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β-Bungarotoxin, a pre-synaptic toxin with enzymatic activity

(neurotoxin/phospholipase A_2/synaptic transmission)

PETER N. STRONG*, JON GOERKE†‡, STEPHEN G. OBERG*, AND REGIS B. KELLY*†

Departments of * Biochemistry and Biophysics, † Physiology, and the ‡ Cardiovascular Research Institute, University of California, San Francisco, San Francisco, Calif. 94143

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ABSTRACT **β-Bungarotoxin, a pre-synaptic neurotoxin isolated from the venom of the snake *Bungarus multicinctus*, has been shown to modify release of neurotransmitter at the neuromuscular junction. In this communication, we demonstrate that β-bungarotoxin is a potent phospholipase A_2 (phosphatide 2-acyl hydrolase, EC 3.1.1.4), comparable in activity with purified phospholipase enzymes from *Naja naja* and *Vipera russellii*. The phospholipase activity of β-bungarotoxin requires calcium and is stimulated by deoxycholate. When strontium replaces calcium, no phospholipase activity is detected. Since neuromuscular transmission is not blocked when calcium is replaced by strontium, it was possible to examine the effects of the toxin on neuromuscular transmission in the presence of strontium. Under these conditions, when the phospholipase activity should be inhibited, the toxin has little or no effect on neuromuscular transmission. If β-bungarotoxin owes its toxicity in part to its enzymatic activity, then it must be placed in a different class from those toxins which produce their effect by binding passively to an appropriate receptor.**

The release of packaged secretions in response to a sudden demand occurs in many diverse cells (e.g., hormones in endocrine glands, digestive enzymes in exocrine glands, and neurotransmitters at nerve endings). It has been suggested that such processes occur by exocytosis—fusion of the membrane of the secretory granule with the cell membrane (1). The similar calcium requirements of neural and nonneural secretory systems (2) have strengthened the idea that many secretory processes are the same at the molecular level. However, the molecular basis for any release system is unknown.

The most well-understood secretory process is probably the release of acetylcholine at the neuromuscular junction, thanks to quantitative electrophysiological measurements (3). Even so, electrophysiology by itself cannot test the molecular models that it proposes. In recent years, the discovery of highly specific neurotoxins which interfere with the release of transmitter from nerve terminals [e.g., botulinum toxin (4, 5), black widow spider venom (6, 7), β-bungarotoxin (8–10), and notexin (11, 12)] has provided us with potentially valuable tools for elucidation of the molecular basis of neurotransmitter release via biochemical technology.

In this paper we examine some of the biochemical and electrophysiological properties of one such neurotoxin, β-bungarotoxin, which has been shown by electrophysiological measurements to act on the pre-synaptic terminal and to have no post-synaptic effects (8, 9). We find that β-bungarotoxin is a powerful calcium-dependent phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4), comparable in activity to purified phospholipase A enzymes from *Naja naja* and *Vipera russellii*. Additionally, we observe that β-bungarotoxin can modify synaptic transmission only when the ionic requirements of the phospholipase activity have been met. Since we find that other phospholipases at equivalent concentrations are not toxic, we propose that the phospholipase activity is a necessary but perhaps not sufficient facet of β-bungarotoxin's mode of action. We suggest that the phospholipase acts preferentially on pre-synaptic neuronal membranes to modify release of transmitter by altering the probability of fusion of transmitter-containing vesicles with the nerve terminal membrane.

Abbreviations: SoyPC, soybean phosphatidylcholine; SEPC, saturated egg phosphatidylcholine; [^{3}H]SEPC, 1-acyl-2-[^{3}H]acyl-*sn*-glycero-3-phosphorylcholine; TLC, thin-layer chromatography; m.e.p.p., miniature endplate potential.

