Deenergization of Nerve Terminals by β-Bungarotoxin†

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ABSTRACT: β-Bungarotoxin is a snake venom protein that has neurotoxic and phospholipase A activities. The toxin causes a neuromuscular blockade by inhibiting the evoked release of acetylcholine from motor nerve terminals. β-Bungarotoxin also reduces ATP stores in synaptosomes from rat brain and inhibits several different synaptosomal transport processes without lysing the synaptosomes. Evidence is presented that these effects of β-bungarotoxin on synaptosomes are secondary to a toxic-induced depolarization of the synaptosomes. In toxin-treated synaptosomes the rate of decrease in ATP levels approximately coincided with the rate of decrease in membrane potential, which was monitored fluorimetrically with a cyanine dye. A similar pattern of coincidental decrease in membrane potential and ATP level was obtained by treatment with gramicidin D, which depolarizes by making membranes permeable to Na^+. Oligomycin and sodium azide, which inhibit ATP synthesis, did not affect synaptosomal membrane potential, although they decreased synaptosomal ATP levels to a greater extent than did β-bungarotoxin. The toxin had less of an effect on synaptosomal ATP stores when the synaptosomes were treated with β-bungarotoxin in the presence of ouabain, an inhibitor of (Na^+,K^+)ATPase; however, ouabain did not affect the toxin-induced depolarization of the synaptosomes. These results indicate that the initial effect of β-bungarotoxin on synaptosomes is a decrease in membrane potential and that the synaptosomes degrade ATP in an attempt to reestablish the original membrane potential. The action of the toxin is not dependent on external Na^+ concentration, suggesting that the depolarization is not due to an action of the toxin on Na^+ channels or (Na^+K^+)ATPase. Ethoxyformic anhydride inactivated the phospholipase activity and the neurotoxicity of β-bungarotoxin. Ethoxyformic anhydride also eliminated β-bungarotoxin-induced reduction of synaptosomal membrane potential, ATP levels, and transport processes. The phospholipase A activity of β-bungarotoxin was protected against ethoxyformic anhydride by dihexanoyllecithin, a substrate for phospholipase A. However, after treatment with ethoxyformic anhydride and dihexanoyllecithin, β-bungarotoxin lost its ability to cause neuromuscular blockade and to deenergize synaptosomes. Thus, the neurotoxicity of β-bungarotoxin and its effects on synaptosomes are not caused solely by its measurable phospholipase A activity. The substrate specificity of β-bungarotoxin phospholipase A was studied. The extent of hydrolysis of phosphoglycerides with different head groups and the composition of fatty acids liberated from rat brain synaptosomes by β-bungarotoxin did not differ significantly from those affected by two nonneurotoxic phospholipases A.

The 21 500-dalton neurotoxic phospholipase A, β-bungarotoxin, is from the venom of the snake *Bungarus multicinctus* (Lee, 1972; Kondo et al., 1978). The toxin acts at the nerve terminals of neuromuscular junctions and inhibits the induced release of acetylcholine (Chang et al., 1973). In addition, β-bungarotoxin has been shown to act on synaptosomes prepared from rat brain and inhibit synaptosomal transport and storage of several neurotransmitters and nontransmitter compounds without lysing the synaptosomes (Wernicke et al., 1974). Wernicke et al. (1975) have presented evidence that the action of β-bungarotoxin on synaptosomes is due to a depletion of synaptosomal energy stores. The initial deenergizing effect was thought to be an inhibition of ATP synthesis caused by fatty acids liberated by the toxin's phospholipase A activity.

Sen & Cooper (1978) have found that treatment of a synaptosome fraction with β-bungarotoxin in the presence of a cyanine dye increased the fluorescence of the dye. Changes in the fluorescence of cyanine dyes have been shown to reflect changes in membrane potential in erythrocytes (Hoffman & Laris, 1974; Sims et al., 1974); treatment of purified synaptosomes with depolarizing agents also causes a change (increase) in the fluorescence of the cyanine dyes (Blaustein & Goldring, 1975). Therefore, the finding of Sen & Cooper (1978), mentioned above, suggests that β-bungarotoxin depolarizes synaptosomes and raises the question of whether the depolarization is a consequence of the reduced ATP level in toxin-treated synaptosomes (Wernicke et al., 1975) or a cause of it. In this paper, we report evidence that β-bungarotoxin initially acts to reduce synaptosomal membrane potential and that the synaptosomes utilize ATP in an attempt to reestablish the original membrane potential.

We have also studied the role of β-bungarotoxin's phospholipase A activity in its neurotoxicity. β-Bungarotoxin is only one of several snake venon neurotoxins for which a phospholipase A activity has been implicated in the ability to inhibit acetylcholine release (Karlsson, 1973; Howard, 1977). Each of these toxins exhibits a low level of phospholipase A activity, and where the amino acid sequence has been determined, a homology to well-established phospholipases A has been found (Breithaupt et al., 1975; Halpert & Eaker, 1975; Fohlman et al., 1976; Kondo et al., 1978). Furthermore, where examined, agents that inactivate the phospholipase A activity of these toxins also cause a loss of their neurotoxicity (Halpert et al., 1976; Strong et al., 1976b; Howard & Truog, 1977; Abe et al., 1977). Paradoxically, most known phospholipases A are not neurotoxic in spite of having greater specific enzyme activity.

Howard and Truog (1977) reported that β-bungarotoxin lost its lethality for mice and its phospholipase A activity after modification with EOFA.[1] When the EOFA treatment was

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[1] Abbreviations used: DiC_6, L-α-dihexanoyllecithin; EOFA, ethoxyformic anhydride; GABA, γ-aminobutyric acid; SE, standard error of the mean; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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performed in the presence of DiC_6, which is a substrate for β-bungarotoxin phospholipase A, the toxin lost its lethality for mice but not its phospholipase A activity, as tested with bacterial membranes as substrate. Thus, the effect of the treatment with EOFA and DiC_6 was to convert β-bungarotoxin from a neurotoxic phospholipase A to a nonneurotoxic phospholipase A.

In the present study, we have examined whether the nonneurotoxic β-bungarotoxin phospholipase A obtained after treatment with EOFA and DiC_6 differs from native β-bungarotoxin with respect to its ability to deenergize synaptosomes and to hydrolyze synaptosomal phosphoglycerides. We have also examined whether synaptosomal membranes possess a specific phosphoglyceride substrate for β-bungarotoxin phospholipase A. We find that the ability of β-bungarotoxin to deenergize synaptosomes cannot be due to the toxin's measurable phospholipase A activity, alone.

