Identification of a Crotoxin-Binding Protein in Membranes from Guinea Pig Brain by Photoaffinity Labeling

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Abstract

Crotoxin is a neurotoxic phospholipase A_2 capable of blocking synaptic transmission by inhibiting the release of neurotransmitters. The photoaffinity labeling technique was used to identify the neural membrane molecules involved in the binding of crotoxin. A photoactivatable, radioactive derivative of crotoxin was synthesized by reacting crotoxin with N-hydroxysuccinimidyl-4-azidobenzoate and with $Na[^{125}I]$. Photoirradiation of synaptosomes from guinea pig brains in the presence of the crotoxin derivative resulted in the formation of a major radioactive conjugate of 100,000 daltons as revealed by autoradiography of a sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern. Pretreatment of the synaptosomes with trypsin, *Staphylococcus aureus* protease, or papain prevented the formation of this conjugate. The conjugate was not detected when plasma membranes from several nonneural tissues replaced the brain synaptosomes. Unmodified crotoxin inhibited the formation of this adduct with an IC_{50} of about 10^{-8} M. Mojave toxin, caudoxin, notexin, *Naja naja* PLA, and taipoxin also inhibited adduct formation with different potencies, while β-bungarotoxin and pancreatic PLA were ineffective. We concluded that an 85,000-dalton protein is the major component responsible for the binding of crotoxin to synaptosomal membranes.

Key Words: Crotoxin; phospholipase A_2; neurotransmitters; photoaffinity labeling; synaptosomes.

Introduction

Certain phospholipases A_2 (PLA$_2$, E.C. 3.1.1.4.)[5] are highly neurotoxic, owing to their blockage of synaptic transmission by their inhibition of the

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[5] Abbreviations: BSA, bovine serum albumin; HSAB, N-hydroxysuccinimidyl-4-azidobenzoate; PLA, phospholipase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

release of neurotransmitters (Howard and Gunderson, 1980). While the pharmacological and electrophysiological aspects of the toxic effects have been extensively studied, the molecular mechanism of action has not been established. Crotoxin from the Brazilian rattlesnake *Crotalus durissus terrificus* is the first neurotoxin purified to a high degree and one of the first crystallized neurotoxic proteins exhibiting enzymatic activities (Slotta and Fraenkel-Conrat, 1938). This toxin is composed of two subunits: a basic subunit B of 14,400 dalton possessing phospholipase A_2 activity, and an acidic subunit A (also called crotapotin) of 9,000 dalton (Rubsamen *et al.*, 1971; Hendon and Fraenkel-Conrat, 1971; Aird *et al.*, 1985, 1986).

It has been established that enzyme activity is required for synaptic blockage by the neurotoxic PLA_2s, (Howard and Gunderson, 1980). More recently, we and others have demonstrated that the neurotoxic PLA_2s show high-affinity specific binding to synaptic membranes, whereas only weak, nonspecific binding was found with nontoxic PLA_2 (Rehm and Betz, 1982; Othman *et al.*, 1982; Tzeng *et al.*, 1986). Therefore, it is reasonable to assume that strong binding to neural membranes is a necessary condition for neurotoxic PLA_2s' effectiveness. It would be desirable to obtain information about the membrane components involved in binding the neurotoxic PLA_2s. In the present study, we have used the photoaffinity labeling technique to identify a protein of apparent mol. wt. 85,000 as the major molecule in the synaptic membrane required for crotoxin binding. To our knowledge, this report identifying a specific binding protein for presynaptically active crotoxin is the first successful use of the photoaffinity labeling technique in delineating presynaptic toxin binding sites. Rehm and Betz (1983) have applied the photochemical crosslinking technique in their identification of a β-bungarotoxin binding peptide.

Materials

Porcine pancreatic PLA_2 and the crude venoms of the South American rattlesnake *Crotalus durissus terrificus*, the Formosan banded krait *Bangarus multicinctus*, the Australian taipan *Oxyuranus scutellatus*, and the horned puff adder *Bitis caudalis* were purchased from Sigma Chemical Co. (St. Louis). Notexin purified from the Australian tiger snake *Notechis scutatus scutatus* was a kind gift of Dr. C. C. Yang, National Tsing Hua University. *N*-Hydroxysuccinimidyl-4-azidobenzoate (HSAB) was obtained from Pierce Chemical Co. (Rockford). Na[^{125}I] was from New England Nuclear (Boston). Sephadex G-25 was from Pharmacia. Molecular weight standards used to calibrate SDS-PAGE were also purchased from Sigma. All other chemicals were of reagent grade.

