Rat cortical synaptosomes have more than one mechanism for Ca^{2+} entry linked to rapid glutamate release: studies using the *Phoneutria nigriventer* toxin PhTX2 and potassium depolarization

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PhTX2, one of the components of the venom of the South American spider *Phoneutria nigriventer*, inhibits the closure of voltage-sensitive Na^+ channels. Incubation of cerebral-cortical synaptosomes with PhTX2 causes a rapid increase in the intrasynaptosomal free Ca^{2+} concentration and a dose-dependent release of glutamate. This release is made up of a slow component, which appears to be due to reversal of Na^+ -dependent glutamate uptake, and more rapid component that is dependent on the entry of extrasynaptosomal Ca^{2+} . It has previously been shown that membrane depolarization using KCl can cause rapid Ca^{2+} -dependent release of glutamate from synaptosomes. This requires Ca^{2+} entry through a specific type of Ca^{2+} channel that is

sensitive to Aga-GI, a toxic component of the venom of the spider *Agelenopsis aperta*. We have compared the effects of PhTX2 and KCl on elevation of intrasynaptosomal free Ca^{2+} and glutamate release, and a number of differences have emerged. Firstly, PhTX2-mediated Ca^{2+} influx and glutamate release, but not those caused by KCl, are inhibited by tetrodotoxin. Secondly, KCl produces a clear additional increase in Ca^{2+} and glutamate release following those elicited by PhTX2. Finally, 500 μM MnCl_2 abolishes PhTX2-mediated, but not KCl-mediated, glutamate release. These findings suggest that more than one mechanism of Ca^{2+} entry may be coupled to glutamate release from nerve endings.

INTRODUCTION

The recognition of glutamate as a major excitatory neurotransmitter in the central nervous system (see Monaghan et al., 1989) has focused attention on the mechanisms by which its release from nerve terminals may be controlled. The critical role of elevated intracellular Ca^{2+} in promoting neurosecretion of many neurotransmitters, including glutamate, is now well established (see Augustine et al., 1987), and recent developments have indicated that localized increases in Ca^{2+} may stimulate the release of specific neurotransmitters (Augustine and Neher, 1992). McMahon and Nicholls (1991) have shown that depolarizing concentrations of KCl are much more effective in promoting release of glutamate from synaptosomes than is the Ca^{2+} ionophore ionomycin. They have hypothesized that, whereas ionomycin causes a general entry of Ca^{2+} through the plasma membranes, the concentration of Ca^{2+} in the region of glutamate-containing vesicles may not reach the level required to cause exocytosis. In contrast, there may be a close physical association between the glutamate-containing vesicles and a type of voltage-sensitive Ca^{2+} channel that is opened during membrane depolarization. Entry of Ca^{2+} through these channels could produce the high local Ca^{2+} concentrations required to stimulate exocytosis. Attempts to characterize the presynaptic voltage-sensitive Ca^{2+} channel associated with glutamate release as belonging to the N, L or T type classification proposed by Fox et al. (1987) have been unsuccessful (see Nicholls, 1993). However, the channel is sensitive to one of the components (Aga-GI) of the

venom of the spider *Agelenopsis aperta* (Pocock and Nicholls, 1992).

Although it is clear that opening of voltage-sensitive Ca^{2+} channels may produce a localized rise in Ca^{2+} and exocytosis of neurotransmitters, there is some evidence that other mechanisms of Ca^{2+} entry should also be considered. For example, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which normally transports Ca^{2+} out of the cell in exchange for extracellular Na^+ , can be reversed by removal of extracellular Na^+ or a rise in the intracellular Na^+ concentration, producing a rise in intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$). There have been a number of suggestions that this route of Ca^{2+} entry may play a significant role in neurotransmitter release (see Carvalho et al., 1991). Studies of excitation–secretion coupling in heart muscle have suggested that small increases in intracellular Na^+ can cause large transient increases in intracellular Ca^{2+} when voltage-sensitive sarcolemmal Ca^{2+} channels are blocked, and it has been suggested that this may occur by reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (Levesque et al., 1991). In order to investigate the effects of such a route for Ca^{2+} entry on synaptosomal glutamate release, an agent capable of inducing Na^+ -dependent Ca^{2+} entry under non-depolarizing conditions would be of considerable value. With this aim in view, we have investigated the actions of the toxin PhTX2 purified from the venom of the Brazilian spider *Phoneutria nigriventer* (Rezende et al., 1991), which appears to block the closure of Na^+ channels without causing significant membrane depolarization (Araujo et al., 1993). Preliminary work (M. A. Romano-Silva, unpublished work) has suggested that PhTX2 may also promote neuro-

