Proc. Natl. Acad. Sci. USA
Vol. 73, No. 11, pp. 4055–4059, November 1976
Cell Biology

Sea anemone toxin: A tool to study molecular mechanisms of nerve conduction and excitation–secretion coupling

(neurotoxins/axonal ionic channels/neurotransmitter release)

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Communicated by Josef Fried, July 27, 1976

ABSTRACT **The effects of a polypeptide neurotoxin from *Anemonia sulcata* on nerve conduction in crayfish giant axons and on frog myelinated fibers have been analyzed. The main features of toxin action are the following: (*i*) the toxin acts at very low doses and its action is apparently irreversible. (*ii*) The toxin selectively affects the closing (inactivation) of the Na^+ channel by slowing it down considerably; it does not alter the opening mechanism of the Na^+ channel or the steady-state potassium conductance. (*iii*) The tetrodotoxin–receptor association is unaffected by previous treatment of the axonal membrane with the sea anemone toxin. (*iv*) Conversely, the sea anemone toxin can only associate with the membrane when the Na^+ channel is open for Na^+; it does not bind when the channel is previously blocked by tetrodotoxin. (*v*) Besides its effect on the action potential, the sea anemone toxin displays a veratridine-type depolarizing action at low Ca^{2+} concentration which can be suppressed by tetrodotoxin. The sea anemone toxin greatly stimulates the release of γ-[^{3}H]aminobutyric acid from neurotransmitter-loaded rat brain synaptosomes. The apparent dissociation constant of the neurotoxin–receptor complex in this system is 20 nM. The sea anemone toxin effect is antagonized by tetrodotoxin.**

Neurotoxins are essential tools for the analysis of molecular aspects of nerve conduction and transmission. Toxic molecules already available for study of molecular aspects of conduction include: (*i*) tetrodotoxin and saxitoxin, which are highly specific for blocking the Na^+ channel in most axons (1, 2); (*ii*) veratridine and batrachotoxin, which depolarize nerve membrane by a selective increase in the resting sodium permeability (2–5); and (*iii*) scorpion neurotoxin, a miniprotein which affects reversibly the closing of the Na^+ channel and the opening of the K^+ channel (6–8).

A series of neurotoxins was recently isolated in the pure form from the sea anemone *Anemonia sulcata* (9–11). The toxins all are small polypeptides. The sequence of one of these neurotoxins, ATX_{II}, has now been established. It is a miniprotein comprising only 47 amino acids crosslinked by three disulfide bridges (12). We analyze in this paper the specificity of action of this neurotoxin and the physico-chemical properties of its association with its receptor site.

MATERIALS AND METHODS

Purification of sea anemone toxins (*Anemonia sulcata*) was carried out according to Béress *et al.* (9, 10). ATX_{II} is the most abundant of the three neurotoxic polypeptides (9, 10, 12).

Giant axons used in this work were those of the crayfish *Astacus leptodactylus* and of a cephalopod, the cuttlefish *Sepia officinalis* (axon diameter 200–400 μm). Giant axons from crustacea were isolated from circumesophageal nerve connectives, those of *Sepia* from stellar nerves (13). Resting and action potential recordings and voltage clamp experiments have been previously described (8).

Abbreviations: ATX_{II}, sea anemone neurotoxin; TTX, tetrodotoxin.

Physiological solutions were Mediterranean sea water for *Sepia* axons and a Van Harreveld solution (207 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, 5.3 mM $MgCl_2$, 10 mM Tris·HCl) at pH 7.5 for crayfish axons.

Single myelinated nerve fibers were dissected from the sciatic nerve of the frog *Rana esculenta* according to Stämpfli (14). Voltage-clamp experiments on the node of Ranvier were carried out as described by Nonner (15).

Rat brain synaptosomes were prepared according to Gray and Whittaker (16) with minor modifications (17). The experimental procedure used for preloading synaptosomes with ^{3}H-labeled γ-aminobutyric acid and measuring efflux was described in detail elsewhere (17).

RESULTS

Effects of ATX_{II} on Crayfish Axons. The periesophageal nerve used in this study contains a small number of giant axons (20–100 μm diameter) and several hundred thin axons (13). Thin axons are much more sensitive to ATX_{II} than giant axons. When the nerve is bathed in a solution containing 0.1 nM ATX_{II} some of the thin axons begin to fire spontaneously (Fig. 1A). More axons are affected at a concentration of 1 nM of ATX_{II}.

