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SCORPION NEUROTOXIN

MODE OF ACTION ON NEUROMUSCULAR JUNCTIONS AND SYNAPTOSOMES

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SUMMARY

Electrophysiological analysis of the effects of scorpion toxin I, one of the neurotoxins from the venom of the scorpion *Androctonus australis Hector*, upon crayfish neuromuscular junctions has shown that the toxin strongly associates with the nerve terminal to stimulate release of neurotransmitters.

The biochemical approach has shown that the binding of scorpion toxin I to rat brain synaptosomes is accompanied by a decrease in their capacity to accumulate γ-aminobutyric acid. The main effect of the toxin is to stimulate neurotransmitter release. The apparent dissociation constant of the toxin-receptor complex is 0.1–0.2 μM at 22 °C. The rate of dissociation is so slow that complex formation seems to be quasi-irreversible. The "quasi-irreversibility" has also been observed in electrophysiological experiments with the crayfish neuromuscular junction. Tetrodotoxin prevents scorpion toxin I action if it is incubated with synaptosomes or with crayfish neuromuscular junctions before scorpion toxin I application. Tetrodotoxin does not reverse scorpion toxin action if it is added to the preparation after scorpion toxin I. Prevention of scorpion toxin action by tetrodotoxin permits measurements of binding characteristics of this toxin to synaptosomes. The dissociation constant of the tetrodotoxin-receptor complex is 2.2 nM at 22 °C. No cooperativity is observed in the binding. Because of its high affinity for synaptosomes (and the "quasi-irreversibility" of the binding), scorpion toxin I appears to be a potentially excellent tool for further studies of the molecular mechanism of neurotransmitter secretion.

INTRODUCTION

Scorpion venoms have been shown to exert a variety of effects on excitable tissues. Schmidt et al. [1, 2] have studied the action of *Leirus quinquestriatus* venom on the ionic currents of single myelinated nerve fibers of *Xenopus laevis*. They observed

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Abbreviation: Scorpion toxin I: *Androctonus australis Hector* neurotoxin I.

that scorpion venom decreases sodium permeability by 60 %, that sodium inactivation is markedly slowed and that maximum potassium permeability is reduced to about 35 % of its normal value. Narahashi et al. [3] studied the effect of the venom from the scorpion *Buthus tamalus* on the giant axon of the squid. They found that the peak sodium current was not affected, that the time course of the sodium inactivation was greatly prolonged and that the steady-state potassium current was substantially suppressed. They also showed that the venom is active only when applied externally to the axonal membrane. Different results were found by Cahalan [4] for the action of *Centruroides sculpturatus* scorpion venom on single frog myelinated nerve fibers. In this case, activation and inactivation of the sodium current appear normal. However, upon repolarization, a novel sodium current turns on, reaches a peak within about 25 ms, and then declines over several hundred ms.

The cardiac system is also affected by scorpion venom. The venom of the Brazilian scorpion *Tityus serrulatus*, induces a short-lasting bradycardia followed by an increase in the force and frequency of contractions [5].

Scorpion venoms contain at least 15 different proteins including phospholipase. The lethal effect of the venom is due to neurotoxins. The chemical structure of several scorpion neurotoxins is now known. They are single-chains proteins containing 63–64 amino acids with eight half-cystine residues [6, 7]. One of the pure neurotoxins obtained from the venom of the scorpion *Androctonus australis Hector* has been shown to affect both the closing of the sodium channel and the opening of the potassium channel in giant axons of crayfish and lobster nerves [8]. The same neurotoxin blocks both the sodium and the potassium conductances in *Sepia* giant axons. Dose-response curves have been obtained with all types of axons and have given values of apparent dissociation constants for the receptor-toxin complexes [8]. Pure scorpion neurotoxins are also strongly cardiotoxic. Treatment of embryonic heart cells in culture with scorpion neurotoxins extracted from the venom of *Androctonus australis Hector* provoked an increase in cell beat frequency and a decrease in the amplitude of contraction at low concentrations ($\approx$ 10 nM) and fibrillation and contracture at a higher concentration (1 μM) [9].

We analyze in this paper the mechanism of action and the physicochemical properties of binding of one of the neurotoxins of *Androctonus australis Hector* venom, scorpion toxin I, upon crayfish neuromuscular junction and upon rat brain synaptosomes examining the characteristics of the uptake and release of γ-[^{3}H]amino butyrate.

MATERIAL AND METHODS

Electrophysiological experiments

Neuromuscular preparations of the accessory flexor muscle of the first legs of the crayfish *Astacus leptodactylus* have been used throughout. This muscle, located in the meropodite is innervated by two axons, one excitatory and one inhibitory. The crayfish leg was first dissected at the level of the carpopodite and the exoskeleton was partially removed near the muscle. Electrophysiological experiments were carried out on the fusorial part of the accessory flexor muscle where it is easy to see with dark field illumination the course of motor axons along muscle fibers. The nerve containing motor axons was exposed at the level of the ischiopodite. After fixing the preparation

in the experimental chamber and after gentle stretching of the muscle by opening the joint between carpopodite and meropopodite the nerve was sucked into a fluid electrode connected to a stimulator (Grass S88) or an A.C. amplifier to stimulate or record nerve activity. Intracellular muscle potentials were recorded by means of a glass capillary microelectrode filled with a 3 M solution of KCl (resistance between 5 and 10 MΩ). The microelectrode was connected to a neutralized capacity amplifier. When necessary, a second microelectrode was positioned near the first and used for applying current pulses to measure membrane resistance. Signals were displayed on a storage oscilloscope (Tektronix 5103N). Microelectrodes used for extracellular recordings were filled with a 2 M solution of NaCl (resistance between 2 and 5 MΩ).