MATERIALS AND METHODS

Reagents. Crude venom from the snake *Bungarus multicinctus* was obtained from the Ross Allen Reptile Institute, Silver Springs, Fla. Purified phospholipase A enzymes from *Naja naja* and *Vipera russellii* were the gifts of Dr. J. Salach (Veterans Administration Hospital, San Francisco). Soybean phosphatidylcholine (SoyPC) was a gift of Dr. H. Eikermann (Nattermann and Co., Cologne). Saturated egg phosphatidylcholine (SEPC) was prepared by the catalytic hydrogenation of purified egg phosphatidylcholine. 1-Acyl-2-[^{3}H]acyl-*sn*-glycero-3-phosphorylcholine ([^{3}H]SEPC), obtained by catalytic tritiation of egg phosphatidylcholine, was a gift of Dr. R. Mason (University of California, San Francisco).

Purification of β-Bungarotoxin (Molecular Weight 21,800). The toxin was purified as previously described (9, 13). The purity of β-bungarotoxin was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (9), isoelectric focusing techniques using broad range (pH 3–10) Ampholynes (9, 14), and phosphocellulose (Whatman P-11) ion-exchange chromatography [12 × 0.7 cm diameter 0.3–0.7 M potassium phosphate gradient, pH 6.2, modified from the procedure of Cooper and Reich (15)]. Purified β-bungarotoxin was stored frozen in distilled water or Tris·HCl buffer, pH 7.6 at −20°. The toxin retains full neurotoxicity and phospholipase activity for many months after repeated freeze–thaw cycles.

Phospholipase A Assay. Purified β-bungarotoxin was desalted by overnight dialysis (4°, distilled water) prior to assaying for phospholipase activity. Phospholipase activities were determined using a pH-stat essentially according to published procedures (16, 17). As is found for pancreatic phospholipase A_2, activity was greatly enhanced by the presence of the emulsifying agent sodium deoxycholate (17). For optimal activities, equimolar concentrations of deoxycholate and phosphatidylcholine were required. Substrate [0.2 ml of SoyPC in ethanol, 8 μmol, containing less than 2% lyso products as determined by thin-layer chromatography (TLC)] was added under a constant stream of nitrogen to 4

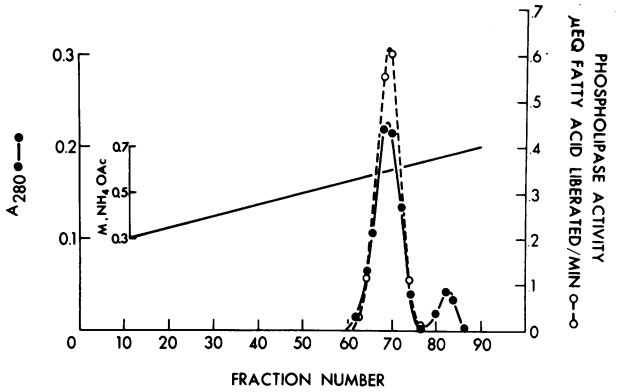

FIG. 1. CM-cellulose ion-exchange chromatography of β-bungarotoxin. Toxic peak fractions from a CM-Sephadex ion-exchange column (9) were concentrated and desalted using an Amicon UM-2 filter and then applied to a CM-cellulose column (Whatman CM-32, 25 cm × 1.6 cm diameter), previously equilibrated with 0.05 M NH_4OAc (pH 5.0). The column was eluted with a 200 ml linear salt gradient, 0.3 M NH_4OAc (pH 5.5) to 0.7 M NH_4OAc (pH 6.5) and 2.0 ml fractions were collected. Fractions absorbing at 280 nm were dialyzed against distilled water and the phospholipase activity was measured as described (Fig. 2). All chromatographic procedures and dialysis operations were carried out at 4°.

