

Noxiustoxin, A Short-Chain Toxin from the Mexican Scorpion *Centruroides noxius*, Induces Transmitter Release by Blocking K⁺ Permeability

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Noxiustoxin (NTX), a 39 amino acid peptide purified from the venom of the Mexican scorpion *Centruroides noxius*, has been shown to block voltage-dependent K⁺ currents in the squid giant axon (Possani et al., 1982; Carbone et al., 1982). Although several other drugs known as K⁺ channel blockers in the squid axon also act on isolated nerve terminals to produce an increase in transmitter release, these releasing effects have not been shown to be related to a decrease of K⁺ permeability in synaptosomes (Vizi et al., 1977; Tapia and Sitges, 1982). In this work we show that NTX increases ³H-GABA release from perfused mouse brain synaptosomes. This effect was not blocked by TTX. Ca²⁺ channel blockers (verapamil or Co²⁺) or the absence of external Ca²⁺ prevents the releasing effect of this toxin. NTX does not seem to induce transmitter release by directly increasing Ca²⁺ permeability: The K⁺ ionophore valinomyacin completely inhibits the release induced by NTX, as well as that evoked by the K⁺ channel blocker 4-aminopyridine; in contrast, the release evoked by a Ca²⁺ ionophore is not blocked by valinomyacin. These findings strongly suggest that the releasing effect of NTX is mediated by a decrease in K⁺ permeability. External Ca²⁺ is needed only in order to couple this stimulus with the release process. Consistent with this hypothesis, we present evidence that NTX blocks the efflux of ⁸⁶Rb⁺ from synaptosomes. An extended comparison of the effect of 4-aminopyridine with that of NTX is also reported.

Neurotoxins from several sources, including those from scorpion venoms, have proved to be valuable tools for neurobiological studies (Catterall, 1982; Couraud and Jover, 1984; Narahashi, 1974). Recently, several toxins from the venom of the Mexican scorpion *Centruroides noxius* Hoffmann, have been purified and their amino acid sequences determined (Possani et al., 1981, 1982, 1985). These are basic polypeptides with a molecular weight of approximately 7000; some, like noxiustoxin (NTX), are short-chain polypeptides with a molecular weight of about 4000. NTX has been studied on the voltage-clamped squid axon, in which it specifically depressed potassium permeability (Carbone et al., 1982).

Drugs like 4-aminopyridine (4-AP) and tetraethylammonium, known K⁺ channel blockers in the squid axon, also act on nerve terminals to increase transmitter release. However, their releasing effect has not been shown to be related to a decrease in K⁺ permeability across the synaptolema (Armstrong

and Binstock, 1965; Lundh et al., 1976; Tapia and Sitges, 1982; Vizi et al., 1977; Yeh et al., 1976). Here, we show that NTX increases the release of labeled GABA from perfused mouse brain synaptosomes. We have characterized this action by studying how it is affected by the ion composition of the medium or by the presence of other drugs that modify specific ion permeabilities. Our results indicate that the releasing effect of NTX is due to a reduction of K⁺ permeability in synaptosomes.

Materials and Methods

Release experiments

In each experiment the brains, without the cerebellums, of 4 adult albino mice (CDI strain) were used to obtain a purified synaptosomal fraction following the method of Hajós (1975) with slight modifications. The method used to load synaptosomes with labeled GABA and to study its release has been described previously (Tapia and Sitges, 1982). Briefly, aliquots (0.8 mg protein) of the final synaptosomal pellet resuspended in dextrose (0.32 M) were incubated (10 min) in the uptake medium (127 mM NaCl, 1.18 mM KH₂PO₄, 3.73 mM KCl, 1.8 mM CaCl₂, 1.18 mM MgSO₄, 20 mM NaHCO₃, 5.6 mM dextrose, aminooxyacetic acid 0.1 mM, ³H-GABA 0.83 μCi, 0.5 μM final concentration; the medium was bubbled with O₂:CO₂, 95:5, to give pH 7.4). ³H-GABA-loaded synaptosomes (0.2–0.3 mg protein) were placed on Millipore filters (0.65 μm) lying on perfusion chambers aligned in parallel. The uptake medium was washed out, and perfusate fractions (0.5 ml) were collected by peristaltic pumping at 1 min intervals. After 6 min a test medium was substituted for the initial control medium. The radioactivity released into each collected fraction and that remaining in the filters at the end of the experiment was counted in Tritosol (Fricke, 1975). The release of labeled GABA per minute was expressed as the percentage of the total radioactivity present in the synaptosomes at the beginning of the perfusion (i.e., the radioactivity released during 12 min of perfusion plus that remaining in the filter at the end of the experiment).

