Modulation of Ca^{2+} -stimulated glutamate release from synaptosomes by Na^+ entry through tetrodotoxin-sensitive channels

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Tityustoxin (TsTX), a toxin obtained from the venom of the Brazilian scorpion *Tityus serrulatus*, stimulates Na^+ influx through tetrodotoxin (TTX)-sensitive Na^+ channels which, in turn, promotes both Ca^{2+} -dependent and Ca^{2+} -independent release of glutamate from rat cerebrocortical synaptosomes. The level of Ca^{2+} -dependent glutamate release after addition of $0.5 \mu\text{M}$ TsTX is greater than that produced by a maximally depolarizing concentration of KCl. This effect of TsTX, which is entirely dependent on Na^+ entry, suggests that Na^+ has a role in modulating Ca^{2+} entry and glutamate release that is not simply related to membrane depolarization. In order to investigate possible modulatory role(s) of Na^+ on Ca^{2+} -dependent glutamate

release, we compared the effects of TsTX with those of KCl and the Na^+ ionophore gramicidin D. When used alone, 100 nM gramicidin D produced a larger increase in intrasynaptosomal free Na^+ than did $0.5 \mu\text{M}$ TsTX, and a similar rise in intrasynaptosomal free Ca^{2+} , but was much less effective in promoting glutamate release. Even the combination of membrane depolarization (by 33 mM KCl) and elevation of intrasynaptosomal free Na^+ (by 100 nM gramicidin) was still less effective than TsTX at causing Ca^{2+} -dependent glutamate release. These data suggest that localized Na^+ entry, through TTX-sensitive Na^+ channels, exerts a modulatory role on Ca^{2+} -dependent glutamate release from nerve endings in the cerebral cortex.

INTRODUCTION

Voltage-sensitive Ca^{2+} channels (VSCC) play a pivotal role in the functioning of neurons. Pharmacological and molecular-cloning studies in a number of laboratories have revealed the existence of an ever-increasing number of distinct types of channels (see Zhang et al., 1993), and considerable effort is being expended in attempting to define the relationship between these VSCCs and the release of individual neurotransmitters. It has recently become clear that more than one type of VSCC may be required to regulate the secretion of a single neurotransmitter (Luebke et al., 1993), and that different types of channel may regulate the release of different neurotransmitters (Turner et al., 1993).

In recent years, we have been using spider and scorpion neurotoxins to investigate the relationships between Ca^{2+} entry through VSCCs and glutamate release from rat cerebrocortical synaptosomes (Romano-Silva et al., 1993, 1994). The toxins that we have been using act either by stimulating opening of Na^+ channels or by delaying their closing (Araujo et al., 1993; Linden and Raftery, 1976). Our initial hypothesis was that the enhanced Na^+ entry in the presence of the toxins would cause membrane depolarization and stimulate opening of VSCCs. However, we have recently discovered that the Ca^{2+} -dependent release of glutamate caused by tityustoxin (TsTX), from the scorpion *Tityus serrulatus*, is greater than can be accounted for solely on the basis of Na^+ entry simply causing membrane depolarization (Romano-Silva et al., 1994). The present study was designed to clarify the relationship between TsTX-mediated Na^+ entry and the activity of Ca^{2+} channels linked to glutamate release.

EXPERIMENTAL

Materials

Na^+ -binding benzofuran isophthalate (SBFI) was obtained from

Molecular Probes, Eugene, OR, U.S.A. Nifedipine, glutamate dehydrogenase (type II, $3 \times$ crystallized), fura-2 AM (acetoxymethyl ester), tetrodotoxin (TTX), Percoll and ionomycin were all obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Purification of TsTX

This was carried out by Dr. T. Moraes-Santos in the Faculty of Pharmacy, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil, by the method of Gomez and Diniz (1966). We are indebted to Dr. Moraes-Santos for his generous gift of TsTX. The toxin was stored at -20°C in distilled water at a concentration of $300 \mu\text{M}$ until required.

Isolation of synaptosomes

Adult male Wistar rats were killed by cervical fracture and their cerebral cortices dissected out on ice and homogenized in 9 vol. of 0.32 M sucrose/ 1 mM EDTA/ 0.25 mM dithiothreitol. Synaptosomes were then prepared from the homogenates by Percoll density-gradient centrifugation (Dunkley et al., 1988). The synaptosomes were resuspended in Krebs/Ringer/Hepes (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. 7.0 mg of protein/ml, divided in $200 \mu\text{l}$ portions and kept in ice until loaded with fura-2 AM, SBFI AM or used for measurement of glutamate release.

