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A STUDY ON THE INTERACTION OF CROTAPOTIN WITH CROTOXIN PHOSPHOLIPASE A_2, NOTEXIN AND OTHER PRESYNAPTIC NEUROTOXINS

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1 Crotapotin, the acidic subunit of crotoxin, greatly potentiated the presynaptic effect of isolated basic phospholipase A (PLA) of crotoxin in both mouse diaphragm and chick biventer cervicis muscles whereas the myotoxic effect was not affected significantly.

2 In contrast to crotoxin PLA, the presynaptic effects of notexin and notechis-5, self-active single chain toxins, were antagonized by crotapotin while actions of β-bungarotoxin were not affected.

3 By assaying PLA activity, crotoxin PLA was found to be unstable in physiological salt solution, especially when in contact with muscle, due to massive non-specific binding to and destruction by the muscle.

4 The decline of crotoxin PLA was greatly reduced by the presence of crotapotin but not by another acidic protein, volvatoxin A_2, or heparin.

5 Notechis-5 was found to be stable even when in the presence of muscles.

6 [^{3}H]-acetylated crotoxin PLA, which retained about 40% of its original enzyme and presynaptic blocking activities, also bound rapidly to the mouse diaphragm on incubation and this binding was greatly hindered by the simultaneous addition of crotapotin.

7 The prevention of binding of crotoxin PLA by crotapotin occurred mostly at those sites where the binding was easily dissociable on washing. No antagonism of binding occurred at the firmly binding site.

8 The binding of [^{3}H]-acetylated crotapotin was much less than that of crotoxin PLA, and interestingly, the binding was increased by the latter, suggesting that crotapotin may be first bound to the diaphragm together with crotoxin PLA.

9 No specific binding at the endplate zone was found either for crotoxin PLA or for crotapotin.

10 It is concluded that crotapotin potentiates the presynaptic effect of crotoxin PLA by curtailing its non-specific affinity with muscles, minimizing its dispersal and destruction *en route* to the nerve terminal, but not by acting as an affinity probe for the nerve terminal.

Introduction

Crotapotin is the acidic subunit of crotoxin, a highly lethal presynaptic neurotoxin isolated from the venom of the South American rattlesnake, *Crotalus durissus terrificus* (Rübsamen, Breithaput & Habermann, 1971; Hendon & Fraenkel-Conrat, 1971). Although it is nontoxic itself, crotapotin increases the lethality of the other subunit of crotoxin, a basic phospholipase A_2 (PLA), by as much as 10 fold when the two units are given together to mice (Hendon & Fraenkel-Conrat, 1971; Rübsamen *et al.*, 1971). This potentiation of the lethality has been shown to be due to a parallel increase of the presynaptic neuromuscular blocking effect (Chang & Lee, 1977). It is generally agreed that the presynaptic blocking effect of crotoxin is due to its enzymatic breakdown of the phospholipid of axolemma by PLA action (Chang, Lee, Eaker & Fohlman, 1977). In a previous study of the mechanism of crotapotin's potentiation of crotoxin PLA, it was found that crotoxin PLA lost its presynaptic activity very rapidly in physiological salt solution at 37°C bubbled with 95% O_2 and 5% CO_2 (Chang & Su, 1978), probably due to its instability and non-specific binding. The presence of isolated tissues, such as mouse diaphragm, in the medium further accelerated the disappearance of active crotoxin PLA, whereas the decline of active crotoxin PLA was completely prevented if an equimolar concentration of crotapotin was also added. It was therefore concluded that crotapotin of the crotoxin complex functions as a 'protector' to minimize dispersal and destruction *en route* to the site of action at the nerve terminal. In accordance with this, Jeng, Hendon & Fraenkel-Conrat (1978) have found that the binding of iodinated crotoxin PLA to

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rabbit erythrocytes was decreased by 25% by crotapotin whereas the distribution of a labelled crotoxin PLA to skeletal muscle after systemic administration was increased by simultaneous administration of crotapotin (Habermann, Walsch & Breithaput, 1972), indicating that crotapotin indeed affects the pharmacokinetics of crotoxin PLA. Whether the complex formed between crotoxin PLA and crotapotin affects the affinity of crotoxin with the target site at the nerve terminal, i.e., crotapotin acts as an affinity probe, has not yet been clarified.

In contrast to crotoxin, notexin and notechis-5 isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*, consist only of a basic phospholipase A molecule (Halpert & Eaker, 1975) but are nearly twice as lethal as crotoxin and potent in presynaptic effect (Harris, Karlsson & Thesleff, 1973; Cull-Candy, Fohlman, Gustavsson, Lüllman-Ranch & Thesleff, 1976). The present experiments were designed to investigate the reason for the difference between the pharmacological potencies of crotoxin PLA and notexin and to shed more light on the role of crotapotin in crotoxin complex by comparing the interactions of crotapotin with these basic PLAs. Interactions in relation to presynaptic as well as myotoxic effects, stability as revealed by their PLA activity and binding with the mouse diaphragm were studied. Interactions of other acidic molecules such as volvatoxin A_2 and heparin with crotoxin PLA were also compared since a synergistic lethal effect was reported for volvatoxin A_2 (Jeng & Fraenkel-Conrat, 1976) whereas an antagonistic effect on myotoxicity was observed for heparin (Ho, 1980).

