Relationship between the Neurotoxicity and Phospholipase A Activity of β-Bungarotoxin†

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ABSTRACT: β-Bungarotoxin is a protein neurotoxin that exhibits phospholipase A activity. The neurotoxin and phospholipase A activities were similarly affected by several agents that modify proteins in various ways. Both activities were very thermostable and resistant to treatment with proteases, 6 M urea, phenylmethylsulfonyl fluoride, and *N*-acetylimidazole. Both activities were sensitive to β-mercaptoethanol, and to *N*-bromosuccinimide and ethoxyformic anhydride, which previously had been shown to inactivate rattlesnake venom phospholipase A by modifying selective amino acids. Dihexanoyllecithin, which acts as a substrate for the β-bungarotoxin phospholipase A, and Ca^{2+} protect the phospholipase A activity against inactivation by ethoxyformic anhydride but not the neurotoxicity. Treatment of intact membranes with proteases reduces hydrolysis of the membranes' lipids by the toxin phospholipase A.

β-Bungarotoxin, a snake venom polypeptide neurotoxin of molecular weight 21 800, causes neuromuscular blockade by inhibiting acetylcholine release (Lee and Chang, 1966; Chang et al., 1973). Studies in this laboratory have shown that β-bungarotoxin also affects the storage of several putative neurotransmitters and nontransmitter compounds in mammalian brain synapses in vitro (Wernicke et al., 1974, 1975). We have proposed and provided evidence that the effects of β-bungarotoxin on brain and neuromuscular synapses result from an interference with energy metabolism caused by a phospholipase A activity that was found to be associated with the toxin (Wernicke et al., 1975). The β-bungarotoxin phospholipase A was shown to be distinct from the other nonneurotoxic phospholipase A present in the snake venom.

Besides β-bungarotoxin at least two other snake venom neurotoxins have phospholipase A activity. Notexin, from the Australian tiger snake *Notechis scutatus scutatus,* has been shown to have a marked homology with both porcine pancreatic phospholipase A and a phospholipase A from the venom of *Naja melanoleuca* (Halpert and Eaker, 1975). Crotoxin, from *Crotalus terrificus,* is a complex of two noncovalently linked proteins: a phospholipase A and an acidic protein. The acidic protein, by itself, lacks neurotoxicity and phospholipase activity but potentiates the neurotoxicity and strongly inhibits the enzyme activity of the phospholipase (Hendon and Fraenkel-Conrat, 1971; Rübsamen et al., 1971; Breithaupt et al., 1974).

In this paper we describe more detailed studies of β-bungarotoxin phospholipase A. As part of these studies we have treated β-bungarotoxin with a variety of agents in order to determine whether the phospholipase A activity of the toxin is required for its toxicity, in vivo. We report that all agents found to inactivate the toxin's phospholipase A activity also inactivate its neurotoxicity.

Materials and Methods

Chemicals. β-Bungarotoxin was purified from *Bungarus multicinctus* venom as described (Wernicke et al., 1974, 1975). *Vipera russelli* phospholipase A, Pronase, egg yolk lecithin, *N*-bromosuccinimide, phenylmethylsulfonyl fluoride, and *N*-α-*p*-tosyl-1-lysine chloromethyl ketone hydrochloride were obtained from Sigma Chemical; ethoxyformic anhydride and 1-acetylimidazole were from Eastman Kodak; trypsin and chymotrypsin were from Worthington; dibutyryl-1-α-lecithin and dioctanoyl-1-α-lecithin were from Supelco; and dihexanoyl-1-α-lecithin was from Applied Science.

Toxin Lethality. Swiss white mice (20–25 g) were injected intraperitoneally with β-bungarotoxin diluted 1.4-fold serially. For each dose of toxin, 4–7 mice were injected. The LD_{50} of our control β-bungarotoxin preparations was 0.05–0.1 μg per g of mouse.

Phospholipase A Assays. Except where indicated, the substrates for the phospholipase A assays were bacterial membranes that had [^{14}C]palmitic acid incorporated into the phosphoglycerides. The membranes were prepared as described (Wernicke et al., 1975), except they were stored frozen in 10 mM Tris[1]-HCl (pH 7.5)–1 mM EDTA. The material to be assayed was added to the membranes (300 μg of protein, 0.01 μCi of ^{14}C) in 10 mM Tris-HCl (pH 7.5)–2.5 mM $CaCl_2$. The reaction mixture (0.1 ml) was incubated at 37 °C with shaking for 60 min unless stated otherwise. The fatty acids were extracted from the reaction mixture by the phase separation procedure described by Gatt and Barenholz (1969) and added to toluene scintillation fluid for measurement of radioactivity.