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

Fura-2 AM (stock solution 1 mM in dimethyl sulphoxide) was added to the synaptosomal suspensions to give a final con-

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; $[\text{Na}^+]_i$, intracellular free Na^+ concentration; TTX, tetrodotoxin; SBFI, Na^+ -binding benzofuran isophthalate; TsTX, tityustoxin; VSCC, voltage-sensitive Ca^{2+} channel.

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centration of $5.0 \mu\text{M}$ and the mixture was incubated at 37°C for 30 min. This was followed by dilution to 1.5 ml (approx. 5 mg of protein/ml) with Krebs/Ringer/Hepes ($-\text{Ca}^{2+}$) and another incubation for 30 min at 37°C . The interval between processing samples was fixed at 30 min, which was approximately the length of one experiment. In this way all samples could be processed under similar conditions. After loading with fura-2, synaptosomal suspensions were transferred to u.v.-transparent disposable plastic cuvettes (1 cm light path) and placed in the cuvette holder of a Cairn research spectrofluorimeter that was maintained at 37°C . Fluorescence emission was recorded at 500 nm by using 320/390 nm or 340/380 nm excitation ratios as previously described (Romano-Silva et al., 1993). The fluorescence was recorded for a period of 2 min, followed by transfer of the synaptosomes to a 1.5 ml plastic (Eppendorf) centrifuge tube and centrifugation for 30 s at 15000 rev./min. The supernatant, containing extracellular fura-2, was discarded, and the pellet resuspended in 1.5 ml of fresh medium and transferred to a plastic cuvette in the cuvette holder. Recording was restarted, and after 1 min $1.5 \mu\text{l}$ of 1 M CaCl_2 was added, giving a final concentration of 1.0 mM. Additions of KCl, TsTX and other materials were made as described in the text and calibration was carried out as previously described (Romano-Silva et al., 1993).

Measurement of intrasynaptosomal free Na^+ ($[\text{Na}^+]_i$)

This was accomplished by using the fluorescent indicator SBFI (Minta and Tsien, 1989). Synaptosomes were loaded with SBFI-AM ($10 \mu\text{M}$) for 1 h at 37°C in Krebs/Ringer/Hepes medium (see above) with NaCl replaced by an equimolar concentration of choline chloride, followed by centrifugation and resuspension in normal (NaCl-containing) medium (see above). Experiments were performed by using excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm.

Measurement of glutamate release

This was done by monitoring the increase in NADPH⁺ fluorescence after adding 1 mM NADP⁺ and 50 units of glutamate dehydrogenase (Nicholls et al., 1987; Romano-Silva et al., 1993).

Reproducibility

Traces shown are representative data from at least three independent experiments; the range of variation between groups of experiments was less than 10%.

RESULTS

In our initial experiments, we examined the effect of TsTX on $[\text{Na}^+]_i$, using the fluorescent Na^+ indicator SBFI (see Figure 1). We have shown previously that $0.5 \mu\text{M}$ TsTX causes a maximal increase in $[\text{Ca}^{2+}]_i$ (Romano-Silva et al., 1994). Figure 1(a) shows that addition of $0.5 \mu\text{M}$ TsTX to SBFI-loaded synaptosomes caused a rapid increase in $[\text{Na}^+]_i$. This was completely inhibited in the presence of $5 \mu\text{M}$ TTX, demonstrating that the Na^+ was entering via voltage-sensitive Na^+ channels. In the absence of TTX, addition of KCl (final concn. 33 mM) 3 min after TsTX caused a rapid fall in $[\text{Na}^+]_i$. This is almost certainly due to the closing of voltage-sensitive Na^+ channels during KCl-mediated depolarization, and suggests that TsTX-mediated Na^+ entry may not depolarize the synaptic plasma membrane as effectively as 33 mM KCl. In the presence of TTX, addition of KCl after TsTX causes a small spike in $[\text{Na}^+]_i$. We have shown subsequently that, whereas the effect of TsTX on $[\text{Na}^+]_i$ is completely inhibited

Figure 1 Effect of TsTX, gramicidin and KCl on intrasynaptosomal free Na^+

Rat cerebrocortical synaptosomes were isolated, loaded with SBFI and incubated at 37°C as described in the text. After establishing the baseline fluorescence ratio, TsTX ($0.5 \mu\text{M}$) or gramicidin (Gram.; 100 nM) was added (a and b respectively) followed, 3 min later, by 33 mM KCl. Incubations were performed after prior addition of $5 \mu\text{M}$ TTX at point t (broken lines) or in the absence of TTX (continuous lines). Results are the mean traces from at least three separate experiments.

by $5 \mu\text{M}$ TTX, 33 mM KCl causes a transient TTX-resistant spike in $[\text{Na}^+]_i$ (results not shown). It thus appears that synaptosomes have a depolarization-activated TTX-insensitive route of Na^+ entry.