The giant axon having the maximum diameter (about 100 μm), which has been used for microelectrode and voltage clamp analysis, is sensitive to ATX_{II} at concentrations higher than 0.1 μM (Fig. 1C). Toxin action on this axon provokes a marked plateau phase of the action potential (Fig. 1B). The dose–effect curve (Fig. 1C) shows an all-or-none effect over a range of about 2–3 in the toxin concentration.

At saturating concentrations of the neurotoxin (0.5–1 μM), for short incubation times of 1 or 2 min one only observes a change of shape of the action potential with no change of the resting potential (Fig. 1B). For longer times (10–30 min) one also observes a depolarization of the axonal membrane accompanied by a decrease in the amplitude of the action potential. The toxin-induced depolarization can ultimately block nervous conduction completely. The effect of ATX_{II} upon the resting potential for the giant axon is calcium dependent. At a concentration of 13.5 mM Ca^{2+}, the magnitude of the depolarization induced by 1 μM ATX_{II} is 10 mV, whereas at 1 mM Ca^{2+} the depolarization can reach 40 mV. It is interesting that neither the effect of ATX_{II} on the action potential nor the effect on the resting potential can be reversed by a prolonged washing of 60 min with the Van Harreveld solution free of toxin.

ATX_{II}, even at a concentration of 10 μM, has no effect on *Sepia* giant axons.

ATX_{II} is positively charged at pH 7.5 (12). By the iontophoretic technique with a glass microelectrode filled with 1 mM ATX_{II}, it is possible to apply the neurotoxin exclusively on the external or on the internal (cytoplasmic) face of the membrane.

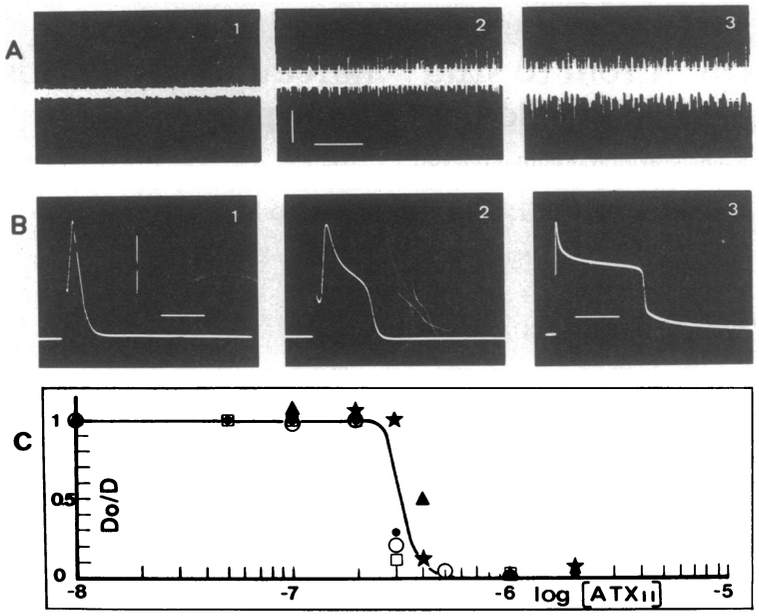

FIG. 1. (A) Spontaneous activity of the crayfish periesophageal nerve. 1, Control; 2, in the presence of 0.1 nM ATX_{II}; 3, in the presence of 1 nM ATX_{II}. 18°C. Time scale (horizontal bar): 2 sec; voltage scale (vertical bar): 200 μV. (B) ATX_{II} effect on the action potential of the crayfish giant axon. 1, Control; 2, 5 min after; and 3, 10 min after the application of 0.5 μM ATX_{II}. 18°C. Time scale for 1 and 2: 2 msec; time scale for 3: 5 msec. Voltage scale: 50 mV. (C) Dose–response curve of ATX_{II} action on the crayfish giant axon at 18°C. D_0 represents the spike duration in the control measured at half height of the action potential; D is the spike duration measured after 40-min treatment with a given concentration of ATX_{II}. The different symbols represent different series of experiments.

