Identification of a New Binding Protein for Crotoxin and Other Neurotoxic Phospholipase A_2s on Brain Synaptic Membranes†

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ABSTRACT: Crotoxin and other neurotoxic phospholipase A_2s exert neurotoxicity by acting primarily at the presynaptic level. Strong binding of crotoxin and several others to synaptic membranes has been demonstrated previously. In this study we used simple chemical cross-linking techniques to identify the neuronal membrane molecules involved in the binding of these toxins. After ^{125}I-crotoxin had bound to synaptosomes from guinea pig brain, treatment with disuccinimidyl suberate, disuccinimidyl dithiobis(propionate) or ethylene glycol bis(succinimidyl succinate) resulted in the formation of a predominant radioactive conjugate of ~60 kDa, which was different from the conjugate formed by photoaffinity labeling technique in a previous report. The membrane component in the conjugate was shown to be a single-chain protein of ~45 kDa. In subfractions of synaptosomes, this binding protein was mostly found in the synaptic membrane fraction and was not present in the mitochondrial fraction. Plasma membranes from several nonneural tissues also did not contain this binding protein. Unmodified crotoxin inhibited the formation of this adduct with an IC_{50} of around 1×10^{-8} M. Mojave toxin and some other phospholipase A_2s were also highly inhibitory to this conjugation, and notexin and others were less effective, while β-bungarotoxin and pancreatic PLA_2 were totally ineffective. We concluded that a new protein of 45 kDa specifically present in neuronal membranes is another major molecule responsible for the binding of crotoxin and other phospholipase A_2s.

Crotoxin, the major toxic component of the South American rattlesnake *Crotalus durissus terrificus* (Slotta & Fraenkel-Conrat, 1938; Rubsamen et al., 1971; Hendon & Fraenkel-Conrat, 1971; Aird et al., 1985, 1986), and other neurotoxic phospholipase A_2s (PLA_2s)[1] exert neurotoxicity by acting primarily at the presynaptic level, although most of them also show postsynaptic toxicity and other effects [see Hawgood and Bon (1991) for review]. At the neuromuscular junction, the effects in the early phases vary with the toxins and experimental conditions, an initial decrease in neurotransmission followed by increase being observed with crotoxin. But the ultimate blockade of transmission is the major cause of neurotoxicity common to the neurotoxic PLA_2s. As the pharmacological and electrophysiological aspects of the effects of the neurotoxic PLA_2s have been extensively studied, it is generally held that the PLA_2 activity of these toxins is a necessary but not sufficient condition for the neuromuscular blocking effect under normal conditions. Among the toxic PLA_2s the degrees of toxicity are not proportional to the levels of enzyme activity, and many PLA_2s exhibit little, if any, toxicity despite high enzymatic activity (Lee & Ho, 1982). Recently, others and we have shown that the neurotoxic PLA_2s show specific binding of high affinity to synaptic membranes, whereas only weak, nonspecific binding was found with nontoxic PLA_2s (Yang & Tzeng, 1983; Tzeng et al., 1986; Rehm & Betz, 1982; Othman et al., 1982; Lambeau et al., 1989). It would be of interest to learn about the membrane components responsible for the binding of the neurotoxic PLA_2s. In a few cases, identification of specific toxin-binding protein on synaptosomal membranes by photoaffinity labeling or simple chemical cross-linking techniques has been reported (Tzeng et al., 1986, 1989; Hseu et al., 1990; Rehm & Betz, 1983; Lambeau et al., 1989; Schmidt & Betz, 1989). Furthermore, the β-bungarotoxin binding protein has been shown to be a K^+ channel (Halliwell et al., 1986; Rehm & Lazdunski, 1988). Although work from this laboratory using photoaffinity labeling technique has identified a membrane protein of 85 kDa as a (subunit of the) major binding protein for crotoxin in neuronal membranes (Tzeng et al., 1986; Hseu et al., 1990), in the present report we used simple chemical cross-linking technique instead and identified a new binding protein on synaptic membranes from guinea pig brain for crotoxin and other PLA_2s. This report may mark the discovery of a protein important to neuronal membranes.