Experiments to study the effect of NTX on the release (leakage) of the nontransmitter α-amino isobutyric acid (AIB) were carried out by the same procedure as GABA, except that 2.5 μCi of ³H-AIB (22 μM final) was substituted for the labeled GABA in the uptake medium.

Perfusion media

The composition of standard Ringer's medium (SRM) used to perfuse the synaptosomal preparation was (mM): NaCl, 127; KH₂PO₄, 1.18; KCl, 3.73; CaCl₂, 1.8; MgSO₄, 1.18; NaHCO₃, 20; dextrose, 11.2; and 0.1% BSA. Aminooxyacetic acid, 0.1 mM, was present to block GABA metabolism. The final pH was adjusted to 7.4 by gassing the medium with O₂:CO₂ (95:5, vol/vol).

The GABA-releasing effect produced by NTX was first studied with regard to its dependence on the presence of external Na and Ca ions. The effect of NTX was also tested in the presence of Na⁺ or Ca²⁺ channel blockers. Na⁺-free media were prepared by substituting NaCl for choline and NaHCO₃ for Tris buffer, keeping the pH at 7.4. Ca²⁺-free media were prepared by excluding this ion from standard Ringer's and adding 50 μM EGTA. TTX, 0.5 μM, was used to block Na⁺ channels. In order to block Ca²⁺ channels, low concentrations of verapamil (20 μM) or Co²⁺

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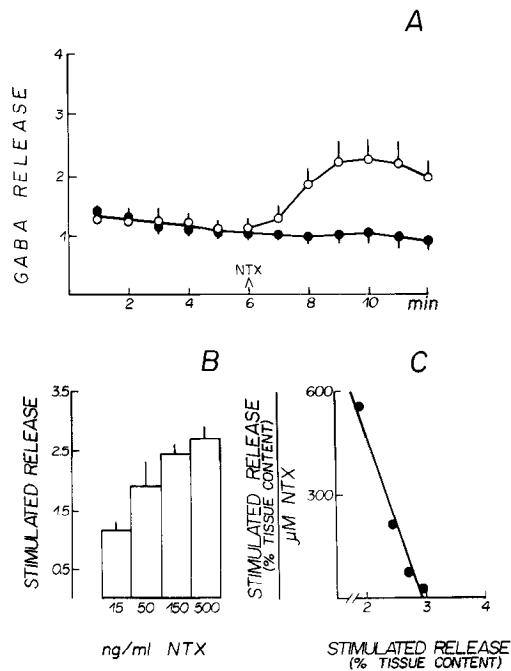


Figure 1. Effect of NTX on the resting release of GABA. *A*, Synaptosomes were loaded with labeled GABA and perfused as described in Materials and Methods. At 6 min (arrow), the standard, low-potassium, Ringer's medium (SRM) was rapidly substituted by the same medium (●) or by a SRM containing 0.1 μM NTX (○). ³H-GABA release, in this and in the following figures, is expressed as the percentage of the total radioactivity present in the synaptosomes at the beginning of the perfusion (see Materials and Methods). Results are means ± SEM of 3 independent experiments. *B*, NTX dose-response plot. Stimulated release is expressed as the difference between the percentage total radioactivity released in the presence of NTX and in the absence of NTX. Total radioactivity refers to the total label contained in synaptosomes at the beginning of the perfusion. *C*, Scatchard plot of the stimulated release evoked by increasing concentrations (3–100 nM) of NTX. For *B* and *C*, results are means of 4 independent experiments. SEM shown in *B*.