A typical plateau phase in the action potential developed in less than 1 min when the toxin was applied at the external surface, and no effect developed at all when the toxin was applied from the cytoplasmic side. The ATX_{II} receptor is thus situated on the external face of the membrane.

Tetrodotoxin (TTX), which specifically blocks the sodium channel of untreated axons (1, 2), also blocks sodium entry in axons treated with ATX_{II}. As shown in Fig. 2, the action potentials in both the axon used as a control and the axon treated with ATX_{II} are suppressed by TTX. The dose–response curve due to the application of TTX (Fig. 3A) to the axon treated with ATX_{II} gives a value of the apparent dissociation constant $K_{0.5}$ = 3 nM identical to that found with the untreated axon. TTX can also suppress the ATX_{II} effect upon the resting potential. It easily repolarizes the membrane previously depolarized by 1 μM ATX_{II} in 1 mM Ca^{2+}. The dose–response curve gives an apparent dissociation constant of 10 nM (Fig. 3B).

The order of introduction of the two neurotoxins ATX_{II} and TTX is crucial. In the experiments presented in Fig. 2A, ATX_{II} was introduced first and TTX second. In this case binding of

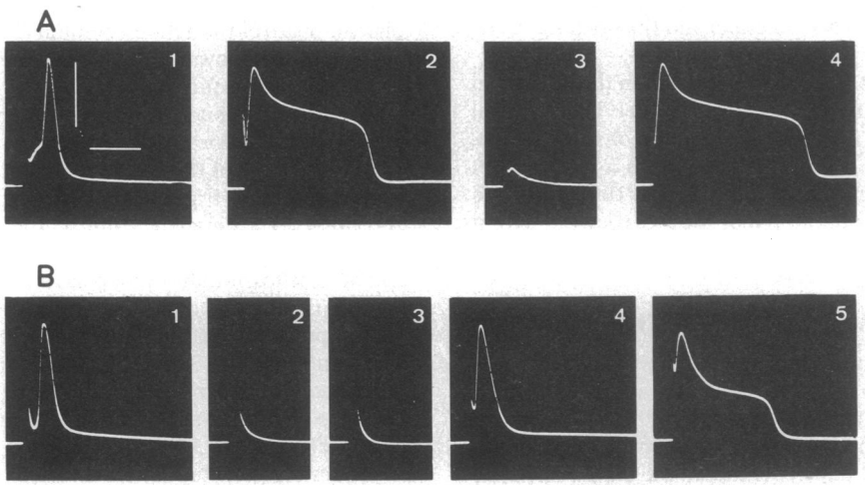

FIG. 2. Mutual effect of ATX_{II} and TTX. (A) TTX action when it is introduced after ATX_{II}. 1, Control action potential; 2, action potential after a 5 min application of 0.5 μM ATX_{II}; 3, TTX (50 nM) was then added in a mixture with 0.5 μM ATX_{II} and blocked the action potential; 4, reappearance of the plateau phase after a 20-min washing with the toxin-free physiological solution. Washing caused the dissociation of TTX but not that of ATX_{II} from the axonal membrane. (B) TTX action when it is introduced before ATX_{II}. 1, Control action potential. 2, Disappearance of the action potential after treatment with 50 nM TTX. 3, The action potential remains blocked if ATX_{II} (0.5 μM) is then applied for 5 min in the presence of 50 nM TTX. 4, After a 20 min washing with the toxin-free physiological solution, recovery of an action potential identical to the control was observed. 5, A new application of 0.5 μM ATX_{II} to the same preparation for 5 min provokes an action potential with a plateau phase. For A and B, time scale (horizontal bar): 2 msec; voltage scale (vertical bar): 50 mV. All experiments were carried out at 18°C.