Experiments were carried out at 20±1 °C. The normal immersion medium for the nerve-muscle preparation is a Van Harreveld solution at pH 7.5 containing 207 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 5.4 mM $MgCl_2$ and 10 mM Tris · Cl.

Preparation of synaptosomes

Adult male Sprague-Dawley rats (150–200 g) were killed by decapitation. Their brains were rapidly removed and homogenized in 10 volumes of ice-cold 0.32 M sucrose, buffered at pH 7.4 with 5 mM Tris · Cl, in a Thomas glass homogenizer fitted with a teflon pestle (10 passes at 700 rev./min). The homogenate was centrifuged at $1200 \times g$ for 5 min and the precipitate was discarded. The supernatant fluid was centrifuged at $23\,000 \times g$ for 20 min. The resulting pellet was resuspended in 1 ml of a solution of 0.32 M sucrose in 5 mM Tris · Cl at pH 7.4 and layered on a discontinuous sucrose gradient buffered by 5 mM Tris · Cl at pH 7.4 and consisting of three 10 ml layers of 1.2 M, 1 M and 0.8 M sucrose respectively. Centrifugation was carried out at $83\,000 \times g$ during 60 min in a SW 27 swinging buckets rotor in a Beckman LS 50 refrigerated ultra-centrifuge. After centrifugation, the synaptosomes which had accumulated at the interface between the 1 M and the 1.2 M sucrose layers were collected and kept at 0 °C.

γ-Aminobutyric acid influx measurements

The filters which were used in γ-amino butyric acid transport measurements (Millipore EHWP 02500 and Sartorius SM 11106, 0.45 μm pore size) were kept in 1 % bovine serum albumin for 2 h, then washed twice with 5 ml of the standard incubation buffer before use. This incubation buffer consisted of 140 mM NaCl, 5 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 20 mM Tris · Cl, pH 7.4 & 1 % BSA. Synaptosomes were suspended in this medium after addition of 0.1 mg of the γ-aminobutyric acid-transaminase inhibitor, sodium dipropylacetate [10], to prevent γ-aminobutyric acid transformation. Influx measurements were carried out at 22±0.1 °C. The influx reaction was initiated by addition of γ-[^{3}H]aminobutyric acid. Aliquots of 0.1 to 0.15 ml were taken from the incubation medium at appropriate times and synaptosomes loaded with γ-[^{3}H]aminobutyric acid were isolated by filtration. Filters were then washed twice with 5 ml of the incubation buffer at 22 °C and placed in counting vials containing 10 ml of Bray's solution which totally dissolves them. Radioactivity measurements were carried out in Packard 3390 and 2450 liquid scintillation spectrometers. The efficiency of radioactivity measurements was routinely determined by the automatic external standard.

Blanks were obtained by passing the same incubation medium containing

appropriate concentrations of γ-[^{3}H]aminobutyric acid, but lacking synaptosomes, through the same filters. The radioactivity which remains on the filter under these conditions is subtracted from the radioactivity obtained in the experiment involving synaptosomes. The radioactivity in the blank never exceeded 5 % of the total radioactivity measured for loaded synaptosomes.

γ-Aminobutyric acid efflux measurements

Synaptosomes were preloaded with γ-[^{3}H]amino butyric acid by incubation at 22 °C for 15 min under conditions described in the preceeding section. The synaptosome suspension was then diluted 10 times in the usual incubation medium (see previous section on influx) to trigger γ-aminobutyric acid efflux. To measure scorpion toxin action, the dilution is carried out in the presence of the toxin. Efflux kinetics are followed by taking aliquots at different times after the dilution. The γ-[^{3}H]amino butyric acid remaining in the synaptosomes was determined by the filtration technique described in the previous section.

Another technique has also been used for measuring efflux. In this technique, synaptosomes which have first been preloaded with γ-[^{3}H]aminobutyric acid during 15 min, as previously described, are separated from external γ-aminobutyric acid by centrifugation at 20 000 × g for 10 min at 1 °C. The pellet containing the synaptosomes is then washed twice with 5 ml of ice-cold sucrose (0.32 M) buffered with 5 mM Tris · Cl, pH 7.4, and finally resuspended in the same sucrose solution. This suspension is kept at 0 °C. For efflux measurements, 0.2 ml of this synaptosomal suspension preloaded with γ-[^{3}H]aminobutyric acid were added to 0.8 ml of the standard incubation medium, free of γ-aminobutyric acid efflux were then followed using the filtration technique. The two techniques give identical results.

Chemicals

γ-[^{3}H]Amino butyric acid (10 Ci/mmol) was obtained from New-England Nuclear Corporation. Non radioactive γ-amino butyric acid was obtained from Sigma. Tetrodotoxin was obtained from Sankyo.

