Neurotoxic Phospholipases A_2 Ammodytoxin and Crotoxin Bind to Distinct High-Affinity Protein Acceptors in *Torpedo marmorata* Electric Organ†

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ABSTRACT: We studied the binding of radioiodinated ammodytoxin C, a monomeric phospholipase A_2 neurotoxin from *Vipera ammodytes*, and of radioiodinated crotoxin, a dimeric phospholipase A_2 neurotoxin from *Crotalus durissus terrificus*, to presynaptic membranes from the electric organ of *Torpedo marmorata*. In both cases, two different families of specific binding sites were identified and characterized. The high-affinity binding sites for both toxins have been shown to be proteins. The low-affinity binding sites were not affected by proteinases or heat, suggesting the involvement of certain lipid structures in this type of binding. By affinity-labeling, [^{125}I]ammodytoxin C was shown to be associated predominantly with membrane proteins of apparent molecular masses of 70 000 and 20 000 Da and to a lesser extent with several proteins of apparent molecular masses ranging between 39 000 and 57 000 Da. [^{125}I]crotoxin, on the other hand bound primarily to a 48 000 Da membrane protein. All phospholipases A_2 tested, except β-bungarotoxin, inhibited the low-affinity specific binding of ammodytoxin C, whereas only neurotoxic phospholipases A_2 prevented the high-affinity binding and the cross-linking of ammodytoxin C and crotoxin. The inhibition profiles of high-affinity binding for [^{125}I]crotoxin and for [^{125}I]ammodytoxin C were quite different. Ammodytoxin C and crotoxin did not inhibit each other on their respective high-affinity binding sites. These observations indicate that at least high-affinity binding sites of these two toxins are different. In contrast with crotoxin, the isolated basic subunit CB of crotoxin was able to completely inhibit the high-affinity binding of [^{125}I]ammodytoxin C. Therefore, the acidic subunit CA of crotoxin does not simply act as a *chaperone* for CB subunit, but it also confers a distinct binding specificity to the crotoxin.

Phospholipases A_2 (PLA_2)[1] are enzymes (EC 3.1.1.4) which catalyze the hydrolysis of 1,2-diacyl-3-*sn*-phosphoglycerides at the 2-acyl ester bond. Some of them, primarily those found in snake venoms, are also potent neurotoxins (β-neurotoxins), which block the release of the neurotransmitter, ACh, from peripheral neurons [reviewed by Hawgood and Bon (1991)], by a mechanism which is not yet well understood. The PLA_2 enzymatic activity of β-neurotoxins was shown to be necessary but not sufficient to block neuromuscular transmission [Marlas & Bon, 1982; reviewed by Chang (1985)]. When neurotoxic PLA_2s were shown to bind with high affinity to specific sites in different neuronal preparations (Tzeng, 1993; Lambeau et al., 1989; Délot & Bon, 1993; Križaj et al., 1994, 1995), it became clear that PLA_2-neurotoxicity is an acceptor-mediated process.

The pathophysiological and electrophysiological effects of diverse β-neurotoxins are very similar, if not identical, in spite of their distinctly different structural organizations [reviewed by Hawgood and Bon (1991)]. The physiological role of a neuronal acceptor has been clarified only in the case of β-Butx from the venom of the Taiwan banded krait (*Bungarus multicinctus*); this toxin binds to a subpopulation of α-Dtx neuronal receptors which were shown to be voltage-dependent K^+ channels (Black & Dolly, 1986; Scott et al., 1990). β-Butx is a two-chain PLA_2 neurotoxin, in which subunits A and B are linked by a disulfide bond. The A-chain is a PLA_2, while the B-chain, which is an α-Dtx homologue, is responsible for the binding specificity of β-Butx. All other β-neurotoxins so far tested were unable to compete with β-Butx for its neuronal binding site. In single-chain β-neurotoxins, like Atx, the toxin domain implicated in the toxin binding to its acceptor is obviously located on this unique polypeptide. The situation is, however, still unclear in the case of multichain PLA_2 neurotoxins, such as crotoxin, in which subunits are noncovalently associated.