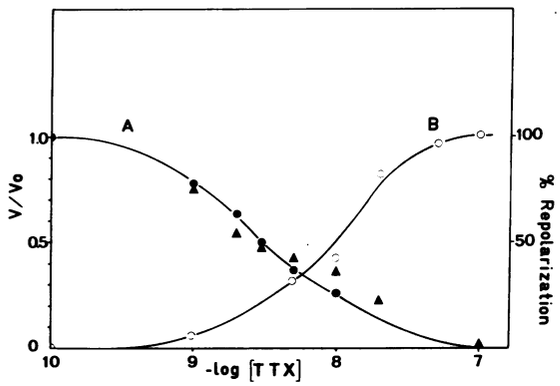

FIG. 3. Dose–response curves for TTX action. (A) (left ordinate) Variation of the maximum rate of the ascending phase of the action potential on a giant crayfish axon first treated for 5 min with 0.5 μM ATX_{II}. V_0 is the control value of the rate without TTX; V is the rate in the presence of different amount of TTX. Different symbols represent different series of experiments. (B) (right ordinate) Repolarization of a crayfish axon first depolarized by 36 mV with 1 μM ATX_{II} in the presence of 1 mM Ca^{2+}.

ATX_{II} does not prevent TTX association with its receptor. Conversely, however, previous treatment of the axonal membrane with TTX prevents ATX binding. This is shown in Fig. 2B. In this experiment, the crayfish axon is first treated with 50 nM TTX, and this bathing solution is then replaced by a mixture of TTX (50 nM) and ATX_{II} (0.5 μM). The incubation period of 5 min would be sufficient for the manifestation of ATX_{II} action in the absence of TTX (Fig. 1B). The preparation is then washed with the physiological solution. Since TTX binding is reversible, whereas ATX_{II} binding is irreversible, one would expect to observe an action potential with a plateau phase. In fact the action potential reappears but without the plateau phase typical of ATX_{II} action. If the preparation is then incubated with an ATX_{II} solution of 0.5 μM, the plateau phase reappears after a few minutes. The experiments in Fig. 2 demonstrate that pretreatment of the membrane to block all Na^+ channels with TTX prevents binding of ATX_{II} to its specific sites.

The voltage clamp analysis provides information concerning the rates of opening and closing of the Na^+ channel, the maximal fluxes of sodium when the Na^+ channel is open (I_p) and of potassium when steady-state is attained (I_{ss}). In a typical series of experiments presented in Fig. 4, the giant axon was submitted to a voltage jump from a holding potential of −80 mV to −10 mV. Treatment of the axonal membrane with TTX suppresses the Na^+ current but leaves the steady-state K^+ current unchanged. In consequence, whereas curve A represents the contribution of both the Na^+ and K^+ currents, curve C is only due to the K^+ current. Subtraction of the K^+ contribution (curve C) from the control (curve A) represents the time-course of the intensity of the Na^+ current (curve D). Curve B is the voltage clamp profile after treatment of the axonal membrane with ATX_{II}. The descending part of the voltage-clamp profile is unchanged by ATX_{II}, whereas the ascending part is drastically affected. Blocking of the Na^+ channel by the addition of TTX to the ATX_{II}-treated axon gives curve C, which is identical to the curve obtained after treatment of the control axon by TTX. In consequence, ATX_{II} does not alter the opening of the K^+ channel. Curve E, which is obtained by subtraction of curves B and C in Fig. 4, demonstrates that whereas ATX_{II} has little effect on the opening of the Na^+ channel, it considerably slows down the closing of this channel. The Na^+ channel remains partially opened even after 6 msec (inward current: 2 mA/cm^2).

Another toxin, which has been partially purified from the Bermuda sea anemone *Condylactis gigantea*, has a molecular weight of 10,000–15,000 (18), very different from that of ATX_{II}. Similarly to ATX_{II}, this toxic compound prolonged the action potential of crayfish axons. The effect of the toxin on the K^+ channel could not be unambiguously studied, but it was demonstrated that sodium inactivation was slowed by the action of the toxic material at a concentration of 0.2 mg/ml (19).

