Inhibition of glutamate uptake by a polypeptide toxin (phoneutriatoxin 3-4) from the spider *Phoneutria nigriventer*

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Glutamate concentration increases significantly in the extracellular compartment during brain ischaemia and anoxia. This increase has an important Ca^{2+} -independent component, which is due in part to the reversal of glutamate transporters of the plasma membrane of neurons and glia. The toxin phoneutriatoxin 3-4 (Tx3-4) from the spider *Phoneutria nigriventer* has been reported to decrease the evoked glutamate release from synaptosomes by inhibiting Ca^{2+} entry via voltage-dependent Ca^{2+} channels. However, we report here that Tx3-4 is also able to inhibit the uptake of glutamate by synaptosomes in a time-

dependent manner and that this inhibition in turn leads to a decrease in the Ca^{2+} -independent release of glutamate. No other polypeptide toxin so far described has this effect. Our results suggest that Tx3-4 can be a valuable tool in the investigation of function and dysfunction of glutamatergic neurotransmission in diseases such as ischaemia.

Key words: brain ischaemia, calcium, glutamate transport, synaptosomes.

INTRODUCTION

Glutamate, the major excitatory neurotransmitter in the central nervous system, has key roles in brain function and dysfunction. The release and homeostasis of glutamate involve synthesis, packaging into synaptic vesicles, release via exocytosis, and subsequent reuptake of glutamate by specialized transporters in the plasma membrane of neurons and glial cells. There is no evidence for an enzyme that inactivates glutamate at the synaptic cleft; termination of its action therefore relies on reuptake by neurons and glia [1]. Prolonged contact of neurons with glutamate results in neurotoxicity and cell death [2].

Several isoforms of amino acid transporters, named EAAT1 (GLAST), EAAT2 (GLT-1), EAAT3 (EAAC1), EAAT4 and EAAT5, have been identified in the central and peripheral nervous systems of different species [3–8]. Their function relies on the Na^+ gradient across the plasma membrane and on the countertransport of K^+ and OH^- [1]. Therefore changes in the concentration of these ions can alter transport function up to the point of reversing its activity, resulting in net glutamate release [9]. This release is distinguished from exocytotic release by the fact that it is independent of Ca^{2+} influx via selective voltage-dependent channels. In brain cortical synaptosomes depolarized with KCl, approx. 50 % of measured glutamate release is Ca^{2+} -independent and is mediated by reversal of the glutamate carrier [10,11]. The transporter can be inhibited by several glutamate analogues such as dihydrokainate and β -D,L-threo-hydroxyaspartate. A polyamine from spider venom was shown to inhibit glutamate uptake [12] but no further investigation has been reported since.

The venom of the spider *Phoneutria nigriventer* contains several neurotoxic peptides with actions such as inhibition of the inactivation of Na^+ channels [13], blockage of K^+ channels [14]

and blockage of Ca^{2+} channels [15,16]. Among these peptides, the toxin phoneutriatoxin 3-3 has been shown to inhibit the evoked Ca^{2+} -dependent release of glutamate from synaptosomes [17]. Further studies showed that another toxin, phoneutriatoxin 3-4 (Tx3-4), had an inhibitory effect on Ca^{2+} uptake in synaptosomes [18]. Here we report that synaptosomes preincubated with Tx3-4 for 30 min and then depolarized with KCl have a decreased Ca^{2+} -independent release of glutamate, probably owing to inhibition of the activity of glutamate transporters. This opens interesting perspectives in the study of protein–protein interactions to shed more light on the transporter function and to allow the development of new drugs with therapeutic value in ischaemia and anoxia.

EXPERIMENTAL

Materials

Tx3-4 was purified as described by Cordeiro et al. [19], dissolved in water (80 μM) and stored in aliquots at -20°C . L-[^3H]Glutamate was purchased from Amersham International (Little Chalfont, Bucks., U.K.). Glutamate dehydrogenase (EC 1.4.1.3), β -NADP⁺ (1 mM stock solution in deionized water) and all other chemicals, unless stated otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of synaptosomes

Adult Wistar rats were killed by decapitation without the use of anaesthetics. Approval for this procedure was given by the local ethics committee for animal research. The brains were extracted, the hippocampi were dissected and the tissue was kept on ice throughout subsequent processing. Tissue was homogenized

Abbreviations used: GPs, glial plasmalemmal vesicles; Tx3-4, phoneutriatoxin 3-4.