Methods

Preparation of Toxins

Purification of crotoxin, β-bungarotoxin, taipoxin, caudoxin, and *Naja naja* PLA from the crude venoms was carried out according to published procedures (Rubsamen *et al.*, 1971; Fohlman *et al.*, 1976; Hanley *et al.*, 1977; Viljeon *et al.*, 1982; Cate and Bieber, 1978; Lo *et al.*, 1966). Synaptosomes were prepared from guinea pig brain according to Whittaker (1959); plasma membrane fractions from the liver, kidney, and erythrocyte of the guinea pig were obtained according to published procedures (Neville, 1960; Booth and Kenny, 1976; Steck *et al.*, 1970). The protein content of the synaptosomes and plasma membrane fractions was determined as described by Lowry *et al.* (1951), using bovine serum albumin (BSA) as a standard.

Preparation of Photoactive Crotoxin

N-Hydroxysuccinimidyl-4-azidobenzoate (HSAB) in dimethyl sulfoxide was added to crotoxin in 50 mM sodium phosphate buffer, pH 7.4, at various ratios. The reaction mixture was thoroughly mixed and then incubated at room temperature for 20 min in the dark. After incubation, 1 N glycine in 50 mM Tris HCl, pH 7.4, was added to make a final concentration of 0.1 N and the incubation continued for another 10 min so as to scavenge free HSAB. The concentrations of HSAB and crotoxin were quantitatively determined spectrophotometrically (Hendon and Fraenkel-Conrat, 1976; Buckland *et al.*, 1985). The reaction mixture was then applied to a Sephadex G-25 column (0.8 × 50 cm) and eluted with 50 mM Tris-HCl, pH 7.4. To calculate the efficiency of the reaction between HSAB and crotoxin, the crotoxin derivative, in quartz cells, was subjected to photoirradiation using a short-wave U.V. lamp at a distance of 5 cm. Irradiation was carried out at room temperature for short periods of time. Absorbance at 275 nm was measured every 30 sec, giving an index of azido activation. Mathematical calculations were performed according to a published method (Buckland *et al.*, 1985; Ji, 1977). Under the reaction conditions employed, at molar ratio, up to 3 : 1 for HSAB : crotoxin, 20% of HSAB reacted with crotoxin. The modified toxin showed the same neurotoxicity as that of the unmodified preparations.

Radioiodination of Crotoxin

Crotoxin or photoactive crotoxin was labeled with Na[^{125}I] using the chloramine-T method (Hunter and Greenwood, 1962). Free ^{125}I was removed by separation on a sephadex G-25 column (0.8 × 50 cm), equilibrated with 1% BSA in 50 mM Tris-HCl, pH 7.4. The peak in the void volume

was collected. In the case of photoactive crotoxin, all glassware was wrapped in aluminum foil and all manipulations were conducted in a dark room. Both ligands were radioiodinated to a specific activity of 50–100 Ci/g.