Methods

Toxins

The procedures used for isolation of crotoxin and β-bungarotoxin were the same as previously described (Chang & Lee, 1977). The crotoxin complex was further separated into crotoxin PLA and crotapotin by chromatography on a DEAE cellulose column in the presence of 5 M urea as described by Hendon & Fraenkel-Conrat (1971). Notexin and notechis-5 were isolated from the venom of *Notechis s. scutatus* by means of chromatography on CM-Sephadex C-25 and Sephadex G-75. Volvatoxin A_2 was prepared according to Lin, Jeng, Chen, Shi & Tung (1973).

Labelled crotoxin phospholipase A_2 and crotapotin

Crotoxin PLA was acetylated with [^{3}H]-acetic anhydride (100 mCi/mmol, Amersham). To 5 mg crotoxin PLA, dissolved in 0.5 ml distilled water, 1.5 mg (1.5 mCi) of [^{3}H]-acetic anhydride was added at 0–2°C, and pH adjusted to 8.0 with NaOH solution. The reaction was stopped at 45 min by diluting the mixture with 4.5 ml of cold H_2O. Free [^{3}H]-acetic acid was removed by dialysis against 200 ml 0.1 M ammonium acetate and then distilled water in a refrigerator. Water for dialysis was changed every hour for 16 h. The [^{3}H]-acetylated crotoxin PLA was then freeze-dried. The contamination of [^{3}H] acetic acid in [^{3}H]-acetylated crotoxin PLA was less than 0.1%. In each molecule of crotoxin PLA, 1.2 [^{3}H]-acetyl groups were incorporated. Crotapotin was acetylated under the same conditions except that 3 mg (3 mCi) of [^{3}H]-acetic anhydride was used and the pH adjusted to 7.2–7.6. About 2.3 [^{3}H]-acetyl groups were incorporated into one molecule of crotapotin.

Nerve-muscle preparations

The biventer cervicis nerve muscle preparation (Ginsborg & Warriner, 1960) was isolated from male Leghorn chicks, 4–10 days old, and suspended in an organ bath filled with 20 ml modified Tyrode solution (composition mM: NaCl 137, KCl 2.8, $CaCl_2$ 2.7, $MgCl_2$ 1.1, NaH_2PO_4 0.33, $NaHCO_3$ 11.9 and glucose 11.2). Phrenic nerve diaphragm preparations (Bülbring, 1946) were isolated from mice weighing 18–25 g and set up in an organ bath containing 12 ml Tyrode solution ($CaCl_2$ 1.8 mM). The organ bath was maintained at 37°C and aerated with 95% O_2 and 5% CO_2. Contractions of muscle were elicited by stimulation of the nerve with supramaximal rectangular pulses of 0.05–0.1 ms duration or by direct stimulation of muscle with pulses of 0.5 ms.

Toxins binding in isolated diaphragm of mouse

Two hemidiaphragms were incubated with labelled toxins for 2 h and washed for 0.5 to 1.5 h with 6–15 exchanges of Tyrode solution. The hemidiaphragm was then stretched on paper, dried and cut into 3 segments in parallel with the central endplate zone. Each segment was digested with 0.2 ml 0.5 N KOH at 35°C and the radioactivity measured in a Packard liquid scintillation spectrometer with 10 ml phosphor solution consisting of toluene 7 ml, ethylene glycol monomethylether 3 ml, PPO 20 mg, POPOP 0.5 mg and naphthalene 300 mg. Quenching was corrected for by an external standard.

Assay of phospholipase A_2 activity

An aliquot (0.1 to 0.2 ml) of the incubation medium containing PLA toxins was added to 5 ml of PLA activity assay medium composed of (mM): synthetic dipalmitoyl 2-α-phosphatidylcholine 2.5, Na deoxycholate 2.5, NaCl 100, $CaCl_2$ 10, disodium

edetate (Na_2EDTA) 0.05. The PLA activity was measured by pH-stat titration at pH 8.0 of the free fatty acid released in 5 min with 2 mM NaOH.

Drugs

Heparin (Sigma), synthetic dipalmitoyl 2-α-phosphatidyl choline (Sigma) and Na deoxycholate (Difco) were used.

Standard error of the mean and Student's *t* test for statistical significance were used.