Where indicated, enzymic hydrolysis of dispersed lecithin was measured with Radiometer pH stat equipment. The reaction mixture contained substrate–0.5 mM Tris-HCl (pH 7.4)–5 mM $CaCl_2$ in a volume of 2 ml. The reaction, which was initiated by the addition of β-bungarotoxin in a 25-μl volume, was carried out at 37 °C under an atmosphere of N_2. Titration was with 0.028 N NaOH. Nonenzymic hydrolysis was negligible under the conditions used. The final concentration of β-bungarotoxin was 7 μg/ml.

Protein Modification. NBS. β-Bungarotoxin was incubated

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EOFA, ethoxyformic anhydride; NBS, *N*-bromosuccinimide; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tos-LysCH_2Cl, *N*-α-*p*-tosyl-1-lysine chloromethyl ketone hydrochloride; SD, standard deviation; Dip-F, diisopropyl fluorophosphate.

TABLE I: Activity of β-Bungarotoxin Phospholipase A on Various Lecithin Substrates.[a]

Substrate	Additions to Buffer	Activity[b]
Egg yolk lecithin[c]		0
	0.1% Triton X-100	0
	2.5 mM deoxycholate	0.27
	0.5 mM deoxycholate	0.08
Dioctanoyllecithin[d]		0.41
Dihexanoyllecithin[d]		0.15
Dibutyryllecithin[d]		0.03

[a] Enzyme activity was measured with a pH stat as described in Materials and Methods. [b] μmol of fatty acids released per min per sample assayed. [c] Amount: 1.25 mg/ml. [d] Amount: 0.3 mg/ml.

with eightfold molar excess of NBS in 10 mM TES (pH 6.0)–50 mM KCl at 25 °C for 10 min.

N-Acetylimidazole. The toxin was incubated with 60-fold molar excess of *N*-acetylimidazole in 50 mM sodium borate (pH 7.5) at 25 °C for 90 min.

EOFA. The toxin was incubated with 30 mM EOFA in 10 mM TES (pH 6.0)–50 mM KCl at 25 °C for 60 min.

Protease. The toxin was incubated with trypsin, chymotrypsin, or Pronase at 40 μg/ml in 10 mM Tris-HCl (pH 7.5)–5 mM $CaCl_2$ at 37 °C for 30 min.

β-Mercaptoethanol. The toxin was incubated with 1% β-mercaptoethanol in 5 mM Tris-HCl (pH 7.5)–2.5 mM $CaCl_2$ at 37 °C for 6 h.

Urea. The toxin was incubated with 6 M urea in 8 mM Tris-HCl (pH 7.5)–4 mM $CaCl_2$ at 37 °C for 8 h.

Phenylmethylsulfonyl Fluoride. The toxin was incubated with 0.1 mM phenylmethylsulfonyl fluoride in 5 mM Tris-HCl (pH 7.5)–2.5 mM $CaCl_2$ at 25 °C for 30 min.

Heat Treatment. The toxin was incubated in 10 mM Tris-HCl (pH 7.5)–5 mM $CaCl_2$ for 5 min in a boiling H_2O bath.

After incubation with the above reagents, the reaction mixture was diluted with 10 mM Tris-HCl (pH 7.5)–5 mM $CaCl_2$ or, in the case of treatment with *N*-acetylimidazole or EOFA, the reaction mixture was dialyzed against 10 mM Tris-HCl (pH 7.5)–5 mM $CaCl_2$.

Results

Dispersed Lecithin Substrates. We have reported the difficulty with which β-bungarotoxin phospholipase A can be assayed using dispersed natural phosphoglycerides as substrates in a variety of assay conditions (Wernicke et al., 1975). This was also the case when activity was measured by continuous titration of released fatty acids with a pH stat. As shown in Table I, no enzyme activity was measured with egg yolk lecithin as a substrate in the presence of 0.1% Triton X-100. A low level of activity was detected with this substrate when deoxycholate was added.

