The Role of Crotapotin in Potentiating the Presynaptic Effect of Phospholipase A in Crotoxin Complex

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Abstract

Crotoxin complex, isolated from the venom of South American rattlesnake, is a presynaptic neurotoxin composed of a basic phospholipase A and an acidic subunit crotapotin. Crotapotin alone does not hydrolyse phospholipids, nor activate the enzyme activity of phospholipase A and has no neuromuscular blocking activity. Phospholipase A alone is 10 times less potent in blocking the neuromuscular transmission than crotoxin. When incubated *in vitro* with an isolated mouse diaphragm, phospholipase A loses 94% neurotoxicity in the absence of crotapotin in 30 min. No loss of activity can be detected if incubated with crotapotin. When incubated alone without the diaphragm, phospholipase A also loses 54% of neurotoxicity. It is concluded that crotapotin functions as 'chaperone' sharpening the specificity by decreasing the non-specific binding and increasing the stability of the toxic phospholipase A so as to minimize distraction and destruction *en route* to the nerve ending.

Since the isolation of β-bungarotoxin from the venom of Formosan krait in 1963[4] there have been four presynaptic toxins isolated from snake venoms [2]. Compared with the post synaptic neurotoxins, as exemplified by α-bungarotoxin, these presynaptic neurotoxins are 30 to 50 times more toxic to mammalians and block the release of acetylcholine from the motor nerve terminal, resulting in paralysis of respiratory muscle. All of these toxins are composed of basic phospholipase A (PLA)[18]. While notexin[9,11,13] isolated from the venom of Australian tiger snake (*Notechis scutatus scutatus*) contains only the enzyme molecule, other presynaptic toxins, including taipoxin [6,9,10,17] of Taipan snake (*Oxyuranus s. scutellatus*), crotoxin[5,14,20] of South American rattlesnake (*Crotalus durissus terrificus*) and β-bungarotoxin[3,4,19] of Formosan Krait (*Bungarus multicinctus*), also contain one or two non-PLA subunits or chain in addition to the enzyme. In these complex toxins, the PLA component alone is much less toxic than the native toxins, its toxicity being only one hundredth for the PLA of taipoxin[10] and one tenth to one fifth for the PLA of crotoxin[14,20]. The non-PLA moieties are non-toxic in all cases.

It has been of central interest that what property of the non-PLA subunits make the toxin complex highly toxic. There is accumulating evidence that the toxic effect of presynaptic neurotoxins is due to an enzyme action on the axolemma[8,12,16,21]. The non-PLA subunits of each toxin, however, have been shown to be either without effect or inhibit the enzyme activity when tested on isolated phospholipids[1,15,16,18,20] rather than to potentiate the enzyme activity. Hendon et al.[15] studied the binding of iodinated crotoxin-PLA with rabbit erythrocytes and found the binding of PLA was decreased by about 25% when the acidic subunit of crotoxin (crotapotin) was present. This reduction of nonspecific binding, however, cannot account for the 5-10 fold enhancement of toxicity of PLA by crotapotin.

The present communication reports the *in vitro* effect of skeletal muscle on the PLA of crotoxin. It was found that even in the isolated nerve-muscle preparations, such as mouse phrenic nerve-diaphragm and baby chick biventer cervicis, the PLA was more than ten times less potent than PLA-crotapotin complex in blocking presynaptically the neuromuscular transmission. Since no other organs are included in this isolated nerve-muscle system, the possibility can be narrowed down to (a) if the complex has higher affinity for the nerve terminal than PLA, (b) if the free PLA molecule has higher non-specific affinity with the muscle than the complex or (c) if PLA is unstable without crotapotin[7].