Effect of ATX_{II} on Myelinated Axons. The effect of ATX_{II} on the action potential of myelinated fibers is very similar to that obtained in Fig. 1B for crayfish giant axons. The toxin provokes the appearance of a plateau phase. Fig. 5 gives voltage clamp data obtained with the node of Ranvier of myelinated

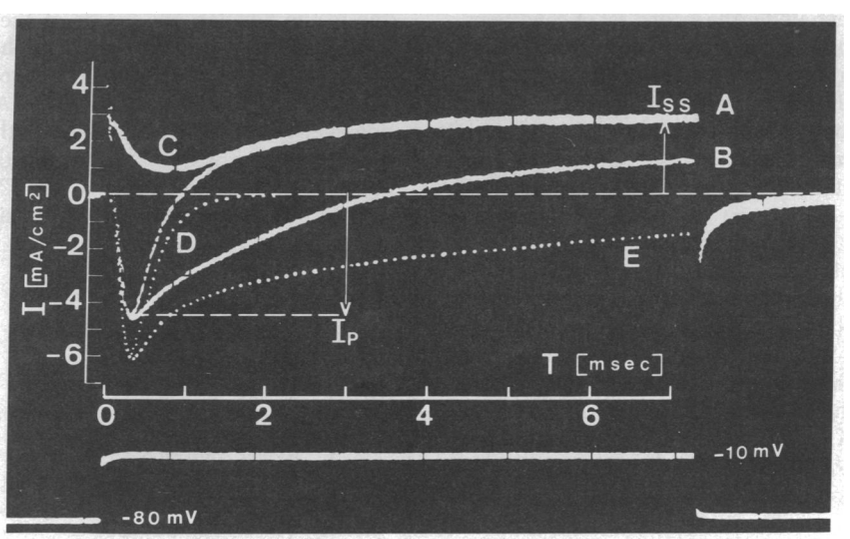

FIG. 4. Voltage clamp analysis of ATX_{II} action on a crayfish giant axon. The membrane potential was clamped at −10 mV after a voltage jump from a holding potential of −80 mV. 15°C. Traces correspond to the membrane currents associated with the voltage jump. (A) Without toxin; (B) after 5-min application of 0.5 μM ATX_{II}; (C) after treatment with 0.1 μM TTX as well as after treatment with 0.5 μM ATX_{II} followed by application of 0.1 μM TTX. The dotted curves D and E correspond to differences between A and C and B and C, respectively. They represent the time course of the Na^+ current in the absence and in the presence of ATX_{II}. Fast speed recordings (0.1 msec/division instead of 1 msec/division for the results shown in this figure) of Na^+ activation kinetics (i.e., the rate of opening of the Na^+ channel) are not shown here but were found to be superimposable in the absence and in the presence of ATX_{II}.

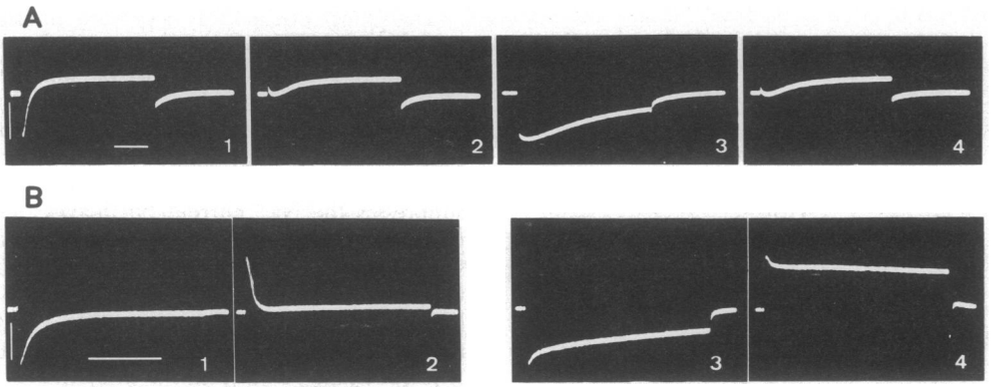

FIG. 5. Voltage clamp analysis of ATX_{II} action on the node of Ranvier of myelinated fibers. 15°C. (A) Current response to a depolarizing voltage jump of 70 mV. 1, In the absence of toxin; 2, after addition of 10 nM TTX; 3, after reversion of the TTX effect by washing with the physiological solution followed by a 2-min application of 1 μM ATX_{II}; 4, after application of 10 nM TTX to the ATX_{II}-treated axon. Time scale (horizontal bar): 5 msec; current scale (vertical bar): 10 nA. The steady-state intensity of the K^+ current (I_K) in experiments 2 and 4 is 3.8 nA; the half-lives ($t_{1/2}$) of the K^+ activation kinetics are 3.8 msec and 4.3 msec, respectively. (B) Na^+ current responses to depolarizing voltage jump of 60 mV (traces 1 and 3) and 200 mV (traces 2 and 4). The K^+ current was blocked with 10 mM tetraethylammonium ion. Traces 1 and 2 are obtained in the absence of ATX_{II}; traces 3 and 4 are obtained in the presence of 1 μM ATX_{II}. Time scale: 5 msec; current scale: 10 nA. No neurotoxin effect was observed at 0.1 μM ATX_{II}.