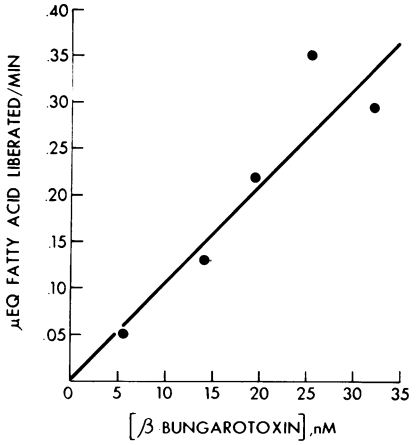

FIG. 2. Determination of phospholipase activity of β-bungarotoxin using a pH-stat technique. Dialyzed toxin was added to an incubation mixture consisting of 8 μmol of phosphatidylcholine and 7.5 μmol of sodium deoxycholate in 4 ml of a salt medium (10 mM $CaCl_2$, 100 mM NaCl, 0.05 mM EDTA). Fatty acid liberated in the presence of toxin was titrated to pH 8 (37°) with 4 mM NaOH, using an autotitrator. Linear kinetics were observed during the first 5 min after toxin addition and from the initial slope, phospholipase activity was computed, expressed as μeq of fatty acid liberated per min.

ml of a salt reaction mixture (100 mM NaCl, 10 mM $CaCl_2$, 0.05 mM EDTA) at 37° in the assay chamber (18). Typically 15 μmol of sodium deoxycholate was introduced and the reaction was started by addition of toxin (<3 μg) in unbuffered distilled water. Fatty acid liberated in the incubation cell was titrated with 4 mM NaOH to pH 8.0 (Radiometer model TTT-11 Autotitrator). The rates of SoyPC hydrolysis were linear for at least the initial 5 min and it was within this time period that reaction rates were computed. Activity is expressed as the liberation of fatty acid in μeq/min. Specific activity is given by the number of μeq of fatty acid liberated per min/mg of protein. Duplicate assays were reproducible within 5%.

Thin-Layer Chromatography. Aliquots from the incubated assay mixture were extracted according to the method of Bligh and Dyer (19) and subjected to TLC using solvent systems previously described (20). Reaction products were identified after exposure to iodine vapor or under UV light after spraying with an aqueous solution of 8-anilino-1-naphthalene sulfonic acid (2.5 mg/ml). When [^{3}H]SEPC was used as a substrate (97.8% radiochemically pure by *Crotalus adamanteus* phospholipase A_2 degradation and TLC), the radioactivity of reaction products was assayed by scraping off regions of silica gel and suspending them in a 50% (vol/vol) aqueous solution of Aquasol (New England Nuclear). The resultant stiff, translucent gels were kept in the dark at 4° for 12 hr and then counted in a Beckman LS-233 liquid scintillation counter.

Toxicity Estimation. β-Bungarotoxin was assayed for lethality by intraperitoneal injection of white mice (9).

Electrophysiological Recording. Synaptic events were recorded intracellularly from muscle fibers of the rat diaphragm as previously described (21).

RESULTS

β-Bungarotoxin has phospholipase activity

Previous work in this laboratory employed β-bungarotoxin isolated from crude venom by chromatography on CM-50 Sephadex (9). When this fraction was applied to a CM-cellulose column and eluted with a linear NH_4OAc salt gradient, two protein peaks were resolved (Fig. 1). The minor protein component (11%), eluted after the bulk of the material, was less than 2.5% as toxic as the major component, whose minimum lethal dose was 0.01 μg/g of mouse. The peak of phospholipase activity coincided with the major component; from an estimate of the detection limits of our assay, the specific activity of the minor component must be less than 0.05% of the major component.

In order to be sure that the phospholipase activity of β-bungarotoxin was not due to a contaminant which coelutes with the toxin, we had to demonstrate the purity of our sample of β-bungarotoxin. The major component of the CM-cellulose column was homogeneous as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence of reducing agents. In the presence of 2% 2-mercaptoethanol, two bands were observed (9); these correspond to the two subunits held together in the native toxin by disulfide bridges. The toxin also appeared as a single band after isoelectric focusing in a polyacrylamide gel containing broad range (pH 3–10) Ampholynes. When β-bungarotoxin was analyzed for purity by chromatography on a phosphocellulose ion-exchange column, 98.5% of the protein eluted as a single peak on application of a phosphate salt gradient.