Preparation of neurotoxin I

The venom of *Androctonus australis Hector* contains several neurotoxins [11]. Neurotoxin I (M_r = 6800) purified as previously described by Rochat et al. [6] was shown to be homogeneous by equilibrium chromatography on Amberlite CG 50, by starch gel and disc electrophoresis, by determination of the N-terminal sequence and by ultracentrifugation. Toxicity measured by intravenous injection in 20 g mice corresponds to LD_{50} = 10 μg/kg mice [12].

RESULTS

Electrophysiological analysis of scorpion toxin I effect upon crayfish nerve-muscle preparations

There are two types of neuromuscular junctions in this preparation: excitatory junctions in which the probable neurotransmitter is glutamate and inhibitory junctions in which the neurotransmitter is probably γ-aminobutyric acid [13].

Typical recordings of junction potentials in the presence of scorpion toxin I

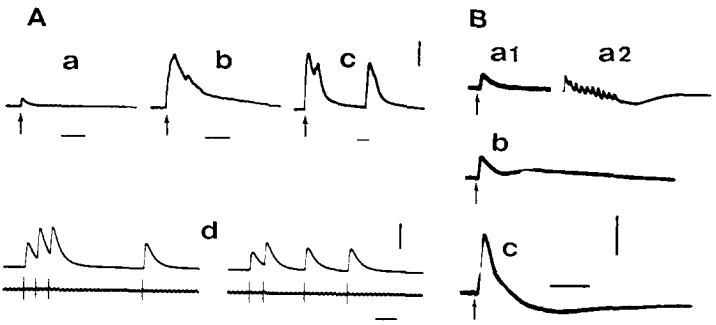

Fig. 1. Effect of scorpion toxin I on excitatory and inhibitory junction potentials. A(a): Excitatory junction potential control to a single stimulus of the nerve; (b): increase of the electrical response 30 s after addition of scorpion toxin I (0.2 μM); (c): repetitive response after 4 min incubation with scorpion toxin I. The stimulus in each case is indicated by an arrow. Horizontal calibration: 100 ms; vertical calibration: 10 mV; (d): simultaneous recordings of excitatory junction potential (top traces) and nerve activity (bottom trace), 10 min after neurotoxin application and in the absence of electrical stimulation. Horizontal calibration: 100 ms; vertical calibration: 10 mV. (B): electrical response after a single stimulus (a_1), and after a train of stimuli (a_2) in the absence of scorpion toxin I. In the case of a_2 the electrical response is observed to include an inhibitory junction potential in addition to the excitatory junction potential which is clearly seen in a_1. (b): After 1 min incubation in the presence of 0.1 μM scorpion toxin I, the excitatory junction potential increases as in (A). The excitatory junction potential is very clearly followed by an inhibitory response which could barely be seen after a single stimulus in the absence of toxin (a_1). (c): excitatory and inhibitory junction potentials are considerably increased after a 5 min application of the toxin. Horizontal calibration: 100 ms., vertical calibration: 10 mV.

are presented in Fig. 1. At 0.2 μM toxin, the effects on neuromuscular junctions can be chronologically classified into three different periods (Fig. 1A): (i) first, in less than 1 min, the toxin induces an important increase of the amplitude of the excitatory junction potentials (Fig. 1Ab), (ii) then, between 1 and 5 min, a single electrical stimulation of the nerve evokes repetitive response (Fig. 1Ac); (iii) finally, after a longer period of exposure to scorpion toxin I (Fig. 1Ad), one observes at the level of the junction an intense spontaneous activity interrupted by rest periods. Knowing that scorpion toxin treatment of the axon induces long lasting pre-junctional spikes [8], the most immediate interpretation of results presented in Fig. 1Ac and in Fig. 1Ad is that the repetitive response and the spontaneous activity are mainly provoked by scorpion toxin I action upon motor nerve fibers. Direct demonstration of the correctness of this interpretation is presented in Fig. 1Ad. The figure shows a typical experiment in which there is simultaneous recording of excitatory junction potentials in the muscle fibre and of the electrical activity of the nerve itself. It is clear from this figure that each new junction potential is triggered by a scorpion toxin I induced spike in the motor axon. Spontaneous inhibitory potentials have also been observed after scorpion toxin I treatment.

Fig. 1B shows a typical situation in which there is simultaneous recording of an excitatory and an inhibitory junction potential. Scorpion toxin I clearly increases both kinds of junction potentials.