Materials and Methods

Isolation of crotoxins

The venom (0.5gm) of Brazilian rattlesnake (*Crotalus durissus terrificus*) which was kindly collected for us by Dr. W. Bucherl was dissolved in 25 ml 0.1 M ammonium acetate (pH.8.3) and subjected to chromatography on the column of DEAE-cellulose (1.7× 55cm) with NaCl gradient (0-0.4 M) and pH gradient (pH8.3-4.0). The chromatography profile is illustrated in Fig. 1. Fractions IV, V and VI were

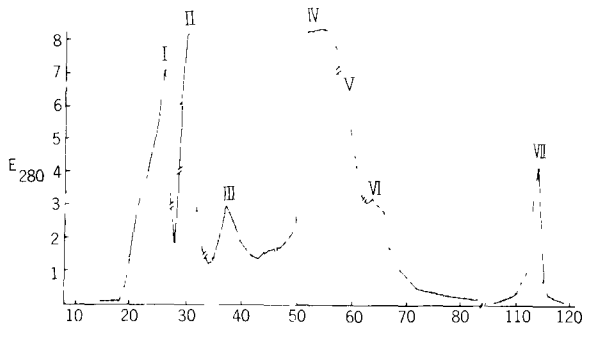

Fig. 1 Chromatography of *Crotalus durissus terrificus* venom on DEAE-Sephadex. EDAE-Sephadex A-25 was equilibrated with 0.05 M NH_4CO_3. CH_3 (pH 8.5) and the fractionation of venom (0.8 g) was carried out by increasing NaCl concentration to 0.4 M and pH to 6. 8.

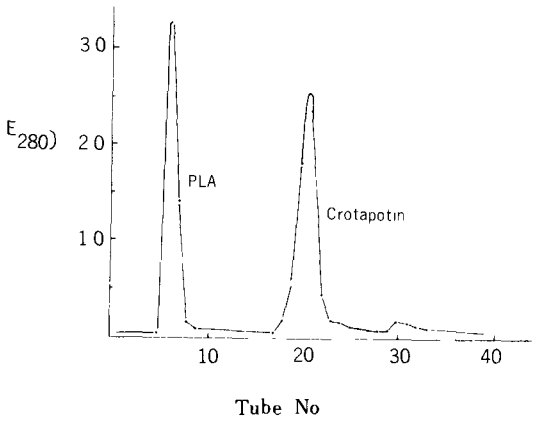

Fig. 2 Separation of crotoxin into phospholipase A_2 and crotapotin. Crotoxin (Fraction VI, Fig. 1) was chromatographed on DEAE-Sephadex, equilibrated with 0.05 M $NH_4CO_3.CH_3$ (pH 6.8) and 6 M urea, by increasing the gradient of NaCl (0.4 M).

all found to be crotoxin complex from their presynaptic effect on the isolated mouse phrenic nerve-diaphragm preparation as described below. They were indistinguishable on the basis of pharmacological effects qualitatively or quantitatively, but fraction IV was much less soluble than fraction V and VI. On Sephadex G-75 gel filtration, all three fractions appeared as single peaks with molecular weight corresponding to about 22,000 daltons.

Fraction I was found to contain crotamines and fraction VII crotapotin not complexed with phospholipase A.

Separation of crotapotin and PLA

Each crotoxin complex was dissolved in 2 ml 0.1 M ammonium acetate (pH6.8) containing 6 M urea and chromatographed with a DEAE-cellulose column (1.6 × 35 cm) equilibrated with the same solution. After the basic PLA fraction had emerged out, the gradient of NaCl(0-0.4 M) was introduced which eluted out the acidic crotapotin. All of the crotoxins showed the same chromatography pattern as illustrated in Fig. 2.

Measurement of PLA activity

L-α-phosphatidyl choline from egg yolk (Sigma Type II-E) was further purified on a column of aluminum oxide (45 × 8 cm) using chloroform: methanol (1:1, v/v) as eluant. After evaporation of the solvent the fraction was dissolved in diethyl ether to a concentration of 10 mg/ml. For use in the enzyme assay the stock solution was evaporated and the residual substrate was emulsified at a concentration of 2.5 mM in a solution containing 2.5 mM Na-deoxycholate, 0.05 mM Na_2-EDTA, 1.8 mM $CaCl_2$ and 100 mM NaCl. The enzymatic hydrolysis was started by adding 1 μg of the appropriate toxin to 4 ml of the lecithin solution. The rate of hydrolysis at 37° was followed by pH-stat titration at pH8.0 with 4 mM NaOH. Spontaneous hydrolysis and CO_2 uptake were corrected for. The rate was calculated from the alkali consumption during the first 5 minutes and was expressed as mmoles fatty acid liberated per minute per g protein.