Results

Effects of crotapotin on the presynaptic action of basic phospholipase A_2s

Crotoxin PLA alone at a high concentration (10 μg/ml) depressed the contractile responses of mouse isolated diaphragms to both indirect and direct stimulation gradually, taking more than 5 and 6 h, respectively, for 90% depression of twitch contraction evoked by indirect and direct stimulation (Table 1). The slight difference in the time required to block these two types of contraction indicates that the depression by crotoxin PLA alone was largely due to a direct effect on the muscle, even at the high concentration used. When an equimolar concentration of crotapotin (6.4 μg/ml) was added simultaneously, the time needed for 90% depression for indirect stimulation was decreased from 5 h to 75 min whereas that for direct stimulation was only shortened from about 6 h to 5 h (Table 1), indicating a specific potentiation of the presynaptic effect of crotoxin PLA by crotapotin. Since crotoxin PLA at 0.5 μg/ml was able to block the indirect response by 90% within 3 h in the presence of an equimolar concentration of crotapotin, it may be calculated that the potentiation by crotapotin of the presynaptic effect is more than 20 fold. The time to cause 90% block of the indirect response was further shortened if the ratio of the concentration of crotapotin to crotoxin PLA was raised from 1:1 to 3:1 (Table 1). A similar shortening of block time occurred if the ratio was altered to 1:3 by raising the concentration of crotoxin PLA (Table 1). These results suggest that 1:1 ratio of crotapotin is not sufficient for full potentiation, probably because there is still some destruction of crotoxin PLA *en route* to the nerve terminal due to dissociation of the complex.

Potentiation of the presynaptic effect of crotoxin PLA by crotapotin was also observed in the isolated biventer cervicis muscle of the chick. In the absence of crotapotin, 90% blockade of the indirect response by 6.1 μg/ml crotoxin PLA took 60 ± 8 min ($n = 4$), whereas the same degree of blockade was produced by less than 0.1 μg/ml of crotoxin PLA in the presence of crotapotin, a potentiation of 60 fold.

The effects of crotapotin on the presynaptic neuromuscular effect of notexin, notechis-5 and β-bungarotoxin in the mouse diaphragm are shown in Table 2. In contrast to the marked potentiation obtained with crotoxin PLA, crotapotin did not change

Table 1 Potentiation by crotapotin of the neuromuscular blocking action of crotoxin phospholipase A_2 (PLA) on the mouse diaphragm

	Time to block (min)			
	Indirect		*Direct*	
Toxins	70%	90%	70%	90%
Crotoxin PLA, 10 μg/ml	216 ± 27 (4)	306 ± 33 (4)	308 ± 36 (4)	385 ± 36 (4)
Crotapotin, 10 μg/ml	390 <	—	390 <	—
Crotoxin PLA, 10 μg/ml plus crotapotin, 6.4 μg/ml	45 ± 8 (6)*	75 ± 17 (6)*	221 ± 49 (6)	301 ± 50 (6)
Crotoxin PLA, 0.5 μg/ml plus crotapotin, 0.32 μg/ml	123 ± 22 (5)*	177 ± 27 (5)*	390 <	390 <
Crotoxin PLA, 0.5 μg/ml plus crotapotin, 0.96 μg/ml	95 ± 13 (5)*	128 ± 19 (5)*	390 <	390 <
Crotoxin PLA, 1.5 μg/ml plus crotapotin, 0.32 μg/ml	99 ± 12 (4)*	137 ± 11 (4)*	390 <	390 <

*$P < 0.05$ vs crotoxin PLA alone.

Crotoxin PLA was mixed with crotapotin before addition to the organ bath. Twitch responses to direct and indirect stimulation were elicited at 0.1 Hz. Time to 70% or 90% block of the twitch response was compared. Means ± s.e.

Table 2 Effect of crotapotin on the neuromuscular blocking action of other presynaptic neurotoxins on the mouse diaphragm: indirect stimulation at 0.1 Hz

Toxins	*Time to block* (min) 70%	90%	100%
3-Bungarotoxin, 1 μg/ml	117 ± 12 (4)	139 ± 16 (4)	160 ± 18 (4)
β-Bungarotoxin, 1 μg/ml plus crotapotin, 1 μg/ml	119 ± 11 (4)	145 ± 11 (4)	171 ± 8 (4)
Notexin, 1 μg/ml	82 ± 6 (17)	98 ± 6 (17)	104 ± 7 (4)
Notexin, 1 μg/ml plus crotapotin, 1 μg/ml	109 ± 7 (4)*	121 ± 7 (4)*	145 ± 10 (4)*
Notechis-5, 5 μg/ml	69 ± 6 (4)	88 ± 2 (4)	103 ± 4 (4)
Notechis-5, 5 μg/ml plus crotapotin, 5 μg/ml	110 ± 18 (5)*	135 ± 17 (5)*	160 ± 14 (5)*

*$P < 0.05$ vs in the absence of crotapotin.

the activity of β-bungarotoxin to any appreciable extent, whereas the activities of notexin and notechis-5, two self-active basic PLAs, were significantly reduced by the presence of crotapotin at 1–2 equimolar concentrations, indicating crotapotin inhibits rather than potentiates the presynaptic activity of notexin and notechis-5.