nerve fibers of *Rana esculenta*. Part A of Fig. 5 shows the influence of TTX on the control and on the giant axon treated with ATX_{II}. In the presence of TTX only the characteristics of the K^+ channel are seen. The near identity of traces 2 and 4 indicates that the steady-state outward current due to K^+ efflux (I_K) is essentially unaffected by ATX_{II}. I_K was also measured after a series of membrane potential depolarizing jumps (ΔV). The I_K versus ΔV representation is identical for the control and for the ATX_{II}-treated axon. The tetraethylammonium ion is a selective blocker of the K^+ channel (20). In consequence one sees in Fig. 5B the time course of the Na^+ current before (traces 1 and 2) and after (traces 3 and 4) treatment with ATX_{II}. Two sets of voltage jump conditions have been used in Fig. 5B: the first jump, of 60 mV, (traces 1 and 3) brings the membrane potential to a value inferior to the equilibrium potential for sodium, E_{Na}; the second jump, of 200 mV (traces 2 and 4) brings the membrane potential to a value higher than E_{Na}. Under the first experimental conditions, Na^+ enters the axon and one measures an inward current; under the second set of conditions, Na^+ goes out and one observes an outward current. The Na^+ gate is closed in the control (traces 1 and 2) after 5 msec; after treatment with ATX_{II}, it is still open after 15 msec (traces 3 and 4). The voltage-clamp analysis with the node of Ranvier confirms that the neurotoxin strongly affects closing of the Na^+ channel. The ATX_{II} effects are irreversible even after prolonged washing (1 hr).

Effect of ATX_{II} upon γ-Aminobutyric Acid Release from Synaptosomes. Neurotoxic compounds such as veratridine and batrachotoxin stimulate the release of neurotransmitters from synaptosomes (21–23). The effect of ATX_{II} on γ-aminobutyric acid release from rat brain synaptosomes is presented in Fig. 6. In this case ATX_{II} binding is reversible and stimulates neurotransmitter release. The dose–response curve of this stimulation indicates that ATX_{II} acts at very low concentrations and gives an apparent value of 20 nM for the dissociation constant of the ATX_{II}–receptor complex.

DISCUSSION

The main effect of ATX_{II} binding to membranes of myelinated or nonmyelinated axons is to specifically affect the closing of the Na^+ channel without affecting the opening of this channel or the functioning of the K^+ channel. Binding of the toxin occurs at the external face of the membrane. A secondary effect of the toxin has been observed with crayfish giant axons; it appears after longer times of incubation and results in a change of the resting potential towards depolarization. This secondary effect is favored at low Ca^{2+} concentrations. It resembles the action of veratridine or batrachotoxin (24). ATX_{II} binding to the membrane site which controls closing of the Na^+ channel can only occur when the channel is open for Na^+. When TTX causes the channel to be closed to Na^+ entry, no binding of ATX_{II} can occur.

Electrophysiological studies (25, 26) have provided convincing evidence that transmitter release is controlled by a depolarization-dependent calcium permeability increase at the presynaptic terminals. Synaptosomes fulfill all of the criteria for stimulus-secretion coupling defined from the electrophysiological approach (21, 23, 27).

Similarly to veratridine (21–23), batrachotoxin (22), or scorpion toxin (17), the sea anemone toxin stimulates release of neurotransmitters from synaptosomes. This action is probably due to the depolarizing action of the neurotoxin (similar to veratridine and batrachotoxin), which should indeed provoke Ca^{2+} entry in the synapse and release of neurotransmitter. The apparent dissociation constant of the ATX_{II}–receptor interaction is 20 nM. However, binding in that case is reversible by washing. Similarly to what happens with axons, the ATX_{II} effect on synaptosomes is suppressed by TTX.