Photoaffinity Labeling of ^{125}I-Labeled Photoactive Crotoxin to Synaptosomes

The ^{125}I-labeled crotoxin derivative was mixed with synaptosomes or plasma membranes from other tissues (0.2 mg protein per ml) in 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% BSA, 150 mM NaCl, 10 mM SrCl$_2$, and 1.0 mM EGTA at 30°C for 2 h, and samples were diluted with ice-cold 0.2 M Tris-HCl, pH 7.4, and centrifuged at 10,000 × g for 5 min. The pellet was washed with the buffer and finally resuspended with 600 μl of ice-cold 50 mM sodium phosphate buffer, pH 7.4. The 1.5-ml Eppendorf vial containing 600 μl of synaptosome suspension was dipped into ice-cold water. Photo-irradiation (4 min) was conducted using a Minerallight (UV-254/366 nm, 0.16 A) positioned 5 cm above the vials. Following a 2-min pause the photo-irradiation was repeated. The mixture was diluted with 400 μl of ice-cold 0.2 M Tris-HCl buffer, pH 7.4, and photoirradiation continued for an additional 10 min in order to quench the unreacted ligand (Hazum, 1983; Wong et al., 1978). The samples were centrifuged as above and the pellets dissolved in 60 μl of 0.1 M Tris-HCl buffer, pH 6.8, containing 5% glycerol and 2% SDS by incubation for 1 hour at 30°C (Iyengar and Herberg, 1984).
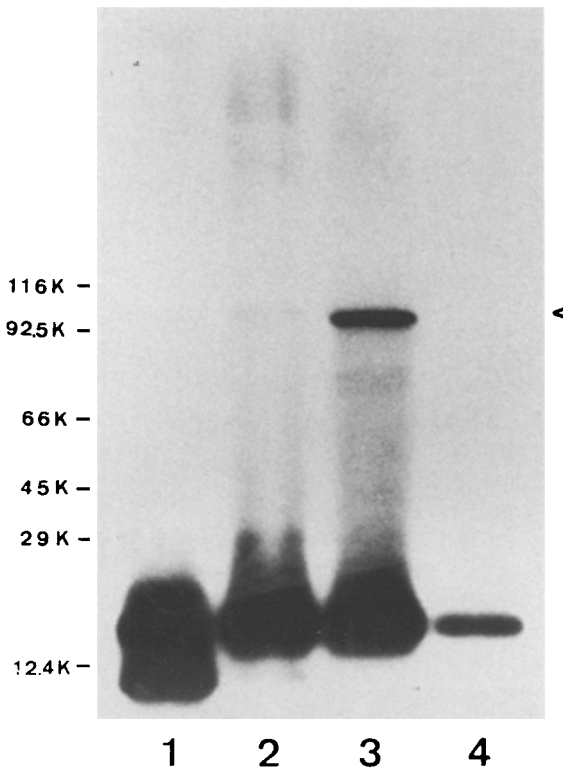
SDS-PAGE and Autoradiography

SDS-polyacrylamide gel electrophoresis was performed using a 5–15% gradient of polyacrylamide and the discontinuous buffer system of Neville (1971). Electrophoresis was carried out at room temperature for 5–8 h at a constant current density of 1 mA/cm. Following the electrophoresis the gel was dried on filter paper under vacuum at 80°C for 1 h. Agfa-Gaevart film was layered over the dried gel and allowed to remain at −80°C for 6–8 h using an intensifying screen. Precolored proteins prepared according to Tzeng (1983) were used as molecular weight standards. For determination of incorporation efficiency, radioactive bands localized by autoradiography were cut from the dried gels and counted by a gamma counter. Autoradiographs shown are representative of at least three experiments.

Treatment with Proteases

Synaptosomes (500 μg of protein/ml) were exposed to the appropriate protease (25–200 μg/ml) in 50 mM phosphate buffer (Hunter and Greenwood, 1962), pH 8.0. Incubations were carried out at room temperature (25°C) for

30 min in a final volume of 200 μl. The proteolytic reaction was stopped by addition of either trypsin inhibitor to a final concentration of 200 μg/ml or by dilution with ice-cold phosphate buffer, cooling with ice water for 10 min, and centrifugation at 10,000 $\times$ g for 5 min to remove the inactivated protease. The pellets were resuspended in 50 mM Tris- HCl, pH 7.4, containing 150 mM NaCl, 10 mM SrCl$_2$ and 1.0 mM EGTA.

Fig. 1. Photoaffinity labeling pattern as revealed by autoradiography following SDS-PAGE. Crotoxin was coupled with N-hydroxysuccinimidyl-4-azidobenzoate and then labeled with [^{125}I]. The modified toxin (1.3 nM) was incubated with a synaptosome preparation from guinea pig brains and irradiated with UV light as described under Methods. Autoradiography was carried out following SDS-PAGE using a 5–15% acrylamide gel. Lane 1: photoactive [^{125}I]crotoxin alone without photoirradiation. Lane 2: photoactive [^{125}I]crotoxin and synaptosomes without photoirradiation. Lane 3: synaptosomes irradiated in the presence of the [^{125}I]crotoxin photoprobe analogue. Lane 4: as with Lane 3 except that 10^{-7} M unmodified crotoxin was present during the binding phase of the experiment.