Fig. 2 illustrates another type of demonstration of scorpion toxin I induced increase of the post-junctional potential. In this experiment, an extracellular electrode

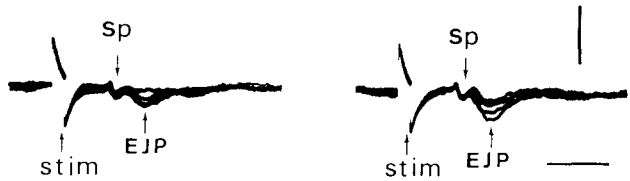

Fig. 2. Extracellular recording of excitatory junction potential (EJP) before (left) and after (right) application of scorpion toxin I (0.2 μM). The diphasic deflection preceding that caused by the excitatory junction potential is due to the nerve terminal spike (Sp). The excitatory junction potential amplitude increases in the presence of toxin while the presynaptic spike is hardly changed. Horizontal calibration: 5 ms; vertical calibration: 500 μV. Stim.: stimulation artifact. The nerve terminal spike (Sp) is the first or second derivative of the presynaptic action potential depending upon microelectrode localisation on the nerve terminal. This signal is mainly caused by the fastest part of the action potential, i.e. the rising phase, which is not affected by scorpion toxin I [8].

was placed in the immediate vicinity of a single neuromuscular junction to record simultaneously the pre-junctional spike potential, which signals the arrival of the nerve impulse at the nerve terminal, and the externally derived post-junctional potential.

It was separately shown that scorpion toxin I treatment leaves the electrical resistance of the muscular membrane unchanged (30 kΩ for a fiber diameter of 200–300 μm). Moreover, the response of the muscular membrane to application of L-glutamate and γ-aminobutyric acid at concentrations of 1 mM is totally unaffected in the presence of 0.5 μM scorpion toxin I. Such an experiment shows that toxin treatment does not provoke "sensitization" of post-junctional receptors to the excitatory and inhibitory transmitters.

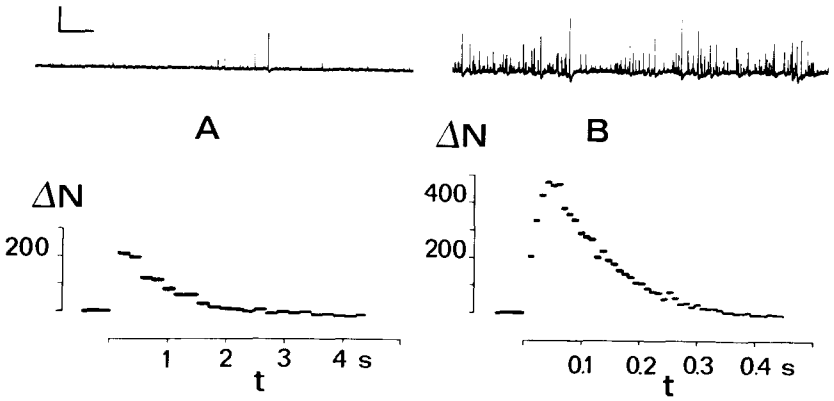

Fig. 3. Influence of scorpion toxin I on spontaneous miniature junction potentials. (A): control without toxin. (B): situation after successive additions of scorpion toxin I (0.2 μM) and tetrodotoxin (50 nM). Tetrodotoxin was added only after the appearance of the spontaneous response (see Fig. 1Ad). Top: Recordings of miniature potentials. Horizontal calibration: 2 s; vertical calibration: 500 μV. Bottom: Distribution of intervals between successive miniature potentials. ΔN: number of intervals whose duration falls within the time increment between t and $t+\Delta t$. In A, Δt = 200 ms; in B, Δt = 10 ms. This type of distribution is fitted by an experimental curve corresponding to the equation [14]: $\Delta N = N\Delta t/T\, e^{-t/T}$

N: total number of observations, T: mean interval between two successive junction potentials. Calculation of T with this equation gives the following values T = 0.84 s in the control and T = 0.1 s in the presence of scorpion toxin I.

Fig. 3 shows that scorpion toxin I increases the frequency of spontaneous miniature potentials. In this type of experiment, the nerve-muscle preparation was treated first by scorpion toxin I (0.2 μM) and then after a few minutes by tetrodotoxin (50 nM). Under these conditions, tetrodotoxin blocks nerve conduction by a selective action on the sodium channel even in the presence of scorpion toxin I [8]. However, the mean frequency on miniature potentials is about 8 times higher in the presence of 0.2 μM scorpion toxin I than it is in the control. This experiment suggests: (i) that scorpion toxin I binds not only to the axonal membrane but also at the level of the nerve terminal, and that this binding provokes neurotransmitter release, (ii) that the scorpion toxin I effect upon terminals cannot be reversed by tetrodotoxin (when tetrodotoxin is added after scorpion toxin I application).

Binding of scorpion toxin I to the terminal is very tight since it cannot be reversed at all by prolonged washing (30 min). When the nerve-muscle preparation is treated by tetrodotoxin (50 nM) before applying scorpion toxin I (0.2 μM) there is a complete prevention of the scorpion toxin effect upon miniature potentials.

γ-Aminobutyric acid accumulation and release in rat brain synaptosomes in the presence of scorpion toxin I

Synaptosomes have been particularly useful in studying the localization, synthesis, uptake, metabolism and, recently the release of neurotransmitters. Fig. 4A shows the kinetics of γ-[^{3}H]aminobutyric acid uptake by rat brain synaptosomes. Incubation of scorpion toxin I with synaptosomes for 30 min decreases the maximum uptake of transmitter by about 40 %.