Effects of crotapotin on the myotoxic effect of crotoxin phospholipase A_2

Although the direct effect of crotoxin on the skeletal muscle at the time of neuromuscular block is insignificant (Chang & Lee, 1977), high concentrations of and prolonged treatment with crotoxin affect the skeletal muscle directly (Breithaupt, 1976). Isolated crotoxin PLA also shared this myotoxic effect. The result in Table 1 shows that the myotoxic effect of crotoxin PLA, as judged by the time to cause 90% depression of contraction evoked by direct stimulation, was neither reduced nor enhanced to a significant extent.

In the chick biventer cervicis muscle, crotoxin PLA evoked a slow progressive contracture even in the presence of (+)-tubocurarine (Figure 1). The time for the toxin-induced contracture to attain a tension equal to that produced by a single supramaximal stimulation of nerve was measured. Crotoxin PLA alone at 6.1 μg/ml took 84 ± 18 min ($n = 5$) whereas crotoxin PLA 6.1 μg/ml with crotapotin 3.9 μg/ml took 68 ± 8 min ($n = 5$), indicating a non-significant degree of enhancement. Since crotoxin PLA alone at 12.2 μg/ml took less than 30 min to cause the same contracture, it is evident that the enhancement, if any, of the myotoxic effect by crotapotin is much less than 2 fold. The crotoxin-induced contracture was abolished by increasing the concentration of Ca^{2+} from 1.8 mM to 10.8 mM even after the contracture had developed (Figure 1). Heparin (1 mg/ml) also antagonized the contracture but only when applied before the toxin.

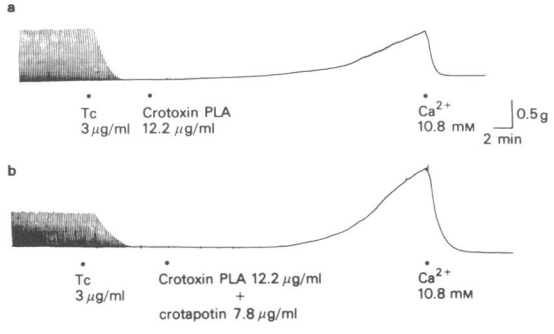

Figure 1 Contracture induced by crotoxin complex and crotoxin phospholipase A_2 (PLA) in the chick biventer cervicis muscle. The chick muscle was first treated with (+)-tubocurarine (Tc, 3 μg/ml) in order to eliminate any presynaptic effect. (a) Crotoxin PLA (12.2 μg/ml); (b) crotapotin (7.8 μg/ml) plus crotoxin PLA (12.2 μg/ml). At Ca^{2+}, $CaCl_2$ was increased to 10.8 mM.

Effects of crotapotin on the stability of phospholipase A_2s

In a previous experiment the stability of crotoxin PLA in an incubation medium (Tyrode solution) at 37°C was monitored by means of its presynaptic activity in the chick muscle preparation after complex formation with crotapotin (Chang & Su, 1978). In order to know whether the rapid decline of the presynaptic effect of crotoxin PLA on incubation is due to a loss of any property specifically concerned

with its presynaptic effect or the loss is accompanied by reduction of PLA activity, we studied the change of enzyme activity during incubation under the same conditions. As illustrated in Figure 2, crotoxin PLA lost its enzyme activity rapidly at a similar rate to that previously found for the decline of the presynaptic effect. The decline was very rapid (60%) during the first 30 min of incubation followed by a slower phase. The presence of two mouse hemidiaphragms in the incubation medium greatly accelerated the rate of decline so that 85% of the activity was lost within 30 min. In contrast, crotoxin PLA was quite stable in the presence of crotapotin. The presence of muscle also accelerated the rate of decline but to a much smaller extent.

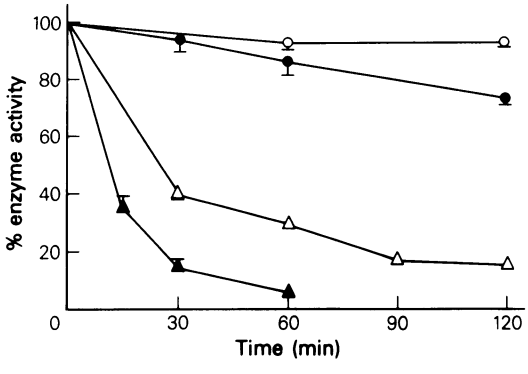

Figure 2 Effect of crotapotin on the inactivation of crotoxin phospholipase A_2 (PLA) incubated in Tyrode solution with or without mouse diaphragms, monitored by its enzyme (PLA) activity. Crotoxin PLA (10 μg/ml) was incubated at 37°C in solution aerated with 95% O_2 plus 5% CO_2. The enzyme activity immediately after addition of PLA to the incubation mixture was taken as 100%. (△) Crotoxin PLA without muscle; (▲) crotoxin PLA with muscle; (○) crotoxin PLA plus crotapotin (6.4 μg/ml) without muscle; (●) crotoxin PLA plus crotapotin with muscle. Values are mean of $n = 4 - 6$; vertical lines show s.e. mean.

