KCl was not blocked by any of the toxins tested (not shown), even though it showed sensitivity to Cd^{2+} (Figure 5b). This suggests that a voltage-gated calcium channel resistant to known toxins (Turner & Dunlap, 1995), or another mechanism for calcium entry, such as the reversion of the Na^{2+}/Ca^{2+} exchanger (Romano-Silva *et al*., 1993; Reuter & Porzig, 1995), may contribute to exocytosis in 60 mM KCl depolarized nerve-endings.

Discussion

Tx3-3 is a protein of 6.3 Kd the partial amino acid sequence of which shows weak homology with other toxins known to block calcium channels (Cordeiro *et al*., 1993). The present experiments were aimed to investigate the action of this *Phoneutria nigriventer* toxin on "exocytotic' calcium channels. Previous work has shown Tx3-3 to affect the calcium-dependent glutamate release induced by KCl, by interfering with a step related to the entry of calcium in synaptosomes, as glutamate release evoked by calcium ionophore was not altered (Prado *et al*., 1996). However, these previous experiments provided little information concerning calcium channels that could be blocked by Tx3-3.

We have investigated the pharmacological action of Tx3-3 on intracellular calcium increase and exocytosis induced by depolarization in nerve endings. The increase in the levels of intracellular calcium is totally dependent on external calcium (Romano-Silva *et al*., 1993), suggesting that it reflects calcium entry into synaptosomes. We have focused on two main questions: whether Tx3-3 interacts with targets of known calcium channel blockers; and whether the channels inhibited by Tx3-3 contribute to exocytosis of neurotransmitters.

The phoneutria toxin was very potent at blocking intracellular changes of calcium in depolarized synaptosomes (IC_{50} 0.9 nM). In an attempt to determine the types of calcium channels that can be blocked by Tx3-3 at nerve terminals, we took advantage of the action of several toxins which inhibit the increase of intracellular calcium induced by KCl.

Block of N type channels by ω-CgTX GVIA induced a modest reduction of KCl evoked calcium changes (12%), but this was not statistically significant. In contrast, Tx3-3 and the P/Q calcium channel blocker ω-Aga IVA were able to decrease calcium entry in synaptosomes, as seen by their block of calcium increases induced by KCl. The joint effect of these two spider toxins was somewhat larger than that of the Tx3-3, yet this difference was not statistically significant. Thus, the results reflect some overlapping population of channels as target for both toxins, but it is possible that the Tx3-3 may also influence other channels.

Measurements of exocytosis provide a good indication of transmitter release. Capacitance measurements can be used to follow exocytosis in cells that can be recorded with microelectrodes (reviewed by Matthews, 1996), but the small size of nerve endings in mammalian brain makes it difficult to use this method. However, the optical techniques introduced by Betz and co-workers, with the fluorescent dye FM1-43, can be used to follow exocytosis in a variety of nerve-endings, including isolated nerve terminals (Betz *et al*., 1992; Meffert *et al*., 1994). As FM1-43 labels all activated nerve endings, this method provides information on calcium channels that are relevant for exocytosis in the majority of purified nerve terminals.

We tested whether FM1-43 labelling and release can give a good indication of exocytosis from synaptosomes in our conditions. The results obtained confirm that FM1-13 release elicited by chemical depolarization (KCl and TsTX) is dependent on entry of calcium, and in the case of TsTX also on sodium influx. In addition, labelling of synaptosomes was dependent on active exocytosis, as it was greatly diminished in the absence of calcium influx. Therefore, our data agree well with earlier experiments performed by Meffert *et al*. (1994) and also with the proposed mechanism for FM1-43 labelling of synaptic vesicles (Betz *et al*., 1996).

