

CROTOXIN EFFECTS ON TORPEDO CALIFORNICA CHOLINERGIC EXCITABLE

VESICLES AND THE ROLE OF ITS PHOSPHOLIPASE A ACTIVITY

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SUMMARY. The phospholipolytic neurotoxin from Crotalus durissus terrificus, crotoxin, is able to produce a dose- and time-dependent block of carbachol-stimulated ^{22}Na efflux from pre-loaded Torpedo californica excitable vesicles. The blocking activity is dependent on calcium and is abolished by chemical modification with p-bromophenacyl bromide. The isolated basic subunit, crotoxin B, produces an identical block, whereas the isolated acidic subunit, crotoxin A, has no detectable effect. Neither crotoxin nor crotoxin B antagonizes the binding of [^{125}I]- α -bungarotoxin to purified acetylcholine receptor, although, at high concentrations, they antagonize its binding to acetylcholine receptor-rich membrane fragments. Certain phospholipase A_2 enzymes and the fatty acid products of their digestion can mimic the crotoxin action. It is therefore suggested that, although considered a pre-synaptic neurotoxin, crotoxin can have *in vitro* post-synaptic effects, possibly mediated by its endogeneous phospholipase A_2 activity.

INTRODUCTION: Crotoxin, the major neurotoxin of Crotalus durissus terrificus venom, is unusual in that its activity results from the synergism of two dissimilar subunits (1). The mechanism of its neuromuscular blockade appears to be an interruption of acetylcholine release similar to that elicited by the pre-synaptic snake toxins; taipoxin, notexin, and β -bungarotoxin (2,3). There are, however, indications of a post-synaptic aspect in its action (4). To test this possibility, I have used the acetylcholine receptor-rich excitable vesicles from Torpedo californica electroplaques as a purely post-synaptic preparation and examined the ability of crotoxin to block cholinergic acceleration of ^{22}Na efflux (5,6). Since crotoxin was shown to block this *in vitro* response, preliminary experiments are reported indicating that crotoxin's phospholipase A_2 activity (EC 3.1.1.4) may be involved in this effect.

Abbreviations used: AChR, acetylcholine receptor; EGTA, ethyleneglycol-bis-(β -aminoethyl ether)N,N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride

MATERIALS AND METHODS. Crotoxin and its subunits were isolated as described elsewhere (1,7). p-Bromophenacyl bromide-modified crotoxin B was a gift of Dr. T.-W. Jeng (8).

[¹²⁵I]-α-bungarotoxin was prepared and purified as described by Lukasiewicz et al (9). α- and β-bungarotoxins were purified as described earlier (10). Torpedo receptor in Triton X-100 was a gift of Dr. David Devore. Binding of [¹²⁵I]-α-bungarotoxin to purified receptor was determined by the technique of Schmidt and Raftery (11), and to Torpedo membrane fragments by the technique of Weber and Changeux (12). Vipera russellii phospholipase A₂ isoenzymes were purified as described elsewhere (13).

Excitable vesicles were prepared from liquid-nitrogen frozen Torpedo californica electroplaques (Pacific Bio-Marine, Venice, Calif.; stored at -70°C) by the procedure of Hazelbauer and Changeux (5) and further purified by centrifugation onto a discontinuous gradient of 0.9M-1.2M sucrose (containing 0.02% NaN₃ and 0.1 mM PMSF) in a Beckman SW27 rotor for 25,000 rpm for 2 hours.

Vesicles were taken from the 0.9M-1.2M sucrose interface and purity was checked by negative staining electron microscopy. Vesicles were resuspended to a final concentration of 10 mg/ml (by Lowry protein determination) in 0.5M sucrose containing 0.02% NaN₃, 0.1 mM PMSF, 10 mM NaCl, and 100 μCi/ml of ²²NaCl (new England Nuclear, carrier free). This was equilibrated at 4°C for 16-18 h and 100 μl aliquots were diluted 80 X with Torpedo Ringer (5) and maintained at 4°C for 30 min to stabilize the resting efflux (6). One hundred μl of either control (Torpedo Ringer) or test (Ringer with appropriate additions) solutions were added and incubations continued for 15 min at 4°C unless otherwise indicated. At time 0, carbachol in Ringer was added to a final concentration of 100 μM and the efflux of ²²Na followed by rapid filtration of 1 ml samples at timed intervals on Millipore HAWP 02500 filters (equilibrated with cold Torpedo Ringer). Filters were rinsed with 3 X 5 ml of ice-cold Torpedo Ringer, and then immediately dissolved with agitation in 10 ml of Beckman Filter-Solv. Samples were counted in a Packard Tri-Carb scintillation spectrometer with third-channel settings for ²²Na. Excitable vesicles had a specific activity of 0.5-0.7 nM of [¹²⁵I]-α-bungarotoxin binding sites /mg protein. Levels of ²²Na efflux have been normalized to a percentage owing to variations in V_{app} with toxin treatment. Typically, 1 ml aliquots of vesicle preparations gave 1400-1800 cpm immediately after dilution and 600-800 cpm after 30 min equilibration. V_{app}, θ, and θ₀ have been defined by Popot et al (20).