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; TTX, tetrodotoxin; PhTX1, PhTX2, PhTX3, *Phoneutria nigriventer* toxins 1, 2 and 3; Aga-GI, Agatoxin, glutamate-release inhibiting, isolated from *Agelenopsis aperta*; SBFI, Na^+ -binding benzofuran isophthalate.

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transmitter release. In the present study, we have investigated the effects of PhTX2 on intracellular Ca^{2+} levels and glutamate release in rat cortical synaptosomes.

EXPERIMENTAL

Fraction GVIA from the marine snail *Conus geographus* (ω -conotoxin) was obtained from Peninsula Laboratories, Belmont, CA, U.S.A., and Na^+ -binding benzofuran isophthalate (SBFI) from Molecular Probes, Eugene, OR, U.S.A. Nifedipine, glutamate dehydrogenase (type II, 3-times crystallized), tetrodotoxin, Percoll and ionomycin were all obtained from Sigma Chemical Co., Pool, Dorset, U.K.

Purification of PhTX2

This was carried out in the Fundacao Ezequiel Dias, Belo Horizonte, Brazil, by the method of Rezende et al. (1991). PhTX2 is one of three neurotoxic fractions, lethal to mice (PhTX1, PhTX2, PhTX3), obtained from the crude venom of *Phoneutria nigriventer* by gel filtration and reverse-phase chromatography. These toxins have molecular masses in the range 6000–9000 Da, and have different amino acid compositions and N-terminal amino acid sequences. PhTX2 was stored in batches at -20°C in distilled water at a concentration of 1 mg/ml.

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 into 200 μl portions and kept in ice until loaded with fura-2 acetomethoxy ester (AM) or SBFI AM, or used for measurement of glutamate release.

Measurement of intrasynaptosomal free Ca^{2+}

Fura-2 AM (stock solution 1 mM in dimethyl sulphoxide) was added to the synaptosomal suspensions to give a final concentration of 5.0 μM and the mixture 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 period of 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 . The excitation source was a 75 W xenon lamp, from which light was transmitted through a rotating filter wheel containing six excitation filters (320, 340, 360, 380, 390 and 430 nm) and subsequently through a fibre-optic link to the cuvette holder. The emitted light was detected by a photomultiplier after passage through a 500 nm filter. The signals were processed and the 320 nm/390 nm and 340 nm/380 nm fluorescence ratios continuously displayed by using an IBM-compatible microcomputer running dedicated Cairn software. Data was collected every 1 s from all six excitation filters. 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 was 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 μl of 1 M CaCl_2 was added, giving a final concentration of 1.0 mM. Addition of drugs and/or toxin was made 10 min after that of CaCl_2 . At the end of each experiment, calibration was performed by recording the maximum and minimum fluorescence values after addition of 15 μl of 10% (w/v) SDS followed by 40 μl of 400 mM EGTA/3 M Tris, pH 8.6, as previously described (Adamson et al., 1989). Autofluorescence was determined by using synaptosomes that had not been loaded with fura-2.

Following the suggestion of Grynkiewicz et al. (1985), most workers use a fluorescence-excitation ratio to measure $[\text{Ca}^{2+}]_i$, using fura-2. Most commonly, fluorescence emission at around 500 nm is monitored while continually switching excitation wavelengths between 340 and 380 nm. The '340 nm/380 nm ratio' is calculated from each pair of observations. As we routinely use six excitation wavelengths (see above), we examined the performance of a number of excitation wavelength ratios in determining $[\text{Ca}^{2+}]_i$. Our data showed that, of the various ratios investigated, 320 nm/390 nm and 340 nm/380 nm yielded very similar Ca^{2+} values, but that the 320 nm/390 nm ratio was often superior in terms of reproducibility and signal-to-noise ratio. For this reason the 320 nm/390 nm ratio was routinely used to compute free Ca^{2+} concentrations.