Mouse diaphragm preparation

The phrenic nerve-diaphragm preparation was taken from mice (NIH strain) weighing 18-25 g. The organ bath contained 12 ml Tyrode solution (composition, in mM: NaCl, 147; kcl, 2.8; $CaCl_2$, 1.8; $MgCl_2$, 1.1; NaH_2PO_4, 0.33; $NaHCO_3$, 11.9; glucose, 11.2) at 37°C and was oxygenated with 95% O_2 + 5% CO_2. The phrenic nerve was stimulated by supramaximal rectangular pulses of 0.1 msec at a rate of 0.1 Hz throughout the experiments. The resting tension was 0.5 g. The presynaptic blocking activities of the neurotoxins were determined by following the blockade of the isometric contraction of the diaphragm evoked indirectly by stimulation of the nerve.

In order to see the effect of incubation with the diaphragm, PLA fraction from crotoxin VI was added to the organ bath at 2 μg/ml with or without further addition of crotapotin at 2.5 μg/ml.

Assay of presynaptic activity

Advantage was taken of the high sensitivity of the baby chick biventer cervicis muscle to crotoxin (5). PLA after incubation with the mouse diaphragm was added to the chick muscle preparation bathed in 10 ml Tyrode solution. Two molar excess of crotapotin was added for the assay if it was not added during incubation with the diaphragm. The time to cause complete neuromuscular block of the chick muscle was compared with a calibration curve using

non-incubated crotoxin complex.

Results and Discussion

Neuromuscular blocking action

The presynaptic neuromuscular blocking effect of crotoxin complex on the mouse diaphragm nerve-muscle and baby chick biventer cervicis preparations has been described (5). The separated subunits alone were found to be inactive or much less potent in this respect. Thus crotapotin was completely without effect even at a concentration as high as 10 μg/ml. No blockade of neuromuscular transmission occurred in the mouse preparation with PLA at 2 μg/ml in the absence of crotapotin whereas 0.2 μg/ml of PLA was sufficient to cause a complete block in 300 min if crotapotin was present. The presynaptic effect of PLA was thus potentiated by crotapotin by more than 10 fold.

The immediate depression and augmentation of contraction, characteristic of the action of crotoxin complex and other complex neurotoxins such as β-bungarotoxin and taipoxin[5,6,8] in the bathing media with low Ca^{2+} or Sr^{2+} Tyrode, was very slight or negligible when PLA alone was added. This result suggests that the binding of PLA with the nerve terminal is not as efficient as the crotoxin complex.

Phospholipase A_2 activity

The phospholipase A_2 activity of the PLA fraction was 295 mmole fatty acid liberated per min per g protein at 37°C. As previously reported[1,15], crotapotin had neither enzyme activity nor influence on the activity of PLA, It is thus unlikely that crotapotin potentiates the presynaptic effect by an augmentation of the enzyme activity.

Effect of crotapotin on the stability and non-specific binding of PLA

The presynaptic activities remaining in the bath media after incubation with the mouse diaphragm were assayed after suitable dilution by the neuromuscular blocking action on the chick biventer cervicis muscle which is highly sensitive to crotoxin[5]. Crotapotin was further added at 2 molar ratio before assay when PLA was incubated alone in order to convert the remaining active PLA to crotoxin. Excess of crotapotin was shown not to inhibit the biological activity of crotoxin. It was found, as shown in Fig. 3, that almost no decline of the biological activity occurred when PLA was incubated with the mouse diaphragm for 30 min in the presence of crotapotin, indicating that crotoxin complex is stable and that no non-specific binding with muscle occurs to the extent serious enough to reduce the toxin concentration. By contrast, when the PLA was incubated with the diaphragm alone, there occurred a rapid and marked reduction in the amount of biologically active PLA. After 30 min incubation the biological activity in the bath media was reduced to about 6%, 15-fold less than that obtained in the presence of crotapotin. When the PLA was incubated alone without the mouse diaphragm, the biological activity was also found to be decreased by about 58% in 30 min (Fig.3). It is obvious that the basic PLA is not only