It is well known that γ-aminobutyric acid transport into synaptosomes closely follows Michaelis-Menten kinetics [15]. Lineweaver-Burk plots of kinetics data concerning γ-aminobutyric acid transport in the absence and in the presence of

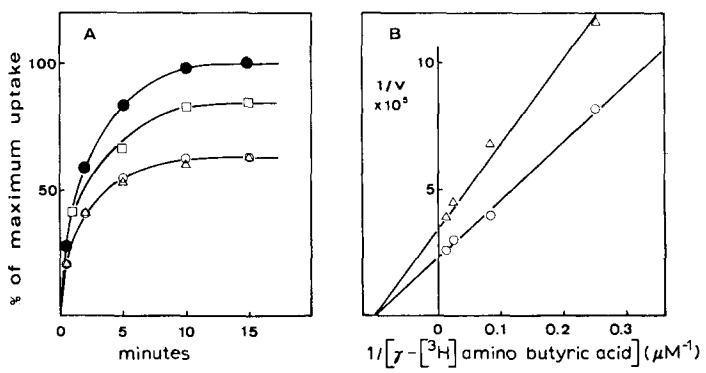

Fig. 4. (A) The influence of scorpion toxin I upon kinetics of γ-[^{3}H]aminobutyric acid uptake into rat brain synaptosomes. Synaptosomes (150 μg of protein/ml) were incubated at pH 7.4, 22 °C with 54 μM γ-[^{3}H]aminobutyric acid after different incubation times with scorpion toxin I (0.8 μM). No preincubation with the toxin (●); preincubation of 20 min before starting γ-aminobutyric acid uptake experiments (□); preincubation of 30 min with scorpion toxin I (○); preincubation of 40 min with scorpion toxin I (△). (B) Lineweaver-Burk representation of kinetics of γ-aminobutyric acid uptake. Synaptosomes treated with 5 μM scorpion toxin I for 30 min (△); synaptosomes left in the same medium for 30 min but without toxin (○). v is expressed in pmol/mg of protein/min, and represents initial uptake rate.

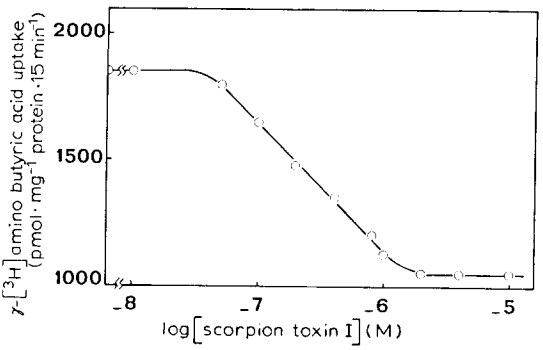

Fig. 5. Scorpion toxin I concentration dependence of maximum γ-[^{3}H]aminobutyric acid uptake into rat brain synaptosomes. Synaptosomes were preincubated with different toxin concentrations for 30 min at 22 °C, pH 7.4. A γ-[^{3}H]aminobutyric acid concentration of 54 μM was used for uptake measurements.

scorpion toxin I are shown in Fig. 4B. The toxin has no effect on the K_m value for the neurotransmitter, 10 μM; the maximal rate for γ-aminobutyric acid transport is 430 pmol/min/mg of protein in the absence of toxin and 290 pmol/min/mg of protein in the presence of 5 μM scorpion toxin I.

Fig. 5 shows the toxin concentrations dependence of the inhibition of γ-amino butyric acid uptake. The results indicate that the toxin prevents neurotransmitter accumulation at very low concentrations. The value of the apparent dissociation constant of the scorpion toxin I-receptor complex derived from these results is 0.26 μM.

The dissociation of the scorpion toxin I-receptor complex is too slow to be seen. Scorpion toxin I action under our conditions appears as "quasi-irreversible". The demonstration of this result was made as follows: synaptosomes were first incubated at 22 °C, pH 7.4 with 3 μM of scorpion toxin I for 30 min; after this time they were isolated by centrifugation as described in the Methods section and carefully washed twice with 5 ml of ice-cold sucrose solution (0.32 M) buffered with 5 mM Tris · Cl, pH 7.4. Since each washing was followed by centrifugation at 20 000 × g, for 10 min, the total washing period was about 30 min long. After washing, synaptosomes were resuspended in 0.32 M sucrose buffered at pH 7.4, brought at 22 °C and incubated with γ-[^{3}H]aminobutyric acid under standard conditions to measure neurotransmitter uptake. The kinetics of uptake of γ-[^{3}H]aminobutyric acid by this preparation of synaptosomes which has been initially incubated with scorpion toxin I were compared to kinetics of uptake of γ-[^{3}H]aminobutyric acid by synaptosomes which have undergone exactly the same manipulations except that during the first 30 min period they were left without scorpion toxin. The plateau value giving maximal γ-[^{3}H]amino butyric acid uptake between 10 and 15 min (see Fig. 4A) is 40 % lower for the synaptosomes preparation which had been initially incubated with the toxin. This experiment demonstrates that dissociation of scorpion toxin I from its receptor does not occur in a medium free of toxin after 30 min at 1 °C (washing period) plus 15 min at 22 °C (period of incubation with γ-[^{3}H]aminobutyric acid).