Experimental Procedures

Materials

Proteases, porcine pancreatic PLA_2, and various snake venoms were purchased from Sigma. Notexin, *Pseudechis australis* PLA_2 (Pa-11), and pseudexin A from *Pseudechis porphyriacus* are gift of Dr. Inn-Ho Tsai. Disuccinimidyl dithiobis(propionate) (DSP), disuccinimidyl suberate (DSS), and ethylene glycol bis(succinimidyl succinate) (EGS) were purchased from Pierce Chemical Co. $Na^{125}I$ was from New England Nuclear. Electrophoresis calibration standards for molecular weight determination of low molecular weight proteins were from Pharmacia. All other chemicals were of reagent grade.

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[1] Abbreviations: DSS, disuccinimidyl suberate; DSP, disuccinimidyl dithiobis(propionate); EGS, ethylene glycol bis(succinimidyl succinate); PLA_2, phospholipase A_2.

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Methods

Preparation of Toxins. Purification of crotoxin, taipoxin, β-bungarotoxin, caudoxin, Mojave toxin, and *Naja naja atra* PLA_2 from the crude venoms was carried out according to published procedures (Hendon & Tu, 1979; Fohlman et al., 1976; Hanley et al., 1977; Viljeon et al., 1982; Cate & Bieber, 1978; Lo et al., 1966).

Preparation of Synaptosomes and Other Membrane Fractions. Crude synaptosome fraction (P_2) was prepared from guinea pig brain according to Whittaker (1959). In certain experiments, seven protease inhibitors, i.e., EDTA (1 mM), phenylmethanesulfonyl fluoride (0.3 mM), iodoacetamide (1 mM), 1,10-phenanthroline (1 mM), benzamidine (1 mM), soybean trypsin inhibitor (0.005%), and aprotinin (0.005%), were included throughout the whole experiment starting from tissue homogenization. Subfractions of P_2 were prepared by suspending and stirring the crude synaptosome in ice-cold 6 mM Tris-HCl, pH 8.1, at 4 °C for 45 min. The membranes were collected by sedimentation at 20000*g* for 40 min and resuspended in 2 mL of 0.32 M sucrose per gram of wet membrane. The suspension was then layered on a discontinuous sucrose gradient containing 2.5-mL layers of 1.3, 1.0, and 0.85 M sucrose. The samples were sedimented at 40000*g* for 120 min. The membrane fractions collected from 0.32–0.85, 0.85–1.0, and 1.0–1.3 M sucrose interface were designated as fractions A, B, and C, respectively. The synaptosomal mitochondria were pelleted at the bottom of the centrifuge tube as fraction D. Plasma membrane fractions from the liver, kidney, and erythrocyte of the guinea pig were obtained according to published procedures (Neville, 1960; Booth & Kenny, 1976; Steck et al., 1970). The protein contents of the synaptosomes and plasma membrane fractions were determined as described by Lowry et al. (1951) using bovine serum albumin as standard.

Iodination of Crotoxin. Crotoxin was labeled with $Na^{125}I$ using the chloramine T method (Hunter & Greenwood, 1962). $Na^{125}I$ (20 μM) was allowed to react with crotoxin (0.2 mg/mL) in 10 mM Tris-HCl, pH 7.4, containing 1 mM Ca^{2+} and 0.02% chloramine T. After 30 s of incubation at room temperature, $Na_2S_2O_3$ in twice the amount of chloramine T was added. The reaction mixture was then diluted 10-fold with 2 mM tyrosine solution. Free ^{125}I was removed by chromatography on a Sephadex G-10 column (0.8 × 40 cm) equilibrated with 0.1% BSA in 10 mM Tris-HCl, pH 7.4, and 0.15 M NaCl. The peak in the void volume was collected. Crotoxin was radioiodinated to a specific activity of about 100 Ci/g (1 iodine/molecule of crotoxin). When an average of 1 atom of iodine was incorporated into 1 crotoxin molecule, the time to 90% neuromuscular blockade was 54 ± 1 min at 0.5 μg/mL, being comparable to a control value of 55 ± 1 min. The iodinated toxin was used within 1 week after preparation.