In order to examine the effect of the locus of Na^+ entry on $[\text{Ca}^{2+}]_i$ and glutamate release, we compared the effects of TsTX (which promotes entry of Na^+ via TTX-sensitive channels) with those of the Na^+ ionophore gramicidin D, which causes a general increase in plasma-membrane permeability to Na^+ . Figure 1(b) shows that 100 nM gramicidin caused a rapid increase in $[\text{Na}^+]_i$, which was much larger than that observed with TsTX. Subsequent addition of 33 mM KCl had only a small effect on $[\text{Na}^+]_i$, and prior addition of TTX was without effect. These data show that gramicidin D promotes Na^+ entry into synaptosomes via a TTX-insensitive mechanism.

Figure 2 shows the data that were obtained when the same experiments were performed and $[\text{Ca}^{2+}]_i$ was measured with the indicator fura-2. Figure 2(a) shows that $0.5 \mu\text{M}$ TsTX caused a rapid spike in $[\text{Ca}^{2+}]_i$. This was completely abolished by prior addition of TTX. Addition of KCl (33 mM) 3 min after TsTX caused a further spike in $[\text{Ca}^{2+}]_i$, followed by a fall to a plateau level about 33% lower than that seen with TsTX. Addition of

Figure 2 Effect of TsTX, gramicidin and KCl on intrasynaptosomal free Ca^{2+}

Experiments were performed exactly as described in the legend to Figure 1, but using synaptosomes loaded with fura-2 AM rather than SBFI in order to facilitate measurement of $[\text{Ca}^{2+}]_i$. Results are mean values from at least three separate experiments.

Figure 3 Effect of TsTX, gramicidin and KCl on glutamate release from rat cerebrocortical synaptosomes

Synaptosomes were isolated as described in the text and incubated at 37 °C. Glutamate release was measured as described in the text by monitoring NADPH fluorescence after addition of NADP⁺ (1 mM) and glutamate dehydrogenase (50 units). Glutamate release was determined in the presence (broken lines) or absence (continuous lines) of 5 μM TTX after addition of 0.5 μM TsTX (a) or 100 nM gramicidin (Gram.) (b), followed, 6 min later, by 33 mM KCl. Curves are the mean results from at least three separate experiments.

33 mM KCl after TTX and TsTX caused a response identical with that seen with KCl alone. These data confirm our previous observations that TsTX is more effective at causing a sustained elevation of $[Ca^{2+}]_i$ than membrane depolarization by KCl (Romano-Silva et al., 1994). They also show that the decrease in $[Na^+]_i$ caused by addition of KCl after TsTX is accompanied by a parallel decrease in $[Ca^{2+}]_i$, i.e. that the additional effect of TsTX over that of KCl on $[Ca^{2+}]_i$ is associated with Na⁺ entry into synaptosomes. Figure 2(b) shows the results obtained when TsTX was replaced by gramicidin. Here, gramicidin alone had a large effect on $[Ca^{2+}]_i$. However, in contrast with the results with TsTX, the effect of 33 mM KCl was additive with that of

gramicidin. There was little effect of prior addition of TTX on these responses.

In Figure 3, the effects of TsTX and gramicidin on glutamate release are shown. Figure 3(a) shows that TsTX causes a large release of glutamate. In the presence of TTX, TsTX has no effect on glutamate release, and subsequent addition of KCl causes a similar response to that seen with KCl alone, showing that TsTX-mediated, but not KCl-mediated, glutamate release is dependent on Na⁺ entry through TTX-sensitive channels. Addition of 33 mM KCl after TsTX decreases the rate of release of glutamate. This effect of KCl is not well shown in Figure 3, where the rate of glutamate release was very low at the point where KCl was added, but has been confirmed by shortening the time interval between addition of TsTX and KCl. Figure 4 shows that 33 mM KCl, when added only 20 s after TsTX, greatly inhibits TsTX-mediated glutamate release.

Figure 3(b) shows that, although gramicidin promotes glutamate release, it is much less effective in doing so than TsTX. Furthermore, the effect of subsequent addition of KCl is to produce a further release of glutamate similar to that seen with KCl alone. TTX has little effect on these responses.