We have explored whether synthetic short-chain lecithins, which are more easily dispersed in water or even water soluble, would serve as better substrates. Such substrates have been used for kinetic studies of phospholipases (de Haas et al., 1971; Wells, 1972). With dioctanoyllecithin as a substrate, enzyme activity was somewhat greater than with egg yolk lecithin. Dihexanoyllecithin and dibutyryllecithin were poorer substrates for the enzyme.

Membrane Substrate. A more sensitive assay for the toxin-associated phospholipase employs, as a substrate, bacterial membranes in which the phosphoglycerides are radioactively labeled with [^{14}C]palmitic acid. As shown in Figure 1A, the enzyme activity was not linear with respect to toxin concentration over the range examined. Since this range of toxin was used in the studies described in this paper, a standard curve of activity vs. enzyme concentration of control enzyme had to be determined to measure the enzyme activity of any toxin sample. The nonlinearity of activity with enzyme concentration is not a simple case of enzyme saturation because, even at higher enzyme concentrations, activity is linear with time of incubation for almost 60 min (Figure 1A). We have not further examined this aspect of the kinetic behavior of the toxin-associated phospholipase, but it is not unique to this enzyme; the activity of phospholipase A from *V. russelli* venom is also nonlinear with enzyme concentration (Figure 1B). All subsequent phospholipase A assays employed the radioactive bacterial membranes as substrates.

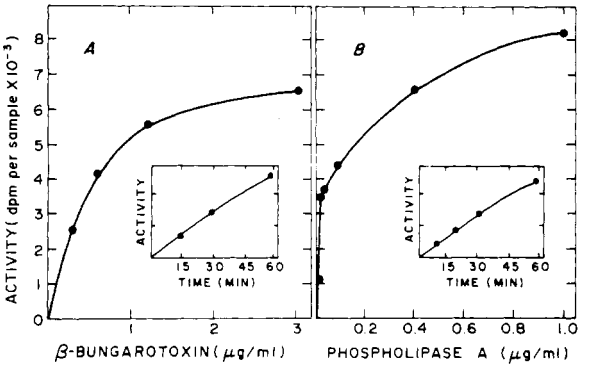

FIGURE 1: Dependence of β-bungarotoxin and *V. russelli* phospholipase A activities on enzyme concentration and duration of incubation (inserts). Radioactively labeled bacterial membranes were incubated with the enzymes and the liberated fatty acids were extracted and counted as described in Materials and Methods. The incubations were either for a constant time of 60 min or at a constant enzyme concentration of 3 μg/ml for β-bungarotoxin or 1 μg/ml for the *V. russelli* enzyme. (A) β-Bungarotoxin; (B) *V. russelli phospholipase A.*

β-Bungarotoxin did not hydrolyze the bacterial membrane phosphoglycerides in the absence of Ca^{2+}, and the enzyme activity was markedly inhibited by 15 mM Mg^{2+} or 15 mM Mn^{2+} (Table II). These results explain the finding that the toxin's ability to cause neuromuscular blockade requires Ca^{2+} and is inhibited by 12 mM Mg^{2+} (Chang et al., 1973).

Protein Modification. Information on the role of β-bungarotoxin phospholipase A was obtained from protein modification studies. We reasoned that agents that irreversibly inactivate the toxin's phospholipase A activity should also inactivate its neurotoxicity if the phospholipase A activity were necessary for neurotoxicity. For these studies the toxin was treated with an agent and then phospholipase A activity and neurotoxicity (mouse lethality) of the treated toxin was compared with those of control toxin, which had been similarly incubated but in the absence of the agent. The chemical agents were sufficiently removed by dilution or dialysis to ensure that the agents themselves had no effect on the bacterial membranes or the mice during the assays. Our methods were sensitive enough to detect a 30% difference in the neurotoxicity of two toxin samples and a 20% difference in phospholipase activity.