Till now, the most useful neurotoxin for the identification

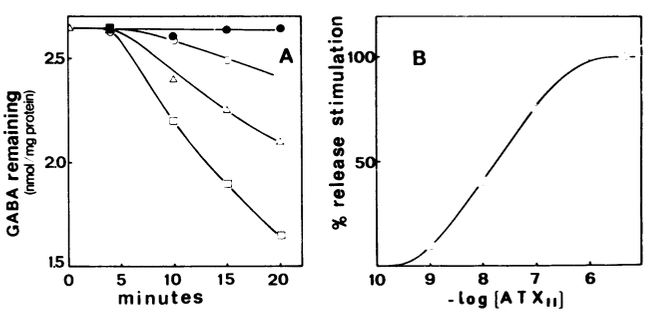

FIG. 6. (A) Kinetics of ^3H-labeled γ-aminobutyric acid (GABA) release from preloaded synaptosomes: control (○); efflux in the presence of 10 nM ATX_{II} (△); 3 μM ATX_{II} (□); 3 μM ATX_{II} plus 1 μM TTX (●). (B) ATX_{II}-concentration dependence of the stimulation of ^3H-labeled γ-aminobutyric acid release from preloaded synaptosomes exposed 20 min to ATX_{II}.

and the analysis of the properties of the Na^+ channel has been tetrodotoxin (24, 28–31). ATX_{II} is complementary to TTX: (*i*) it specifically affects another mechanism of the Na^+ channel machinery, i.e., the closing of the Na^+ channel; (*ii*) its receptor site is distinct from that of TTX [the dissociation constant of the TTX-receptor complex is the same in a control axon and in an ATX_{II}-treated axon (Fig. 3)].

The importance of protein neurotoxins in the study of molecular aspects of nerve conduction and transmission is beautifully illustrated by the wide use which has been made of postsynaptic toxins, snake neurotoxins. These neurotoxins are now essential tools for the identification, for the localization, and for the isolation of the acetylcholine receptor (for reviews see refs. 32–35). This success is due to three factors: (*i*) the toxin action is very specific; (*ii*) the snake toxin associates very tightly with its receptor and in the case of the so-called "long" neurotoxins the rate of dissociation of the toxin–receptor complex is sufficiently low to make binding quasi-irreversible (36, 37); (*iii*) the toxin is a protein. This property permits radioactive labeling (in a variety of ways) essential for receptor purification and histochemical techniques; it also permits the use of crosslinkers to irreversibly graft the toxin to its membrane receptor; finally, it allows the easy preparation of an affinity column for the purification of the acetylcholine receptor. The sea anemone toxin should have an importance among presynaptic toxins equivalent to that of snake neurotoxins among the postsynaptic toxins.

The authors are very grateful to Mrs. M. T. Cavey for expert assistance; to Prof. C. Bergman for introducing one of us (G.R.) to the use of the myelinated nerve fiber preparation; to Drs. R. Chicheportiche, J. P. Vincent, M. Balerna, and M. Fosset for fruitful discussions; and to Prof. T. Kaiser for very careful reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Commissariat à l'Energie Atomique, the Délégation Générale à la Recherche Scientifique et Technique and the Fondation pour la Recherche Médicale.