The inset of Fig. 6 indicates that the presence of tetrodotoxin can reverse the effect of scorpion toxin I provided that it is added to the incubation medium simultaneously with or before scorpion toxin I. The dose-response curve of the tetrodotoxin

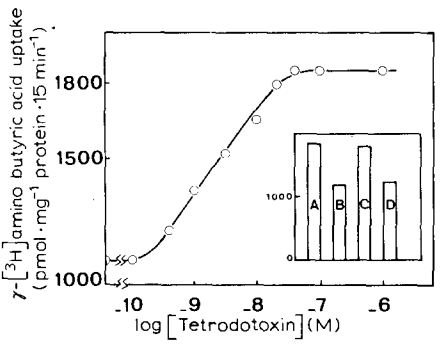

Fig. 6. Reversibility by tetrodotoxin of the inhibition by scorpion toxin I of γ-[^{3}H]aminobutyric acid uptake by rat brain synaptosomes, pH 7.4, 22 °C. Inset. Conditions before starting γ-[^{3}H]amino butyric acid uptake. (A) Synaptosomes incubated 30 min in the standard medium, without toxin (B) Synaptosomes incubated 30 min with 0.6 μM scorpion toxin I. (C) Synaptosomes incubated 30 min with 0.6 μM scorpion toxin I and 0.7 μM tetrodotoxin (added at the same time). (D) Synaptosomes first incubated 28 min with 0.6 μM scorpion toxin I, then 2 min in the presence of 0.6 μM scorpion toxin I and 0.7 μM tetrodotoxin. The curve represents the titration of the tetrodotoxin receptor. Before starting γ-aminobutyric acid uptake, synaptosomes were preincubated 30 min at 22 °C, pH 7.4, with a mixture of 2 μM scorpion toxin I and different concentrations of tetrodotoxin.

induced prevention of scorpion toxin I inhibition of synaptosomal accumulation of γ-aminobutyric acid is presented in Fig. 6. This curve gives the characteristic features of tetrodotoxin binding to synaptosomes i.e. a Hill coefficient of 1.0 and an apparent dissociation constant of 2.2 nM for the tetrodotoxin-receptor complex. It should be noted, however, that if synaptosomes are preincubated with scorpion toxin I before tetrodotoxin addition, the inhibitory effect of the scorpion toxin upon γ-aminobutyric acid accumulation cannot be reversed.

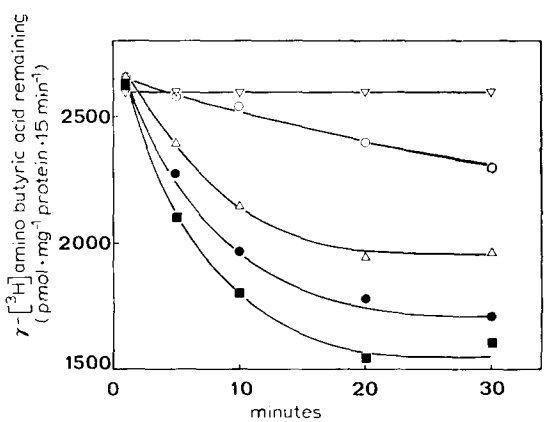

Fig 7. Effect of scorpion toxin I on the kinetics of γ-[^{3}H]aminobutyric acid release from preloaded, syn.aptosomes. Synaptosomes were preloaded with γ-[^{3}H]aminobutyric acid (54 μM) at 22 °C pH 7.4. After 15 min loading, γ-aminobutyric acid efflux was triggered by a ten-fold dilution as described under Material and Methods in the presence of increasing scorpion toxin I concentrations. Scorpion toxin I concentrations in the figure are (○) zero, (△) 0.1 μM, (●) 0.3 μM, (■) 2 μM. In one experiment (▽), the dilution medium contained both scorpion toxin I 2 μM and tetrodotoxin 1 μM.

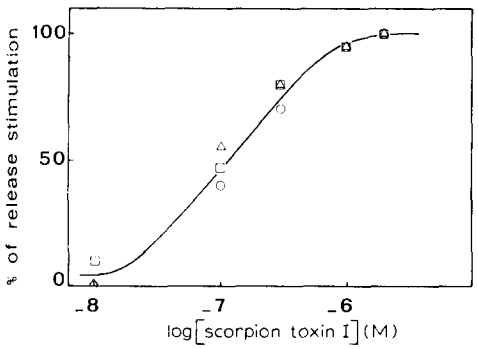

Fig. 8. Scorpion toxin I concentration dependence of the stimulation of γ-[^{3}H]aminobutyric acid release. Experimental points were obtained at p_{Ii} 7.4, 22 °C after release times of 5 min (○), 10 min (△) and 30 min (□).

The scorpion toxin I effect upon γ-amino butyric acid efflux from the synaptosomes is presented in Fig. 7. The data clearly indicate that the scorpion toxin stimulates γ-aminobutyric acid release. This stimulation is suppressed when synaptosomes are treated by scorpion toxin I in the presence of tetrodotoxin. The scorpion toxin I concentration dependence of the release of γ-aminobutyric acid is presented in Fig. 8. The dose-response curve indicates again a high affinity of the toxin for its receptor. The apparent dissociation constant of the scorpion toxin I-receptor complex is 0.12 μM.