The results show that the Tx3-3, ω-AGA IVA, and ω-CmTX MVIIC can abolish the exocytotic response induced by KCl (25 mM). The small effect of the ω-CgTX GVIA (13%), which causes a persistent block of N-type calcium channels, may indicate that these channels do not contribute much to exocytosis, a result that agrees with the small interference of ω-CgTX GVIA (12%) with changes on intracellular calcium induced by KCl in synaptosomes. However, cortical synaptosomes are likely to be heterogeneous regarding their population of channels, and the results with ω-CgTX GVIA may also be explained if the toxin could suppress calcium entry and exocytosis in a small number of nerve terminals (12–13%).

Nonetheless, the results point to a major contribution of ω-AGA IVA sensitive channels (P/Q family) to exocytosis measured with FM1-43 in synaptosomes. The ω-AGA IVA has been shown to interact selectively with non-L and non-N calcium channel populations in rat dorsal root ganglion neurones (Mintz *et al*., 1992a), and it abolishes most of the calcium currents in cerebellar Purkinje cells (Mintz *et al*., 1992b). Recently, Randall and Tsien (1995) showed that ω-AGA IVA-sensitive currents found in rat cerebellar granule neurones have two components: one blocked with an IC_{50} of 90 nM (called Q-type current) and a second component which is more potently inhibited by ω-AGA IVA with IC_{50} of 2 nM (P-type current, Llinás *et al*., 1989, Mintz *et al*., 1992b). If it is accepted that calcium channels in nerve terminals resemble their counterparts from cell bodies, the two classes of channels above (P/Q) may take part in controlling exocytosis evoked by KCl (25 mM). Experiments performed with ω-AGA IVA at a lower concentration (30 nM) indicate that there is still a major component of exocytosis that can be blocked. Yet, by increasing the concentration of ω-AGA IVA, exocytosis could be totally abolished (Figure 6), suggesting that P-type channels may contribute more than Q-type for exocytosis. Therefore, inhibition of both channels is necessary to impair stimulated synaptic vesicle fusion. Whether the biophysical and pharmacological properties of channels in nerve terminals are similar to those in cell bodies remains to be determined and, in fact, there have been suggestions that channels in nerve endings have pharmacological differences when compared to those in neuronal bodies (Turner *et al*., 1995). Since the molecular counterparts of P and Q channels have not been clearly identified, and the distinction between these two classes may depend on subunit composition or alternative splicing (see review by Dunlap *et al*., 1995), there is a possibility that ω-AGA IVA targets only one population of channels in nerve endings with an intermediate potency between that which interferes with P or Q type channels in neuronal bodies.

The similarity of effect seen for Tx3-3 and ω-AGA IVA on exocytosis suggests that they are able to inhibit the same ca-

tegories of voltage-gated channels. Thus, Tx3-3 in a concentration that produces maximal inhibition on intracellular calcium changes also impaired exocytosis. We thus conclude that Tx3-3 is able to interact with ω-AGA-sensitive channels (perhaps P/Q class of calcium channels), although it is possible that other channels can also be inhibited. It should be noted that the present experiments provide no information as to whether Tx3-3 totally inhibits the passage of calcium through channels. Thus, it is conceivable that the toxin had a partial effect on ω-AGA-sensitive channels and affected other channels also partially.

In summary, ω-AGA-sensitive channels, likely of the P/Q class, contribute largely to calcium entry coupled to exocytosis of synaptic vesicles in isolated nerve endings from rat brain cortex. Tx3-3 from the *Phoneutria nigriventer* venom can block "exocytotic' calcium channels similar to those blocked by ω-AGA IVA. These results agree with recent observations that Tx3-3 can inhibit distinct neuronal calcium currents (Leão *et al*., unpublished observations), thus supporting the hypothesis that Tx3-3 is a novel ω-toxin.

Toxins that interfere with discrete populations of calcium channels are proving to be useful in regulating transmitter release in pathological conditions, such as ischaemic injuries and pain in terminal patients (Miljallich & Ramachandran, 1995). Therefore, this and other toxins from *Phoneutria nigriventer* with calcium channel blocking activities (Prado *et al*., 1996), may provide a new source of drugs for therapeutic intervention of neuronal calcium channels.

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