Similar experiments with notechis-5 (Figure 3) disclosed that notechis-5, an active toxin without a subunit, was much more stable than crotoxin PLA. Although there was a rapid initial decline with approx. 35% activity being lost in the first 30 min, unlike crotoxin PLA, the later progressive decline did not occur. Moreover, no acceleration of decline was observed by adding mouse diaphragm to the incubation medium, indicating that the muscle tissue does not appreciably bind or destroy this toxin. Interestingly, however, addition of crotapotin also significantly reduced the initial decline of notechis-5 PLA activity, indicating that crotapotin does complex with notechis-5.

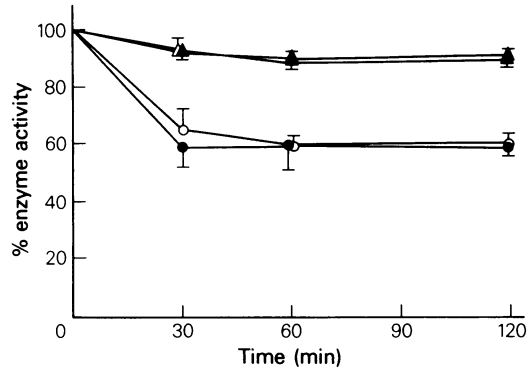

Figure 3 Effect of crotapotin on the inactivation of notechis-5, incubated in Tyrode solution with or without mouse diaphragms, monitored by its enzyme activity. Other experimental conditions were the same as those described in Figure 2. (○) Notechis-5 (10 μg/ml) without muscle; (●) notechis-5 with muscle; (△) notechis-5 plus crotapotin (10 μg/ml) without muscle; (▲) notechis-5 plus crotapotin with muscle. Values are mean of $n = 4 - 6$; vertical lines show s.e. mean.

In order to determine whether the rapid decline of crotoxin PLA in the Tyrode solution, especially in the presence of muscle tissue, is due to a non-specific binding and/or to destruction, the decline of radioactivity of ^{3}H-labelled crotoxin PLA and crotapotin was assayed in the presence of two mouse hemidiaphragms. The labelled crotoxin PLA was found to have 1.2 acetyl groups per molecule (specific activity = 61.2 Ci/mol) and retained 38% enzyme activity and 30–40% of its presynaptic effect (assayed with native crotapotin) on the phrenic nerve-diaphragm preparation of mice. A precipitate was formed on addition of crotapotin, indicating the unchanged affinity for the latter. On the other hand, the labelled crotapotin was found to have 2.3 acetyl groups per molecule (specific activity = 117 Ci/mol) and retained the full activity of potentiating the presynaptic effect of crotoxin PLA. As illustrated in Figure 4, there was also a rapid decline of isotopic concentration of the labelled crotoxin PLA, about 45% being lost in the initial period of 20 min after incubation. However, in contrast to the enzyme activity, the concentration of crotoxin PLA as measured by its radioactivity in the medium remained unchanged thereafter. These results in Figures 2 and 4 together suggest that the muscle tissue not only binds non-specifically a great amount of crotoxin PLA very rapidly but also accelerates the destruction of crotoxin PLA. In the presence of crotapotin, the initial rapid decline corresponding to binding of crotoxin PLA was almost completely abolished and a slow decline of radioactivity, similar to the decline of enzyme activity, was observed, suggesting that the decline of PLA in the presence of crotapotin is due to

binding to muscle. On the other hand, the labelled crotapotin alone was found to be quite stable and inert under the same conditions. In the presence of crotoxin PLA, the decline of radioactivity of the labelled crotapotin was somewhat enhanced (Figure 4), suggesting an increased binding of crotapotin with the diaphragm muscle in the presence of crotoxin PLA.