Lactate dehydrogenase is a classical marker of cytoplasm occluded within synaptosomes. This enzyme is completely released after 30 min treatment of synaptosomes at 22 °C with 1 % of the detergent Triton X 100 well known to destroy plasma membranes. The amount of lactate dehydrogenase released by 1 μM scorpion toxin I after a 30 min treatment with the toxin at 22 °C is less than 1 % of the amount released by Triton X 100. It is identical to the release of lactate dehydrogenase observed in the control in the absence of Triton X 100 or scorpion toxin I. Thus scorpion toxin I does not appear to cause any synaptosome disruption.

DISCUSSION

Electrophysiological analysis of the effect of scorpion toxin I upon the crayfish neuromuscular junction has shown that the toxin, which was already known to affect axons [8], also associates strongly with the nerve terminal to stimulate the release of the excitatory and inhibitory transmitters of the neuromuscular junction.

These results obtained with the pure toxin are in accord with other results obtained with venoms from various scorpions which have been shown to release acetylcholine [16, 17], serotonin [18] or norepinephrine [19].

It is now well accepted that the synaptosomal system is particularly suited for the evaluation of the molecular mechanisms of, and drug effects upon, transmitter secretion. The in vitro study of the scorpion toxin I effect upon the accumulation of γ-aminobutyric acid by rat brain synaptosomes has confirmed the electrophysiological results; the main effect of the toxin is to stimulate neurotransmitter release. Scorpion toxin I binds very strongly to synaptosomes. The apparent dissociation

constant of the toxin-receptor complex is 0.1–0.2 μM (Figs. 5 and 8), and the rate of dissociation is so slow that complex formation seems to be quasi-irreversible. This "quasi-irreversibility" of scorpion toxin I binding to its receptor has also been observed in electrophysiological experiments.

It is worthwhile to compare characteristics of binding of scorpion toxin I to synaptosomes with those which have been found for binding of this toxin to axonal membranes. Apparent dissociation constants for the receptor-toxin complexes were 0.25 μM and 0.7 μM for crayfish and lobster axons [8]. A higher dissociation constant was found for *Sepia* axons, 2–4 μM [8]. There are two important differences between scorpion toxin I binding to axons and scorpion toxin I binding to synaptosomes. First, dose-response curves for toxin action are markedly cooperative with all types of axons (Hill coefficient higher than 2) whereas very little positive cooperativity is observed for scorpion toxin I action on synaptosomes (Hill coefficient of 1). This value of the Hill coefficient denotes the absence of strong interactions between scorpion toxin I binding sites. Second, the action of scorpion toxin I on membranes of giant axons is reversible whereas on neuromuscular junctions or synaptosomes it is not. It would obviously be of great interest to know the mechanism by which scorpion toxin I stimulates neurotransmitter release. Electrophysiological studies by Katz and Miledi [20–22] on the squid giant synapse provide convincing evidence that transmitter release is controlled by a depolarization-dependent calcium permeability increase at the presynaptic terminals. Recent in vitro studies with synaptosomes have confirmed that depolarization provokes the entry of Ca^{2+} and the release of transmitter [23–25].

In fact, synaptosomes fulfill all of the criteria for stimulus-secretion coupling defined from the electrophysiological approach. As expected, the stimulation of γ-aminobutyric acid release by scorpion toxin I is accompanied by a stimulation of Ca^{2+} uptake by synaptosomes. Scorpion toxin I at a concentration of 3 μM stimulates the rate of $^{45}Ca^{2+}$ entry (measured according to Blaustein [23]) by a factor of 2 at 25 °C. There are two interpretations for the scorpion toxin I stimulation of γ-amino butyric acid release or of Ca^{2+} entry: (i) scorpion toxin I increases Ca^{2+} permeability by direct interaction with the calcium channel without affecting the membrane potential; (ii) scorpion toxin I increases Ca^{2+} permeability by simply depolarizing the presynaptic membrane. It is not yet possible to choose unequivocably between these two interpretations. At first sight the effects of scorpion toxin I have great similarities with those of veratridine, a typical depolarizing agent, at concentrations of 0.01–1 mM, on a variety of excitable tissues [26–28]. Similarly to scorpion toxin I, veratridine stimulates calcium uptake and neurotransmitter release by synaptosomes [23]; moreover, the veratridine effect is also prevented by tetrodotoxin [26–29]. Veratridine and scorpion toxin I have also similarities of action on chick embryo heart cells. They both stimulate the passive uptake of both Na^+ [30] and Ca^{2+}*. However there are major differences in the mode of action of scorpion toxin I and veratridine on axonal membranes. Veratridine depolarizes nerve membranes by a selective increase in resting sodium permeability [27], scorpion toxin I leaves the resting potential of giant axons nearly unchanged (variations of 3–5 mV at 8 °C and high toxin concentrations) and selectively affects both the closing of the Na^+ channel and the opening of

* Fosset, M., De Barry, J. and Lazdunski, M., unpublished.

the K^+ channel [3, 8]. There are differences in the mode of action of the two toxic compounds even on synaptosomes; the veratridine stimulation can be reversed by tetrodotoxin even when tetrodotoxin is added after veratridine. This is not the case for scorpion toxin I.