(replacing Ca²⁺) were present in the media. The role of K⁺ permeability on the effect of NTX was studied using synaptosomes perfused with a SRM containing 1 μg/ml of the K⁺ ionophore valinomyacin. In order to compare the effect of NTX in the presence of valinomyacin with the effects of the K⁺ channel blocker 4-AP (20 μM) or the Ca²⁺ ionophore A-23187 (5 μM), these drugs were also tested in the presence of vali-

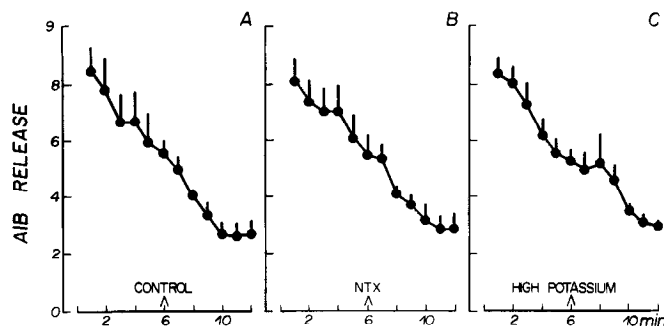


Figure 2. Passive leakage of ³H-AIB in the presence of NTX or high concentrations of external potassium. ³H-AIB-loaded synaptosomes were perfused with SRM. At 6 min (arrows), the SRM was substituted by the same medium (*A*), by SRM containing 0.2 μM NTX (*B*), or by a 50 mM K⁺ depolarizing medium (*C*). Results are means ± SEM of 4 independent experiments.

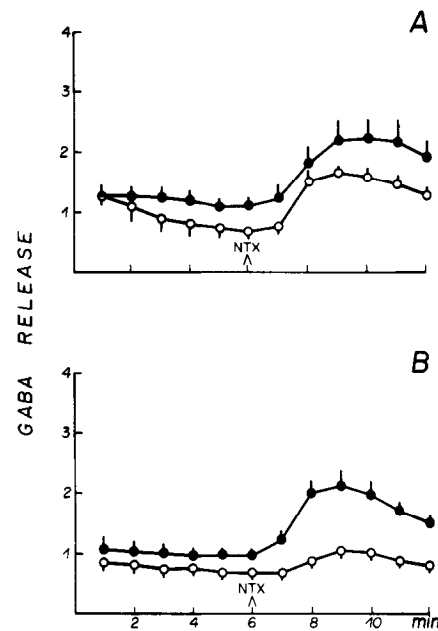


Figure 3. Release of GABA evoked by NTX in the presence of TTX and in the absence of external sodium. *A*, Synaptosomes were perfused with SRM (●) or with SRM containing 0.5 μM TTX (○); NTX (0.1 μM) was added to these perfusing media from min 6 (arrowhead) to min 12. *B*, Synaptosomes were perfused with SRM (●) or with a modified SRM in which Na⁺ was replaced by choline (○); NTX was added as indicated in *A*. Results (expressed as a percentage of ³H-GABA in synaptosomes before perfusion), are means ± SEM of 3 independent experiments.

nomycin. Experiments were designed to study the combined effects of NTX and a depolarizing high-potassium (15 mM) perfusion medium, substituting K⁺ for an equimolar concentration of Na⁺. The combined actions of NTX and veratrine (2.5 μg/ml) and of NTX and 4-AP (1 mM) on GABA release were also studied.