Cross-Linking of ^{125}I-Crotoxin with Membrane Components. ^{125}I-Crotoxin was mixed with synaptosomes or other membrane preparations (0.25 mg of protein/mL) in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% BSA, 150 mM NaCl, 10 mM $SrCl_2$, and 0.5 mM EGTA at 37 °C for 2.5 h. After diluting with 10 mM Tris-HCl, the mixture was centrifuged at 8000*g* for 10 min. The pellet was then washed and resuspended in phosphate-buffered saline. The bifunctional reagents, DSS, DSP, or EGS, in dimethyl sulfoxide were added to the resuspended membrane, and the mixture was incubated for 4 min at 25 °C. The reaction was stopped by the addition of 100 μL of 1 N glycine to each vial of reaction mixture. After centrifugation at 10000*g* for 15 min, the membrane pellet was solubilized with 0.1 M Tris-HCl buffer, pH 6.8, containing 5% glycerol and 2% SDS, under either nonreducing or reducing (5% β-mercaptoethanol present) conditions, and analyzed by SDS–polyacrylamide gel electrophoresis (Neville, 1971). Precolored proteins prepared according to Tzeng (1983) were used as molecular weight markers. After electrophoresis for 5–6 h the gel was dried, and then autoradiography was carried out at −80 °C using X-ray film and an intensifying screen.

Treatment with Proteases. Proteolysis of synaptosomes (1 mg/mL) by trypsin (150 μg, 1800 units), proteinase K (100 μg, 1 unit), α-chymotrypsin (100 μg, 5 units), thermolysin (18 μg, 1 unit), subtilisin (145 μg, 1 unit), protease of *Staphylococcus aureus* V8 (10 μg, 5 units), or carboxypeptidase Y (55 μg, 5 units) was carried out in 0.5 mL of 50 mM Tris-HCl, pH 7.4. Treatment with leucine aminopeptidase (355 μg, 5 units) or elastase (85 μg, 1 unit) was performed in 50 mM Tris-HCl, pH 8.8; and 50 mM phosphate buffer, pH 6.2, was used for digestion by papain (100 μg, 5 units). After incubating at 25 °C for 2 h, the proteolytic reaction was stopped either by addition of soybean trypsin inhibitor (500 μg) for trypsin incubation or by dilution with ice-cold Tris-HCl buffer. The mixtures were centrifuged at 10000*g* for 10 min to remove the proteases. The membranes were washed twice by centrifugation (at 10000*g* for 10 min) and then resuspended in binding assay buffer (10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 10 mM $SrCl_2$, and 0.5 mM EGTA). The definitions of enzyme unit of the proteases are according to Sigma.

RESULTS

Crotoxin is a dimeric protein with two different subunits: a 9000-Da subunit A and a 14400-Da subunit B exhibiting PLA_2 activity (Hendon & Fraenkel-Conrat, 1971; Rubsamen et al., 1971). Previous equilibrium binding studies have established that only subunit B of crotoxin can bind to the neuronal membranes, whereas subunit A cannot (Tzeng et al., 1986; Bon et al., 1979). On the basis of the observation that nondissociable crotoxin complex is inactive, Hendon and Tu (1979) have also proposed that only subunit B is involved in binding to membranes. Since in the present study the material unbound to membrane was removed by centrifugation after binding had reached equilibrium, only subunit B of ^{125}I-crotoxin was available for the subsequent cross-linking reaction. As revealed by autoradiography following SDS–PAGE, after subunit B of ^{125}I-crotoxin had bound to synaptosomal membrane, chemical cross-linking with DSS resulted in a predominant band of conjugation with a molecular weight of about 60000 (Figure 1). This band was absent when the reagent or membrane was omitted. The pattern of the autoradiogram was not affected by the presence of protease inhibitors in the preparation of the synaptosomes and in the coupling procedure (data not shown). Since only subunit B of crotoxin was cross-linked to the membrane, and it is reasonable to assume that 1 mol of the 60-kDa conjugate contains only 1 mol of the 14400-Da subunit B, then the molecular weight of the membrane entity in the conjugate would be ~45 kDa. When the affinity-labeled membranes were solubilized in the presence of 5% β-mercaptoethanol prior to electrophoresis, the electrophoretic mobility of the conjugate was only slightly slower than when the membranes were not treated with reducing agent (Figure 1). No other adducts were found when the amounts of ^{125}I-crotoxin mixed with 50 μg (in protein) of membrane were varied from 5×10^5 to 3×10^7 cpm. Three bifunctional reagents, DSS, DSP, and EGS, in different concentrations (from 0.05–1.5 mM) were employed in the cross-linking experiments. All these experiments gave results