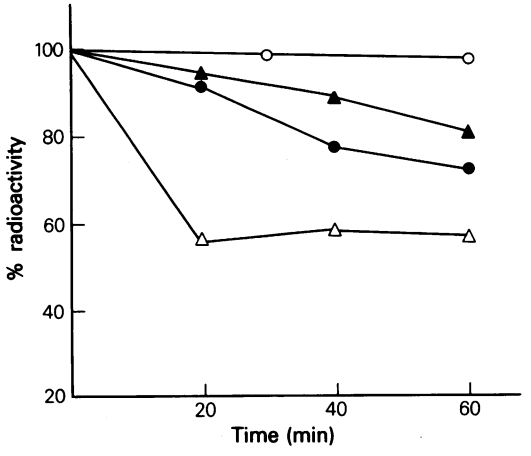

Figure 4 The effects of crotapotin and crotoxin phospholipase A_2, (PLA), respectively, on the decline of radioactivities of [^{3}H]-crotoxin PLA and [^{3}H]-crotapotin in the incubation medium containing mouse diaphragms. The incubation medium was 10 ml Tyrode solution aerated with 95% O_2 plus 5% CO_2 at 37°C and contained two mouse hemidiaphragms. (△) [^{3}H]-crotoxin PLA (2 μg/ml) alone; (▲) [^{3}H]-crotoxin PLA plus native crotapotin (1.3 μg/ml); (○) [^{3}H]-crotapotin (1.3 μg/ml) alone; (●) [^{3}H]-crotapotin plus native crotoxin PLA (2 μg/ml). The radioactivity immediately after mixing was taken as 100%. Mean of 2 observations. Due to the shortage of materials, experiments could not be repeated.

Binding of the labelled crotoxin phospholipase A_2 and crotapotin with the mouse diaphragm

The mouse hemidiaphragm was incubated with labelled crotoxin PLA (2 μg/ml) for 2 h and then repeatedly washed for 30 or 90 min. The radioactivity remaining bound to the diaphragm after sectioning the muscle into three segments in parallel with the central endplate zone is shown in Figure 5. There was a great decrease of radioactivity between the 30 min-wash and the 90 min-wash, indicating that most of the binding was reversible. When incubated together with native crotapotin (1.3 μg/ml), the binding of crotoxin PLA after the 30 min-wash was reduced to about a half, whereas the binding remaining after the 90 min-wash was almost the same. This result suggests that crotapotin interferes chiefly with

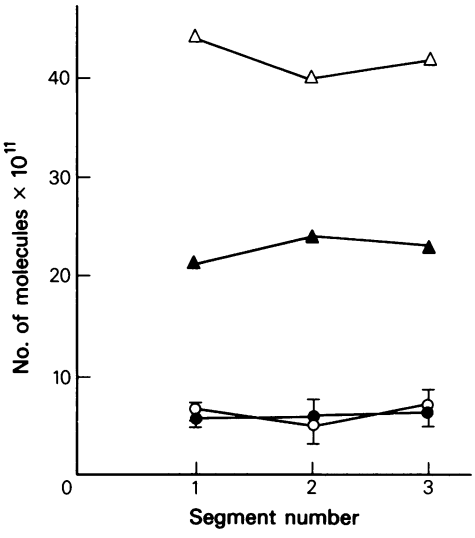

Figure 5 Effect of crotapotin on the binding of [^{3}H]-crotoxin phospholipase A_2 (PLA) to the mouse diaphragm. Two mouse hemidiaphragms were incubated in 10 ml Tyrode solution containing 2 μg/ml [^{3}H]-crotoxin PLA with or without crotapotin for 2 h. The radioactivities remaining bound to the diaphragm of segment 1 (central tendon side), 2 (endplate zone) and 3 (rib side) after washing were calculated as the number of molecules bound. (△), (○): [^{3}H]-crotoxin PLA alone; (▲), (●): with crotapotin (1.3 μg/ml); (△), (▲): after 30 min wash; (○), (●): after 90 min wash. Mean of n = 2 – 4; vertical lines show s.e. mean.

the binding of crotoxin PLA at the site where the binding is easily reversible. The distribution of radioactivity on the central segments with endplates of the diaphragms was not different from other non-endplate segments even incubated together with crotapotin and after repeated washing for 90 min, indicating that binding of crotoxin PLA took place not only to the nerve terminal but also to the muscle. The binding of the labelled crotapotin with the diaphragm was only about one fifth of that of crotoxin PLA in comparable conditions. The binding was also reversible and characterized by non-specific distribution (Figure 6). However, the effect of native crotoxin PLA on crotapotin binding was interesting since an enhancement was obtained instead of a reduction of binding as seen in the effect of crotapotin on PLA binding. This result is in accordance with the enhanced decline of labelled crotapotin in the medium in the presence of PLA (Figure 4).