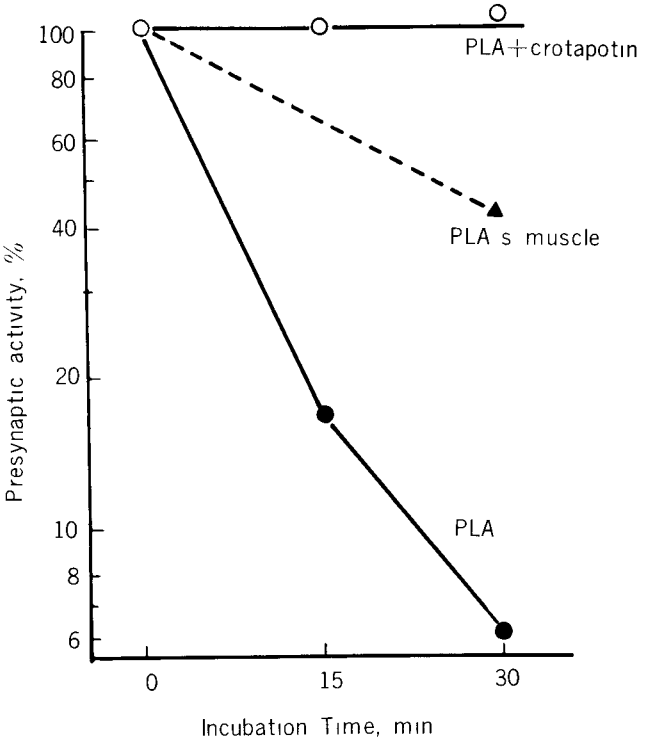

Fig. 3 The presynaptic blocking activity of a basic PLA remaining unchanged in the media after incubation with a mouse diaphragm with or without addition of crotapotin. Assayed of the chick biventer cervicis muscle after converting the free PLA to crotoxin with crotapotin.
○, PLA with crotapotin; •, PLA only
△. PLA only without mouse diaphragm. Mean of 3-4 experiments.

unstable in its free form but is also readily bound with and/or decomposed by the skeletal muscle. The protection of these inactivations by crotapotin seems sufficient to account for the potentiation of PLA toxicity by 5-10 fold. It was also found that the depressant effect of large doses of PLA on the direct stimulation of the mouse diaphragm was curtailed in the presence of crotapotin, indicating the decrease of nonspecific effect of PLA. It may be concluded from the present result that crotapotin potentiates the toxicity of basic PLA in the crotoxin complex by increasing the stability as well as by reducing the non-specific binding and decomposition by the skeletal muscle. It is quite likely that PLA is much less active in its free form because

it is bound or decomposed by the muscle before reaching the nerve terminal which is embedded in the muscle at end-plate. It has been shown by M. Hanley (unpublished) that the interaction of crotapotin with PLA leads to considerable conformational changes. Since the basic PLA alone can be bound with the nerve terminal at high concentrations (5-10 μg/ml) and cause the immediate depression of neuromuscular transmission in the mouse diaphragm in Sr^{2+}-Tyrode though less marked than the complex neurotoxin do, it may be suggested that crotapotin plays no important role once the basic PLA is bound with the axolemma. The present result substantiates the speculation made by Fohlman et al[10] that crotapotin and probably the β and γ units of taipoxin function as 'Chaperones' to minimize distraction and destruction *en route* to the site of action at nerve terminal.

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於 Crotoxin 神經毒中 Crotapotin 加強磷脂酶甲之鍵前神經毒性之角色

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摘　要

南美響尾蛇毒之 Crotoxin 乃作用於運動神經末端之鍵前神經毒素，由酸性多肽 Crotapotin 及鹼性磷脂酶構成。Crotapotin 本身無磷脂酶活性，並不加強磷脂酶活性，亦缺少神經毒性。磷脂酶甲本身卽只有 Crotoxin 一成以下之神經毒活性，磷脂酶甲以離體小鼠橫膈膜處理時其神經毒性於30分鐘內消失94%，但 Crotapotin 同時存在時却無任何活性之消退。無橫膈膜時磷脂酶甲單獨亦減弱54%，由上可結論Crotapotin在Crotoxin中之角色乃爲，「Chaperone」卽保護磷脂酶甲不受非特異性結合及破壞，護送其順利到達作用點卽神經末端結合產生作用。