Materials and Methods

Chemicals. β-Bungarotoxin[2] and fraction IVa phospholipase A were purified from *B. multicinctus* venom as described (Wernicke et al., 1974, 1975). Ouabain, sodium azide, oligomycin, gramicidin D, valinomycin, veratridine, choline chloride, *Vipera russelli* phospholipase A, and phosphoglycerides used as standards were purchased from Sigma Chemical Co. Fatty acid methyl esters, DiC_6, and L-α-[2-*palmitoyl*-9,10-^{3}H]dipalmitoylphosphatidylcholine (13 Ci/mmol) were obtained from Applied Science. EOFA was purchased from Eastman Kodak Co. [U-^{14}C]GABA (224 mCi/mmol) and 2-deoxy-D-[1-^{3}H]glucose (22 Ci/mmol) were obtained from Amersham Corp. Firefly luciferase was purchased from Worthington. The cyanine dye, 3,3′-dihexyloxacarbocyanine iodide (di-O-C_6) was a gift from A. S. Waggoner of Amherst College. All other chemicals were of reagent grade.

Preparation of Synaptosomes and Synaptosome Plasma Membranes. All fractions were prepared from the cerebral cortex of male Sprague-Dawley rats (170–200 g). A crude synaptosome-mitochondrial fraction (P_2') was prepared as described (Wernicke et al., 1974) with the modification that the cerebral cortices were homogenized in 10 volumes of sucrose medium (0.32 M sucrose and 10 mM Tris-HCl, pH 7.4). The synaptosomes were further purified by centrifugation of the P_2' fraction through a Ficoll density gradient as described by Goodkin & Howard (1974).

Synaptosome plasma membranes were prepared as described by Cotman & Taylor (1972).

ATP Determination. A 0.2-mL sample of purified synaptosomes (0.7 mg of protein) was incubated with 2 mL of 20 mM Tris-HCl (pH 7.4), 83 mM NaCl, 74 mM sucrose, 3 mM KCl, 3 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM glucose at 37 °C for 15 min or other specified lengths of time. The medium also contained β-bungarotoxin or other agents as indicated under Results. After incubation, synaptosomal ATP was extracted (Bradford, 1969) and measured with the firefly luciferase-luciferin system using a scintillation counter to measure the emitted light as described (Stanley & Williams, 1962; Wernicke et al., 1975).

Fluorescence Measurement. A 0.1-mL sample of purified synaptosomes (0.5 mg of protein) was incubated at 37 °C for 5 min or other specified lengths of time in 3 mL of the same medium used in the ATP experiment. The cyanine dye 3,3′-dihexyloxacarbocyanine iodide (5 μL; 1.5 mg/mL of ethanol) was added and the incubation was continued for 5 min, after which the fluorescence of the suspension was measured on an Aminco-Bowman spectrofluorometer (excitation, 475 nm; emission, 500 nm). The experiments described in Figures 6 and 8 were performed as above except that a crude synaptosome-mitochondrial fraction (0.8 mg of protein) was used. Almost all of the fluorescence increase obtained with this fraction under depolarizing conditions can be attributed to depolarization of synaptosomes rather than to effects on contaminating mitochondria or other membrane fragments (Blaustein & Goldring, 1975).

Synaptosomal Uptake of GABA and Deoxyglucose and Retention of GABA. A crude synaptosome-mitochondrial fraction was used for these experiments rather than Ficoll-purified synaptosomes; it has been shown that the accumulated GABA and deoxyglucose in this fraction were located in synaptosomes (Oberjat & Howard, 1973; Wernicke et al., 1974) and that β-bungarotoxin produced the same effects on the storage of GABA and deoxyglucose with purified synaptosomes as with the crude synaptosome-mitochondrial fraction (Wernicke et al., 1975). To measure GABA uptake, a 0.2-mL suspension of the crude synaptosome-mitochondrial fraction (0.4 mg of protein) was added to 1.8 mL of 25 mM Tris-HCl (pH 7.4), 2 mM $CaCl_2$, 10 mM glucose, 124 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, and 1.2 mM KH_2PO_4, with and without β-bungarotoxin or phospholipase A IVa. The mixture was incubated at 37 °C on a Dubnoff metabolic shaker under an atmosphere of 95% O_2–5% CO_2. After 10 min, [^{14}C]GABA (20 μL, 0.2 μCi) was added until a concentration of 0.2–0.4 μM was reached, and the incubation was continued for 5 min. GABA uptake was terminated by the addition of 5 mL of 10% sucrose and rapid filtration on Millipore filters (AAWP, 0.8-μm pore size), which trap synaptosomes and their accumulated GABA. The filter was washed with 15 mL of 10% sucrose and placed directly in 10 mL of 3a70B liquid scintillation cocktail (Research Products International) for several hours prior to counting by liquid scintillation spectrometry. GABA uptake was linear with time under these conditions.

To measure retention of previously accumulated GABA, one-half of a rat cerebral cortex was placed in 3 mL of the Tris-salts buffer used for GABA uptake and minced into pieces of approximately 1 mm in diameter. The tissue was preincubated for 5 min at 37 °C on a Dubnoff metabolic shaker under an atmosphere of 95% O_2–5% CO_2. [^{14}C]GABA was then added until a concentration of 3.3 μM (0.7 μCi/mL) was reached, and the incubation was continued for 10 min. After incubation, the sample was mixed with 10 mL of 0.32 M sucrose and centrifuged at 1000*g* for 5 min to remove the salts of the incubation buffer. The pellet was washed twice with 10 mL of 0.32 M sucrose and centrifuged. The final pellet was homogenized and a crude synaptosome-mitochondrial fraction was prepared. A 0.2-mL sample (0.3 mg of protein) of this fraction was added to 1.8 mL of the Tris-salts buffer, with or without β-bungarotoxin. The mixture was incubated for 15 min at 37 °C as described above. After incubation, GABA efflux was terminated by rapid filtration and the amount of GABA retained in the synaptosomes was determined as described in the uptake procedure.

Under the conditions used in these experiments most of the ^{14}C accumulated and retained in control synaptosomes or lost from toxin-treated synaptosomes is associated with GABA (Oberjat & Howard, 1973; Wernicke et al., 1974).

The experimental procedure for measuring deoxyglucose uptake was the same as that for GABA uptake with the following modifications: (1) glucose was omitted from the incubation mixture, (2) 2-deoxy-D-[1-^{3}H]glucose was added in-

[2] The β-bungarotoxin used in these experiments corresponds to the β_3-bungarotoxin fraction of Abe et al. (1977).

TABLE I: Depolarization of Synaptosomes by β-Bungarotoxin, KCl, and Gramicidin D [a]

additions to synaptosome[b]	fluorescence[c]
control	48.6 ± 0.6
β-bungarotoxin	56.8 ± 0.4
KCl	59.1 ± 0.6
gramicidin D	62.1 ± 0.4
KCl + β-bungarotoxin	62.9 ± 0.7
gramicidin D + β-bungarotoxin	62.4 ± 0.2

[a] The decrease in synaptosomal membrane potential was detected as described under Materials and Methods by an increase in the fluorescence of 3,3'-dihexyloxacarbocyanine iodide. [b] Purified synaptosomes were incubated for 15 min at 37 °C. KCl and gramicidin D were added at the start of the incubation at a final concentration of 55 mM and 3 μg/mL, respectively. β-Bungarotoxin was added 5 min after the start of the incubation at a final concentration of 66 ng/mL. The dye was added 5 min before the incubation ended. [c] Arbitrary units. Values are means ± SE for duplicate incubations.

stead of [^{14}C]GABA, and (3) a solution of 10% sucrose and 0.1 M D-glucose was used to terminate the reaction and wash the Millipore filters. Extraction and separation of the accumulated deoxyglucose and its metabolite, deoxyglucose phosphate, were as described by Colby & Romano (1975).