We investigated the interaction of Atx and crotoxin with their neuronal targets, taking advantage of the considerable information available concerning their structure, the existence of variants and their homology. Atx is a single-chain

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[1] Abbreviations: ACh, acetylcholine; Agtx, agkistrodotoxin; $AtnI_2$, ammodytin I_2; Atx, ammodytoxin; Bo, maximal binding capacity; Bns, non-specific binding; Bsp, specific binding; Bt, total binding; BSA, bovine serum albumin; β-Butx, β-bungarotoxin; CA, acidic subunit of crotoxin; CB, basic subunit of crotoxin; α-Dtx, α-dendrotoxin; DSS, disuccinimidyl suberate; DTT, dithiothreitol; IC_{50}, concentration of an inhibitor which causes 50% inhibition; Kd, dissociation constant; PLA_2, phospholipase A_2; OS_2, *Oxyuranus scutellatus* phospholipase A_2; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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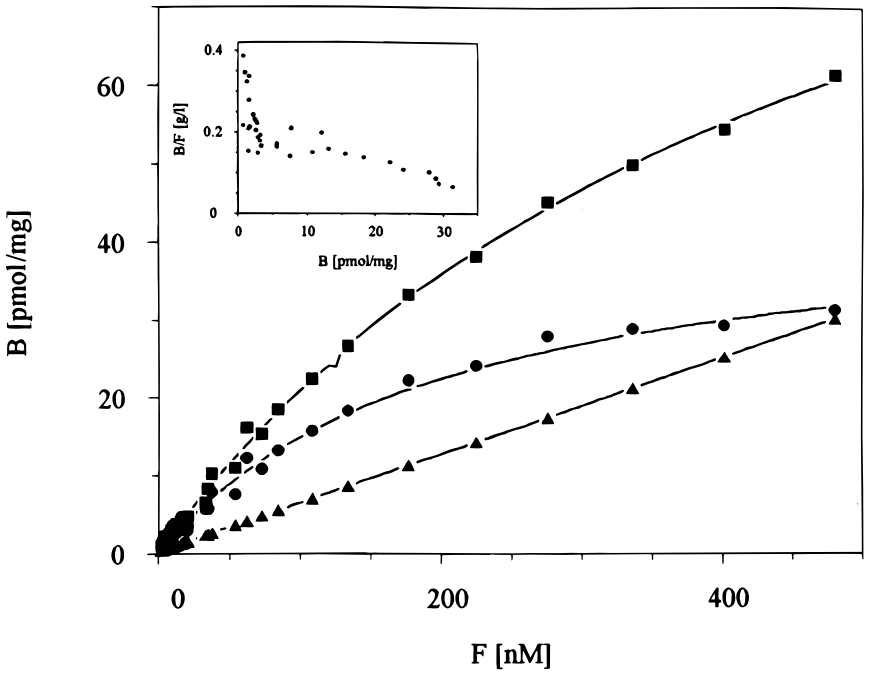

FIGURE 1: Equilibrium binding of [^{125}I]AtxC to *Torpedo marmorata* synaptic membranes. Concentrations of membrane-bound [^{125}I]AtxC (B) and free toxin (F) were determined at equilibrium as described in Experimental Procedures. Specific binding (●) is defined as the difference between total (■) and nonsaturable binding (▲), determined in the presence of a 200-fold excess of native over labeled AtxC. Average values of duplicate determinations are plotted. Inset: Scatchard plot of the specific binding.