Rubidium efflux

Experimental procedures were similar to those used to study the release of GABA. Synaptosomes were resuspended in SRM (2.5 mg synaptosomal protein/ml), preincubated 5 min, and incubated in the presence of ⁸⁶Rb⁺ (1.5 × 10⁶ cpm/ml) for 20 min. Aliquots (250 μl) from this suspension were placed in microporous filters and rapidly washed 3 times with 5 ml of cold SRM. This preparation was perfused until a constant ⁸⁶Rb⁺ efflux was attained, before testing the effect of NTX. Perfusate fractions were collected every 30 sec. ⁸⁶Rb⁺ efflux is expressed as the percentage of the total radioactivity present in synaptosomes at the beginning of the perfusion.

NTX (fraction II-11 from the venom of *C. noxius* Hoffmann) was purified according to Possani et al. (1982). Other toxins from this venom, II-9.2.2 and II-10, were purified according to Possani et al. (1981). 2,3-³H-GABA (specific activity 34.7 Ci/nmol) and α-methyl-³H-AIB (specific activity, 2.2 Ci/nmol) were obtained from New England Nuclear. ⁸⁶Rb⁺ (specific activity, 0.24 Ci/nmol) was from Amersham. Veratrine hydrochloride, TTX, 4-AP, valinomyacin, aminooxyacetic acid, and the calcium ionophore A23187 (cat. no. 7522) were from Sigma Chemical Co. (St. Louis, MO). Verapamil HCl was a gift of Knoll Laboratories (México, D.F.).

Results

Effect of NTX on resting release of GABA

Synaptosomes were perfused with SRM and then exposed to the same medium containing various concentrations of NTX. There was no observable delay in the onset of the response (Fig. 1*A*). A dose-dependent increase of labeled GABA release was observed. Maximal stimulation was obtained with 500 ng/ml (0.1 μM) NTX (Fig. 1*B*); higher concentrations produced the

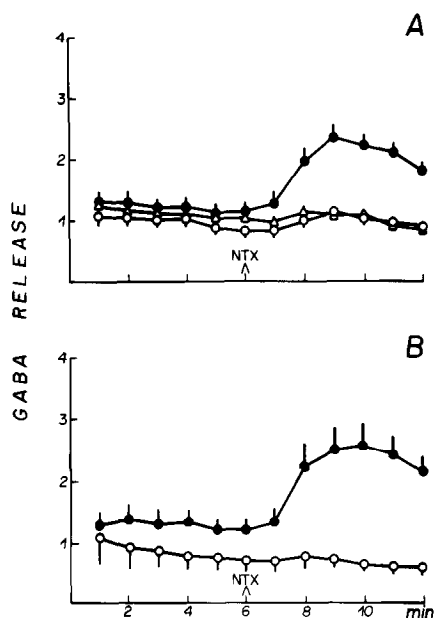


Figure 4. Inhibition of the releasing effect of NTX in the absence of external Ca^{2+} or in the presence of verapamil or Co^{2+} . Synaptosomes were perfused with SRM (●, control), SRM containing $20 \mu\text{M}$ verapamil (O, in A), SRM Ca^{2+} -free containing $50 \mu\text{M}$ EGTA (Δ), or with a SRM in which Ca^{2+} was replaced for Co^{2+} (O, in B). In all cases, $0.1 \mu\text{M}$ NTX was added at 6 min (arrow). Each point is the mean of 3 experiments \pm SEM.

same response. An ED_{50} of 2.9 nM NTX was calculated from the Scatchard plot of the dose-response data (Fig. 1C).

Figure 2 shows that the passive leakage of a nontransmitter amino acid, AIB, from nerve terminals is not altered by the presence of NTX nor by chemical depolarization (50 mM K^+).

Effect of NTX in the presence of TTX and in the absence of external Na^+

Although the presence of TTX in the perfusing medium decreases the resting release of GABA, it does not affect the release evoked by NTX (Fig. 3A); nor can the absence of external Na^+ completely block the NTX releasing effect (Fig. 3B).