Results. Incubation with micromolar levels of crotoxin eradicated the carbachol increased efflux of ²²Na from pre-loaded vesicles without affecting the resting efflux rate. From Fig. 1, it is apparent that this effect was identical to that observed with the demonstrated post-synaptic antagonist α-bungarotoxin. To see whether crotoxin and α-bungarotoxin competed for the same sites, competitive binding experiments against [¹²⁵I]α-bungarotoxin (Table 1) were performed. Crotoxin could inhibit α-toxin binding only at high levels and only to membrane fragments. However, double reciprocal plots of crotoxin/α-toxin

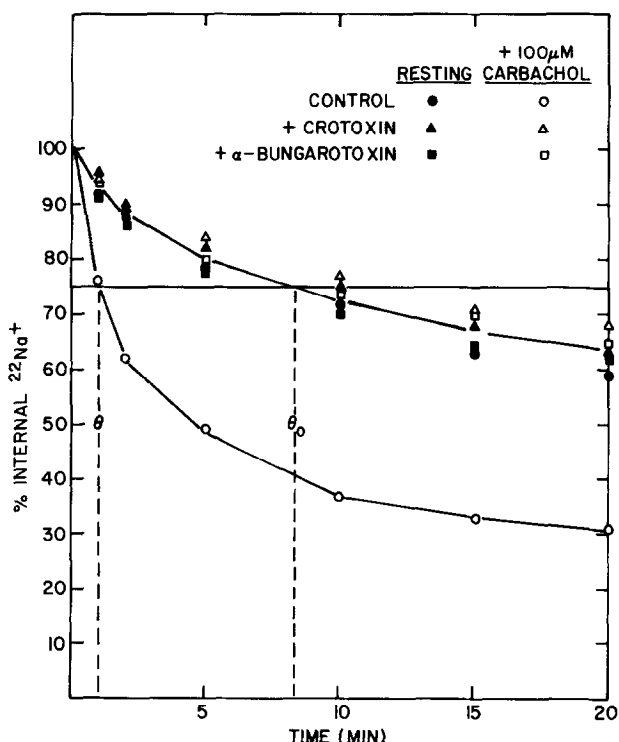


Figure 1. Carbachol acceleration of ^{22}Na efflux from *Torpedo* excitable vesicles and its blockade by crotoxin and α -bungarotoxin. Toxins were added after the 30 min equilibration of diluted ^{22}Na -loaded vesicles (see Materials and Methods) to a final concentration of $1\ \mu\text{M}$ and further incubated for 15 min at 4°C . The time reference points θ_0 and θ occur where 25% of the total vesicle bound ^{22}Na has been lost without or with carbachol addition.

binding-inhibition experiments suggested a non-competitive mechanism. To obtain quantitative information on the blocking activity, both the time and concentration-dependence were determined (Fig. 2). With 15 min incubations at 4°C , $1\ \mu\text{M}$ crotoxin is the lowest tested dose capable of achieving a complete block. Similarly, with $1\ \mu\text{M}$ crotoxin, the block is complete by 7.5 min at 4°C . Interestingly, at high doses or long incubation times, the resting efflux rate (measured by $1/\theta_0$) begins to increase, although the θ_0/θ ratio remains constant owing to a coordinate change in the stimulated efflux rate. Thus, the vesicles were becoming more "leaky", possibly

TABLE I. Competition of crotoxin and its subunits with [125 I]- α -bungarotoxin at Torpedo californica acetylcholine receptor sites

Toxin	Ki app.*	
	Membrane-Bound AChR	Purified AChR
α -Bungarotoxin	$9 \cdot 10^{-9}$ M	$3.5 \cdot 10^{-9}$ M
Crotoxin	$17 \cdot 10^{-6}$ M	$> 10^{-4}$ M
Crotoxin B	$5 \cdot 10^{-6}$ M	$> 10^{-4}$ M
Crotoxin A	$> 10^{-4}$ M	$> 10^{-4}$ M

*Ki app. is the apparent inhibition constant assuming mass action interaction of the competing species at a single, non-interacting site and is given by $IC_{50} (1 + S/K_d)^{-1}$ where IC_{50} is the point of 50% displacement for a log-probit plot, S = concentration of [125 I]- α -bungarotoxin (5 nM for membrane-bound AChR, 3.9 nM for purified AChR), and K_d = dissociation constant for [125 I]- α -bungarotoxin determined from saturation analysis (10 nM for membrane-bound AChR, 8 nM for purified AChR).