The assay used to determine phospholipase activity was essentially the pH-stat technique used by Wells (16) and de Haas *et al.* (17). Minor modifications of their published procedures are as indicated in *Methods*. Under our assay conditions, the hydrolysis of phosphatidylcholine was essentially zero-order and a linear relationship between velocity of substrate hydrolysis and the concentration of β-bungarotoxin was found (Fig. 2). When rates were calculated using higher concentrations of toxin, the measured activity was no longer directly proportional to toxin concentration.

The phospholipase activity of the toxin required calcium and deoxycholate; removal of either from the incubation mix virtually eliminated release of fatty acid (Table 1). Addition of 50 mM EDTA in the presence of 10 mM Ca^{++} also abolished activity. Strontium was not able to substitute for

Table 1. Requirements for phospholipase activity

	Relative activity*
Complete system*	1.00
— β-Bungarotoxin	0.02
— Ca^{++}	0.03
— Deoxycholate	0.04
— Ca^{++}, deoxycholate	0.03
+ 2 mM Sr^{++} (Ca^{++}-free)	0.02

* Activities are expressed in relation to the activity of the complete system. The complete system (specific activity, 72 μeq of fatty acid per min/mg of protein) consisted of 8 μmol of SoyPC, 7.5 μmol of sodium deoxycholate, and 0.001 μmol of β-bungarotoxin in a salt solution (10 mM $CaCl_2$, 100 mM NaCl, 0.05 mM EDTA), total volume 4 ml.

calcium, either at 10 mM or at 2 mM.

The phospholipase activity of β-bungarotoxin was characterized using the phospholipid substrate [^{3}H]SEPC, specifically labeled in the 2-acyl side chain. When [^{3}H]SEPC was used as substrate in the assay, 95% of the radioactive hydrolysis products were found comigrating with free fatty acid by TLC (Table 2). Using SoyPC as a substrate, TLC hydrolysis products were visualized as fluorescent derivatives. The only observed components migrated with free fatty acid, lysophosphatidylcholine, and unreacted SoyPC. No neutral lipids other than fatty acid were detected (via anilinonaphthalene sulfonic acid fluorescence) in the hydrolysis mixture when the same sample was analyzed by neutral lipid TLC using Et_2O:petroleum ether:HOAc as the solvent system.

The above results argue that β-bungarotoxin is specifically a phospholipase A_2 (i.e., hydrolyzes uniquely the 2-acyl side chain in a 1,2-diacyl-*sn*-glycero-3-phosphorylcholine). The appearance of lysophosphatidylcholine as a reaction product suggests that neither phospholipase A_1 activity (concomitant with phospholipase A_2 activity) nor phospholipase B activity is present in β-bungarotoxin in significant amounts. The absence of any neutral lipids as hydrolysis products similarly eliminates possible contributions from phospholipase C activity. The presence of phospholipase D activity is also unlikely since no spot corresponding to phosphatidic acid was seen on TLC.

Comparison of toxicity and phospholipase activity between β-bungarotoxin and purified phospholipase enzymes

Because phospholipase A activity is assayed in many diverse ways (see, *inter alia*, refs. 17, 22–25), there is no absolute standard with which to compare the phospholipase activity of β-bungarotoxin. We chose, therefore, to relate the toxin's phospholipase activity to that of two available, highly purified phospholipase A enzymes (23): *Naja naja* enzyme IA (pI 4.63) and *Vipera russellii* enzyme peak 3 (pI 9.52). For convenience, the nomenclature used is that of Salach *et al.* (23). Table 3 shows β-bungarotoxin to possess one-third the phospholipase activity of *V. russellii* enzyme but to be twice as potent as *N. naja* enzyme. Comparison of the toxicity to mice indicates that both of these phospholipase enzymes are much less toxic than β-bungarotoxin. These data also support our contention that β-bungarotoxin and not some undetected impurity has the phospholipase activity. If the putative impurity had the same specific activity as the more potent enzyme (*V. russellii*) then it would have to account for 31% of the protein in the sample. A contaminant of this magnitude would most likely have been detected.