Protein Modification. β-Bungarotoxin or phospholipase IVa (22 μg) was incubated at 25 °C for 15 min in 0.2 mL of 10 mM Tes buffer (pH 6.0) containing 50 mM KCl and, where indicated, 70 mM DiC_6. EOFA was added to 30 mM and the incubation continued for 45 min. Control samples were incubated in the absence of EOFA but in the presence of DiC_6. All samples containing DiC_6 also contained 5 mM $CaCl_2$. After incubation the reaction mixtures were diluted to 2 mL with 10 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$; DiC_6 was added to those samples to which it had not been added during incubation. All samples were dialyzed against the Tris buffer for 22 h at 4 °C.

In Vitro Neurotoxicity Assay. Phrenic nerve-diaphragm preparations were isolated from Swiss Webster male mice weighing 25–30 g. The neuromuscular preparation was suspended at 37 °C in pH 7.4 Ringer-bicarbonate buffer (composition in mM: NaCl, 138; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; KH_2PO_4, 1; $NaHCO_3$, 12; glucose, 10) and gassed with 5% CO_2–95% O_2. The nerve was stimulated with supramaximal rectangular pulses of 2-ms duration every second, and the amplitude of muscle twitch was measured isometrically with a force transducer led to an oscilloscope. Following a 30-min preincubation of the neuromuscular preparation, β-bungarotoxin was added. At the time of addition of β-bungarotoxin each nerve impulse-induced muscle twitch resulted in a deflection of the oscilloscope beam of 16 to 26 mm. Paralysis time was independent of initial twitch amplitude. Neuromuscular blockade was taken to occur when each of several impulses caused a deflection of less than 2 mm. When blockade developed, the muscles of the paralyzed preparations were tested in all cases for their ability to contract upon direct electrical stimulation of the muscle to assure that the paralysis was not due to an effect on the muscle itself.

Analysis of Synaptosomal Phosphoglycerides Hydrolyzed. The reaction mixture (0.55 mL) consisted of purified synaptosomes (4 mg of protein) in 10 mM Tris-HCl (pH 7.4), 0.32 M sucrose, 4 mM $CaCl_2$, with and without β-bungarotoxin, *B. multicinctus* phospholipase A IVa or *V. russelli* phospholipase A at the concentration indicated. The mixture was incubated at 30 °C with shaking for 60 min, after which the membrane lipids were extracted (Kates, 1972) and separated by two-dimensional thin-layer chromatography (Rouser et al., 1966) on silica gel plates with the following solvent pairs: chloroform–methanol–28% aqueous ammonia (13:7:1), followed by chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5). The phospholipid spots were analyzed quantitatively as described (Rouser et al., 1966).

Analysis of Fatty Acids Released. A suspension of purified synaptosomal plasma membranes (1 mg of protein) in 10 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$ was incubated with and without β-bungarotoxin (1 μg), *B. multicinctus* phospholipase A IVa (0.25 μg) or *V. russelli* phospholipase A (0.33 μg) for 60 min at 37 °C. The volume of the reaction mixture was 0.5 mL. After incubation, fatty acids were extracted as described (Folch et al., 1957) with chloroform–methanol (2:1, v/v), washed with water, and dried over $MgSO_4$. To obtain methyl esters from the fatty acids, the samples were treated with diazomethane (Schlenk & Gellerman, 1960). The methyl esters were then extracted into pentane and purified by elution from a silica gel column with 4% ether in pentane (Fulco, 1969). For fatty acid analysis the samples were redissolved in benzene, and equal portions were analyzed by gas–liquid chromatography on a 6-ft column of 15% FFAP (Varian) either isothermally (204 °C) or with a linear temperature program using 190–240 °C at 2 °C/min; identical results were obtained by each method. The peaks of the samples on the chromatograms were identified by comparison with those of a standard mixture containing methyl esters of saturated and unsaturated fatty acids analyzed under the same conditions.

Phospholipase A Assays. When [^{3}H]phosphatidylcholine was used as the substrate, the reaction mixture (0.2 mL) consisted of the material to be assayed in 10 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$. The reaction was initiated by the addition of 10 μL of [^{3}H]phosphatidylcholine (12.5 nCi) in ethanol (final phosphatidylcholine concentration was 0.85 mM), and the samples were incubated at 37 °C with shaking for 4 min. The fatty acids were extracted from the reaction mixture by the phase-separation procedure described by Gatt & Barenholz (1969) and added to 3a70B scintillation fluid for measurement of radioactivity. When synaptosomes were used as the substrate, the synaptosomes (4 mg of protein) were incubated for 60 min at 30 °C, and lysophosphatidylcholine was extracted and analyzed as described above.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

Depolarization of Synaptosomes. As shown in Table I, treatment of purified synaptosomes with β-bungarotoxin in the presence of the dye 3,3'-dihexyloxacarbocyanine iodide caused an increased fluorescence of the dye. This effect is not caused by lysis of the synaptosomes (Wernicke, 1974). Exposure of synaptosomes to the depolarizing agents K^+ (55 mM) and gramicidin D, which depolarizes by making membranes permeable to Na^+ (Pressman, 1976), also caused an increased fluorescence of the dye. None of the agents listed in Table I affected the fluorescence of the dye in the absence of synaptosomes.

The increased fluorescence obtained after treatment of the synaptosomes with gramicidin D should be a maximum because gramicidin D could be expected to fully depolarize the synaptosomes. Therefore, if β-bungarotoxin does, in fact, increase dye fluorescence by decreasing membrane potential, treatment of the synaptosomes with β-bungarotoxin in combination with 55 mM K^+ or gramicidin D should cause a flu-