Studies of the release of glutamate from synaptosomes are complicated by the fact that, as well as Ca²⁺-dependent exocytosis, reversal of Na⁺-dependent glutamate uptake can also occur (Nicholls and Sihra, 1986). In order to study Ca²⁺-dependent exocytosis of glutamate, we measured total release (in the presence of 1 mM extra-synaptosomal free Ca²⁺) as well as Ca²⁺-independent release (in the presence of excess EGTA) in the presence of 33 mM KCl, 100 nM gramicidin + 33 mM KCl and 0.5 μM TsTX (5–11 separate experiments in each case). The differences between total and Ca²⁺-independent release were used to determine Ca²⁺-dependent release. The levels of Ca²⁺-dependent release of glutamate caused by KCl, KCl + gramicidin and TsTX were 4.407 ± 0.172 , 4.896 ± 0.260 and 5.726 ± 0.331 nmol/mg of protein respectively (means $\pm$ S.E.M.). These data indicated that, whereas TsTX caused significantly higher release of glutamate than did KCl alone ($P < 0.001$), KCl + gramicidin did not. The Ca²⁺-dependent glutamate release evoked by TsTX was also significantly greater ($P < 0.05$) than that caused by gramicidin + KCl.

Figure 4 Inhibition of TsTX-mediated glutamate release by subsequent addition of 33 mM KCl

Glutamate release was measured as described in Figure 3 after addition of TsTX (0.5 μM). The upper curve indicates release in the absence of 33 mM KCl and the lower curve shows that when 33 mM KCl was added 20 s after TsTX.

DISCUSSION

The current findings reinforce our previous conclusions: (i) that TsTX causes Na⁺ entry into synaptosomes through TTX-sensitive Na⁺ channels, (ii) that the Na⁺ entry results in rapid elevation of $[Ca^{2+}]_i$ and release of glutamate, and (iii) that TsTX is more efficient than depolarizing levels of KCl in stimulating Ca²⁺-dependent glutamate release (Romano-Silva et al., 1994). When 33 mM KCl is added after a maximally effective concentration of TsTX, it causes an immediate fall in $[Na^+]_i$ that is accompanied by a parallel fall in $[Ca^{2+}]_i$ and a decrease in the rate of glutamate release. Tibbs et al. (1989) have suggested that depolarization with KCl will cause a single transient firing of Na⁺ channels, with no subsequent re-activation after closure. This suggestion would agree well with our observations, which lead us to the conclusion that the effects of TsTX-mediated Na⁺ entry on Ca²⁺-stimulated glutamate release are not explicable on the basis that the sole effect of Na⁺ entry is to produce membrane depolarization. In that event, the effects of TsTX would not exceed those of KCl, which effectively depolarizes the synaptic membrane. It could be argued that TsTX, which produces transient depolarizations, would allow Ca²⁺ channels rapidly inactivated during depolarization to recover, permitting further Ca²⁺ entry and glutamate release. However, McMahon and

Nicholls (1991) and our own studies (M. A. Romano-Silva and M. J. Brammer, unpublished work) have shown that depolarization of synaptosomes by KCl in Ca^{2+} -free medium does not lead to glutamate release, but normal KCl-mediated release is observed when Ca^{2+} is added back to the extracellular medium several minutes later. These data demonstrate that rapidly inactivated Ca^{2+} channels apparently do not contribute to depolarization-induced glutamate release in rat cerebrocortical synaptosomes. It would thus seem unlikely that TsTX exerts its additional effect on glutamate release via repeated opening and closing of such Ca^{2+} channels. Furthermore, McMahon and Nicholls (1991) have shown that the K^+ -channel inhibitor 4-aminopyridine, which also produces a 'flickering' depolarization, produces a maximal glutamate release very similar to that seen with KCl. Interestingly, although the effects of 4-aminopyridine, like those of TsTX, are TTX-sensitive, 4-aminopyridine causes a much smaller elevation in the intrasynaptosomal free Na^+ concentration (M. A. Romano-Silva and M. J. Brammer, unpublished work).