Table 2. TLC analysis of β-bungarotoxin reaction with [^{3}H]SEPC*

	Lysophosphatidylcholine	Phosphatidylcholine	Fatty acid
Initial (cpm)	14	3788	96
Final (cpm)	101	636	2021

Above spots account for 94% of total radioactive material applied to TLC plate. Solvent: $CHCl_3$–MeOH–H_2O–HOAc, 60:35:4.5:0.5; support: silica gel G; visualization: I_2 vapor.

* The reaction was performed in an analogous manner to that described in the legend to Fig. 2, except that the substrate consisted of 6.5 μmol of SoyPC and 1.5 μmol of [^{3}H]SEPC and that samples were removed from the incubated assay mixture 1 hr after the reaction was initiated.

β-Bungarotoxin exhibits similar ionic requirements for phospholipase activity and for modification of transmitter release

The characteristic changes in synaptic transmission at the neuromuscular junction in response to incubation with β-bungarotoxin have already been documented (9, 21). In summary, the toxin first enhances the probability of transmitter release, then reduces it until no release is detectable. If the phospholipase activity of β-bungarotoxin is involved in producing these changes then they should not occur when the phospholipase activity is inhibited. Since the calcium requirement for synaptic transmission at the neuromuscular junction can be satisfied by strontium (26), whereas the calcium requirement of the phospholipase activity cannot (Table 1), substitution of strontium for calcium in the bathing medium provides a convenient method for inhibiting phospholipase activity without disrupting the physiological release process.

Intracellular recordings were made from fibers of rat phrenic nerve–diaphragm preparations in which the average miniature endplate potential (m.e.p.p.) frequency had been elevated by raising the extracellular potassium. When toxin was added to a preparation bathed in calcium-containing solutions, the average m.e.p.p. frequency rose about 3-fold in the first 40 min. After peaking, the frequency slowly fell and by 2 hr was significantly below the average frequency in a control preparation, analyzed simultaneously, which lacked toxin (Fig. 3, open bars). If the toxin was added to a preparation bathed in strontium-containing solutions, the increase in frequency was much smaller and was perhaps not significant ($P > 0.025$). In contrast to the result with calcium, at later times no drop in frequency was ob-

Table 3. Comparison of phospholipase activity and toxicity between phospholipase enzymes and β-bungarotoxin

	Specific activity (μeq of fatty acid per min/mg of protein)	Minimum lethal dose (μg/g of mouse)
β-Bungarotoxin	133	0.01
N. naja enzyme	76	4
V. russellii enzyme	424	0.48

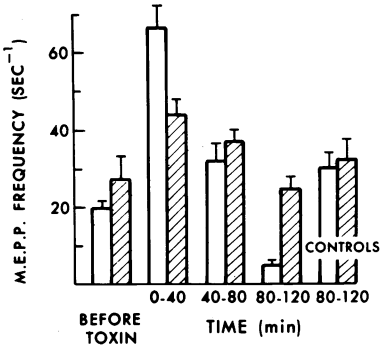

FIG. 3. Comparison of spontaneous release rates in Ca^{++} (□) and Sr^{++} (▨) after treatment of a rat phrenic nerve–diaphragm preparation with β-bungarotoxin. The preparation was depolarized by K^+ to increase the m.e.p.p. frequency either in 2 mM Ca^{++} (19 mM K^+) or 2 mM Sr^{++} (15 mM K^+). Measurements of m.e.p.p. frequency were begun 30 min after transferring to depolarizing solutions. M.e.p.p. frequencies of successively impaled muscle fibers were pooled and averaged for the 40-min period before application of toxin and for three successive 40-min periods after toxin application. Control experiments (identically treated preparations except for the omission of toxin) gave constant m.e.p.p. frequencies at each time period. For convenience, we only include control data from the last period. Each bar represents data recorded from 15 to 28 muscle fibers and the error brackets represent the SEM.

served relative to the controls ($P > 0.2$) (Fig. 3, hatched bars). In the complete absence of extracellular calcium, the toxin caused no change in m.e.p.p. frequency (11).