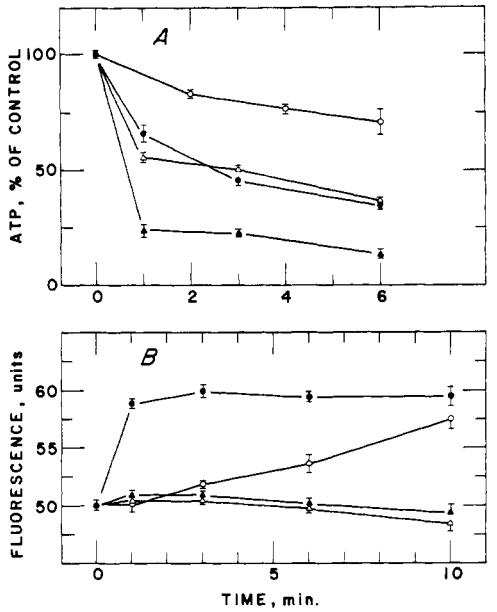

FIGURE 1: Time courses of the effects of β-bungarotoxin, gramicidin D, sodium azide, and oligomycin on synaptosomal ATP level and membrane potential. All synaptosome samples were incubated for 10 min; agents were added at the specified lengths of time before the incubation ended. Control synaptosomes were incubated in the absence of any agent. (A) Synaptosomal ATP level. The values are expressed as a percentage of ATP level of control synaptosomes, which contained 3.4 nmol of ATP/mg of protein. Each data point represents the mean ± SE for triplicate incubations. (B) Depolarization of purified synaptosomes was detected by an increase in fluorescence of the dye 3,3′-dihexyloxacarbocyanine iodide. Each data point represents the mean ± SE for four incubations: (○) β-bungarotoxin (65 ng/mL); (●) gramicidin D (1 μg/mL); (△) oligomycin (1 μg/mL); (▲) sodium azide (5 mM).

orescence increase that is no greater than that obtained with gramicidin D alone. The results given in Table I show that this is the case. The toxin-induced depolarization of synaptosomes is both time and concentration dependent, as is shown below in other contexts.

Depolarization and Depletion of ATP Stores. Wernicke et al., (1975) found that there is a decreased level of ATP in synaptosomes treated with β-bungarotoxin. As shown in Figure 1, the time course of the decrease in ATP in toxin-treated synaptosomes was approximately the same as the time course of the decrease in membrane potential. We have found that the ATP content of synaptosomes is also substantially decreased by two other depolarizing agents, gramicidin D and veratridine, which acts on Na^+ channels (Narahashi, 1974). Treatment of synaptosomes for 12 min with 75 μM veratridine caused a 40% decrease in ATP levels. The results obtained with gramicidin D are shown in Figure 1. Gramicidin D produced a pattern similar to that for β-bungarotoxin in that there was an approximately coincidental decrease in synaptosomal membrane potential and ATP content.

As shown in Figure 1, both oligomycin (Lardy et al., 1975) and sodium azide, which inhibit mitochondrial ATP synthesis, caused a greater and more rapid decrease in synaptosomal ATP content than did β-bungarotoxin. However, neither oligomycin nor sodium azide caused any decrease in synaptosomal membrane potential under the conditions used. These results demonstrate that the ATP decrease in β-bungarotoxin-treated synaptosomes could not be the cause of the decreased membrane potential in these synaptosomes.

The effects of β-bungarotoxin on synaptosomes are intriguingly similar to those produced on bacterial cells by colicins K and El, which are protein toxins made by *Escherichia coli*. These colicins interfere with several bacterial transport processes, decrease membrane potential, and lower ATP levels (Brewer, 1974; Holland, 1975). It is believed that the primary effect of the colicins is a deenergization (i.e., depolarization) of the bacterial membrane. Secondarily, ATP is utilized in an attempt by the cell to repolarize its membrane. Under conditions in which the membrane (Ca^{2+},Mg^{2+})ATPase is made inactive in colicin-treated cells, the membranes become deenergized without an accompanying decrease in ATP levels (Feingold, 1970; Plate et al., 1974).

TABLE II: Effect of Ouabain and β-Bungarotoxin on the Synaptosomal ATP Level.

treatment[a]	ATP level[c]
control	100 ± 4
ouabain	97 ± 2
β-bungarotoxin	39 ± 3
ouabain, + β-bungarotoxin[b]	67 ± 1

[a] Purified synaptosomes were incubated with ouabain (0.1 mM) for 15 min or β-bungaratoxin (50 ng/mL) for 13.5 min at 37 °C. [b] β-Bungarotoxin (50 ng/mL) was added 1.5 min after the addition of ouabain (0.1 mM). [c] Values are means ± SE for triplicate incubations. Control synaptosomes contained 2.2 nmol of ATP/mg of protein.

We reasoned that the decrease in ATP levels in β-bungarotoxin-treated synaptosomes may also result from an attempt of the synaptosomes to reestablish their original membrane potential by activation of the (Na^+,K^+)ATPase that acts as a Na^+ pump. As a test of this hypothesis, synaptosomal ATP content was measured after treatment with β-bungarotoxin in the presence of ouabain, an inhibitor of the (Na^+,K^+)-ATPase. As shown in Table II, ouabain prevented much of the ATP decrease caused by β-bungarotoxin. However, ouabain did not affect the ability of β-bungarotoxin to depolarize synaptosomes (Table III). Ouabain did not completely block the ability of β-bungarotoxin to deplete synaptosomal ATP stores perhaps in part because only a portion of synaptosomal (Na^+,K^+)ATPase activity is inhibited by ouabain under these conditions (Albers et al., 1965).

Table III shows that, as expected, the ability of gramicidin D to depolarize synaptosomes is sensitive to variations in external Na^+ concentration. However, depolarization of synaptosomes by β-bungarotoxin is independent of external Na^+ concentration (Table III), suggesting that β-bungarotoxin does not depolarize by acting on Na^+ channels or the Na^+ pump.

Inhibition of Synaptosomal Transport Processes. β-Bungarotoxin inhibits synaptosomal transport of several compounds, e.g., choline, GABA, norepinephrine, and deoxyglucose (Wernicke et al., 1974, 1975). β-Bungarotoxin-induced depolarization of synaptosomes itself may account for inhibition of the transport of most of these compounds but not of deoxyglucose. Synaptosomal transport and storage of deoxyglucose are not affected by ouabain (Diamond & Fishman, 1973; Wernicke et al., 1975) or by a buffer of high K^+ concentration (Diamond & Fishman, 1973) under conditions in which they depolarize synaptosomes[3] (Blaustein & Goldring, 1975).

[3] Ouabain at 0.1 mM causes a slight depolarization of synaptosomes (Marchbanks & Campbell, 1976), but the depolarization is not enough to cause a significant increase in dye fluorescence under the conditions described in Table III. However, at higher doses ouabain does produce a substantial increase in dye fluorescence (Blaustein & Goldring, 1975). Even at 0.1 mM, ouabain strongly inhibits the synaptosomal uptake of several compounds, e.g., biogenic amines and amino acids (Tissari et al., 1969; Bennett et al., 1973).