Effect of volvatoxin A_2 and heparin on neurotoxicity and phospholipase A_2 activity of crotoxin phospholipase A_2

Volvatoxin A_2, which forms a complex with crotoxin PLA and potentiates the lethality of crotoxin PLA

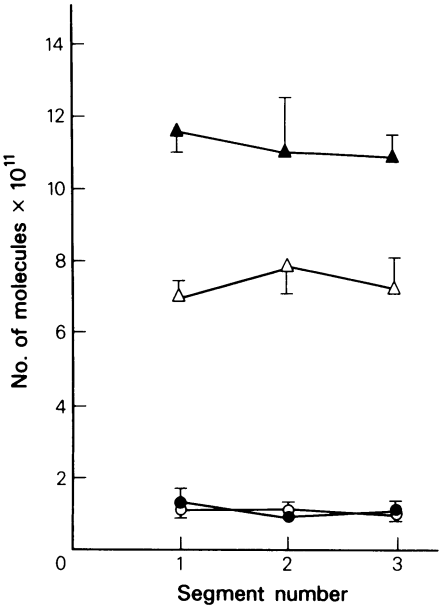

Figure 6 Effect of crotoxin phospholipase A_2 (PLA) on the binding of [^{3}H]-crotapotin to the mouse diaphragm. Two mouse hemidiaphragms were incubated in 10 ml Tyrode solution containing 1.3 μg/ml [^{3}H]-crotapotin with or without crotoxin PLA for 2 h. Other experimental conditions were the same as those described in Figure 5. (△), (○): [^{3}H]-crotapotin alone; (▲), (●): with crotoxin PLA (2 μg/ml); (△), (▲): 30 min wash; (○), (●): 90 min wash. Mean of $n = 4$; vertical lines show s.e. mean.

(Jeng & Fraenkel-Conrat, 1976), was without effect on the presynaptic toxicity and myotoxicity of crotoxin PLA in the isolated phrenic nerve diaphragm preparation of the mouse. Heparin was found to antagonize the myotoxic effect of crotoxin PLA and crotoxin complex in chick biventer cervicis muscle. However, the neurotoxic and myotic effect of crotoxin PLA in the mouse isolated diaphragm preparation was not affected by heparin (Table 3). In addition to their ineffectiveness in potentiating the neurotoxicity of crotoxin PLA, both volvatoxin A_2 and heparin were ineffective in preventing the decline of PLA activity of crotoxin PLA in the incubation medium.

Discussion

The presynaptic neurotoxins of snake venom are either basic phospholipase A or contain a subunit or chain which is a basic phospholipase A (Karlsson, 1979). These single unit phospholipase A toxins such as notexin (Halpert & Eaker, 1975; Cull-Candy *et al.*, 1976) and notechis-5 (Halpert & Eaker, 1976) are highly active on the nerve terminal structure when given alone, whereas the basic phospholipase As of complex neurotoxins such as crotoxin (Hendon & Fraenkel-Conrat, 1971; Rübsamen *et al.*, 1971; Breithaupt, 1976; Hawgood & Smith, 1977) and taipoxin (Fohlman, Eaker, Karlsson & Thesleff, 1976) are much less active on the nerve terminal when given alone and need the help of the other subunits in the complex to be fully active.

The isolated basic phospholipase A of crotoxin has been shown to lose its presynaptic blocking activity rapidly in physiological salt solution and this instability is greatly increased in the presence of tissues such as skeletal muscle (Chang & Su, 1978). The present results, showing the decline of PLA as well as its radioactivity, further indicate that skeletal muscles can greatly reduce the basic PLA of crotoxin both by non-specific binding and by destruction of the enzyme molecules. Since the motor nerve terminal, the ultimate target of presynaptic toxins, is embedded in the endplate and not directly exposed, the dispersal and destruction *en route* to the target site would make it very difficult for the isolated crotoxin PLA molecule to reach the site of action. With the protection of crotapotin, PLA may effectively reach and act on the nerve terminal. In contrast, the self-active

Table 3 Effects of volvatoxin and heparin on the neuromuscular blocking action of crotoxin phospholipase A_2 (PLA) on the mouse diaphragm: direct and indirect stimulation at 0.1 Hz

	Time to block (min)			
	Indirect		*Direct*	
Toxins	70%	90%	70%	90%
Crotoxin PLA, 10 μg/ml	285 ± 42 (4)	306 ± 33 (4)	308 ± 36 (4)	385 ± 81 (4)
Crotoxin PLA, 10 μg/ml plus volvatoxin A_2, 5 μg/ml	278 ± 40 (5)	312 ± 34 (5)	285 ± 54 (5)	—
Crotoxin PLA, 10 μg/ml plus heparin, 1 mg/ml	281 ± 11 (5)	323 ± 15 (5)	342 ± 38 (4)	406 ± 70 (4)

Means ± s.e.

notechis-5 basic PLA was found not to be bound or destroyed to a large extent by skeletal muscle. This would explain why the toxin is active without the help of a 'protector'. Crotapotin appeared too complex with notechis-5 as well as with crotoxin PLA in view of the formation of a precipitate. However, this complex formation resulted in the reduction of the presynaptic activity of notechis-5 (instead of potentiation as seen between crotapotin and crotoxin PLA) despite the stabilization of the notechis-5 molecule produced by crotapotin as judged from the protection of PLA activity. It is probable from these results that crotapotin is not an affinity probe and has no specific affinity with the nerve terminal. On the contrary, it may reduce the affinity of notexin and notechis-5. In the case of crotoxin PLA, the reduction of affinity with the non-specific binding sites by crotapotin probably far exceeds that with the specific site at the nerve terminal, thus resulting in potentiation of presynaptic activity.