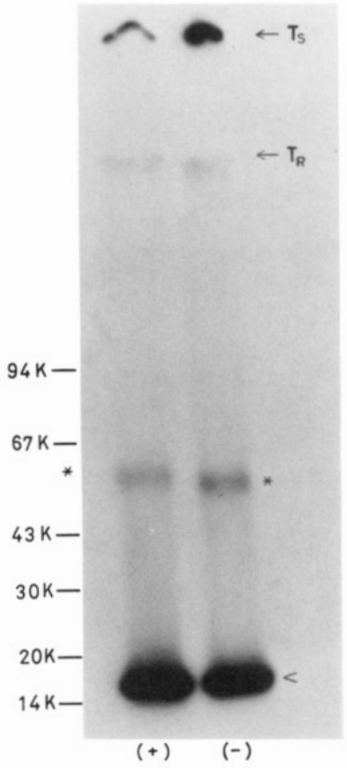

FIGURE 1: Cross-linking of ^{125}I-crotoxin to synaptosomes as analyzed by autoradiography after gel electrophoresis. After ^{125}I-crotoxin (7 ng) had bound to synaptosome preparation from guinea pig brain, DSS was added to a final concentration of 0.6 mM. The pellets after centrifugation were either treated with 5% β-mercaptoethanol or not treated and then subjected to SDS–polyacrylamide gel electrophoresis using a gradient of 7.5–15% acrylamide. Autoradiography was then carried out with 12-h exposure. (+) 5% β-mercaptoethanol treated; (−) untreated. The positions of the M_r markers are indicated (phosphorylase *b*, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 000; α-lactalbumin, 14 400). T_S, top of stacking gel; T_R, top of running gel; *, conjugate; <, subunit B of ^{125}I-crotoxin.

similar to those in Figure 1 except for the intensity of the conjugate bands; the optimal concentrations in our experiments were 0.5, 0.9, and 1.2 mM for DSP, DSS and EGS, respectively. Coomassie blue staining of the gels in control experiments showed that the protein patterns were not appreciably changed under the above conditions of cross-linking except for the appearance of some material retarded at the top of the stacking gel and of the running gel, which is typical of cross-linking experiments (data not shown). The formation of the radioactive conjugate was suppressed by unlabeled crotoxin with an IC_{50} around 10 nM as estimated from the autoradiogram in Figure 2 and like data. Therefore, we may deduce that this band represents the result of covalent joining of specifically bound ^{125}I-crotoxin (subunit B) to a particular toxin-binding molecule within the membrane.

Several other PLA_2s were also tested for their effectiveness in suppressing the formation of the 60-kDa band. Among those examined, Mojave toxin, taipoxin, *P. australis* PLA_2 (Pa-11), and pseudexin A completely inhibited the formation of this band at a concentration of 1 μM. Notexin and caudotoxin were somewhat less effective, while *N. naja atra* PLA_2 was only weakly inhibitive. In contrast, β-bungarotoxin and pancreatic PLA_2 showed no effect at 1 μM (Figure 3) and even at 50 μM (data not shown). From experiments similar to those in Figure 2, the IC_{50}s of the following PLA_2s were estimated to be ~10 nM for Pa-11 and Mojave toxin, ~50 nM for taipoxin and pseudexin A, ~0.5 μM for notexin and caudoxin, and ~5 μM for *N. naja atra* PLA_2. These values are comparable to our unpublished IC_{50} data of these PLA_2s in reversible binding studies. We have also radioiodinated five of those toxins with $IC_{50} \leq 0.5$ μM and examined their conjugation to the membrane, and in each case, results similar to