β-neurotoxin. Several isoforms (AtxA, AtxB, and AtxC) have been isolated from *Vipera ammodytes ammodytes* venom (Gubenšek et al., 1980; Ritonja & Gubenšek, 1985; Ritonja et al., 1986; Križaj et al., 1989). Crotoxin, isolated from the venom of *Crotalus durissus terrificus*, is a noncovalent dimer with a basic, weakly toxic PLA_2 subunit (CB) and an acidic, nontoxic and enzymatically inactive subunit (CA), which enhances the lethal potency of CB (Hendon & Fraenkel-Conrat, 1971; Rübsamen et al., 1971; Horst et al., 1972). Several crotoxin isoforms deriving from the combination of subunit isoforms have been characterized and the origin of their diversity was established (Faure et al., 1991, 1993, 1994). The sequences of Atx and CB show about 60% identity (Ritonja & Gubenšek, 1985; Ritonja et al., 1986; Križaj et al., 1989; Aird et al., 1986). Both crotoxin and Atx, like β-Butx, inhibit K^+ currents at the nerve terminus (Rowan & Harvey, 1988, Križaj et al., 1995). However, the effects of crotoxin on ACh release from *Torpedo marmorata* synaptosomes were different from those of CB, AtxA or Agtx, another single-chain PLA_2 neurotoxin (Délot & Bon, 1992; Choumet et al., 1993).

In previous studies, the membrane binding properties of Atx and crotoxin have been mainly investigated on distinct preparations so that their characteristics could not be directly compared. A specific acceptor for Atx was identified and characterized in bovine cortex synaptic membranes (Križaj et al., 1994, 1995), while crotoxin binding was analyzed on guinea pig brain preparations (Tzeng et al., 1986; Hseu et al., 1990; Degn et al., 1991; Yen & Tzeng, 1991; Tzeng, 1993) and *Torpedo marmorata* synaptic membranes (Délot & Bon, 1993). In all cases, CB was shown to be a potent inhibitor of Atx specific binding, while Atx proved to be a weak inhibitor of crotoxin specific binding. Crotoxin dissociates when it interacts with biological membranes or phospholipid vesicles: CB remains associated with the membranes or the vesicles while CA is released into solution (Bon et al., 1979; Tzeng et al., 1986; Radvanyi et al., 1989). It was speculated that CA acts as a *chaperone* (Habermann & Breithaupt, 1978; Bon et al., 1979), preventing the nonspecific binding of CB. Two possible mechanisms of the interaction of crotoxin with presynaptic membranes were suggested: a dissociation model in which CB alone interacts with acceptor and a transient ternary complex model in which both subunits participate in toxin−acceptor interaction (Délot & Bon, 1993).

In the present work, we investigated and compared the mode of interaction of these two structurally different β-neurotoxins with presynaptic membranes from *Torpedo* electric organ. The corresponding membrane acceptors were identified and their subunit composition was analyzed by chemical cross-linking. This study provides experimental support for the formation of a ternary complex in which CA targets CB to a specific crotoxin acceptor on presynaptic neuronal membranes. It suggests that isolated CB interacts with a different acceptor.

EXPERIMENTAL PROCEDURES

Materials. AtxA, AtxC, and $AtnI_2$ were purified from the venom of *Vipera ammodytes ammodytes* (Gubensek et al., 1980; Križaj et al., 1992). Crotoxin was isolated from the venom of *Crotalus durissus terrificus*, and its subunits CA and CB were separated as described by Hendon and Fraenkel-Conrat (1971). Crotoxin was subsequently reconstituted from isoforms CA_2 and CB_d (Hendon & Fraenkel-Conrat, 1971; Faure & Bon, 1988; Faure et al., 1991) and repurified on a Mono Q column to separate the complex from isolated subunits. Notexin was a generous gift from Dr. S. Gasparini (CEA, Saclay, France). OS_2 was kindly provided by Dr. G. Lambeau (Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, Valbonne, France). Taipoxin, β-Butx, and porcine pancreatic PLA_2 were purchased from Sigma. Agtx was purified from the venom of *Agkistrodon blomhoffii*