Toxins were pre-incubated for 30 min at 20°C with purified AChR before addition of [125 I]- α -bungarotoxin, after which incubations were continued for an additional 45 min at 20°C. Bound toxin was determined by retention on either Millipore HAWP 02500 filters for membrane-bound AChR (12), or Whatman DE81 filters for purified AChR in 0.1% Triton X-100 (11). [125 I]- α -bungarotoxin was the moniodinated derivative of α -bungarotoxin from Bungarus multicinctus venom, prepared and purified as described elsewhere (9).

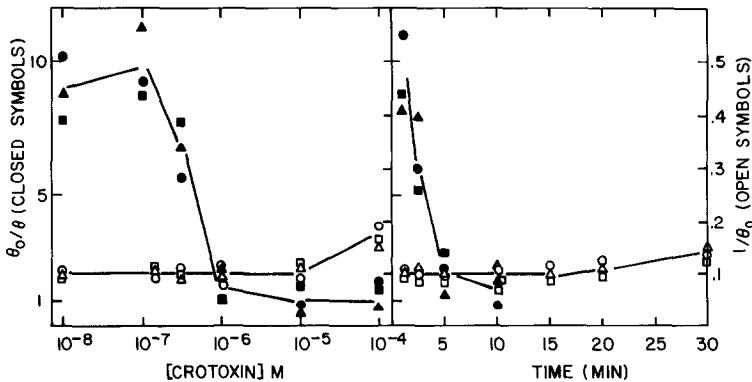


Figure 2. Concentration dependence and time course of crotoxin effects on the carbachol stimulated (indicated by θ_0/θ) and the resting (θ_0) efflux rates of 22 Na.

indicating that crotoxin was acting directly on the membrane. In view of the phospholipase A₂ and hemolytic activities of crotoxin (1), several phospholipase A₂ enzymes and specific modification conditions were tested. In Table II, the data are compared. The effects of crotoxin were mimicked by the isolated basic B subunit, and the isolated acidic A subunit was ineffective. Further, the blocking activity was dependent on calcium, since the inclusion of 0.1 mM EGTA abolished the blocking ability of either crotoxin or crotoxin B. The addition of high levels of calcium introduced another effect, an attenuation of the resting efflux rate. The basic phospholipase from Vipera russellii also produced a calcium-dependent block, but the acidic isoenzyme from the same venom had no inhibitory action. β -Bungarotoxin, a presynaptic neurotoxin (14), can exhibit an in vitro phospholipase A activity (14) although its physiological expression is controversial (15). β -Bungarotoxin produced a calcium-independent depression of the resting efflux rate, and did not block carbachol excitation. p-Bromophenacyl bromide modification is a fairly specific procedure for the alkylation of an important histidine in phospholipase A enzymes (16). Reconstituted crotoxin complex from p-bromophenacyl bromide-modified crotoxin B was inactive on excitable vesicles.

To test whether the products of crotoxin's phospholipolytic digestion could reproduce its effects, 10 μ M crotoxin was incubated for 30 min at room temperature with Torpedo vesicles and the fatty acid and lysophosphatide products were purified. Fig. 3 shows that the fatty acid fraction could suppress carbachol stimulation whereas the lysophosphatide fraction disrupted the vesicles and increased both the resting and stimulated efflux rates. Attempts to extract the products at low concentrations of crotoxin or after shorter incubations were unsuccessful, even though detectable hydrolysis had taken place. The addition of bovine serum albumin, to sequester low levels of any fatty acids, did not alter crotoxin's blocking potency.