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Figure 1 Effect of the toxin Tx3-4 on glutamate release in hippocampal synaptosomes

Synaptosomes prepared as described in the Experimental section were incubated with Tx3-4 at various concentrations indicated for 30 min before depolarization with 33 mM KCl. (A) Time course of glutamate release. After preincubation with Tx3-4 at the concentrations shown (0.16–16 nM) or positive control. (B) Dose–response curve generated from the data in (A) after 6 min of stimulation and expressed as percentages of inhibition of the control. Data are means $\pm$ S.D. for at least three separate experiments.

(0.1 g of tissue/ml) in gradient solution (0.32 M sucrose/1 mM EDTA/0.25 mM dithiothreitol, adjusted to pH 7.4 with 1 M NaOH). Synaptosomes were isolated by Percoll® gradient centrifugation [20] and resuspended in 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 M NaOH) at approx. 5.0 mg/ml protein, as described previously [11].

Preparation of glial plasmalemmal vesicles (GPVs)

GPVs were prepared as described in [21]. In brief, the hippocampi of Wistar rats were dissected and the tissue was kept on ice throughout subsequent processing. Tissue was homogenized (0.1 g of tissue/ml) in modified gradient solution (0.32 M sucrose/1 mM EDTA/0.25 mM dithiothreitol/20 mM Hepes, adjusted to pH 7.4 with 1 M NaOH) and centrifuged at 1000 g for 10 min. The supernatant was layered on a discontinuous gradient composed of 20%, 10%, 6% and 2% (v/v) Percoll in gradient solution. The tubes were centrifuged at 33 500 g for 5 min. The layer between 2% and 6% (v/v) Percoll was diluted (to 10 ml) and centrifuged at 1000 g for 20 min; the supernatant was centrifuged at 33 500 g for 40 min. The precipitate was washed twice with gradient solution and used as the GPV fraction.

Continuous glutamate release assay

Glutamate release was assayed by monitoring the increase in fluorescence due to the production of NADPH in the presence of NADP⁺ and glutamate dehydrogenase [10,11]. In experiments designed to assess the Ca²⁺-independent release of glutamate under different conditions, we omitted CaCl₂ from the assay buffer and added 2.0 mM EGTA.

Glutamate content of synaptosomes

Glutamate content was assayed as described above in samples of the supernatant of centrifuged synaptosomes incubated in the

presence of Tx3-4; the pellet was treated with Triton X-100 [0.05% (v/v) final concentration] before measurement of the glutamate remaining.

Assay for lactate dehydrogenase release

Synaptosomes were incubated for 1 or 30 min with Tx3-4 (8 nM) followed by centrifugation at 10 000 g for 15 s at room temperature. The supernatant was assayed for lactate dehydrogenase as described [22].

Uptake of L-[³H]glutamate

Synaptosomes or GPVs were diluted to a concentration of 0.8 mg/ml protein in HBSS without CaCl₂. Samples (500 μ l) were pipetted into tubes and incubated for 15 min at 35 °C. CaCl₂ (1 mM final concentration) was added to synaptosomes or GPVs and incubated with 0.35 μ Ci of L-[³H]glutamate for 30 s after preincubation with toxins (Tx3-4 or ω -conotoxin MVIIC) or control experiments. To stop the uptake, 5 ml of ice-cold HBSS was added; the synaptosomal or GPV suspension was then vacuum-filtered (2.3 μ m filter pore size). The filters were washed twice with 5 ml of HBSS containing 5 μ M non-radioactive glutamate. The washed filters were then transferred to scintillation vials containing 5 ml of scintillation cocktail [30% (v/v) ethanol/30% (v/v) dioxane/30% (v/v) toluene/1% (v/v) Triton X-100/7% (w/v) naphthalene/0.02% 1,4-bis-(5-phenyloxazol-2-yl)benzene ('POPOP')/0.5% 2,5-diphenyloxazole ('PPO')] and the radioactivity was quantified in a liquid-scintillation spectrophotometer (TR 1600 Tri Carb; Packard). Data in c.p.m. were converted to d.p.m. after correction for sample quenching and background subtraction.