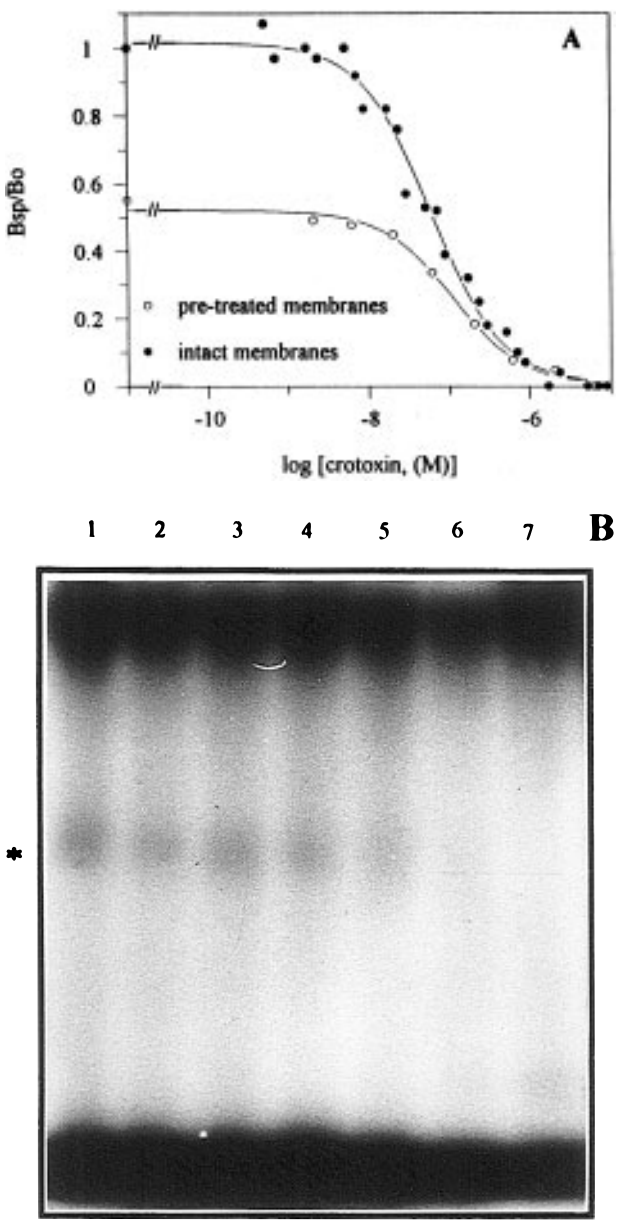

FIGURE 2: Estimation of dissociation constants for [^{125}I]crotoxin binding to *Torpedo* synaptic membranes. (A) The dissociation constant for crotoxin low-affinity binding to synaptic membranes (Kd_2) was obtained from the homologous competition experiment on preheated membranes (○). For the comparison also the homologous competition assay on native membranes is shown (●). (B) The estimate of the dissociation constant for crotoxin high-affinity binding to synaptic membranes (Kd_1) was obtained from the effect of increasing concentrations of unlabeled crotoxin, during the incubation of [^{125}I]crotoxin with synaptic membranes, on the specific adduct formation (lane 1−7: 0 M, 1 nM, 3.34 nM, 10 nM, 33.4 nM, 100 nM, and 1 μM of unlabeled crotoxin). The formation of the conjugate was half-inhibited (IC_{50}) in the presence of approximately 40 nM of unlabeled crotoxin in the incubation mixture. Experimental details are described in Experimental Procedures.

brevicaudus as described by Chen et al. (1981). Na[^{125}I] (carrier-free) was obtained either from Du Pont-New England Nuclear (Les Ulis, France) or from Amersham (Les Ulis, France). Proteinases and glycosidases were from Sigma (St. Louis, MO) or from Boehringer (Mannheim, Germany). Dinonyl phthalate was obtained from Fluka (Buchs, Switzerland) and Dow-Corning MS-550 silicone fluid from BDH (Poole, England). CM-Sepharose CL-6B and Sephadex G-25, fine, were from Pharmacia (Uppsala, Sweden) and DSS was from Pierce (Rockford, IL). DTT was from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade. In order to minimize the adsorption of proteins, particularly at low concentrations, low protein adsorption test tubes (Polylabo, Paris, France) were used in all experiments and pipette tips were silanized with a 2% (v/v) solution of dimethyldichlorosilane BDH (Poole, England) in heptane.