These data show that the toxin has a much more stringent requirement for calcium than has synaptic transmission, where strontium is an acceptable analog. It is difficult to reconcile this observation with modification of the calcium metabolism of the terminal by the toxin; it is of course quite consistent with the involvement of a calcium-dependent phospholipase.

When preparations in calcium-containing media were treated identically to those described above except that 20 μg/ml of the purified phospholipase A_2 from *V. russellii* replaced β-bungarotoxin, the average m.e.p.p. frequency of fibers recorded 2 hr later was not significantly different from untreated controls ($P > 0.6$). It would seem that the phospholipase activity of the toxin is not a complete explanation of its inhibition of transmitter release.

DISCUSSION

We have sought to demonstrate that β-bungarotoxin has phospholipase activity and that this activity is associated with the molecule's physiological role as a pre-synaptic toxin. β-Bungarotoxin modifies release of transmitter at the neuromuscular junction in two distinct ways (21): at first, an initial enhancement of spontaneous, evoked, and delayed release is observed, peaking within 30 min; second, the rate of release declines and transmission fails completely after several hours. Both perturbations are calcium-dependent. When calcium is removed (21) or when strontium replaces calcium in the bathing medium, the toxin does not block release of transmitter; the initial enhancement in the rate of spontaneous transmitter release is reduced, but perhaps not eliminated (Fig. 3). Since the phospholipase activity of β-bungarotoxin requires calcium and is inhibited by strontium, the similarity in ionic requirements suggests that the phospholipase activity is involved in the toxin's modification of synaptic release.

The neurotoxicity of snake venom proteins has often been attributed to phospholipase activity, only to have further purification separate enzymatic from neurotoxic activity. The characteristics of β-bungarotoxin as a phospholipase and the consequent arguments that we wish to construct regarding the toxin's molecular function at the pre-synaptic nerve membrane are therefore critically dependent on the purity of our preparation. We have purified β-bungarotoxin from *Bungarus multicinctus* venom using a combination of CM-Sephadex and CM-cellulose ion-exchange chromatographic procedures. We have subsequently demonstrated that our neurotoxin is homogeneous, using the criteria of sodium dodecyl sulfate-polyacrylamide electrophoresis, isoelectric focusing, and phosphocellulose ion-exchange chromatography.

The characteristics of the phospholipase A_2 activity in β-bungarotoxin share many similarities with other phospholipases A_2 (see ref. 27 for a recent compendium of specific phospholipases A_2 isolated from snake venoms). Addition of deoxycholate to the assay mixture greatly enhances the phospholipase activity of the toxin and a similar requirement for calcium is observed. Although there are exceptions (28), most phospholipases have an absolute requirement for calcium when pure phospholipids serve as substrates (29) and where noted (17, 20, 30, 31), deoxycholate stimulates the activity of basic phospholipases, either by an emulsifying effect or by conferring a negative charge on the micellar substrate. Strontium has also been shown to be an inhibitor of pancreatic phospholipase A_2 (32) and *Crotalus adamanteus* phospholipase A_2 (33).