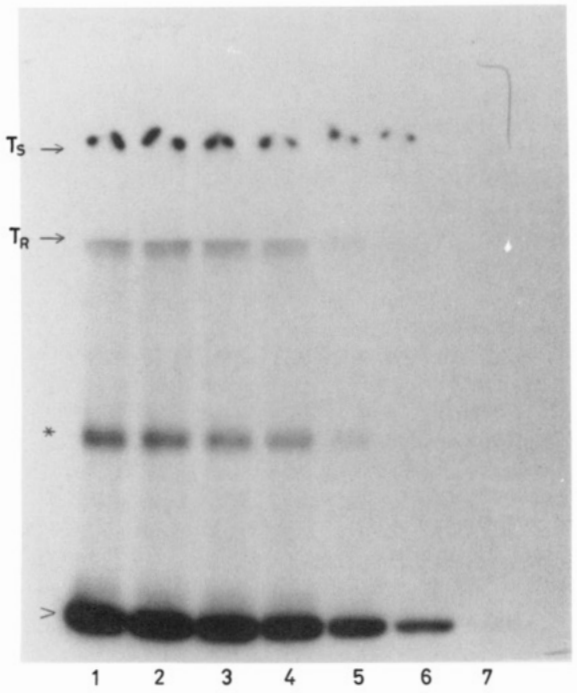

FIGURE 2: Effect of increasing amounts of native crotoxin on the cross-linking of ^{125}I-crotoxin to guinea pig brain synaptosomes. After ^{125}I-crotoxin (7 ng) had bound to synaptosomes, DSP was added to a final concentration of 0.5 mM. The pellets after centrifugation were subjected to SDS–polyacrylamide gel electrophoresis using a gradient of 7.5–15% acrylamide under nonreducing conditions. Autoradiography was then carried out with 12-h exposure. Lanes 1–7 are with 0, 10^{-10}, 10^{-9}, 10^{-8}, 2×10^{-8}, 5×10^{-8}, and 10^{-7} M, respectively, unlabeled crotoxin present during incubation of ^{125}I-crotoxin with synaptosomes. Symbols used are as in Figure 1.

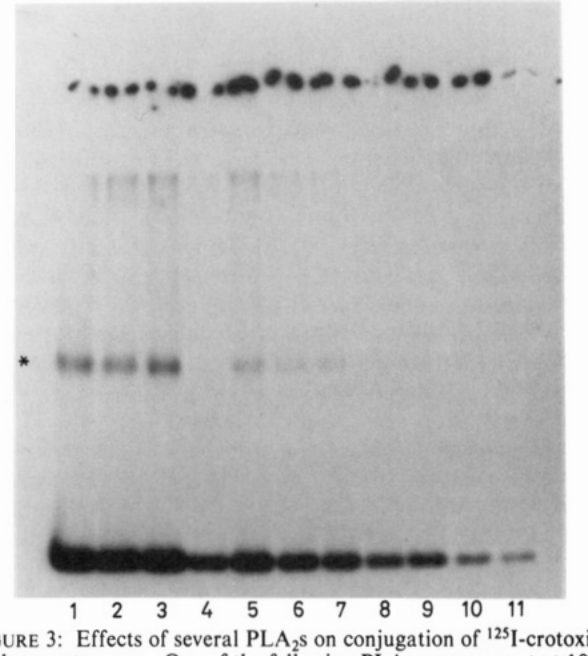

FIGURE 3: Effects of several PLA_2s on conjugation of ^{125}I-crotoxin with synaptosomes. One of the following PLA_2s was present at 10^{-6} M during incubation of ^{125}I-crotoxin (7 ng) with synaptosomes in the binding period. Other conditions are as those of Figure 2. Lanes 1–11: None, pancreatic PLA_2, β-bungarotoxin, pseudexin A from *P. porphyriacus*, *N. naja atra* PLA_2, caudoxin, notexin, *P. australis* PLA_2 (Pa-11), taipoxin, Mojave toxin, and unlabeled crotoxin. (*) indicates the 60-kDa band.