Results

Photoirradiation of the synaptosomal membranes in the presence of the photoactivatable [^{125}I]crotoxin produced one major band of radioactivity having a molecular weight of about 100,000 in addition to the protein band representing subunit B of crotoxin in the autoradiogram following SDS-PAGE of the membrane pellet (Fig. 1, lane 3). When incubated in the presence of 10^{-7} M native crotoxin the incorporation of radioactivity into the 100K band was prevented (Fig. 1, lane 4). Control experiments carried out
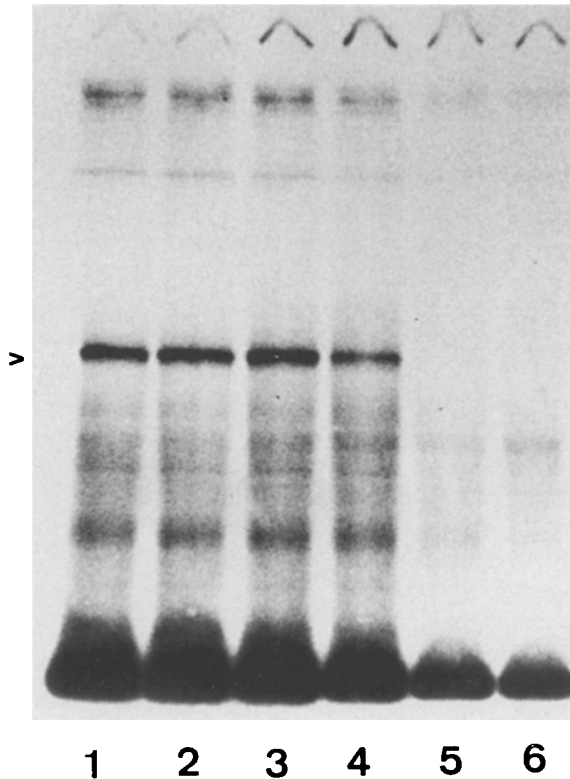
Fig. 2. Effect of increasing amounts of native crotoxin on the photoaffinity labeling pattern of guinea pig brain synaptosomes. Synaptosomes were incubated and photoirradiated in the presence of 5 nM photoactive [^{125}I]crotoxin and varying amounts of native crotoxin. Other conditions were as in Fig. 1. The arrowhead indicates the 100K band. Lane 1 to 6 contain 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M native crotoxin, respectively.

without photoirradiation (Fig. 1, lane 2) or in the absence of synaptosomes (data not shown) did not show this band. Therefore, we may assume that the generation of this labeled band is the result of photoactivated crosslinking of specifically bound [^{125}I]crotoxin to a particular toxin-binding component within the membrane. The apparent molecular weight of this band did not change with chemical reduction of the preparation (data not shown). In addition to the major conjugate, several minor ones were observed whose presence varied depending upon the experimental conditions.

When the influence of varying concentrations of native crotoxin on the formation of the 100K conjugate was examined, the radioactive intensities in the adduct varied in proportion to the toxin concentrations (Fig. 2). Estimated from the figure, the IC_{50} was approximately 10 nM, close to the IC_{50} of 11 nM reported for the binding of [^{125}I]crotoxin to the membrane (Tzeng *et al.*, 1986). The formation of the minor conjugates was also suppressed by the presence of unmodified crotoxin with slightly higher IC_{50}, as judged from lane 5 of Fig. 2.
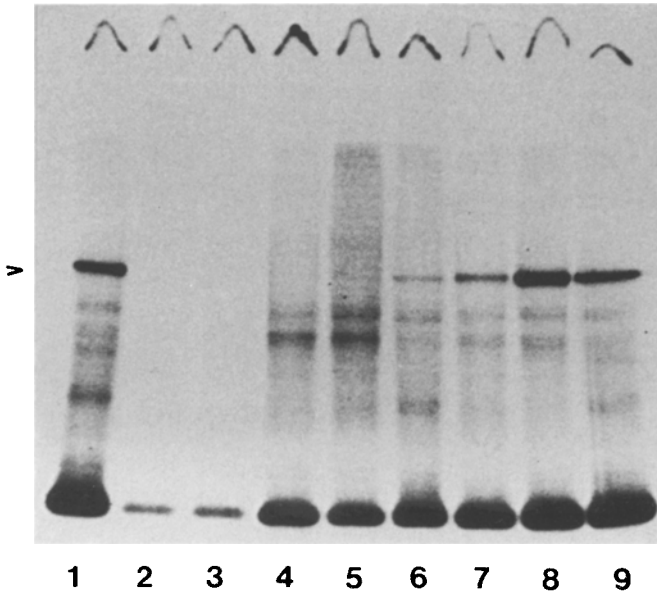
Fig. 3. Effects of several PLA$_2$s on the photoaffinity labeling pattern. Synaptosomes were incubated with 3 nM photoactive [^{125}I]crotoxin together with different PLA$_2$s. Other procedures were as in Fig. 2. (1) Synaptosomes irradiated in the presence of [^{125}I]crotoxin photoprobe analogue; (2) 10^{-7} M crotoxin added; (3) 10^{-7} M Mojave toxin added; (4) 10^{-7} M taipoxin added; (5) 10^{-7} M caudoxin added; (6) 10^{-7} M notexin added; (7) 10^{-5} M *Naja naja* PLA added; (8) 10^{-5} M porcine pancreatic PLA$_2$ added; (9) 10^{-5} M β-bungarotoxin added.