Statistics

The statistical analysis for comparison between means ($\pm$ S.D.) was performed by analysis of variance (Sigma-Stat, Jandel

Corporation). Differences were considered significant at $P < 0.05$.

RESULTS

Concentration compared with effect of Tx3-4 on glutamate release

Figure 1 shows the dose-dependent effect of Tx3-4 on glutamate release evoked by 33 mM KCl. Concentrations of Tx3-4, ranging from 0.16 to 16 nM, progressively decreased the KCl-evoked release of glutamate from hippocampal synaptosomes, achieving a maximal effect (approx. 75% inhibition) at 8.0 nM (Figure 1A). Up to 32 nM Tx3-4 was tested, with results identical with those obtained for 8 and 16 nM, but for clarity these results are not shown. The amount of accumulated glutamate release, as a percentage of the control level, measured after 6 min of incubation with 33 mM KCl, was used to construct the concentration–effect plot (Figure 1B). An IC_{50} of 1.6 nM was calculated from the data shown in Figure 1(B), which corresponds to the inflexion point of the curve.

Time-dependence of the effect of Tx3-4

In the experiments shown in Figure 1, Tx3-4 (8 nM) was added 30 min before stimulation with 33 mM KCl. To characterize the time dependence of the toxin effect, preincubations of 1, 5, 15, 30 and 45 min with Tx3-4 were performed, followed by depolarization with KCl (Figure 2A). The addition of Tx3-4 (8 nM) at 1, 5 or 15 min before KCl caused approx. 50% inhibition of induced glutamate release. However, further inhibition (up to 75%) was observed only at incubations of 30 min or longer with Tx3-4. The KCl-induced release of glutamate from rat cortical synaptosomes has Ca^{2+} -dependent and Ca^{2+} -independent components [10]. The latter can be measured in the presence of the Ca^{2+} chelator EGTA. The Ca^{2+} -independent release evoked by 33 mM KCl is shown in Figure 2(A), as indicated (EGTA), and corresponds to approx. 47% of the total release. This is not different from the KCl-evoked release in the presence of 8 nM Tx3-4 ($P > 0.5$) added up to 15 min before KCl. However, when synaptosomes were preincubated for 30 or 45 min with Tx3-4, the KCl-evoked release was significantly smaller than that in the presence of EGTA alone, suggesting an inhibition of the Ca^{2+} -independent component.

Ca^{2+} -channel blocker ω -conotoxin MVIIC does not inhibit Ca^{2+} -independent glutamate release

Short-term incubation with Tx3-4 seems to block Ca^{2+} channels [18]; for comparative purposes we therefore tested the Ca^{2+} channel blocker ω -conotoxin MVIIC in the same experimental conditions as used for Tx3-4. ω -Conotoxin MVIIC is able to inhibit completely the Ca^{2+} -dependent release of glutamate evoked by KCl in synaptosomes [17,23]. Hippocampal synaptosomes were incubated for 1 or 30 min with ω -conotoxin MVIIC (1 μ M) followed by the addition of 33 mM KCl (Figure 2A). ω -Conotoxin MVIIC was able to inhibit KCl-evoked release after 1 min of incubation. The inhibition was not different from that in nominal Ca^{2+} -free medium (EGTA column; $P > 0.05$), however, no enhancement of the effect was observed when the preincubation period was extended to 30 min (Figure 2A).