The results of these studies are given in Table III. The toxin phospholipase A activity and neurotoxicity were resistant to digestion by trypsin, chymotrypsin, and Pronase. Both activities were resistant to 6 M urea and completely inactivated

TABLE II: Dependence of β-Bungarotoxin Phospholipase A Activity on Buffer Cation Composition.[a]

Additions to Buffer	Activity (dpm)[b]
Complete	3129 ± 526
Omit Ca^{2+}	158 ± 11
15 mM Mg^{2+}	1290 ± 363
15 mM Mn^{2+}	1579 ± 255

[a] Incubation conditions were as described in Materials and Methods. β-Bungarotoxin was at 1.8 μg/ml. [b] Radioactive fatty acids released. Values are means ± SD for triplicate incubations.

by 2-mercaptoethanol, indicating that intact disulfide bonds are necessary for the activities. Neither activity was significantly affected by 0.1 mM phenylmethylsulfonyl fluoride, which, like diisopropyl fluorophosphate (Dip-F), inhibits serine esterases.

Both activities are very thermostable. After being in a boiling H_2O bath for 5 min, the toxin preparation retained 60% of its phospholipase A activity and 70% of its neurotoxicity (Table III).

The toxin was also treated with three agents, NBS, EOFA, and *N*-acetylimidazole, which were used by Wells (1973) to modify specific amino acid residues in the study of the active site of phospholipase A from the venom of the rattle snake *Crotalus adamanteus*. The *C. adamanteus* phospholipase A was inactivated by oxidation of two tryptophan residues with NBS or by acetylation of one lysine residue with EOFA. The enzyme activity was not affected by acetylation of tyrosine residues with *N*-acetylimidazole. As shown in Table III, these reagents had similar effects on the phospholipase A activity and neurotoxicity of our β-bungarotoxin preparation. Both activities were substantially reduced after treatment with NBS or EOFA; neither was affected by treatment with *N*-acetylimidazole. Under the conditions used NBS would be expected primarily to oxidize tryptophan residues and EOFA to acetylate histidine residues and amino groups (Spande et al., 1970; Melchior and Fahrney, 1970). β-Bungarotoxin contains 4 tryptophan, 5 histidine and 13 lysine residues (Lee et al., 1972; Kelley and Brown, 1974).

β-Bungarotoxin phospholipase activity was protected against the effects of EOFA by Ca^{2+} and by dihexanoyllecithin, which both interact at the phospholipase active site. However, the EOFA inactivation of the neurotoxicity was not affected by Ca^{2+} or the short-chained lecithin (Table III). In addition to a loss of lethality for mice, β-bungarotoxin treated with EOFA in the presence of dihexanoyllecithin lost its ability to cause neuromuscular blockade when incubated with an isolated phrenic nerve-diaphragm preparation as described by Howard et al. (1976). The finding that Ca^{2+} and dihexanoyllecithin protect the toxin phospholipase A against the effects of EOFA is similar to that made by Wells (1973) with *C. adamanteus* phospholipase A.

Trypsin Effects on Membranes. In the course of the protein modification studies, it was found that trypsin and Pronase largely inhibited the toxin-associated phospholipase A activity when these proteases were present (at 10 μg/ml) during incubation of the toxin with the bacterial membranes. Neither inactivated the enzyme when incubated with the toxin at an even greater concentration (40 μg/ml) and removed before assay of the phospholipase activity (Table III). Therefore, it appeared that the proteases were interfering with the phospholipase activity by altering the membranes directly. Alternatively the proteases could have acted on the phospholipase,

TABLE III: Effect of Various Agents on Phospholipase A Activity and Neurotoxicity of β-Bungarotoxin.

Treatment	Act., % of Control: Phospholipase A	Act., % of Control: Neurotoxicity
Trypsin	100	100
Chymotrypsin	100	100
Pronase	100	100
Urea	70	70
2-Mercaptoethanol	<10	<10
Phenylmethylsulfonyl fluoride	100	100
Heat	60	70
N-Acetylimidazole	100	100
NBS	15	<10
EOFA	<10	<10
EOFA + 2.5 mM Ca^{2+}	100	<15
EOFA + 70 mM dihexanoyllecithin	100	<15

which had changed to a protease-sensitive conformation upon binding to the bacterial membranes. To distinguish between these two possibilities, the membranes were pretreated with trypsin for 30 min. Then a trypsin inhibitor, Tos-LysCH_2Cl, was added. Ten minutes later β-bungarotoxin was added and the phospholipase activity was measured. By this procedure trypsin was allowed to react with the membranes but not with the toxin. As shown in Table IV, under these conditions the trypsin inhibitor did not totally block the ability of trypsin to inhibit the phospholipase activity. Therefore, the proteases were exerting their effects by acting at least in part on the membranes rather than only on the phospholipase. Trypsin also inhibited the activity on bacterial membranes of phospholipase A from *V. russelli* venom, although the *V. russelli* enzyme was not as sensitive to the action of trypsin.