Preliminary reports (34, 35) have shown that notexin (12) and taipoxin (36), two other highly purified pre-synaptic toxins, also have phospholipase activity toward egg yolk phosphatidylcholine, although neither the details of the assay conditions nor further enzymatic characterization has been given. Using the rather toxic phospholipase from *N. nigricollis* (mouse minimum lethal dose 1 μg/g) as a standard, Eaker (35) has shown that the relative phospholipase activities of the *N. nigricollis* phospholipase, notexin, and taipoxin are 1.00:0.05:0.05, whereas the relative toxicities toward mice are 1:40:500, respectively. Other reports have claimed that β-bungarotoxin has either no (10, 37) or very low (38) levels of phospholipase activity. Although our demonstration of potent phospholipase A_2 activity in β-bungarotoxin may be due to our choice of assay conditions, the toxin acts similarly on natural membranes, since we have shown that both the toxin and *Crotalus adamanteus* phospholipase A_2 inactivate liver mitochondrial function at similar rates (13).

Table 3 shows that β-bungarotoxin is considerably more toxic than either of the other two phospholipase enzymes tested. Since we have also demonstrated that *V. russellii* phospholipase does not modify transmitter release (even at enzyme activity levels three times higher than that of the toxin), we suggest that either the phospholipase activity of β-bungarotoxin plays only a partial role in the toxin's modification of synaptic transmission, or that as a phospholipase, β-bungarotoxin has evolved a specific preference for the pre-synaptic plasma membrane. The toxin's pre-synaptic specificity may be due either to a substrate specificity, for special phospholipids in pre-synaptic plasma membranes for example, or else due to a specific binding site distinct from the enzymatic substrate.

If the toxin makes use of its phospholipase activity in perturbing transmitter release, the mechanism could be either direct or indirect. Indirect effects could include some modification of calcium metabolism, such as the inactivation of

the rate of calcium removal from the terminal as we have suggested earlier (39), or of oxidative phosphorylation, as suggested by Wernicke *et al.* (38). On the other hand, the toxin could modify release directly by binding to the pre-synaptic plasma membrane and then altering the probability that a vesicle will fuse with the membrane. Once bound to the plasma membrane, the enzymatic activity of the toxin might raise the level of fatty acids and lysophospholipids in the membrane, which in turn might alter the probability that a vesicle will fuse with the membrane to release transmitter. Experimental support for such a model comes from the increase of fusion of synthetic liposomes containing fatty acids (40) or of cells in the presence of lysophosphatidylcholine (41). At this present stage it is not possible to distinguish between direct versus indirect mechanisms; however, the detailed information that we have on the biochemical and physiological properties of β-bungarotoxin make it a useful molecular probe of the pre-synaptic nerve terminal. In addition, the data now available on the phospholipase activity of pre-synaptic neurotoxins make us wonder if this is a unique situation, or whether other toxins might have as yet undetected enzyme activity.