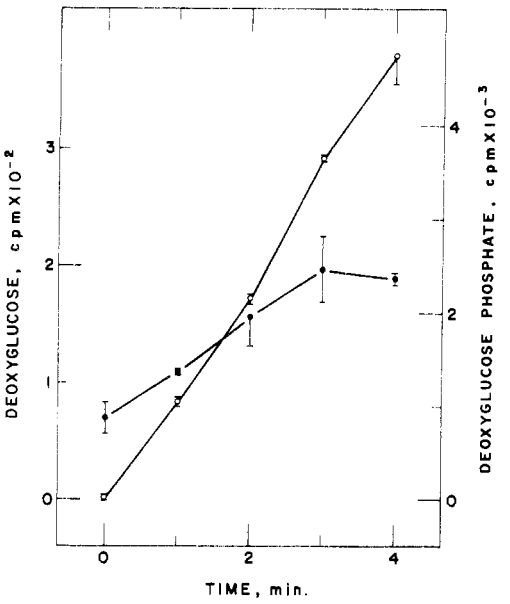

FIGURE 2: Amount of radioactive deoxyglucose and deoxyglucose phosphate in synaptosomes after incubation with deoxy[^{3}H]glucose. A crude synaptosome-mitochondrial preparation was incubated with 1 μCi of deoxy[^{3}H]glucose at 37 °C for various time periods. The incubation mixtures were filtered and the composition of the radioactive compounds retained in the synaptosomes on the filters was analyzed. The values are radioactivity per sample of incubated synaptosomes and the data points represent the means ± SE for duplicate incubations: (●) deoxy[^{3}H]glucose; (○) deoxy[^{3}H]glucose phosphate.

How does β-bungarotoxin inhibit synaptosomal transport of deoxyglucose? We have found that synaptosomal uptake of deoxyglucose is also inhibited by two other depolarizing agents, gramicidin D and veratridine. Under the experimental conditions described under Materials and Methods, the amount of deoxyglucose accumulated by synaptosomes in 3 min was reduced by 42 and 44% in the presence of 5 μg of gramicidin D/mL and 75 μM veratridine, respectively. These agents, like β-bungarotoxin, reduce synaptosomal stores of ATP, while depolarization with ouabain or buffers of high K^+ composition does not affect synaptosomal ATP content (Table II; Wernicke et al., 1975). Thus, the ability of β-bungarotoxin, gramicidin D, and veratridine to inhibit synaptosomal uptake of deoxyglucose could result from their ability to deplete synaptosomal stores of ATP. In many tissues, deoxyglucose is transported by facilitated diffusion (Morgan & Whitfield, 1973) and is metabolized only to deoxyglucose phosphate (Sols & Crane, 1954; Diamond & Fishman, 1973). As shown in Figure 2, most of the accumulated deoxyglucose is quickly converted to the phosphorylated derivative under the conditions used in our experiments. Treatment of synaptosomes with β-bungarotoxin inhibited synaptosomal accumulation of deoxyglucose phosphate but not of unphosphorylated deoxyglucose (Table IV). Similar results were obtained with oligomycin and the K^+ ionophore valinomycin, which inhibits mitochondrial ATP synthesis (Pressman, 1976). Treatment of synaptosomes with valinomycin under the conditions described for Table IV caused a 53% reduction of synaptosomal ATP. These results suggest that deoxyglucose diffuses into synaptosomes and is trapped inside upon being phosphorylated; β-bungarotoxin reduces the amount of phosphorylated deoxyglucose that is trapped within synaptosomes but has no effect, per se, on the transport or storage process for unphosphorylated deoxyglucose.

Neurotoxicity of β-Bungarotoxin after Treatment with EOFA and DiC_6. Treatment of β-bungarotoxin with EOFA and DiC_6 causes the toxin to lose its lethality for mice but not its phospholipase A activity, as tested with bacterial membranes as substrate (Howard & Truog, 1977). Table V shows that this decrease in neurotoxicity can also be demonstrated in vitro. After treatment with EOFA and DiC_6, β-bungarotoxin required a substantially increased time to cause neuromuscular blockade of an isolated neuromuscular preparation. This result indicates that the loss of lethality for mice is not due to an additional alteration of the treated toxin subsequent to its injection into the animals.

Activity of β-Bungarotoxin on Synaptosomes after Treatment with EOFA and DiC_6. We have studied whether the nonneurotoxic β-bungarotoxin obtained by treatment with EOFA and DiC_6 differs from native β-bungarotoxin with respect to activity on synaptosomes. As shown in Figure 3, treatment with EOFA alone largely inactivated the β-bungarotoxin phospholipase A activity, which was measured with two different substrates—dispersed [^{3}H]phosphatidylcholine or synaptosomes. However, when β-bungarotoxin was treated with EOFA in the presence of DiC_6, it had all of the phospholipase A activity detected in the native toxin, even with synaptosomes as substrate. Although this nonneurotoxic (see Table V) β-bungarotoxin apparently retained full phospholipase A activity on synaptosomes, it lost most of its ability to alter synaptosomal uptake of GABA, retention of previously accumulated GABA (Figure 4), and uptake of deoxyglucose (Figure 5A). Nonneurotoxic β-bungarotoxin obtained by treatment with EOFA plus DiC_6 largely lost the ability of

TABLE III: Depolarization of Synaptosomes by β-Bungarotoxin and Gramicidin D in the Presence of Choline, Na^+, and Ouabain.[a]

additives to incubat. medium[b]			increase in fluorescence[c]	
choline	Na^+	Ouabain	β-bungarotoxin	gramicidin D
+	−	−	7.6 ± 1.2	4.5 ± 0.7
−	+	−	6.5 ± 1.0	8.2 ± 1.0
−	+	+	6.1 ± 1.1	8.4 ± 1.4

[a] Purified synaptosomes were incubated in the specified medium for 2 min at 37 °C before the addition of β-bungarotoxin (66 ng/mL) or gramicidin D (1 μg/mL). Incubation was continued for 10 min. The cyanine dye was added 5 min before the incubation ended. [b] The composition of the media was the same as that described under Materials and Methods except that choline chloride (83 mM) and ouabain (0.1 mM) were included where indicated. Values are means ± SE for six incubations. [c] Arbitrary units.

TABLE IV: Amounts of Radioactive Deoxyglucose and Deoxyglucose Phosphate in Synaptosomes after Incubation with Deoxy[^{3}H]glucose and Various Agents.

treatment[a]	total uptake of radioact.[b]	radioact. compds (cpm)[c] deoxyGlc	deoxyGlc phosphate
control	100 ± 1	170 ± 11	3492 ± 23
β-bungarotoxin (50 ng/mL)	34 ± 2	211 ± 11	1034 ± 60
oligomycin (1 μg/mL)	63 ± 1	231 ± 5	2067 ± 29
valinomycin (5 ng/mL)	59 ± 2	240 ± 7	1912 ± 87

[a] A crude synaptosome-mitochondrial preparation was incubated as described under Materials and Methods with 1 μCi of deoxy[^{3}H]-glucose and the specified agent at 37 °C for 3 min. Values are means ± SE for four incubations. [b] Expressed as percent of control values. [c] Radioactivity per sample of incubated synaptosomes.