Dependence of the effect of NTX on external Ca^{2+} and Ca^{2+} permeability

In the absence of calcium ($50 \mu\text{M}$ EGTA) in the perfusion medium, NTX did not increase the release of labeled GABA. Also, the substitution of cobalt for Ca^{2+} or the addition of a low concentration of verapamil ($20 \mu\text{M}$) to the medium blocked the release induced by this toxin (Fig. 4).

Suppression of the NTX effect by increasing K^+ permeability

Although the presence of valinomycin, a K^+ ionophore, increases the resting GABA release, it completely blocks the release evoked by NTX (Fig. 5A). Also 4-AP, a putative K^+ channel blocker, was ineffective in evoking release in the presence of this ionophore (Fig. 5B). In contrast, the release of GABA elicited by a calcium ionophore (A-23187) was not altered by valinomycin (Fig. 5C).

Effect of NTX and 4-AP on $^{86}\text{Rb}^+$ efflux

Experiments were carried out allowing the efflux of $^{86}\text{Rb}^+$ from synaptosomes to reach a steady state (10–15 min perfusion). Under this condition, addition of NTX ($0.2 \mu\text{M}$) rapidly decreased Rb^+ efflux to less than 10% of the resting value. Also, 4-AP ($200 \mu\text{M}$) drastically inhibited Rb^+ permeability (Fig. 6).

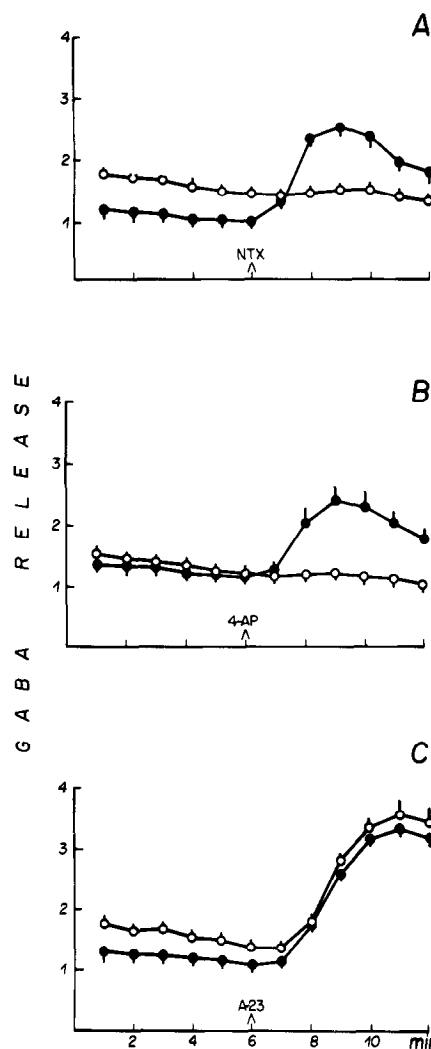


Figure 5. Modification of the release of ^3H -GABA evoked by NTX, 4-AP, and A-23187 in the presence of the K^+ ionophore valinomycin. Synaptosomes were perfused with SRM (●) or with SRM containing $1 \mu\text{g/ml}$ valinomycin (O). Beginning at 6 min these media also contained $0.1 \mu\text{M}$ NTX (A), $50 \mu\text{M}$ 4-AP (B), and $5 \mu\text{M}$ calcium ionophore A-23187 (C). Each point is the mean of 3–4 experiments.