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1. Selinger, Z., Sharoni, Y. & Schramm, M. (1974) in *The Cytopharmacology of Secretion*, eds. Ceccarelli, B., Clementi, F. & Meldolesi, Jr. (Raven Press, New York), pp. 23–28.
2. Rubin, R. P. (1970) *Pharmacol. Rev.* **22**, 389–428.
3. Katz, B. (1969) *The Release of Neural Transmitter Substances* (Liverpool University Press, Liverpool, England).
4. Chang, C. C. & Huang, M. C. (1974) *Naunyn-Schmiedeberg's Arch. Pharmakol.* **282**, 129–142.
5. Habermann, E. & Heller, I. (1975) *Naunyn-Schmiedeberg's Arch. Pharmakol.* **287**, 97–106.
6. Kawai, N., Mauro, A. & Grundfest, H. (1972) *J. Gen. Physiol.* **60**, 650–664.
7. Granata, F., Paggi, P. & Frontali, N. (1972) *Toxicon* **10**, 551–555.
8. Chang, C. C., Chen, T. F. & Lee, C. Y. (1973) *J. Pharmacol. Exp. Ther.* **184**, 339–345.
9. Kelly, R. B. & Brown, F. R., III (1974) *J. Neurobiol.* **5**, 135–150.
10. Wernicke, J. F., Oberjat, T. & Howard, B. D. (1974) *J. Neurochem.* **22**, 781–788.
11. Karlsson, E., Eaker, D. & Rydén, L. (1972) *Toxicon* **10**, 405–413.
12. Harris, J. B., Karlsson, E. & Thesleff, S. (1973) *Br. J. Pharmacol.* **47**, 141–146.
13. Kelly, R. B. Oberg, S. G., Strong, P. N. & Wagner, G. M. (1975) *Cold Spring Harbor Symp. Quant. Biol.* **40**, in press.
14. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021.
15. Cooper, D. & Reich, E. (1972) *J. Biol. Chem.* **247**, 3008–3013.
16. Wells, M. A. (1972) *Biochemistry* **11**, 1030–1041.
17. de Haas, G. H. Postema, N. M., Nieuwenhuizen, W. & Van Deenen, L. L. M. (1968) *Biochim. Biophys. Acta* **159**, 103–117.
18. Batzri, S. & Korn, E. D. (1973) *Biochim. Biophys. Acta* **298**, 1015–1019.
19. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917.
20. Goerke, J., de Gier, J. & Bonsen, P. P. M. (1971) *Biochim. Biophys. Acta* **248**, 245–253.
21. Oberg, S. G. & Kelly, R. B. (1975) *J. Neurobiol.*, in press.
22. Saito, K. & Hanahan, D. J. (1962) *Biochemistry* **1**, 521–532.
23. Salach, J. I., Turini, P., Seng, R., Hauber, J. & Singer, T. P. (1971) *J. Biol. Chem.* **246**, 331–339.
24. Shipolini, R. A., Callewaert, G. L., Cottrell, R. C., Doonan, S., Vernon, C. A. & Banks, B. E. C. (1971) *Eur. J. Biochem.* **20**, 459–468.
25. Vidal, J. C. & Stoppani, A. O. M. (1971) *Arch. Biochem. Biophys.* **145**, 543–556.
26. Dodge, F. A., Jr., Miledi, R. & Rahamimoff, R. (1969) *J. Physiol. (London)* **200**, 267–283.
27. Botes, D. P. & Viljoen, C. C. (1974) *J. Biol. Chem.* **249**, 3827–3835.
28. Augustyn, J. M. & Elliot, W. B. (1970) *Biochim. Biophys. Acta* **206**, 98–10..
29. Salach, J. I., Seng, R., Tisdale, H. & Singer, T. P. (1971) *J. Biol. Chem.* **246**, 340–347.
30. Magee, W. L., Gallai-Hatchard, J., Sanders, H. & Thompson, R. H. S. (1962) *Biochem. J.* **83**, 17–25.
31. van Deenen, L. L. M., de Haas, G. H. & Heemskerk, C. H. Th. (1963) *Biochim. Biophys. Acta* **67**, 295–304.
32. Pieterson, W. A., Volwerk, J. J. & de Haas, G. H. (1974) *Biochemistry* **13**, 1439–1445.
33. Wells, M. A. (1973) *Biochemistry* **12**, 1080–1085.
34. Halpert, J. & Eaker, D. (1975) *J. Biol. Chem.* **250**, 6990–6997.
35. Eaker, D. (1975) *Toxicon* **13**, 90.
36. Kamenskaya, M. A. & Thesleff, S. (1974) *Acta Physiol. Scand.* **90**, 716–724.
37. Lee, C. Y., Chang, S. L., Kau, S. T. & Luh, S. H. (1972) *J. Chromatogr.* **72**, 71–82.
38. Wernicke, J. F., Vanker, A. D. & Howard, B. D. (1975) *J. Neurochem.* **25**, 483–496.
39. Wagner, G. M., Mart, P. E. & Kelly, R. B. (1974) *Biochem. Biophys. Res. Commun.* **58**, 475–481.
40. Kantor, H. L. & Prestegard, J. H. (1975) *Biochemistry* **14**, 1790–1795.
41. Poole, A. R., Howell, J. I. & Lucy, J. A. (1970) *Nature* **227**, 810–813.