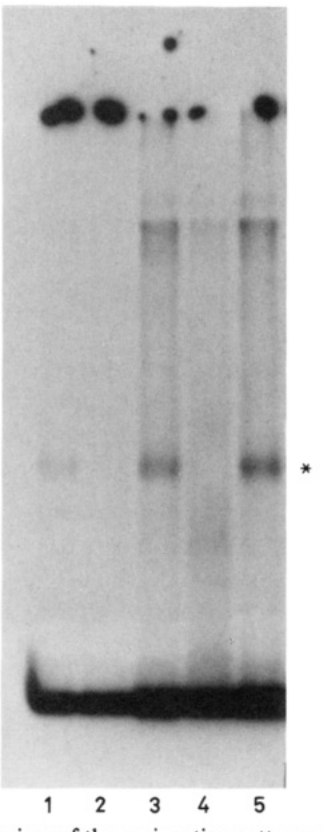

FIGURE 4: A comparison of the conjugation patterns of subfractions of P_2 labeled with ^{125}I-crotoxin. The subfractions of P_2 were incubated with ^{125}I-crotoxin (7 ng) and processed as in Figure 2. Lanes 1–4 are subfractions A, B, C, and D, respectively, of lysed synaptosome. Lane 5 is synaptosome (P_2). (*) indicates the 60-kDa band.

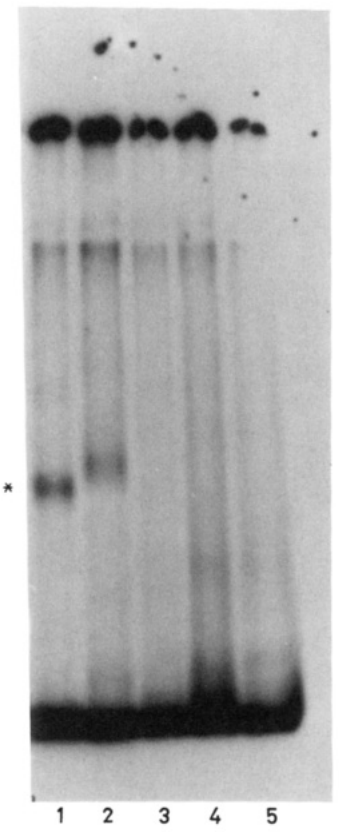

FIGURE 5: A comparison of the conjugation patterns of a variety of membrane preparations labeled with ^{125}I-crotoxin. Membrane preparations from different organs were incubated with ^{125}I-crotoxin (7 ng) and processed as in Figure 2. Lanes 1–5 are synaptosome (P_2), plasma membrane from kidney, resealed erythrocyte ghost, erythrocyte ghost, and plasma membrane from liver, respectively. (*) indicates the 60-kDa band.

those in Figure 1 were observed. When several K^+ channel blockers were tested, no effect on the 60-kDa conjugate formation was observed with quinine, quinidine, and 3,4-diaminopyridine at 10 mM (data not shown).

Pretreating the synaptosomes with proteinase K (200 μg/mL) rendered the membrane incapable of forming the 60-kDa conjugate with ^{125}I-crotoxin, and trypsin, subtilisin, α-chymotrypsin, and elastase showed different degrees of effectiveness in this respect, while thermolysin, leucine aminopeptidase, and protease of *S. aureus* were without effect. Conjugates of lower molecular weights were formed when cross-linking was performed with some protease-treated synaptosomes. Heating the synaptosomal membrane also rendered it incapable of conjugating with crotoxin (data not shown).