Discussion. Crotoxin is one example of a family of snake neurotoxins that block neuromuscular transmission at the pre-synaptic nerve ending and show an in-

TABLE II. Effects of toxins and phospholipase A enzymes on stimulated and resting ^{22}Na efflux from Torpedo californica excitable vesicles

<u>Treatment</u>	θ_o (min)	θ (min)	θ_o/θ
Control (10)	9.3 ± 1.0	$1.0 \pm .1$	9.3 ± 1.0
Crotoxin (12)	11.4 ± 2.8	9.4 ± 1.0	$1.3 \pm .4$
Crotoxin + 10 mM CaCl_2 (2)	13.4	13.0	1.0
Crotoxin + 0.1 mM EGTA (2)	9.7	1.1	8.8
p-Bromophenacyl bromide-modified crotoxin* (2)	10.6	1.1	9.6
Crotoxin A (2)	8.5	.9	9.4
Crotoxin B (2)	8.9	9.9	.9
Crotoxin B + 10 mM CaCl_2 (1)	15.3	17.2	.9
Crotoxin B + 0.1 mM EGTA (1)	10.1	1.2	8.4
β -Bungarotoxin (2)	13.0	.9	14.4
β -Bungarotoxin + 0.1 mM EGTA (2)	14.9	1.1	13.5
Basic <u>V. russellii</u> phospholipase A (2)	11.2	9.9	1.1
Acidic <u>V. russellii</u> phospholipase A (1)	9.8	1.1	8.9

*Prepared by addition of 0.6 μM p-bromophenacyl bromide-modified crotoxin B to 0.4 μM crotoxin A. Complex formation was not affected by this modification (8).

All toxins were incubated for 15 min at 4°C at a final concentration of 1 μM . Numbers in parantheses give number of experimental determinations. θ_o and θ are the times necessary for the stimulated and resting efflux levels respectively to reach 75% of their initial levels (20).

trinsic phospholipase A_2 activity (3). However, crotoxin has also been reported to have post-synaptic action under certain conditions (4). On the exclusively post-synaptic preparation of excitable cholinergic microsacs, crotoxin has been shown, in this communication, to block carbachol stimulation. Further, in the isolated, intact Electrophorus electroplaque, crotoxin exerts an essentially irreversible antagonism to the action of bath-applied carbachol (Bon et al, manuscript in preparation) similar to the effect noted for Agkistrodon piscivorus

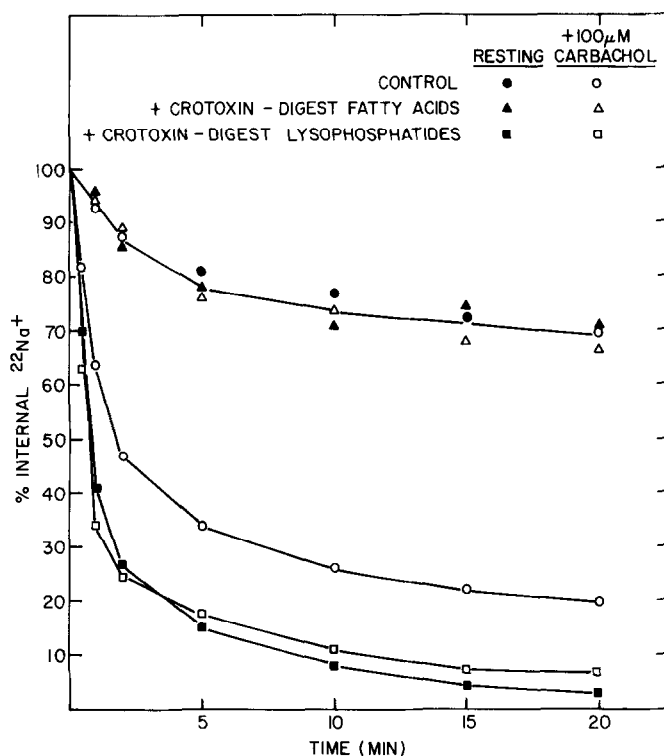


Figure 3. Effects of addition of purified fatty acid and lysophosphatide fractions from a crotoxin digest of *Torpedo* vesicles. 10 μ M crotoxin was incubated with 100 μ l (containing 1 mg protein) of vesicles suspension for 30 min at 20°C. Lipids were extracted with 1 ml of chloroform-methanol (3:1 v/v) for one h at 20°C followed by reextraction of the residue with chloroform-methanol (2:1 v/v) for 1/2 h. One ml each of chloroform and water were added, and the phases separated by centrifugation in a desk centrifuge. The chloroform phase was removed and an equal volume of benzene added before drying under a nitrogen stream. The dry residue was then taken up in 500 μ l acetone with 20 μ l of 5% $MgCl_2$ in methanol and maintained at 4°C for 1 h. The precipitate (containing polar lipids) was spun down at 5000 X g for 10 min, washed twice with 500 μ l acetone, and dried under a nitrogen stream. The acetone supernatants (containing neutral lipids) were pooled and dried. The polar lipid fraction was taken up in 100 μ l chloroform and spotted on a silica gel H plate (Brinkmann). A parallel sample of standard lysolecithin (Sigma Type III) was also applied as a mobility marker. The plate was developed with chloroform-methanol-acetic acid-water (25:15:4:2 v/v) and the marker position ($r_f = .1$) determined by staining with iodine vapors or 0.01% Rhodamine 6G. The position corresponding to standard lysolecithin in the *Torpedo* fraction was removed, eluted with 100 μ l chloroform-methanol (1:1 v/v), dried, and taken up in 20 μ l chloroform. The neutral lipids were taken up in 100 μ l chloroform and applied to a silica gel H plate. The plate was developed in heptane-isopropyl ether-acetic acid (60:40:4 v/v) with marker palmitic acid (Sigma) run in parallel to the *Torpedo* sample. The marker position ($r_f = .45$) was determined