The raw fluorescence data was converted into ASCII files and imported into a spreadsheet (Quattro Pro), and Ca^{2+} values were calculated using a macro (developed by M. A. R.-S.) based on the method described by Grynkiewicz et al. (1985).

Measurement of intrasynaptosomal free Na^+

This was accomplished with the fluorescent indicator SBFI (Minta and Tsien, 1989). Synaptosomes were loaded with SBFI-AM (20 μM) for 2 h at 37°C in Krebs–Ringer–Hepes medium (see above), followed by centrifugation and resuspension in fresh 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 carried out by monitoring the increase in NADPH^+ fluorescence in the presence of glutamate dehydrogenase (Nicholls et al., 1987). Briefly, synaptosomes were first incubated for 1 h at 37°C , and centrifuged (see above) to ensure comparability with experiments using fura-2. After resuspension in fresh medium, the synaptosomes were transferred to the cuvette housing of the fluorimeter, and fluorescence at 450 nm was recorded after excitation at 340 and 360 nm. Then 1 min later, NADP^+ was added (final concn. 1 mM), followed 1 min later by CaCl_2 (final concn. 1 mM). After a further 5 min, glutamate dehydrogenase (50 units) was added and NADPH^+ fluorescence allowed to reach equilibrium after removal of the glutamate present in the enzyme preparation and the extracellular medium. Subsequently, additions were made as described in the Results section and Figure legends. Calibration was performed by adding known amounts of glutamate to the system.

Reproducibility

Traces shown are representative data from at least three in-

dependent experiments; the range of variation between groups of experiments was less than 10%.

RESULTS

Effect of PhTX2 on intrasynaptosomal free Ca^{2+} , and glutamate release; inhibition by tetrodotoxin (TTX), and comparison with the effects of K^+

Addition of PhTX2 to synaptosomal suspensions leads to a dose-dependent increase in $[\text{Ca}^{2+}]_i$ (Figure 1). The EC_{50} value is approx. $0.5 \mu\text{g/ml}$. Subsequent experiments were performed with

Figure 1 Dose-dependence of the effect of PhTX2 on intrasynaptosomal free Ca^{2+} .

Synaptosomes were isolated and loaded with fura-2 AM as described in the text. At 10 min after adding CaCl_2 (final concn. 1 mM), various concentrations of PhTX2 were added and the increases in free Ca^{2+} determined. Data are the mean values from at least three experiments at each PhTX2. Curves a, b, c, d and e show the effects of 0, 0.1, 0.5, 1 and $5 \mu\text{g/ml}$ of PhTX2 respectively.

Figure 2 Effect of PhTX2 on glutamate release from synaptosomes

Synaptosomes were isolated and glutamate release was determined as described in the text after adding PhTX2 ($1 \mu\text{g/ml}$). Data are mean values from nine experiments.

$1.0 \mu\text{g/ml}$ PhTX2/ml, which increased $[\text{Ca}^{2+}]_i$ by around 200 nM and caused a rapid release of glutamate from synaptosomes (Figure 2). These data extend previous results showing that PhTX2 can promote acetylcholine release from brain slices (M. A. Romano-Silva, unpublished work).

Araujo et al. (1993) have shown that PhTX2 acts by preventing the inactivation of Na^+ channels. We therefore examined the effects of the Na^+ -channel blocker TTX on the PhTX2-mediated Ca^{2+} increase and glutamate release. Figures 3(a) and 3(b) respectively show the intrasynaptosomal free Ca^{2+} in the absence and presence of PhTX2 ($1 \mu\text{g/ml}$). Addition of TTX ($5 \mu\text{M}$) 60 s after PhTX2 reversed the increase in $[\text{Ca}^{2+}]_i$ (Figure 3c), and preincubation with TTX for 1 min before adding PhTX2 abolished the Ca^{2+} increase (Figure 3d) and the release of glutamate (Figure 4), confirming the dependence of both these phenomena on entry of Na^+ .

Figure 3 Effect of TTX on the PhTX2-mediated rise in intrasynaptosomal free Ca^{2+} .

Synaptosomes were isolated and loaded with fura-2 as described in the text. The data show the effects of addition of PhTX2 ($1 \mu\text{g/ml}$) and TTX ($5 \mu\text{M}$). (a) Control; (b) PhTX2 alone; (c) PhTX2, followed 1 min later by TTX; (d) TTX, followed 1 min later by PhTX2. Data are the mean values from at least three experiments.