The above inference is further supported by the binding experiments showing that the amount of crotoxin PLA remaining bound after a 30 min-wash was halved by crotapotin. Similar inhibition of non-specific binding of ^{125}I-crotoxin PLA to the erythrocyte membrane (Jeng *et al.*, 1978) and receptor-rich membrane from *Torpedo* (Bon, Changeux, Jeng & Fraenkel-Conrat, 1979) have been described. The uniform distribution of crotoxin PLA on the diaphragm, whether incubated in the presence or absence of crotapotin, reveals that there are many non-specific binding sites for crotoxin PLA on skeletal muscle. Assuming one mouse hemidiaphragm contains 5000 muscle fibres of 15 μm diameter, the density of binding sites for crotoxin PLA may be calculated to be more than 5000 per μm^2 for the binding remaining after a 30 min-wash, indicating that crotoxin PLA is a highly reactive protein having affinity with the molecules of sarcolemma. The specific binding of crotoxin PLA with the nerve terminal would be therefore concealed in the non-specific binding.

A comparison of the binding of crotoxin PLA with that of crotapotin on the diaphragm reveals that the latter was always significantly less than the former, even when the diaphragm was incubated in the presence of their respective 'partner' so that they existed as a complex. This result suggests that crotoxin PLA has a higher affinity than crotapotin does and that, after binding, crotapotin dissociates more easily than crotoxin PLA. This, together with the finding that crotoxin PLA increased the binding of crotapotin, suggests that crotoxin binds with its target in a complex form though thenceforth crotapotin may dissociate leaving crotoxin PLA alone at the site. Hendon & Tu (1979) found that, after crosslinkage of crotoxin PLA and crotapotin with a bifunctional cross-linking agent, the non-dissociable crotoxin was much less lethal than the dissociable crotoxin although the enzyme activity was not reduced. This result was taken to suggest that, for crotoxin PLA to be able to bind, crotapotin has to dissociate first. However, it is possible that crotapotin could bind together with its PLA and the dissociation of crotapotin from crotoxin PLA after binding may be one necessary process for the PLA to exhibit its biological activity.

On the basis of the pharmacological actions and different effects of crotapotin upon crotoxin PLA, i.e., the marked potentiation of presynaptic effect, very slight change of myotoxic effect and marked inhibition of non-specific binding, it may be inferred that crotoxin PLA has at least three different binding sites, i.e., nerve terminal, muscle and unidentified non-specific sites having no pharmacological effect. The reason for the much smaller enhancement of the myotoxic effect of crotoxin PLA by crotapotin may be that the target site on sarcolemma for the myotoxic effect is rather exposed in comparison with the nerve terminal. The binding site at the nerve terminal, which is the most important for the toxic effect of crotoxin, undoubtly comprises only a very small proportion of the binding sites.

Volvatoxin A_2 was previously found to act synergistically with crotoxin PLA by potentiating its lethal toxicity (Jeng & Fraenkel-Conrat, 1976) and was thought to be acting like crotapotin. However, our results show that this is not the case since the presynaptic effect of crotoxin PLA, the major cause of lethal toxicity, was not enhanced. Furthermore, the stability of crotoxin PLA in physiological salt solution was not improved by volvatoxin A_2. Since, unlike crotapotin, volvatoxin A_2 has toxicity of its own, the synergism with crotoxin PLA for the lethal effect observed by Jeng & Fraenkel-Conrat (1976) may be simply due to their different modes of toxicity acting simultaneously. Like volvatoxin A_2, heparin neither enhanced to any appreciable degree the presynaptic effect of crotoxin PLA nor stablized the latter peptide, although the myotoxic effect was reduced. These results suggest that the potentiation by crotapotin of crotoxin PLA's presynaptic effect is not due to a simple complex formation between acidic and basic molecules.

In conclusion, the basic crotoxin PLA is much less active on the nerve terminal than the basic PLA of notexin and notechis-5 because tissues such as skeletal muscle have high non-specific affinity with crotoxin PLA and catalyse its decomposition. Crotapotin, by a specific complex formation, minimizes this dispersal and destruction *en route* to the target site, by decreasing the PLA's affinity with the non-specific site. The binding of crotoxin PLA with tissues in the presence of crotapotin is still predominantly non-specific and the specific binding at the nerve terminal is scarcely revealed.

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