piscivorus phospholipase A (16). Nonetheless, these data do not enable one to predict whether crotoxin would have the same effect on the vertebrate neuromuscular junction under physiological conditions. The available electrophysiological (2) and ultrastructural (Hanley, unpublished observations) evidence point to crotoxin having an exclusively pre-synaptic mechanism at this site.

In view of the continuing search for new chemical agents to study the post-synaptic surface, the origin of this crotoxin effect is particularly interesting. The failure of crotoxin to displace α -bungarotoxin from purified acetylcholine receptor ruled out antagonism at the binding site, suggesting that it must be exerting its effects through the ion channel and/or the membrane. The dose- and time-dependence indicated two stages in crotoxin's action, a quick loss of chemical excitability and a slower onset of membrane destabilization. That these phenomena were not the separate consequences of the two subunits was shown by the inactivity of purified crotoxin A and the full activity of crotoxin B. Thus, subunit synergism is not required for this action, unlike animal lethality (1). Although these could be discrete and unrelated effects, it seems likely that they have a common cause, possibly in the endogeneous phospholipase activity of the B subunit. Consistent with this, detectable membrane digestion has taken place under the conditions of blockade (data not shown), and both the blocking and enzymatic activities require calcium and are eradicated by a selective chemical modification known to be effective on other phospholipases (17). Moreover, a basic isoenzyme of Vipera russellii phospholipase A also prevents vesicle excitability. Lastly, the two stages could be separately imitated by the addition of either a fatty acid or lysophosphatide

by spraying with 0.01% Rhodamine 6G or 0.04% bromocresol green in basic ethanol. The position corresponding to fatty acids was eluted with 100 μ l chloroform, dried, and taken up in 20 μ l ether. The "lysophosphatide" or "fatty acid" fractions were added in their solvents to equilibrated, diluted vesicles and incubated for 1 min before determining the efflux rates. Control additions of 20 μ l either chloroform or ether had no effect.

fraction from a crotoxin digest of Torpedo membrane vesicles; fatty acids inhibiting cholinergic stimulation and lysophosphatides increasing leakiness. Consistent with this, fatty acids have been previously shown to antagonize the effects of cholinergic agonists on electroplaques acetylcholine receptor (18).

However, there are complications to a simple cause-and-effect interpretation. First, neither the acidic V. russellii phospholipase A nor the pre-synaptic toxin β -bungarotoxin blocked carbachol responses. β -Bungarotoxin did exhibit an attenuation of the resting efflux rate, as did crotoxin or crotoxin B in the presence of high calcium. This effect has been noted before in the action of the putative ionophore-specific toxin, ceruleotoxin (19). Second, attempts to reproduce the block by fatty acid extracts from short incubations, or to inhibit it by fatty acid removal through binding to BSA, were unsuccessful. Consequently, it is premature to suggest that the blocking activity results solely from an unselective hydrolysis of membrane lipids. The possibilities of a particular lipid involvement in cholinergic receptor function or of a non-enzymatic, surface-interaction disruption by post-synaptically active phospholipases should be considered.

These data can also be taken to sound a precautionary note on the use of excitable vesicles to screen for post-synaptic activities (19). Noting the detectable phospholipase activity in ceruleotoxin preparations (19), it is possible that this, and not a binding specificity for the ion channel, can account for its effects. In spite of this, the utility of ceruleotoxin, and indeed crotoxin, as a post-synaptic tool remains an interesting question.

While this paper was in preparation, Andreassen and McNamee reported that a purified Naja naja siamensis phospholipase A could inhibit agonist stimulation of ^{22}Na efflux from AChR-rich Torpedo membrane vesicles (21). The striking similarity of crotoxin's action to that of the Naja phospholipase is strongly supportive of the involvement of crotoxin's phospholipase activity.

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