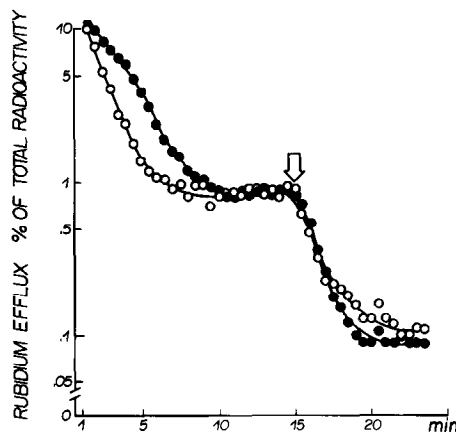


Figure 6. Effect of NTX and 4-AP on $^{86}\text{Rb}^+$ efflux from synaptosomes. Filter holding $^{86}\text{Rb}^+$ -loaded synaptosomes were transferred to parallel perfusion chambers and superfused with SRM. At the time indicated by the arrow, SRM was substituted by a SRM containing $0.24 \mu\text{M}$ NTX (●) or $200 \mu\text{M}$ 4-AP (O). Results are expressed as a percentage of the total radioactivity contained in the synaptosomes.

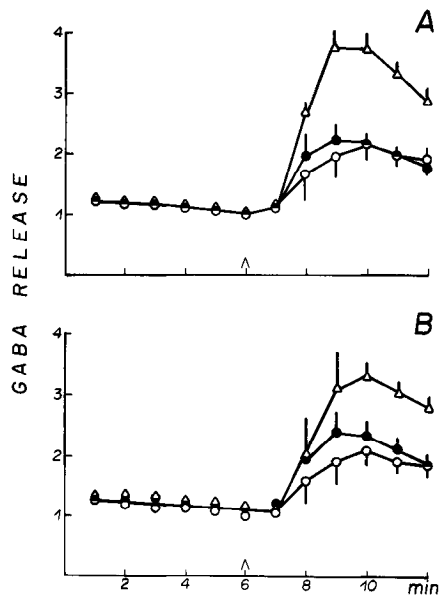


Figure 7. Combined effects of NTX or 4-AP and veratrine. Synaptosomes were perfused with the SRM, and at 6 min the SRM was substituted as follows: *A*, SRM containing 0.1 μM NTX (\bullet), SRM containing 2.5 $\mu\text{g/ml}$ veratrine (\circ), or SRM containing 0.1 μM NTX plus 2.5 $\mu\text{g/ml}$ veratrine (Δ). *B*, SRM containing 20 μM 4-AP (\bullet), SRM containing 2.5 $\mu\text{g/ml}$ veratrine (\circ), or SRM containing 20 μM 4-AP plus 2.5 $\mu\text{g/ml}$ veratrine (Δ). Results are means \pm SEM of 3 independent experiments.

Independent experiments were carried out as a control of the spontaneous release of $^{86}\text{Rb}^+$ for as long as 30 min. The plateau after 10 min remained, showing that the effects of NTX or 4-AP were not artifactual (data not shown).

Combined effects of NTX and veratrine

At doses of NTX and veratrine producing nearly half-maximal effects, their combined actions elicited a releasing response close to the sum of their separate contributions (Fig. 7*A*). Similarly, an additive effect was observed between 4-AP and veratrine (Fig. 7*B*). When these experiments were performed using doses of veratrine evoking the maximal response, NTX or 4-AP did not further increase the stimulated release of GABA (data not shown).

Combined effects of NTX and 4-AP

When NTX and 4-AP were tested at doses producing maximal effects, the release of GABA evoked by 4-AP was higher than that evoked by NTX. Their combined maximal effects were not additive: The release of GABA in the presence of both was

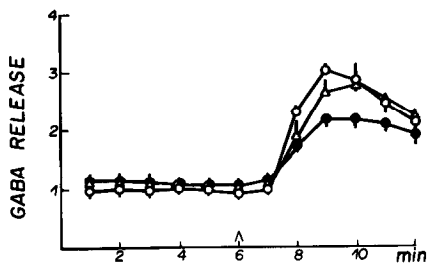


Figure 8. Combined effects of NTX and 4-AP on ^3H -GABA release. Synaptosomes were perfused with the SRM, and at 6 min it was substituted by a SRM containing 0.2 μM NTX (\bullet), 1 mM 4-AP (\circ), or NTX plus 4-AP at the indicated concentrations (Δ). Each point is the mean \pm SEM of 3 independent experiments.