Preparation of Presynaptic Membranes from Torpedo Electric Organ. Live *Torpedo marmorata* were obtained from the Stations de Biologie Marine of Arcachon or Roscoff (France). The method of Morel et al. (1985) was used for purification of *Torpedo* presynaptic plasma membranes from the electric organ. The preparation was carried out on ice or at 4 °C in the presence of the following proteinase inhibitors: 1 mM EDTA, 25 μg/mL bacitracin, 10 μg/mL soybean trypsin inhibitor, 0.2 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride. The concentration of membrane proteins in the synaptic membrane preparations was determined by the Folin-Lowry method, as modified by Markwell et al. (1978), using BSA as a standard.

Preparation of Radioiodinated Toxins. AtxC and crotoxin were radioiodinated by the chloramine T method of Greenwood et al. (1963), as previously described for AtxC (Križaj et al., 1994). [^{125}I]crotoxin was separated from the remaining Na[^{125}I] and free iodine by gel filtration chromatography on Sephadex G-25 fine (0.5 × 12 cm) equilibrated in 10 mM HEPES, pH 7.4, 0.6 M NaCl, 0.02% (v/v) Triton X-100, and 0.005% (w/v) NaN_3. The specific radioactivity, determined as specified by Black et al. (1986), was about 500 Ci/mmol for AtxC and 800 Ci/mmol for crotoxin, corresponding to the mean incorporation of 0.38 atoms of ^{125}I per molecule of crotoxin and 0.3 atoms of ^{125}I per AtxC. For determination of the radioactivity incorporated in each crotoxin subunit, the two subunits were separated in a MonoQ column in 6 M urea; urea was subsequently removed by gel filtration on Fast Desalting Column HR 10/10 (Pharmacia). Radiolabeled toxins were biologically identical to the native toxins. Upon storing at 4 °C, the binding characteristics of [^{125}I]toxins remained unchanged for at least two months.

Binding Studies. Binding experiments were performed at 20 °C using incubation buffer A (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM $SrCl_2$, 0.5 mM EGTA, 0.5% (w/v) BSA and 0.02% (w/v) Triton X-100), in which PLA_2 activity is negligible (1−2%). In competition studies, presynaptic membranes (400 μg of membrane proteins per mL) were incubated with the radiolabeled ligand in the presence or absence of unlabeled competitor for 1 h in a final volume of 250 μL. The concentrations of [^{125}I]crotoxin and [^{125}I]-AtxC were about 1 nM. Membrane-bound and free radioligands were separated by rapid centrifugation of the membranes through a silicone-based oil mixture (Black et al., 1986; Križaj et al., 1994) and their respective concentrations measured by γ-radiation counting. Saturation curves for [^{125}I]crotoxin or [^{125}I]AtxC binding to synaptic membranes were determined by the incubation of membranes (400 μg of membrane proteins per mL) with several concentrations of [^{125}I]toxin (0−40 or 0−400 nM) in binding buffer for 1 h. The total incubation volume was 250 μL. Nonsaturable binding to membranes was determined in parallel incubations containing a 200-fold excess of unlabeled toxin. The nonlinear curve fitting program GRAFIT (Leatherbarrow,