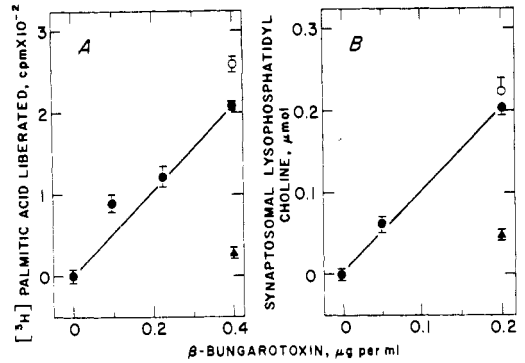

FIGURE 3: Effect of EOFA and DiC_6 on the phospholipase A activity of β-bungarotoxin using two different substrates. (A) Liberation of [^{3}H]-palmitic acid from [^{3}H]phosphatidylcholine. Control β-bungarotoxin released 9.7 μmol of fatty acid min^{-1} (mg of protein)$^{-1}$. (B) Formation of lysophosphatidylcholine in purified synaptosomes. Each data point represents the mean ± SE for three incubations: (●) control β-bungarotoxin treated with DiC_6; (▲) β-bungarotoxin treated with EOFA; (○) β-bungarotoxin treated with EOFA and DiC_6.

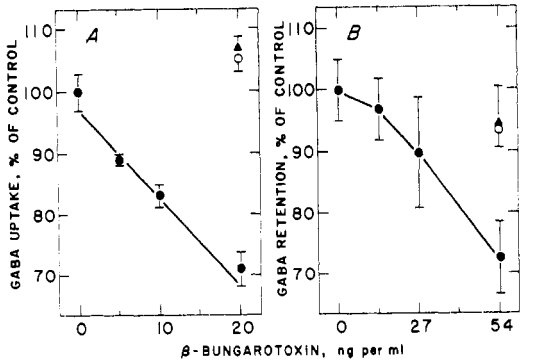

FIGURE 4: Effect of EOFA and DiC_6 on the ability of β-bungarotoxin to alter synaptosomal uptake and retention of GABA. (A) Synaptosomal uptake of GABA. The values are expressed as a percentage of GABA taken up in control synaptosomes, which accumulated 580 pmol of GABA/mg of protein. Each data point represents the mean ± SE for three incubations. (B) Synaptosomal retention of previously accumulated GABA. The values are expressed as a percentage of GABA retained in control synaptosomes, which retained 89 pmol of GABA/mg of protein. Each data point represents the mean ± SE for six incubations: (●) control β-bungarotoxin treated with DiC_6; (▲) β-bungarotoxin treated with EOFA; (○) β-bungarotoxin treated with EOFA and DiC_6.

TABLE V: In Vitro Neurotoxicity of β-Bungarotoxin after Treatment with EOFA and DiC_6.

toxin concn (μg/mL)	paralysis time (min)[a]	
	control toxin	treated toxin
5	85	175[b]
1	110	
0.1	130[b]	>250[c]
0	>250[c]	

[a] β-Bungarotoxin was treated with or without EOFA and DiC_6, dialyzed, and added to an isolated phrenic nerve-diaphragm preparation as described under Materials and Methods. Paralysis time is the interval between the addition of toxin and complete inhibition of muscle response to a single nerve pulse. The treated toxin used in these experiments had as much phospholipase A activity as the control toxin utilizing bacterial membranes as substrate in the enzyme assay as described by Howard & Truog (1977). [b] The values are means for duplicate experiments. The SE was less than 10%. [c] The values were obtained in each of duplicate experiments.

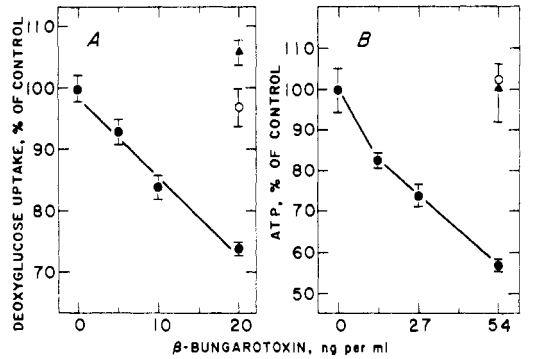

FIGURE 5: Effect of EOFA and DiC_6 on the ability of β-bungarotoxin to alter synaptosomal uptake of 2-deoxyglucose and the synaptosomal ATP level. (A) Synaptosomal uptake of 2-deoxyglucose. The values are expressed as a percentage of 2-deoxyglucose taken up (and phosphorylated) in control synaptosomes, which accumulated 0.9 pmol/mg of protein. Each data point represents the mean ± SE for six incubations. (B) Synaptosomal ATP level. The values are expressed as a percentage of the ATP level of control synaptosomes, which was 3 nmol of ATP/mg of protein. Each data point represents the mean ± SE for three incubations: (●) control β-bungarotoxin treated with DiC_6; (▲) β-bungarotoxin treated with EOFA; (○) β-bungarotoxin treated with EOFA and DiC_6.

native toxin to reduce synaptosome membrane potential (Figure 6) and decrease synaptosomal stores of ATP (Figure 5B). We have repeated these experiments several times with different preparations of β-bungarotoxin treated with EOFA alone or with EOFA plus DiC_6. Similar results were obtained each time.

EOFA Modification of IVa Phospholipase A. B. multicinctus venom contains a nonneurotoxic phospholipase A (fraction IVa phospholipase A), which has a much greater specific enzyme activity than does β-bungarotoxin phospholipase A (Wernicke et al., 1974, 1975; Kondo et al., 1978). Fraction IVa phospholipase also decreases synaptosomal uptake of GABA, but this effect requires a higher concentration of IVa phospholipase A than is required with β-bungarotoxin (Wernicke et al., 1975).

Similar studies of the effects of protein modification with EOFA were performed with this nonneurotoxic phospholipase A, and the results are shown in Figures 7 and 8. The results confirm that the specific phospholipase A activity of the fraction IVa enzyme is greater than that of β-bungarotoxin and that IVa phospholipase A is not as effective as β-bungarotoxin at causing inhibition of synaptosomal uptake of GABA. (Compare the results of Figure 7 with those of Figures 3A and 4A.) As with β-bungarotoxin, EOFA inactivated most of the phospholipase A activity of IVa (Figure 7A) and the ability of IVa to inhibit GABA uptake (Figure 7B) and reduce synaptosomal membrane potential (Figure 8). Another similarity is that DiC_6 protected the phospholipase A activity of IVa against inactivation of EOFA. However, unlike the situation with β-bungarotoxin, DiC_6 also protected the IVa enzyme against EOFA inactivation of the enzyme's ability to inhibit synaptosomal uptake of GABA and reduce synaptosomal membrane potential. Indeed, IVa enzyme treated with EOFA and DiC_6 had as much effect on GABA uptake and membrane potential as untreated enzyme.