Mojave toxin mimicked the action of crotoxin in abolishing the photodependent formation of the conjugates. Among other phospholipase A_2s tested, taipoxin, caudoxin, and notexin each effectively inhibited the formation of the 100K band at a concentration of $0.1\,\mu M$. At $10^{-5}\,M$ *Naja naja* PLA selectively reduced half of the labeling of this band. In contrast, β-bungarotoxin and porcine pancreatic PLA_2 showed no effect even at $10^{-5}\,M$ (Fig. 3).

Figure 4 demonstrates the effect of pretreating the synaptosomes with various proteases on the photodependent formation of this conjugate.
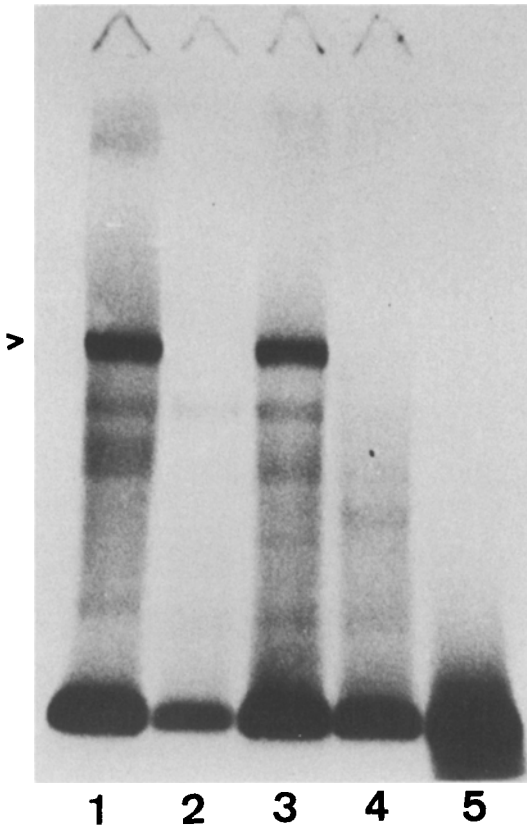
Fig. 4. Effect of proteolytic pretreatment of synaptosomes on the photoaffinity labeling pattern. The synaptosome preparation was preincubated with specific proteases. After removing the proteases by centrifugation, photoaffinity labeling experiments were carried out as described in Fig. 1. (1) No protease treatment; (2) trypsin, $200\,\mu g/ml$; (3) chymotrypsin, $50\,\mu g/ml$; (4) protease of *S. aureus* V8, $25\,\mu g/ml$; (5) papain, $50\,\mu g/ml$.
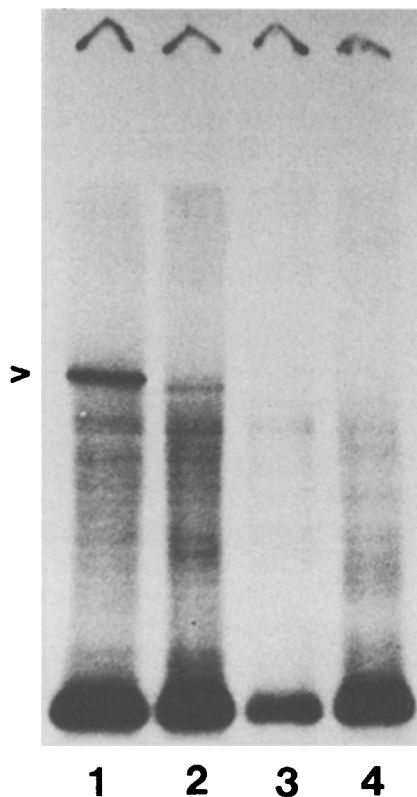
Fig. 5. A comparison of a variety of membranes for their ability to react photochemically with the photoaffinity analogue of [^{125}I]crotoxin. Membrane preparations from different organs were incubated with the photoactive [^{125}I]crotoxin and processed as in Fig. 1. Lane 1: synaptosome. Lane 2: plasma membrane from kidney. Lane 3: plasma membrane from liver. Lane 4: erythrocyte ghost.