Effect of Tx3-4 on glutamate uptake

Many lines of evidence suggest that the Ca^{2+} -independent release of glutamate is due to the reversal of the glutamate carrier of the plasma membrane [10,11,24]. Tx3-4 inhibited Ca^{2+} -independent

Figure 2 Time-dependence of the effect of Tx3-4 on release and uptake of glutamate

Synaptosomes and GPVs were prepared as described in the Experimental section. (A) The release of glutamate from synaptosomes preincubated with Tx3-4 for different periods (1, 5, 15, 30 or 45 min) before depolarization with KCl (33 mM) was compared with control and against synaptosomes preincubated with ω -conotoxin MVIIC (1 μ M) for 1 or 30 min. Ca^{2+} -independent glutamate release was measured in the presence of 2 mM EGTA, as indicated. *Significant difference ($P < 0.001$) from EGTA. (B) Synaptosomes were left to take up L-[3 H]glutamate for 30 s after being treated with 8 nM Tx3-4 for different periods (1, 5, 15 or 30 min) or with ω -conotoxin MVIIC (MVIIC) for 1 or 30 min. **Significant difference ($P < 0.05$) from Tx3-4 at 30 min. (C) GPVs were allowed to uptake L-[3 H]glutamate for 30 s after being treated with 8 nM Tx3-4 for 30 min. Data are means $\pm$ S.D. for at least three separate experiments. Abbreviation: DHK, dihydrokainate.

glutamate release; we therefore tested whether Tx3-4 could inhibit the activity of the glutamate transporter in hippocampal synaptosomes. Preincubation with Tx3-4 (8 nM) was able to inhibit L-[3 H]glutamate uptake by $36.9 \pm 12.5\%$ at 1 min and $34.9 \pm 15.7\%$ at 5 min of preincubation, and by $45 \pm 7.4\%$ and $63 \pm 9.4\%$ when the incubation was extended to 15 and 30 min respectively (Figure 2B). Under the same conditions, ω -conotoxin MVIIC (1 μ M) did not have any effect on uptake at 1 or 30 min of preincubation (Figure 2B).

GPVs possess a high capacity of glutamate uptake via GLT-1 and GLAST glutamate transporters [21,25]. We therefore investigated the effect of Tx3-4 on glutamate uptake in that preparation. Figure 2(C) shows that Tx3-4 (8 nM) was able to inhibit glutamate uptake in GPVs by only 9.5% after 30 min of preincubation with the toxin. Dihydrokainate was able to decrease uptake by 70% under the same conditions, showing that the uptake was due to glial glutamate transporters [21,26].

Additive effects of Tx3-4 and EGTA on glutamate release

To test whether Tx3-4 was able to affect the component of release that is independent of Ca^{2+} , synaptosomes were preincubated

Figure 3 Tx3-4 inhibition of the glutamate release from the Ca^{2+} -independent (cytoplasmic) pool

(A) Veratridine (VER; 10 μM) was incubated with synaptosomes in the presence or absence of EGTA (2 mM) to show that veratridine-evoked release was mainly Ca^{2+} -independent and sensitive to Tx3-4 (8 nM). (B) KCl- or veratridine-evoked glutamate release in the presence of EGTA (Ca^{2+} -independent release) was measured in synaptosomes preincubated with 8 nM Tx3-4 for 1 min (column labelled a) or for 30 minutes (column labelled b). *Significant difference ($P < 0.05$) from KCl + EGTA. Data are means $\pm$ S.D. for at least three separate experiments.

with Tx3-4 (8 nM) for 1 or 30 min, followed by the addition of EGTA (2 mM) and then KCl (33 mM) or veratridine (10 μM).

Veratridine is an alkaloid that binds to toxin site 2 of Na^+ channels and induces the release of glutamate from synaptosomes [27]. However, the release evoked by veratridine is predominantly Ca^{2+} -independent. Veratridine-evoked glutamate release was slightly inhibited (approx. 15%) in nominally Ca^{2+} -free medium. In contrast, it was inhibited by more than 60% when synaptosomes were preincubated with 8 nM Tx3-4 for 30 min (Figure 3A). Tx3-4 (8 nM) inhibited KCl- or veratridine-evoked Ca^{2+} -independent glutamate release by 59% and 64% respectively (Figure 3). As a control, synaptosomes were preincubated with Tx3-4 for 1 min before the addition of EGTA and KCl; the result was not different from that with EGTA plus KCl alone (Figure 3B).