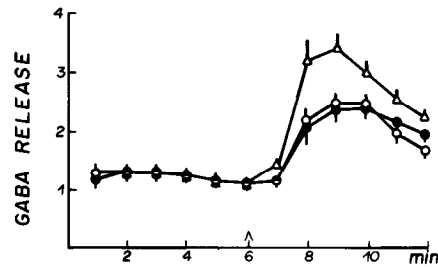


Figure 9. Combined effects of NTX and high K^+ (15 mM) on ^3H -GABA release. Synaptosomes were perfused with the SRM, and at 6 min SRM was substituted by SRM containing 0.1 μM NTX (\bullet), SRM containing 15 mM K^+ (\circ), or 15 mM K^+ Ringer's containing 0.1 μM NTX (Δ). Each point is the mean \pm SEM of 3 experiments.

between that produced by NTX or 4-AP alone (Fig. 8). At lower 4-AP doses, the combined action of NTX and 4-AP always resulted in a releasing response, lower than that evoked by 4-AP alone (data not shown).

Effect of NTX in the presence of high concentrations of external potassium

The effect of NTX on chemically depolarized synaptosomes was studied by adding this toxin to a perfusing medium containing 15 mM K^+ . The releasing effect of NTX (0.1 μM) was superimposed on that produced by the depolarizing medium (Fig. 9). Synaptosomes depolarized in 50 mM external K^+ released 6 times more ^3H -GABA than 15 mM K^+ or NTX alone, and the addition of NTX did not further increase this release (data not shown).

Discussion

We have shown that the exposure of synaptosomes to NTX purified from *C. noxius* venom results in an enhanced release of preloaded labeled neurotransmitter. In contrast, NTX did not increase the release of the nontransmitter amino acid ^3H -AIB. This difference shows that NTX acts on the physiological mechanisms involved in neurotransmitter release and does not have an indiscriminate effect promoting the leakage of synaptosomal contents. It is noteworthy that the effective doses of NTX in the axon ($K_d = 300$ nM) (Carbonc et al., 1982) and in synaptosomes ($\text{ED}_{50} = 2.9$ nM) differ widely. In addition, the delay of several min to the onset of NTX action on the squid axon was not observed in the synaptosomal preparation. These differences could be explained by a difference in the access of NTX to its receptors in each of these preparations.

The releasing effects of many scorpion toxins, including some from *C. noxius* (Table 1), are mediated by an increase of Na^+ permeability that is blocked by TTX. The results given in Table 1 show that toxins II-9.2.2 and II-10, both long-chain toxins

Table 1. GABA release evoked by *C. noxius* toxins in the presence of TTX or veratrine

Scorpion toxins	Evoked release (%)		
	NTX (0.1 μM)	II-9.2.2 (0.2 μM)	II-10 (0.2 μM)
ScTx alone ^a	92 \pm 11 ^b	92 \pm 14	111 \pm 21
ScTx + TTX (0.5 μM)	89 \pm 22	0	9 \pm 3
ScTx + veratrine (2.5 $\mu\text{g/ml}$) (71 \pm 11)	183 \pm 15	297 \pm 32	298 \pm 31

Evoked release is expressed as a percentage of the resting control values.

^a Scorpion toxins (ScTx) were tested either alone or together with TTX or veratrine. Veratrine (2.5 $\mu\text{g/ml}$) alone stimulated release by 71 \pm 11%, and TTX alone slightly decreased (30 \pm 6%) the resting value.

^b SEM calculated from 3–4 independent experiments.

from *C. noxius* venom, affect GABA release from synaptosomes in a similar way as those from *Tityus serrulatus* and *C. suffusus suffusus* (Couraud et al., 1982; Coutinho-Netto et al., 1980). In contrast, the effect of NTX was not blocked by TTX, indicating that NTX does not induce GABA release through the activation of TTX-sensitive Na⁺ channels. However, the releasing action of NTX appeared to depend on the sodium gradient and the sodium equilibrium potential, which are reversed in Na⁺-free media, since the absence of external Na⁺ reduced the effectiveness of NTX.