Trypsin, protease from *S. aureus* V8, and papain all rendered the membrane incapable of forming the 100K conjugate with the derivatized crotoxin, while chymotrypsin was without effect (Fig. 4).

We have also compared four membrane preparations for photodependent labeling by the radioactive crotoxin analogue (Fig. 5). The labeling patterns observed for the four membranes were different. Labeling by the analogue to form a 100K band was not observed with membranes from the liver, kidney, and from erythrocyte ghost. With kidney and with erythrocyte membranes, several weak bands of the conjugates were seen without, however,

a clearly dominantly labeled band. In the case of liver membranes, few conjugates could be observed, although the same conditions were used in all experiments with different membranes. It is possible that labeling intensity observed with liver membranes was decreased because the bound radioactivity was readily dissociated from the liver membrane in the washing step prior to photoirradiation.

Discussion

We have demonstrated that $[^{125}I]$crotoxin carrying a photoactive group can be irreversibly incorporated into synaptosomal membranes in a specific manner. The formation of this covalently linked conjugate of an apparent M_r of 100,000 was inhibited by native crotoxin with an IC_{50} of about 10 nM, quantitatively consistent with the previously demonstrated specific binding of crotoxin to the membrane (Tzeng, 1986). The formation of this conjugate was also reduced to varying extents by several other PLA$_2$s. Pancreatic PLA and β-bungarotoxin were, on the other hand, not effective even at high concentrations (Fig. 2). The variable occurrence of several minor conjugates may represent binding sites for crotoxin with lower affinities, although we could not exclude the possibility that some of the minor conjugates may result from crosslinking of the derivatized crotoxin with proteins that are associated with or in the neighborhood of the crotoxin-binding component(s) in the membranes. Significantly, among the other PLA$_2$s tested, only Mojave toxin could antagonize the formation of these minor bands. Thus, the binding site constituting the 100K conjugate is unique in being of higher affinity and a common binding site for several PLA$_2$s. At a molar ratio of less than 3:1 for HSAB:crotoxin during synthesis of the crotoxin photoprobe analogue, the product contains less than one photoactive group on the average (see Methods). Since only the PLA$_2$ subunit can bind to membranes (Tzeng et al., 1986), it is reasonable to assume that only one PLA$_2$ subunit was incorporated in the 100K conjugate. The M_r of the PLA$_2$ subunit is 15,000; therefore the apparent M_r of the membrane component in the conjugate is judged to be about 85,000. This 85K component appears to be a protein, as pretreatment of the membrane with trypsin, protease V8, or papain destroyed the ability of the membrane to form the conjugate. The 85K protein consists of a single polypeptide chain, since the molecular weight of the conjugate was not affected by reducing agents.

It is significant that the three non-neural membranes tested did not show existence of an 85K component as inferred from the absence of the 100K conjugate. Since specific binding could be demonstrated with each of the three membranes (Tzeng et al., 1986), we may conclude that the 85K protein

is unique for neural membranes and that it represents the major binding site for crotoxin in that tissue. The demonstration that there exists a large number of proteins capable of interaction with crotoxin, although with low affinities, may explain the fact that crotoxin acts on many tissues (Vital-Brazil, 1972; Breithaupt, 1976; Hawgood and Smith, 1977; Bon et al., 1979) in addition to its unique and specific binding to neural tissue.

Acknowledgment

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