Figure 4 Effect of TTX on PhTX2- and KCl -mediated release of glutamate from synaptosomes

Glutamate release was determined after sequential addition of PhTX2 ($1 \mu\text{g/ml}$) and (5 min later) 33 mM KCl . The upper trace shows glutamate release in the absence of TTX and the lower trace glutamate release when TTX ($5 \mu\text{M}$) was added 1 min before PhTX2. Data are mean values from 5 experiments (lower trace) and 9 experiments (upper trace).

Figure 5 Effect of TTX on the PhTX2- and KCl-mediated rises in intrasynaptosomal free Ca^{2+}

Synaptosomes loaded with fura-2 as described in the text were treated with PhTX2 (1 μ g/ml) and the Ca^{2+} increase was allowed to reach equilibrium. KCl (33 mM) was then added 2–3 min later. (a) shows the response to KCl in the absence of TTX, and (b) that when TTX (5 μ M) was added 1 min after PhTX2. Data are mean values from 3 experiments (a) and 4 experiments (b).

A further series of studies was carried out to compare the effects of PhTX2 with those of a depolarizing concentration of KCl, which can promote glutamate release by causing an influx of Ca^{2+} through voltage-sensitive channels. Addition of 33 mM KCl after PhTX2 caused a further spike in $[Ca^{2+}]_i$, followed by a fall to a plateau level somewhat higher than that attained with PhTX2 alone (Figure 5a). This type of response to KCl has been described previously in other laboratories (Nachsen, 1985; Tibbs et al., 1989). However, 33 mM KCl can produce a full Ca^{2+} response under conditions where the response to PhTX2 was reversed by addition of TTX (Figure 5b). PhTX2 and 33 mM KCl also produced distinct episodes of glutamate release (Figure 4), and that caused by addition of 33 mM K^+ was also not affected by TTX.

Effect of PhTX2 on intracellular free Na^+

PhTX2 appears to act initially on Na^+ channels, and we have studied its effect on intracellular Na^+ , using synaptosomes loaded with the Na^+ indicator SBFI. PhTX2 produced a small increase in the 340 nm/380 nm fluorescence ratio, indicating elevation of the intrasynaptosomal Na^+ concentration (Figure 6). In contrast, abolition of the transmembrane Na^+ gradient by subsequent

Figure 6 Effect of PhTX2 and gramicidin D on intrasynaptosomal free Na^+

Synaptosomes were loaded with SBFI as described in the text. The data show the effects of adding PhTX2 (1 μ g/ml) and (3 min later) gramicidin D (2 μ M). Data are shown as the ratios of fluorescence at 500 nm with excitation wavelengths of 340 nm and 380 nm, and are mean values from 4 experiments.

addition of the univalent-cation ionophore gramicidin D (2 μ M) led to a much larger increase in SBFI fluorescence (Figure 6) and a large, rapid, release of glutamate (Figure 7) that was much greater than that caused by either PhTX2 or 33 mM KCl. These data show that PhTX2 addition causes an increase in intrasynaptosomal free Na^+ , but suggest that this does not amount to a collapse of the transmembrane Na^+ gradient, as occurs in the presence of gramicidin D. This conclusion is in agreement with the findings of Araujo et al. (1993) that PhTX2 does not cause substantial membrane depolarization. Lack of substantial membrane depolarization by PhTX2 would explain the ability of subsequent addition of depolarizing concentrations (33 mM) of KCl to promote rapid release of glutamate (Figure 4).

Effects of Ca^{2+} -free medium and Ca^{2+} -channel blockers

Na^+ entry could promote glutamate release from synaptosomes by reversing the Na^+ -dependent neurotransmitter-uptake mechanism. We investigated this possibility by studying the dependence of PhTX2-mediated glutamate release on extracellular Ca^{2+} . It was shown that chelation of extracellular Ca^{2+} by excess EGTA abolished the PhTX2-induced rise in $[Ca^{2+}]_i$ and greatly attenuated the PhTX2-induced release of glutamate (Figures 8a and 8b). Addition of extracellular Ca^{2+} (1 mM excess over EGTA) 3 min after PhTX2 led to an increase in $[Ca^{2+}]_i$ identical with that observed when the toxin was added in Ca^{2+} -containing medium (results not shown). These data show that the PhTX2-mediated increase in $[Ca^{2+}]_i$ and the bulk of the PhTX2-mediated release of glutamate were dependent on the presence of extracellular Ca^{2+} , and that reversal of Na^+ -dependent glutamate uptake was only responsible for a small part of PhTX2-mediated glutamate release. In addition, the fact that delayed addition of extracellular Ca^{2+} can promote a full response to PhTX2 suggests that PhTX2 does not promote Ca^{2+} entry through a rapidly inactivating Ca^{2+} channel.