The analysis of the effect of scorpion toxin I upon both neuromuscular junctions of the crayfish and rat brain synaptosomes has allowed us to study some characteristics of tetrodotoxin action at the nerve terminal membrane. An interesting observation which remains unexplained is that the prevention by tetrodotoxin of the effects of scorpion toxin I only occurs when scorpion toxin I is introduced after tetrodotoxin. When scorpion toxin I is introduced first, the stimulation of neurotransmitter release, as followed by the electrophysiological or the biochemical technique, cannot be reversed by tetrodotoxin. Tetrodotoxin binding to the synaptosomal membrane is characterized by a high affinity nearly identical to that found for the interaction of tetrodotoxin with membranes of axons (dissociation constant of the tetrodotoxin-receptor complex: 2.3 nM) using electrophysiological as well as biochemical techniques [26, 31, 32]. As also observed in axons [26, 32], tetrodotoxin binding to its receptor follows a Michaelis-Menten type law (Hill coefficient of 1.0).

This work clearly demonstrates that scorpion toxin I ranks with tetrodotoxin, saxitoxin, batrachotoxin and, perhaps, veratridine (although this last compound acts at higher concentrations) among the neurotoxic tools which will be of great importance to decipher the molecular mechanism not only of axonal conduction but also of neurotransmitter secretion.

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REFERENCES

1 Adam, K. R., Schmidt, H., Stampfli, R. and Weiss, C. (1966) Br. J. Pharmacol. 26, 666–677
2 Koppenhöffer, E. and Schmidt, H. (1968) Pflügers Arch. 303, 133–149
3 Narahashi, T., Shapiro, B. I., Duguchi, T., Scuka, M. and Wang, C. M. (1972) Am. J. Physiol. 222, 850–857
4 Cahalan, M. D. (1975) J. Physiol. 244, 511–534
5 Corrado, A. P., Antonio, A. and Diniz, C. R. (1968) J. Pharmacol. Exp. Ther. 164, 253–258
6 Rochat, H., Rochat, C., Miranda, F., Lissitzky, S. and Edman, P. (1970) Eur. J. Biochem. 17, 262–266
7 Kopeyan, C., Martinez, G., Lissitzky, S., Miranda, F. and Rochat, H. (1974) Eur. J. Biochem. 47, 483–489
8 Romey, G., Chicheportiche, R., Lazdunski, M., Rochat, H., Miranda, F. and Lissitsky, S. (1975) Biochem. Biophys. Res. Commun. 64, 115–121
9 Fayet, G., Couraud, F., Miranda, F. and Lissitzky, S. (1974) Eur. J. Pharmacol. 27, 165–174
10 Godin, Y., Heiner, L., Marc, J. and Mandel, P. (1969) J. Neurochem. 16, 869–873
11 Miranda, F., Kopeyan, C., Rochat, H., Rochat, C. and Lissitzky, S. (1970) Eur. J. Biochem. 16, 514–523
12 Rochat, C., Rochat, H., Miranda, F. and Lissitzky, S. (1967) Biochemistry 6, 578–585

13 Gerschenfeld, H. M. (1973) Physiol. Rev. 53, 1–119
14 Martin, A. R. (1966) Physiol. Rev. 46, 51–66
15 Martin, D. L. (1973) J. Neurochem. 21, 345–356
16 Gomez, M. V., Dai, M. E. M. and Diniz, C. R. (1973) J. Neurochem. 20, 1051–1061
17 Gomez, M. V., Diniz, C. R. and Barbosa, T. S. (1975) J. Neurochem. 24, 331–336
18 Cunha Melo, J. R., Freire-Maia, L., Tafuri, W. L. and Maria, T. A. (1973) Toxicon, 11, 81–84
19 Moss, J., Colburn, R. W. and Kopin, I. J. (1974) J. Neurochem. 22, 217–221
20 Katz, B. and Miledi, R. (1970) J. Physiol. 207, 789–801
21 Katz, B. and Miledi, R. (1971) J. Physiol. 216, 503–512
22 Miledi, R. (1973) Proc. R. Soc. B 183, 421–425
23 Blaustein, M. P. (1975) J. Physiol. 247, 617–655
24 Levy, W. B., Haycock, J. W. and Cotman, C. W. (1974) Mol. Pharmacol. 10, 439–449
25 Cotman, C. W., Haycock, J. W. and White, W. F. (1976) J. Physiol. 254, 475–505
26 Balerna, M., Fosset, M., Chicheportiche, R., Romey, G. and Lazdunski, M. (1975) Biochemistry 14, 5500–5511
27 Ulbricht, W. (1969) Ergeb. Physiol. Biol. Chem. Exp. Pharmakol. 61, 18–71
28 Ohta, M., Narahashi, T. and Keeler, R. F. (1973) J. Pharmacol. Exp. Ther. 184, 143–154
29 Catterall, W. A. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1782–1786
30 Couraud, F. and Lissitzky, S., Biochim. Biophys. Acta, in the press
31 Cuervo, L. A. and Aldelman, W. J. (1970) J. Gen. Physiol. 55, 309–335
32 Schwarz, J. R., Ulbricht, W. and Wagner, H. H. (1973) J. Physiol. 233, 167–194