1. Evans, M. H. (1972) *Int. Rev. Neurobiol.* **15,** 83–166.
2. Narahashi, T. (1974) *Physiol. Rev.* **54,** 813–889.
3. Ulbricht, W. (1969) *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **61,** 18–71.
4. Ohta, M., Narahashi, T. & Keeler, R. F. (1973) *J. Pharmacol. Exp. Ther.* **184,** 143–154.
5. Albuquerque, E. X., Daly, J. W. & Witkop, B. (1971) *Science* **172,** 995–1002.
6. Koppenhöffer, E. & Schmidt, H. (1968) *Pfluegers Arch.* **303,** 133–149.
7. Narahashi, T., Shapiro, B. I., Duguchi, T., Scuka, M. & Wang, C. M. (1972) *Am. J. Physiol.* **222,** 850–857.
8. Romey, G., Chicheportiche, R., Lazdunski, M., Rochat, M., Miranda, F. & Lissitzky, S. (1975) *Biochem. Biophys. Res. Commun.* **64,** 115–121.
9. Béress, L., Béress, R. & Wunderer, G. (1975) *FEBS Lett.* **50,** 311–314.
10. Béress, L., Béress, R. & Wunderer, G. (1975) *Toxicon* **13,** 359–367.
11. Rathmayer, W., Jessen, B. & Béress, L. (1975) *Naturwissenschaften* **62,** 538–539.
12. Wunderer, G., Machleidt, W. & Wachter, E. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357,** 239–240.
13. Bullock, T. H. & Horridge, G. A. (1965) in *Structure and Function in Nervous System of Invertebrates,* eds. Whitaker, D. M., Emerson, R., Kennedy, D. & Beadle, G. W. (W. H. Freeman and Co., San Francisco, Calif.), Vol. II, pp. 848–868, 1433–1485.
14. Stämpfli, R. (1969) in *Laboratory Techniques in Membrane Biophysics,* eds. Passow, J. & Stämpfli, R. (Springer-Verlag, Berlin, Heidelberg, New York), pp. 157–166.
15. Nonner, W. (1969) *Pfluegers Arch.* **309,** 176–192.
16. Gray, E. G. & Whittaker, V. P. (1962) *J. Anat.* **96,** 79–87.
17. Romey, G., Abita, J. P., Chicheportiche, R., Rochat, H. & Lazdunski, M. (1976) *Biochim. Biophys. Acta,* in press.
18. Shapiro, B. I. (1968) *Toxicon* **5,** 253–259.
19. Narahashi, T., Moore, J. W. & Shapiro, B. I. (1969) *Science* **163,** 680–681.
20. Hille, B. (1970) *Prog. Biophys. Mol. Biol.* **21,** 1–32.
21. Blaustein, M. P. (1975) *J. Physiol. (London)* **247,** 617–655.
22. Holz, R. & Coyle, J. T. (1974) *Mol. Pharmacol.* **10,** 746–758.
23. Levy, W. B., Haycock, J. W. & Cotman, C. W. (1974) *Mol. Pharmacol.* **10,** 438–449.
24. Catterall, W. A. (1975) *J. Biol. Chem.* **250,** 4053.
25. Katz, B. & Miledi, R. (1970) *J. Physiol. (London)* **207,** 789–801.
26. Katz, B. & Miledi, R. (1971) *J. Physiol. (London)* **216,** 503–512.
27. Cotman, C. W., Haycock, H. W. & While, W. F. (1976) *J. Physiol. (London)* **254,** 475–505.
28. Benzer T. I. & Raftery, M. A. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3636–3637.
29. Colquhoun, D., Henderson, R. & Ritchie, J. M. (1972) *J. Physiol. (London)* **227,** 95–126.
30. Henderson, R. & Wang, J. H. (1972) *Biochemistry* **11,** 4565–4569.
31. Balerna, M., Fosset, M., Chicheportiche, R., Romey, G. & Lazdunski, M. (1975) *Biochemistry* **14,** 5500–5511.
32. Changeux, J. P. (1976) in *Proceedings of the Sixth International Congress of Pharmacology,* ed. Klinge, E. (Pergamon Press, Oxford, England), pp. 165–177.
33. Raftery, M. A., Deutsch, J., Reed, K., Vandlen, R. & Lee, T. (1976) in *Proceedings of the Sixth International Congress of Pharmacology,* ed. Klinge, E. (Pergamon Press, Oxford, England), pp. 87–96.
34. Vogel, Z. & Daniels, M. P. (1976) *Proceedings of the Sixth International Congress of Pharmacol,* ed. Klinge, E. (Pergamon Press, Oxford, England), pp. 59–66.
35. Barnard, E. A. & Dolly, J. O. (1976) *Proceedings of the Sixth International Congress of Pharmacol,* ed. Klinge, E. (Pergamon Press, Oxford, England), pp. 77–85.
36. Weber, M. & Changeux, J. P. (1974) *Mol. Pharmacol.* **10,** 1–14.
37. Chicheportiche, R., Vincent, J. P., Kopeyan, C., Schweitz, H. & Lazdunski, M. (1975) *Biochemistry* **14,** 2081–2091.