In order to shed more light on the mechanism of Ca^{2+} entry after addition of PhTX2, we studied the effects of preincubating synaptosomes for 1 min with the channel blockers nifedipine and ω -conotoxin before adding PhTX2. Neither of the channel blockers was able to abolish or significantly decrease the increase

Figure 7 Effect of gramicidin D on glutamate release from synaptosomes

Glutamate release was measured as described in the text after addition of gramicidin D ($2 \mu\text{M}$) to synaptosomes. Data are mean values from 3 experiments.

Figure 8 Dependence of the PhTX2-mediated rise in $[\text{Ca}^{2+}]_i$ and release of glutamate on the presence of extracellular Ca^{2+}

Intrasyntosomal free Ca^{2+} levels (a) and glutamate release (b) were monitored after adding EGTA (2 mM) to chelate extracellular Ca^{2+} and subsequent addition of PhTX2 ($1 \mu\text{g/ml}$). Data are mean values from 3 experiments (a) and 4 experiments (b).

in $[\text{Ca}^{2+}]_i$ that followed addition of PhTX2 ($1.0 \mu\text{g/ml}$) (results not shown). These experiments indicate that PhTX2 does not cause Ca^{2+} entry through N- or L-type Ca^{2+} channels. However, other workers have shown that synaptosomes contain another type of voltage-sensitive Ca^{2+} channel (Pocock and Nicholls, 1992), which is linked to glutamate release following K^+ depolarization.

In order to study Ca^{2+} -channel involvement in PhTX2-mediated glutamate release, we studied the effects of Mn^{2+} , which has long been known to be a blocker of presynaptic voltage-dependent Ca^{2+} channels (Katz and Miledi, 1969; Meiri and Rahamimoff, 1972), over the concentration range $5 \mu\text{M}$ – 5 mM . Our initial studies revealed that millimolar concentrations of MnCl_2 blocked both the KCl- and PhTX2-mediated increases in $[\text{Ca}^{2+}]_i$ and glutamate release, whereas very low concentrations had little effect on either the KCl or the PhTX2 responses (results not shown). However, intermediate concentrations are able to distinguish between the two phenomena. Figure 9 shows the

Figure 9 Effect of MnCl_2 on PhTX2- and KCl-mediated glutamate release and rises in $[\text{Ca}^{2+}]_i$

Synaptosomes were treated with MnCl_2 ($500 \mu\text{M}$), followed by PhTX2 ($1 \mu\text{g/ml}$) and KCl (33 mM) and finally ionomycin ($5 \mu\text{M}$). (a) Data for intrasyntosomal free Ca^{2+} [expressed as the $320 \text{ nm}/390 \text{ nm}$ fluorescence ratio (see the text), because of interference of MnCl_2 with the calibration process]. (b) Glutamate release under similar conditions. Data are mean values from 4 experiments (a) and 3 experiments (b).

results of a series of experiments using $500 \mu\text{M}$ MnCl_2 . Immediately after addition of MnCl_2 , there is a decrease in the $320 \text{ nm}/390 \text{ nm}$ fluorescence ratio (Figure 9a) due to its quenching of the signal from extracellular fura-2. Although Mn^{2+} quenches fura-2 fluorescence excited at both 320 and 390 nm , it has a somewhat larger effect on the (smaller) lower-wavelength signal, causing a fall in the ratio. After the initial sharp fall, the ratio continues to decline at a lower rate, indicating penetration of MnCl_2 into the synaptosomes. Fluorescence-ratio changes due to alterations in $[\text{Ca}^{2+}]_i$ are superimposed on this decline in ratio. It can be seen that $500 \mu\text{M}$ MnCl_2 effectively abolishes the Ca^{2+} increase and glutamate release caused by PhTX2 (Figures 9a and 9b) and the rapid (spike) phase, but not the slower (plateau) phase of the Ca^{2+} increase after addition of 33 mM KCl (Figure 9a). Under these conditions, KCl-mediated glutamate release is unaffected (Figure 9b). At the end of the experiment, ionomycin was added, quenching the intrasyntosomal fura-2 signal and sharply decreasing the $320 \text{ nm}/390 \text{ nm}$ fluorescence ratio.