Tx3-4 is not a competitive inhibitor of glutamate uptake

To characterize the inhibition of glutamate transport by Tx3-4, we analysed the transport kinetics in relation to the concentration of glutamate. L-[^3H]Glutamate uptake was measured as described above but in the presence of increasing concentrations of

Figure 4 The effect of Tx3-4 on glutamate uptake in the presence of increasing glutamate concentrations

Synaptosomes were left to take up L-[^3H]glutamate for 30 s in the presence (○) or absence (●) of Tx3-4. Tx3-4 (8 nM) markedly decreased the uptake of L-[^3H]glutamate independently of the glutamate concentration in the incubation medium. Data are means for at least three separate experiments.

glutamate, from 14 μM (basal free glutamate) to 4 mM. Glutamate uptake was plotted against increasing glutamate concentrations, in the presence (○) or absence (●) of Tx3-4 (Figure 4). In the presence of Tx3-4, glutamate uptake was markedly inhibited, as represented by the lower curve, independently of the glutamate concentration in the incubation medium. That suggests a non-competitive relationship. The values obtained for K_m were 47.6 μM (control) and 40.0 μM (Tx3-4); those for V_{max} were 1.33×10^7 molecules/s (control) and 3.36×10^6 molecules/s (Tx3-4). Thus the V_{max} was decreased but the K_m was essentially unchanged, suggesting that the inhibition of glutamate uptake by Tx3-4 does not rely on competition.

As a last control, to discard possible damage to the membrane by Tx3-4, which could have resulted in a glutamate leakage, we tested the integrity of synaptosomes by assaying the release of lactate dehydrogenase in the presence of Tx3-4. There was no difference between control and synaptosomes incubated with 8 nM Tx3-4 for 1 or 30 min (results not shown).

DISCUSSION

During brain ischaemia, the Ca^{2+} -independent accumulation of glutamate occurs extracellularly in nerve tissue [28–31]. A better comprehension of the mechanisms involved in this process is important in establishing the therapeutic window and prospective treatment procedures. Specific inhibitors of the glutamate carrier could aid these studies.

Our investigation started with the observation that the inhibitory effect of Tx3-4 on KCl-evoked glutamate release from synaptosomes was enhanced when preincubation with the toxin was prolonged from 1 to 30 min. This finding suggested that when Tx3-4 was preincubated for 30 min with synaptosomes, the Ca^{2+} -independent component of release was inhibited. This observation extends previous reports that Tx3-4 can decrease the KCl-evoked Ca^{2+} -dependent (exocytotic) release after a 1 min preincubation [17] and is reinforced by our finding that after 1 min of preincubation Tx3-4 had no effect on Ca^{2+} -independent glutamate release (Figure 3B).

The Ca^{2+} -independent release of glutamate is thought to occur via the reversal of the glutamate transporter of the plasma membrane [10]. It was shown in astrocytes that the Ca^{2+} -

independent release has a fast and a slow phase, when cells were depolarized with 100 mM KCl [32], the first and transient release being due to transporter reversal, and the second via an as-yet uncharacterized anion channel. Concentrations of KCl lower than 70 mM elicited the transient transporter-mediated release preferentially [32,33]. Because the anion channel involved in this phenomenon has not yet been demonstrated in neurons, and the concentration of KCl used in our experiments was 33 mM, we assume that the Ca^{2+} -independent release observed in our conditions, and clearly inhibited by Tx3-4, was mediated by the glutamate transporter.

Ca^{2+} -channel blockers are used as tools to investigate neurotransmitter release mechanisms. ω -Conotoxin MVIIC inhibits the elevation of intracellular $[\text{Ca}^{2+}]$ and the Ca^{2+} -dependent glutamate release evoked by KCl [15,17,23]. However, there are no reports of an effect of ω -conotoxin MVIIC on the Ca^{2+} -independent release of glutamate. We demonstrated, as expected, that ω -conotoxin MVIIC had no effect on L-[^3H]glutamate uptake measured in hippocampal synaptosomes after 1 or 30 min of incubation. Thus the impairment of glutamate uptake mediated by Tx3-4 could not be related to its action on Ca^{2+} channels.