Specificity of Substrate. A conceivable basis for the difference in the neurotoxocity and synaptosomal effects of toxic and nonneurotoxic phospholipases A is that the two classes of phospholipase A might hydrolyze different neuronal phosphoglycerides. We have examined this possibility by comparing the types of synaptosomal phosphoglycerides hydrolyzed and fatty acids liberated by β-bungarotoxin and by two nonneurotoxic phospholipases A—fraction IVa phospholipase A and

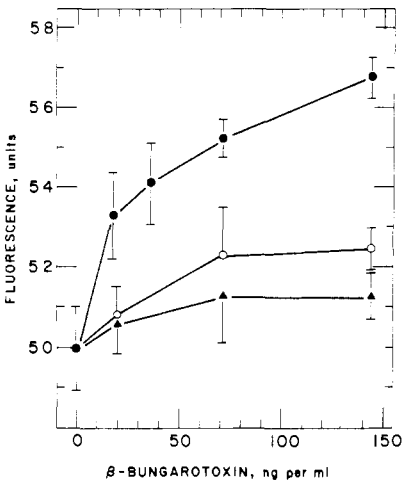

FIGURE 6: Effect of EOFA and DiC_6 on the ability of β-bungarotoxin to alter the membrane potential of synaptosomes. Depolarization of synaptosomes by the toxin was detected by an increase in fluorescence of 3,3′-dihexyloxacarbocyanine iodide. Each point represents the mean ± SE for six incubations: (●) control β-bungarotoxin treated with DiC_6; (▲) β-bungarotoxin treated with EOFA; (○) β-bungarotoxin treated with EOFA and DiC_6.

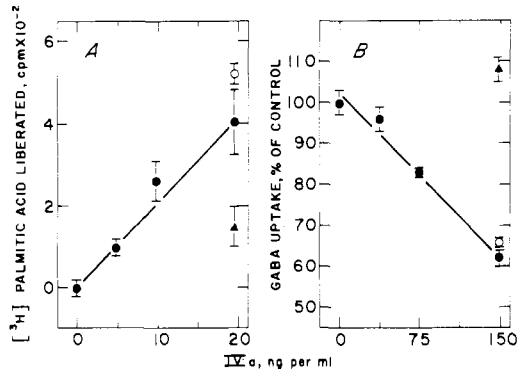

FIGURE 7: Effect of EOFA and DiC_6 on the phospholipase A activity of fraction IVa and on the ability of IVa phospholipase A to alter synaptosomal uptake of GABA. Each data point represents the mean ± SE for three incubations: (●) control IVa treated with DiC_6; (▲) IVa treated with EOFA; (○) IVa treated with EOFA and DiC_6. (A) Phospholipase A activity. The substrate was [^{3}H]phosphatidylcholine. Control IVa released 380 μmol of fatty acid min^{-1} (mg of protein)$^{-1}$. (B) GABA uptake. The results are expressed as a percentage of GABA taken up in control synaptosomes, which accumulated 570 pmol of GABA/mg of protein.

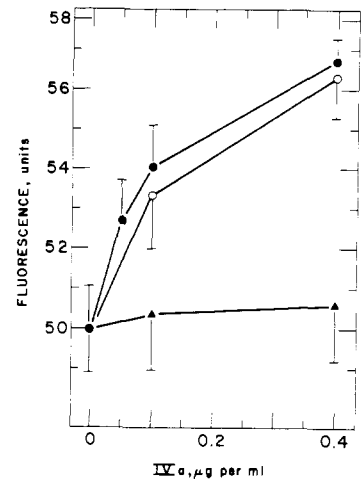

FIGURE 8: Effect of EOFA and DiC_6 on the ability of IVa phospholipase A to alter the membrane potential of synaptosomes. Depolarization of synaptosomes was detected by an increase of fluorescence of 3,3′-dihexyloxacarbocyanine iodide. Each data point represents the mean ± SE for six incubations: (●) control IVa treated with DiC_6; (▲) IVa treated with EOFA; (○) IVa treated with EOFA and DiC_6.

TABLE VI: Hydrolysis of Synaptosomal Phosphoglycerides by Neurotoxic and Nonneuratoxic Phospholipases A.

phospholipase A	concn (ng/mL)	% hydrolysis[a] PE	PS	PC
β-bungarotoxin	26	7	17	5
	262	35	31	39
	2620	32	37	42
B. multicinctus IVa	3	0	8	6
	36	20	11	20
	360	27	25	26
V. russelli	7	0	0	0
	74	13	11	12
	740	52	58	48

[a] Synaptosomes were incubated with phospholipases A, and the phosphoglycerides were extracted and analyzed as described under Materials and Methods. Each value represents a single determination. Abbreviations used: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine.

V. russelli venom phospholipase . As shown in Table VI, β-bungarotoxin phospholipase A did not exhibit any striking difference from the nonneurotoxic phospholipases A with respect to the relative amounts of the three major phosphoglycerides hydrolyzed. There was no apparent specificity based on phosphoglyceride head groups.

After incubation of β-bungarotoxin with synaptosomal plasma membranes, the major types of fatty acids liberated were palmitic, oleic, and arachidonic acids (Figure 9). Also, stearic acid and linoleic acid were easily detectable. With chromatographic elution of extended duration, we detected no additional fatty acids having retention times equal to or less than that of a nervonic acid ($C_{24:1}$) standard (data not shown). Figure 9 also shows that fraction IVa and *V. russelli* phospholipases A liberated fatty acids of approximately the same composition as that by β-bungarotoxin. Fraction IVa phospholipase A liberated a slightly greater proportion of arachidonic acid.

Discussion

We have provided evidence that the initial effect of β-bungarotoxin on synaptosomes is a partial depolarization of the synaptosomes and that, secondarily, there is a decrease in synaptosomal ATP. The decrease in ATP likely results from synaptosomal utilization of ATP in an attempt to reestablish the original membrane potential. The toxin's inhibition of synaptosomal transport processes can be attributed to the initial depolarization and/or to the subsequent drop in ATP levels.

The results presented in this paper strongly indicate that the in vitro effects of β-bungarotoxin on synaptosomes are relevant mechanistically for its neurotoxicity at neuromuscular junctions. Loss of neurotoxicity after treatment with EOFA and DiC_6 was accompanied by loss of the toxin's ability to deenergize synaptosomes, which are of course also nerve terminals. The depolarization of nerve terminals by β-bungarotoxin can also account for its ability to inhibit acetylcholine release from neuromuscular junctions. It is well established that depolarization of nerve terminals decreases the induced release of neurotransmitters; indeed, transmitter-mediated presynaptic inhibition may occur by this mechanism (Eccles et al., 1961;

Katz, 1962; Schmidt, 1971). Any secondary drop in ATP levels in toxin-treated nerve terminals would also contribute to the reduction in transmitter release, as it is known that inhibitors of ATP synthesis can decrease transmitter release (Beani et al., 1966; Nelson-Krause & Howard, 1978).