DISCUSSION

Although there is a slow component of glutamate release which is Ca^{2+} -independent, Ca^{2+} -dependent exocytosis is a major con-

tributor to stimulated release (Nicholls and Sihra, 1986; Nicholls et al., 1987). The relationship between increases in intracellular free Ca^{2+} and neurotransmitter release has been known for many years (Katz, 1969; Llinas, 1977), but recent studies have suggested that highly localized large increases in Ca^{2+} may be required for exocytosis of some neurotransmitters, including glutamate (Simon and Llinas, 1985; Verhage et al., 1991; Augustine and Neher, 1992). Following axonal stimulation, the most significant rise in Ca^{2+} occurs directly under the presynaptic membrane of the squid giant presynaptic terminal (Llinas et al., 1992; Smith and Augustine, 1988). Guthrie et al. (1991) have demonstrated that synaptic activation in hippocampal neurones can produce highly localized rises in Ca^{2+} in dendritic spines. Finally, Verhage et al. (1991) have shown that Ca^{2+} entry through voltage-sensitive channels, opened by K^+ depolarization of the synaptic membrane, is much more effective in releasing glutamate than is generalized Ca^{2+} influx caused by ionomycin.

These and other similar experimental results have led to the hypothesis that transmitter vesicles may be 'docked' close to voltage-sensitive Ca^{2+} channels (see Nicholls, 1993). The studies by Nicholls and his co-workers have suggested that the channel involved in K^+ -mediated Ca^{2+} entry and glutamate release is relatively insensitive to inhibitors of so-called N- and L-type channels, such as dihydropyridines and ω -conotoxin, but is inhibited by one of the components of the venom of *Agelenopsis aperta* (Aga-GI) (Pocock and Nicholls, 1992). This toxin, although completely inhibiting stimulated glutamate release, causes only a partial inhibition of the plateau phase of the K^+ -mediated rise in intrasynaptosomal free Ca^{2+} .

From the outset, our data showed some clear differences between the actions of PhTX2 and depolarizing levels of KCl. Although PhTX2 can also cause rapid release of glutamate from synaptosomes, the primary event in this case is clearly increased Na^+ entry through tetrodotoxin-sensitive channels, as would be predicted from previous findings on the action of the toxin (Araujo et al., 1993). The simplest explanation for Na^+ -dependent release of glutamate would be reversal of Na^+ -dependent glutamate uptake, a process previously shown by Nicholls et al. (1987) to occur in synaptosomes. Indeed, this reversal may be a major contributor to glutamate release from synaptosomes when the transmembrane Na^+ gradient is totally collapsed by veratridine, which prevents Na^+ -channel inactivation (see Nicholls, 1993). However, it is clear that most of the PhTX2-stimulated glutamate release is dependent on extracellular Ca^{2+} , although there is a small but significant Ca^{2+} -independent component. The average rise in $[\text{Ca}^{2+}]_i$ required to cause glutamate release after addition of PhTX2 is comparable with that caused by depolarizing K^+ and much smaller than that required in the presence of ionomycin (Verhage et al., 1991). However, the local effect of PhTX2 on $[\text{Ca}^{2+}]_i$ may, as in the case of depolarizing K^+ , be much larger (see Nicholls, 1993).

As both KCl- and PhTX2-mediated glutamate release require Ca^{2+} entry, it is possible that this is occurring in both cases through a voltage-sensitive channel and that the only difference may be the route to membrane depolarization (Na^+ entry following PhTX2 addition), which may lead to TTX-sensitivity of the PhTX2 effect. On preliminary examination of the evidence, this seems unlikely. Firstly, the electrophysiological studies of Araujo et al. (1993) failed to detect membrane depolarization by PhTX2. Secondly, as KCl must bring about membrane depolarization in order to release glutamate, it is unlikely that this could occur if the membrane had already been depolarized by PhTX2. In fact, 33 mM KCl produces clear additional glutamate release following the response to a maximally effective concentration of PhTX2 (see Figure 4).