The release of GABA induced by NTX was blocked in the absence of external Ca²⁺ or by the presence of Ca²⁺ channel antagonists. This observation indicated that a Ca²⁺ influx through verapamil or cobalt-sensitive Ca²⁺ channels might be directly induced by the toxin or otherwise required for the release process triggered by NTX.

The latter possibility—that NTX can initiate the release process by a mechanism different from direct activation of Ca²⁺ channels—is supported by the fact that this toxin decreases K⁺ permeability in the voltage-clamped squid axon (Carbone et al., 1982). In order to test if the release of GABA evoked by NTX was triggered by a reduction of K⁺ permeability, synaptosomes were treated with valinomycin to increase their permeability to K⁺ ions. In the presence of this K⁺ ionophore, the releasing action of NTX was completely blocked. Also, the release of GABA evoked by 4-AP, a drug that blocks the K⁺ conductance in the presynaptic terminal of the squid giant synapse (Llinás et al., 1976), was inhibited by valinomycin. In contrast, the release evoked by a calcium ionophore was not modified in the presence of valinomycin, making it unlikely that NTX could act by directly increasing Ca²⁺ permeability. Thus, the Ca²⁺ dependence of the releasing effect of NTX appears to be linked to the role of this ion in the coupling of stimulus and secretion. All these results, along with direct evidence that NTX (and also 4-AP) blocks ⁸⁶Rb efflux in the synaptosomal preparation, support the concept that this toxin induces release by decreasing K⁺ permeability.

The effect of many scorpion toxins that bind to Na⁺ channels is potentiated in the presence of steroidal alkaloids like veratridine, which are also activators of Na⁺ currents (Catterall, 1975; Krueger and Blaustein, 1980; Ulbricht, 1969). Neither the releasing effect of NTX nor that of 4-AP was potentiated by veratrine, further supporting the concept that the mechanism of action of NTX differs from that of the scorpion toxins acting on Na⁺ channels (Table 1). However, the observation that the effects of NTX and veratrine appear to be additive suggests that stimuli mediated through K⁺ and Na⁺ channels are capable of acting simultaneously to increase transmitter release.

Although NTX and 4-AP behave as K⁺ channel blockers, several differences between them have been found. The maximal releasing response evoked in their presence differs, suggesting that they might preferentially interact with different populations of K⁺ channels (Bartschat and Blaustein, 1985; Rogawski, 1985) or that they could use different mechanisms to reduce K⁺ permeability. In this regard it is noteworthy that the combined effect of NTX and 4-AP that we observed can be interpreted either as negative cooperativity between them or as competition for the same sites.

In the squid axon, NTX blocks K⁺ permeability in a voltage-independent manner (Carbone et al., 1982), whereas the blockade produced by 4-AP is overcome at large depolarizations (Yeh et al., 1976). Consistent with this observation is the finding that while 4-AP is unable to increase the release of transmitters in a depolarized (20 mM K⁺) synaptosomal preparation (Tapia and Sitges, 1982), NTX-induced release is observed over and above that produced by 15 mM external K⁺.

Finally, among K⁺ channel blockers, NTX is the most potent: In synaptosomes the ED₅₀ of NTX-induced GABA release is

2.9 nM, while 4-AP is required in micromolar concentrations to elicit a measurable release (Tapia and Sitges, 1982). In the squid axon, NTX has a K_d of 300 nM to depress K⁺ permeability (Carbone et al., 1982), whereas 4-AP and TEA are active only in the millimolar range (Armstrong and Binstock, 1965; Yeh et al., 1976).

NTX actions have to be characterized in more detail using other techniques and preparations, but what we know about its mechanism of action on the nerve terminals makes it a promising tool for neurobiological research.

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