Veratridine is a potent inducer of neurotransmitter release that also evokes Ca^{2+} -independent glutamate release owing to an increase in intracellular $[\text{Na}^+]$ [34]. Veratridine was therefore used as a positive control to compare with the depolarization evoked by 33 mM KCl. Tx3-4 (8 nM) inhibited veratridine-evoked release by approx. 65%, in comparison with the 15% inhibition in Ca^{2+} -free medium (Figure 3A). Thus, because veratridine depolarizes the membrane via a mechanism distinct from that of KCl, an artifact related to the conditions produced by depolarization with KCl, such as swelling [35], was less probable.

Our analysis of the kinetic of glutamate transport in hippocampal synaptosomes yielded an apparent K_m of 40 μM (Figure 4). This value is somewhat higher than that reported for synaptosomes from other brain regions. In cortex slices the apparent K_m is 3 μM [36], whereas in cerebellar synaptosomes from GLAST-gene-inactivated mice the affinity is 10 μM [37]. The glutamate transporter EAAC1 was reported to be the major subtype found in hippocampal neurons [38]. In cultured cells, this transporter has an apparent K_m of 17 μM for glutamate [39,40]. Again, this is a somewhat higher affinity than we measured, but within the same order of magnitude. The IC_{50} value obtained for the effect of Tx3-4 on KCl-evoked glutamate release was 1.6 nM, whereas the IC_{50} calculated for the inhibition of Ca^{2+} influx in cerebrocortical synaptosomes was 7.9 nM [18]. Despite the fact that the experiments were performed in different preparations, the calculated constants fall within a narrow range, in agreement with our results, in which the effect of toxin on the Ca^{2+} -dependent and Ca^{2+} -independent release occurs within the same concentration range.

Although it was shown that 1 min of preincubation with Tx3-4 was enough to decrease L-[^3H]glutamate uptake by approx. 40%, the effect on Ca^{2+} -independent release was detected only after preincubation for 30 min or more. This delay could be required to decrease the synaptosomal glutamate content to a critical level so as to impair release. In control conditions, after 1 min of preincubation, synaptosomes are loaded with glutamate to the same level as occurs at 30 min (approx. 80 nmol/mg of protein; results not shown). If basal release is considered, the glutamate content must be in a dynamic steady state in which uptake equals spontaneous release. When uptake is blocked, basal release continues, depleting the terminal of its glutamate. The effect of Tx3-4 on Ca^{2+} -independent release was therefore probably linked to a decrease in synaptosomal glutamate content.

In addition, owing to the high activity of glutamate transporters [41,42], an inhibition of uptake greater than 60% might be required to produce a significant impairment in the overall function. That would explain why no inhibition of Ca^{2+} -independent release was observed after 1 min of preincubation with Tx3-4, at a point at which uptake is already inhibited by 40%.

These observations could reflect the heterogeneity and distinct pharmacological sensitivity of glutamate transporters present in synaptosomes [43,44] and cultured cells [45,46]. In fact, the experiments performed with GPVs, which is a preparation rich in GLAST and GLT-1, reinforce this view, because a much smaller effect of Tx3-4 was observed in comparison with synaptosomes; this suggests that the toxin might have some specificity towards one or more neuronal types of glutamate transporter, such as EAAC1. To shed more light on these questions, further investigation of the effect of Tx3-4 on isolated systems is necessary. The toxin has been cloned by our group (L. Grossi, M. A. Romano-Silva, M. A. M. Prado, M. V. Gomez and E. Kalapothakis, unpublished work); future experiments are therefore aimed at expressing Tx3-4 *in vitro* and at producing variants of the peptide to gain a better understanding of its mechanism of interaction and to identify its binding site.

In conclusion, the toxin Tx3-4 could be the starting point for the development of more selective inhibitors of the glutamate transport, which would have immediate application to research and to potential therapeutic use in conditions in which decreasing the activity of glutamate transporters could be beneficial.

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