Discussion

The protein-modification studies reported here add to other substantial evidence (Wernicke et al., 1975; Howard, 1975) that the effects of β-bungarotoxin are due to its phospholipase A activity. Elsewhere, we have discussed how the phospholipase activity of β-bungarotoxin accounts for all the known pharmacological effects of the toxin (Wernicke et al., 1975). Another intriguing question is why β-bungarotoxin is a neurotoxin while most other phospholipase A enzymes are not. Perhaps the answer lies in the enzymes' specificities for substrates; β-bungarotoxin may bind more readily to neuronal membranes than to other membranes while other nonneurotoxin phospholipase A enzymes do not. By binding to tissues indiscriminately, the nonneurotoxic phospholipases would become too dilute to be effective at neuromuscular junctions. Some evidence for this explanation has been obtained. β-Bungarotoxin phospholipase A released fatty acids from purified nerve-ending plasma membranes much more readily than from erythrocyte ghosts, whereas the activity of the nonneurotoxic phospholipase A from the venom was slighty greater on eythrocyte membranes than on the neuronal membranes (Wernicke et al., 1975).

β-Bungarotoxin was converted from a neurotoxic phospholipase A to a phospholipase A without neurotoxic activity by treatment with EOFA in the presence of Ca^{2+} or dihexanoyllecithin. These results indicate that EOFA alters at least two sites on the toxin: the phospholipase A active site and another site also required for neurotoxicity. Only the phospho-

TABLE IV: Tos-LysCH$_2$Cl Inhibition of Trypsin Activity on Membranes and Phospholipase A.

Additions to Membranes[a]			Phospholipase A Act.	
Period 1	Period 2	Period 3	dpm[b]	% of Control
		β-Bungarotoxin	6079 ± 120	100
Trypsin		β-Bungarotoxin	2210 ± 86	15
Trypsin	Tos-LysCH$_2$Cl	β-Bungarotoxin	4427 ± 16	45
Trypsin + Tos-LysCH$_2$Cl		β-Bungarotoxin	5664 ± 46	80
Trypsin			837 ± 399	<10
Tos-LysCH$_2$Cl			14 ± 3	<10

[a] Incubations were for 70 min at 37 °C. Radioactively labeled bacterial membranes (0.1 ml) in 1.0 mM Tris-HCl (pH 7.5)–2.5 mM $CaCl_2$ were added at the start of incubation. Other additions were made during the periods indicated. Period 1 was 0–30 min; period 2 was 30–40 min; period 3 was 40–70 min. Final volume of the reaction mixture was 0.14 ml. Trypsin, Tos-LysCH$_2$Cl, and β-bungarotoxin were at 30 μg/ml, 0.3 mM, and 5 μg/ml, respectively. [b] Radioactive fatty acids released. Values are means ± mean deviation for duplicate incubations.

lipase A active site could be protected by Ca^{2+} and dihexanoyllecithin. The technique of selectively inactivating one of these two sites may be useful in establishing how a neurotoxic phospholipase differs from other phospholipases. For example, it will be interesting to determine whether the nonneurotoxic β-bungarotoxin phospholipase A retains the native enzyme's preference for neuronal membranes

Of course an alternate explanation of the results with EOFA, Ca^{2+}, and dihexanoyllecithin is that the phospholipase A activity in our β-bungarotoxin preparation is actually due to a contaminating enzyme and is not involved in the neurotoxicity of β-bungarotoxin. This explanation seems most unlikely in view of all the contrary evidence cited above for β-bungarotoxin and the two other neurotoxic phospholipases.

We have not determined the mechanism by which proteases inhibit phospholipase A hydrolysis of membrane lipids. By degrading membrane proteins, the proteases may simply cause the membrane lipids to change to a state that is unfavorable for interaction with the phospholipase A. A more interesting explanation is that the proteases degrade some specific protein(s) with which the phospholipase interacts during its enzymic activity. Such proteins could be the physical basis for the known preference of β-bungarotoxin phospholipase A for neuronal membranes; neuronal membranes might contain an unusually large number of these proteins or their structure in neuronal membranes might allow unusually good interaction with β-bungarotoxin. These possibilities warrant further exploration.

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