Although these findings may imply that PhTX2 is not causing depolarization-mediated opening of a voltage-sensitive Ca^{2+} channel, further proof may be required. Tibbs et al. (1989) studied the effects of the K^+ -channel inhibitor 4-aminopyridine and KCl on synaptosomal glutamate release. Our results using PhTX2 combined with their data on 4-aminopyridine suggest that both substances produce apparently similar results. Both cause TTX-sensitive rises in intrasynaptosomal free Ca^{2+} and glutamate release. Also, following addition of maximally effective concentrations of either 4-aminopyridine or PhTX2, depolarizing concentrations of KCl can produce further effects on $[\text{Ca}^{2+}]_i$ and glutamate release. Tibbs et al. (1989) concluded that 4-aminopyridine, unlike KCl, caused repetitive transient membrane depolarization. They argued that, although fluorescent probes of membrane potential failed to reveal substantial depolarization by 4-aminopyridine, the TTX-sensitivity of the response showed that voltage-sensitive Na^+ channels were opened. Their conclusion was that 4-aminopyridine was promoting Ca^{2+} entry through voltage-sensitive channels that were opened during these transient depolarizations. Subsequently, Pocock and Nicholls (1992) showed that Aga-GI inhibited both the 4-aminopyridine- and the KCl-mediated release of glutamate from synaptosomes, suggesting that 4-aminopyridine and KCl might be causing opening of the same type of Ca^{2+} channel.

The apparent similarity of the PhTX2 and 4-aminopyridine effects suggested to us that PhTX2 might also be activating the same type of channel and that KCl, PhTX2 and 4-aminopyridine might all be causing localized $[\text{Ca}^{2+}]_i$ increases by the same route. However, the ability of 500 μM MnCl_2 to inhibit selectively PhTX2-mediated Ca^{2+} entry and glutamate release, but not the 'slow' phase of KCl-mediated Ca^{2+} entry that is linked to Ca^{2+} influx through Aga-GI-sensitive channels (Pocock and Nicholls, 1992), suggests that the same mechanism of Ca^{2+} entry is unlikely to be operative in the two cases. This raises the possibility that synaptosomes may contain more than one type of Ca^{2+} channel which can produce localized $[\text{Ca}^{2+}]_i$ changes large enough to promote glutamate release. Alternatively, entry of Na^+ could lead to Ca^{2+} influx by reversing the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. A number of reports have suggested that Ca^{2+} entry by reversal of $\text{Na}^+/\text{Ca}^{2+}$ exchange might either contribute to, or promote, γ -aminobutyrate or catecholamine release from nerve endings (Carvalho et al., 1991; Torok and Powis, 1990; Taglialetta et al., 1990). This explanation is compatible with the observations (a) that PhTX2-mediated Na^+ entry precedes the rapid rise in intrasynaptosomal Ca^{2+} and glutamate release, leading to TTX-sensitivity of these responses, and (b) that Mn^{2+} inhibits the effects of PhTX2, as Blaustein and Ector (1976) have shown that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is sensitive to Mn^{2+} . However, this would also imply that Ca^{2+} entry by 'reversed' $\text{Na}^+/\text{Ca}^{2+}$ exchange, as well as through specific voltage-sensitive channels, could produce high enough local Ca^{2+} levels to promote glutamate exocytosis. The route for Ca^{2+} entry into synaptosomes after addition of PhTX2 appears distinct from that after K^+ depolarization.

The previous physiological and biochemical data on PhTX2 are very limited, consisting of the observations made by Araujo et al. (1993) that the toxin affected Na^+ -channel inactivation and that by M. A. Romano-Silva (unpublished work) of its promotion of acetylcholine release. There are no previous reports of interactions between Na^+ and Ca^{2+} entry following PhTX2 addition, and our data suggest that PhTX2 may be a useful pharmacological tool for investigating the links between Na^+ entry, Ca^{2+} entry and glutamate release. However, until further experimentation has been performed, the mechanism by which PhTX2 promotes Ca^{2+} entry must remain the subject of speculation.

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