When the crude synaptosomal fraction P_2 was lysed and further fractionated, fraction C, which is enriched in synaptic membranes, showed the highest density in the membrane component capable of forming the 60-kDa conjugate, whereas this conjugate could not be detected with the intrasynaptosomal mitochondria (fraction D) (Figure 4). The labeling of fraction A may have resulted from the contamination of synaptic membranes. Erythrocyte ghost and plasma membranes prepared from kidney and liver did not form the 60-kDa conjugate, though a 65-kDa band of conjugate was observed with plasma membrane from kidney (Figure 5). The molecule in the kidney membrane appeared also to be a specific binding site for crotoxin, as the radioactive conjugate was inhibited by unlabeled crotoxin with an IC_{50} of 2.5×10^{-8} M.

Discussion

Subunit B of ^{125}I-crotoxin was covalently linked to a 45-kDa entity on synaptic membranes by three bifunctional reagents: DSP, DSS, and EGS. This 45-kDa substance appears to be a protein, as pretreatment of the synaptic membrane with some proteases or heat destroyed its ability to couple with crotoxin (subunit B). The 45-kDa crotoxin-binding protein consists of a single polypeptide chain, since the molecular weight of the conjugate was not significantly affected by reducing agents.

A previous study from our laboratory using the photoaffinity labeling technique revealed a 100-kDa conjugate formed by the coupling of crotoxin (subunit B) and a 85-kDa protein in the synaptosomes of guinea pig brain (Hseu et al., 1990). An adduct of similar size was not obtained in this study. Because the conjugate pattern was not affected by the presence of protease inhibitors in the cross-linking experiments of this study, we can exclude the possibility that the 45-kDa protein is a proteolytic degradation product of the 85-kDa protein. Different polypeptide labeling patterns using different labeling techniques have also occurred in several other cases (Schmid-Antomarchi et al., 1984; Seagar et al., 1985; Barhanin et al., 1988; Lambeau et al., 1989; Schmidt & Betz, 1989). Judging from the difference in the effects of K^+ channel blockers, such as quinine, and the change in molecular weight of the conjugates under reducing or nonreducing conditions, the binding protein identified in the present study is different from the binding proteins for β-bungarotoxin and for OS_2, a neurotoxic PLA_2 from *Oxyuranus scutellatus* venom (Rehm & Betz, 1983; Lambeau et al., 1989). The taipoxin-binding protein identified previously in this laboratory is of essentially the same molecular weight as the crotoxin-binding protein reported here. However, crotoxin shows higher affinity toward the 85-kDa and the 45-kDa crotoxin-binding proteins than toward the taipoxin-binding protein, whereas the

affinities of taipoxin toward the binding proteins are in decreasing order of taipoxin-binding protein, 85-kDa crotoxin-binding protein, and 45-kDa crotoxin-binding, as judged from the potencies of the toxins in suppressing the conjugate formation (Tzeng et al., 1989; Hseu et al., 1990; this study). Therefore, it seems that the 45-kDa binding protein for crotoxin is different from the 45-kDa taipoxin-binding protein. Direct proof must await purification of the binding proteins. Among the other toxic PLA_2s tested, several demonstrated strong to weak ability to inhibit the formation of the 60-kDa conjugate, β-bungarotoxin being exceptional. Thus, the binding protein reported here is a common binding site for several toxic PLA_2s and is different from the K^+ channel protein responsible for β-bungarotoxin binding.

The 60-kDa conjugate was not found when three nonneuronal membranes were used; therefore, virtually no 45-kDa crotoxin-binding protein is present in these membranes. We may conclude that the 45-kDa protein is likely to be uniquely present in neuronal membranes and represents a new binding protein for crotoxin in that tissue. Our finding that crotoxin formed other adducts with some nonneuronal membrane is a reflection of the fact that crotoxin acts on many tissues in addition to the neurons. Apparently, the neuron manufactures "toxin-binding proteins" not for the purpose as such. The physiological roles of the 45-kDa crotoxin-binding protein remain to be explored. Experiments are underway to isolate and purify the 45-kDa binding protein for structural and functional studies.

Registry No. PLA_2, 9001-84-7; crotoxin, 9007-40-3; β-bungarotoxin, 12778-32-4; caudoxin, 82799-95-9; notexin, 37223-96-4; taipoxin, 52019-39-3.

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