A major aim of the present study was to attempt to establish whether localized Na^+ entry was required to produce maximally effective Ca^{2+} -dependent glutamate release. With this goal in mind, we compared the effects of TsTX (which produces Na^+ entry through TTX-sensitive Na^+ channels) with those of 33 mM KCl plus the Na^+ ionophore gramicidin D (which promotes delocalized Na^+ entry). The concentration of gramicidin was titrated to yield a $[\text{Ca}^{2+}]_i$ increase similar to that seen with 0.5 μM TsTX. Under these circumstances, it was found that gramicidin produced a much larger increase in $[\text{Na}^+]_i$ than did TsTX, but was much less effective at releasing glutamate. This suggested that the Ca^{2+} influx caused by gramicidin, although producing the same volume-average increase in $[\text{Ca}^{2+}]_i$ as TsTX, was not localized in those areas of the synaptic terminal close to 'docked' synaptic vesicles containing glutamate (Verhage et al., 1991). The exact route by which gramicidin is causing Ca^{2+} entry into synaptosomes is not clear, and could involve either some depolarization-mediated activation of voltage-sensitive Ca^{2+} channels close to synaptic vesicles, or another route such as reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Carvalho et al., 1991) following large-scale Na^+ entry. In order to obtain delocalized Na^+ entry accompanied by membrane depolarization, we employed a combination of KCl plus gramicidin D. This combination is still less effective than TsTX in causing Ca^{2+} -dependent glutamate release, even though the combination of gramicidin and KCl causes a much larger increase in both volume-average $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ than TsTX does. As TsTX and KCl will both promote opening of voltage-sensitive Ca^{2+} channels, the main difference between the ion entry promoted by TsTX and that brought about by KCl plus gramicidin will be in the locus of the Na^+ entry. We thus suggest that localized Na^+ entry through TTX-sensitive channels causes a (direct or indirect) positive modulation of VSCC activity linked to glutamate release. Less localized ionophore-mediated Na^+ entry appears to be significantly less effective, possibly because the ionophore may cause smaller local changes in $[\text{Na}^+]_i$.

Modulatory roles of $[\text{Na}^+]_i$ in intracellular function have received much less attention than those of $[\text{Ca}^{2+}]_i$, possibly because, as Linden et al. (1993) have recently observed, the maximum possible change in $[\text{Na}^+]_i$ upon stimulation is only from around 12 mM to around 140 mM. This range contrasts sharply with the local changes in $[\text{Ca}^{2+}]_i$ suggested to occur during the release of neurotransmitters (Llinas et al., 1992). The relative lack of interest in possible intracellular signalling roles of Na^+ has thus led to comparatively few reports of changes in $[\text{Na}^+]_i$ during neurotransmitter release. However, a number of

interesting observations have recently appeared. Linden et al. (1993) have shown that Na^+ appears to play a direct part in inducing long-term depression in cultured cerebellar Purkinje cells and that Na^+ entry through voltage-gated Na^+ channels and AMPA (α -amino-3-hydroxy-5-methyl-1-oxazole-4-propionic acid) receptors may both have significant effects. They have discussed a number of possible intracellular targets for Na^+ . These include the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (see Carvalho et al., 1991), phospholipase C (see Gusovsky et al., 1986) and phospholipase A_2 (Dumuis et al., 1993), although stimulation of the last two may be an indirect consequence of Ca^{2+} entry stimulated by $\text{Na}^+/\text{Ca}^{2+}$ exchange. We have some evidence that $\text{Na}^+/\text{Ca}^{2+}$ exchange may contribute to TsTX-mediated Ca^{2+} entry, as 100 μM Gd^{3+} , which effectively blocks voltage-sensitive Ca^{2+} channels, but is ineffective against the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Canzoniero et al., 1993), decreases, but does not abolish, TsTX-mediated increases in synaptosomal $[\text{Ca}^{2+}]_i$ (M. A. Romano-Silva and M. J. Brammer, unpublished work). However, it seems unlikely that $\text{Na}^+/\text{Ca}^{2+}$ exchange could produce the high local $[\text{Ca}^{2+}]_i$ changes in nerve endings to stimulate rapid release of neurotransmitters (Llinas et al., 1992). Direct actions of Na^+ on Ca^{2+} channels may occur, but there is as yet no information on such effects.

In a previous study (Romano-Silva et al., 1993), we have examined the effects of the spider toxin PhTX2 from *Phoneutria nigriventer* on synaptosomal $[\text{Na}^+]_i$, $[\text{Ca}^{2+}]_i$ and glutamate release. Like TsTX, PhTX2 acts on TTX-sensitive Na^+ channels, but it causes less depolarization than TsTX (Araujo et al., 1993). Our data showed that PhTX2 appears to release glutamate from synaptosomes by a mechanism involving Na^+ -dependent Ca^{2+} entry by a route distinct from that stimulated by 33 mM KCl. Molgo et al. (1993) have recently reached similar conclusions using the ciguatoxin from *Gymnothorax javanicus* to investigate acetylcholine release from *Torpedo* synaptosomes, though they consider that activation of the $\text{Na}^+/\text{Ca}^{2+}$ antiporter is implicated.

These data reveal a potentially significant role of Na^+ in modulating Ca^{2+} entry linked to neurotransmitter release. The details of this modulatory interaction now require elucidation.

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