The mechanism by which β-bungarotoxin causes a depolarization of nerve terminals remains to be determined. The fact that the toxin's effects are not dependent on external Na^+ concentration indicates that the toxin does not depolarize by opening Na^+ channels or by interfering with the Na^+ transport activity of the plasma membrane (Na^+,K^+)ATPase. It is possible that the toxin depolarizes by altering the conductance of K^+ or Cl^- channels.

The studies with EOFA and DiC_6 show an important difference between the neurotoxic and nonneurotoxic phospholipases. DiC_6, presumably by binding to the catalytic site of a phospholipase A, protects it against inactivation by EOFA (Wells, 1973; Howard & Truog, 1977). Protecting the catalytic site of the nonneurotoxic IVa phospholipase A with DiC_6 was sufficient for retention of that enzyme's ability to inhibit synaptosomal uptake of GABA and to reduce the synaptosomal membrane potential. However, in the case of β-bungarotoxin, DiC_6 protected the phospholipase A activity without protecting against EOFA inactivation of the toxin's ability to alter several synaptosomal processes. These results show that β-bungarotoxin and IVa phospholipase A deenergize synaptosomes by different mechanisms. The effects of the IVa enzyme on synaptosomes are probably due to the actions of the large amounts of fatty acids and lysophosphoglycerides liberated by the high phospholipase activity of IVa.

β-Bungarotoxin lost its neurotoxicity but not its measurable phospholipase A activity after treatment with EOFA and DiC_6. One interpretation of this result is that the neurotoxicity of β-bungarotoxin is not related to its phospholipase A activity. However, other studies indicate that the phospholipase A activity is neither simply a vestigial and irrelevant activity of the toxin molecule nor is it due to a contaminating enzyme. All known presynaptically acting neurotoxins from snake venoms have phospholipase A activity (Karlsson, 1973; Howard, 1977), and, where examined, these toxins, including β-bungarotoxin, have been found to be homologous in amino acid sequence to well-established phospholipases A such as porcine pancreatic phospholipase A (Breithaupt et al., 1975; Halpert & Eaker, 1975; Fohlman et al., 1976; Kondo et al., 1978). The neurotoxicity and phospholipase A activity of β-bungarotoxin have similar cation requirements (Strong et al., 1976a). All agents reported to inactivate the phospholipase A activity of β-bungarotoxin also inactivate its neurotoxicity (Strong et al., 1976b; Howard & Truog, 1977; Abe et al., 1977). In light of these studies, one should not yet dismiss the possibility that the phospholipase A catalytic site of β-bungarotoxin functions in its neurotoxicity.

While our studies indicate that β-bungarotoxin does not depolarize nerve terminals indirectly by liberating fatty acids nonspecifically from neuronal phosphoglycerides, they do not exclude the possibility that the toxin's phospholipase A activity alters membrane potential by other means. β-Bungarotoxin may exert a direct effect by hydrolyzing a particular membrane phosphoglyceride that has a specific role in the activity of ion channels. Such a phosphoglyceride may be resistant to hydrolysis by nonneurotoxic phospholipases A, including β-bungarotoxin that had been treated with EOFA and DiC_6. Furthermore, its hydrolysis by native β-bungarotoxin phospholipase A could not necessarily be distinguished from hydrolysis of other synaptosomal phosphoglycerides by our methods. This crucial phosphoglyceride could have a unique composition or a unique location, e.g., as an annular lipid for an ion channel. Degradation of membrane phosphoglycerides can inactivate membrane proteins (Fourcans & Jain, 1974).

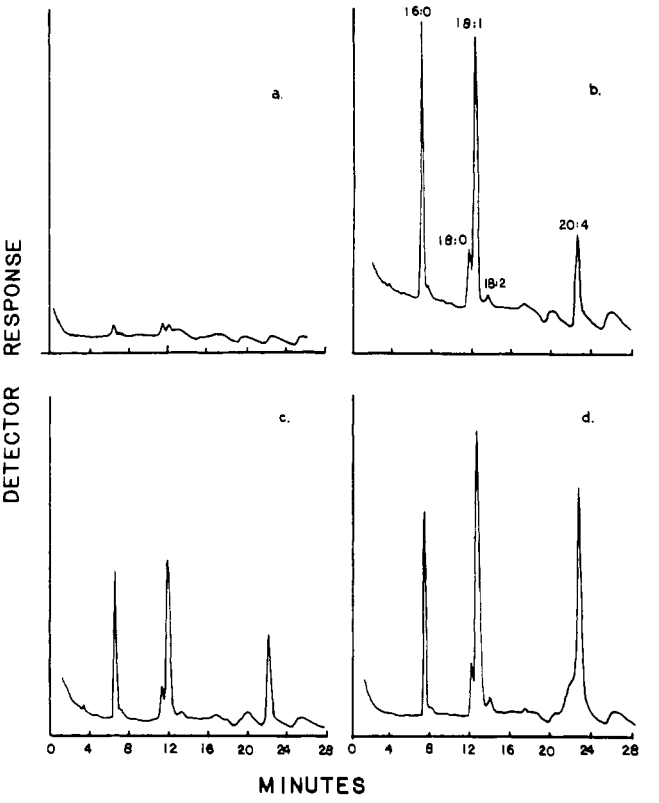

FIGURE 9: Gas-liquid chromatography of fatty acids liberated from synaptic plasma membranes after incubation with (a) no additive, (b) β-bungarotoxin (2 μg/mL), (c) *V. russelli* phospholipase A (0.6 μg/mL), or (d) *B. multicinctus* IVa phospholipase A (0.5 μg/mL). Chromatography was temperature programmed as described under Materials and Methods.

Howard & Truog (1977) proposed that when EOFA reacts with β-bungarotoxin it modifies and inactivates at least two sites,[4] each of which is essential for the toxin's neurotoxicity. One is the phospholipase A catalytic site, which can be protected against EOFA by DiC_6. The second site, which is not protected by DiC_6, may confer neurotoxicity on the phospholipase A catalytic site by enabling it to hydrolyze the specific lipid involved in ion-channel activity.

Alternatively, the phospholipase A catalytic site of β-bungarotoxin may function only to accomplish the specific binding of the toxin to a particular location on a nerve terminal or to expose a membrane receptor for the toxin while the other toxin site affected by EOFA functions to open or close an ion channel.

In any case, the catalytic sites of neurotoxic phospholipases A are likely to possess some very interesting properties. Furthermore, the neurotoxic phospholipases A will likely have much utility, distinct from that of nonneurotoxic phospholipases, as probes of membrane function.

Acknowledgments

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[4] Under the conditions used in these experiments, EOFA would be expected primarily to acylate histidine residues and amino groups (Melchior & Fahrney, 1970; Spande et al., 1970). β-Bungarotoxin contains 5 histidine and 13 lysine residues (Kondo et al., 1978).

somes by β-bungarotoxin, and Dr. A. S. Waggoner for his gift of 3,3′-dihexyloxacarbocyanine iodide.

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