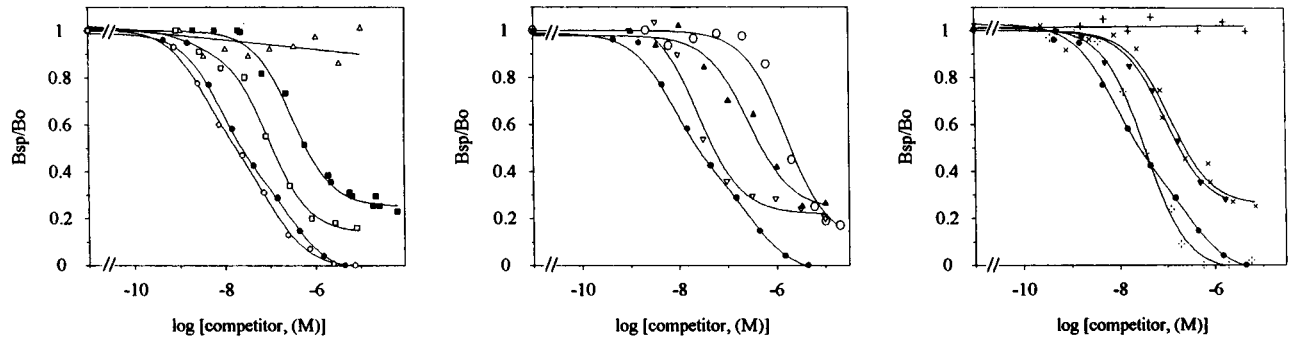

FIGURE 3: Inhibition of the specific binding of [^{125}I]AtxC to *Torpedo marmorata* synaptic membranes. [^{125}I]AtxC (2.7 nM) was incubated with synaptic membranes in the presence of various concentrations of AtxC (●), AtxA (○), β-Butx (△), $AtnI_2$ (■), Agtx (□), OS_2 (○), notexin (▽), taipoxin (▲), CB (broken plus sign), crotoxin (×), CA (+) and porcine pancreatic PLA_2 (▼). Total membrane-bound radioactivity (Bt) was measured as described in Experimental Procedures. Nonspecific binding (Bns) was obtained in experiments where unlabeled AtxC was present in over 1500-fold excess over [^{125}I]AtxC. The specific binding (Bsp=Bt−Bns) is shown relative to the maximal specific binding, determined in the absence of any competing ligand (Bo). Values shown are averages of at least two experiments.

Table 1: Residual Specific Binding of [^{125}I]AtxC in the Presence of Saturating Concentration of PLA_2 Competitors[a]

competitor	$(B_{sp}/B_0 \pm MD) \times 100$ (%)
AtxC	0
AtxA	0
CB	0
Agtx	16.0 ± 1.4
OS_2	18.7 ± 5.6
notexin	19.6 ± 2.5
crotoxin	25.9 ± 4.8
taipoxin	25.2 ± 0.9
$AtnI_2$	25.2 ± 2.3
porcine pancreatic PLA_2	25.0 ± 0.8
β-Butx	100
CA	100

[a] The data indicate the residual specific binding of [^{125}I]AtxC in the presence of respective competitor at its saturating concentration, (B_{sp})relative to the specific binding of [^{125}I]AtxC in the absence of competitor (B_0). The values are averages of at least two independent experiments.

1990) was used for analysis of the data obtained after γ-counting (80% efficiency).

Affinity Cross-Linking Experiments. *Torpedo* presynaptic membranes (800 μg of membrane proteins per mL) were incubated in 250 μL of buffer A (1 h at 20 °C) with 3.4 nM [^{125}I]crotoxin or 10.8 nM [^{125}I]AtxC in the absence or in the presence of an excess of unlabeled competitor. The membranes were then pelleted (14000*g* for 15 min), the supernatant removed by aspiration, and 490 μL of cross-linking buffer (0.5 M triethanolamine, pH 8.2, 150 mM NaCl) was added. DSS, dissolved in 10 μL of dimethyl sulfoxide, was added to a final concentration of 100 μM, and the mixture was incubated for 5 min at room temperature. The cross-linking reaction was stopped by addition of 500 μL 0.5 M Tris-HCl, pH 6.8, followed by subsequent centrifugation (14000*g* for 15 min). Pellets were solubilized in SDS−PAGE sample buffer under reducing conditions (50 mM DTT) and analyzed by SDS−PAGE (Laemmli, 1970) in 10% homogenous precast gels from Novex (San Diego, CA). The gels were stained with Coomassie Brilliant Blue R-250, dried, and autoradiographed at −80 °C using Amersham HYPER-film-MP and two intensifying screens.

Pretreatment of Torpedo Synaptic Membranes. Before some binding or cross-linking studies, *Torpedo* synaptic membranes were treated with proteinase K, trypsin, neuraminidase, or by heating, as already described (Križaj et al., 1994, 1995).

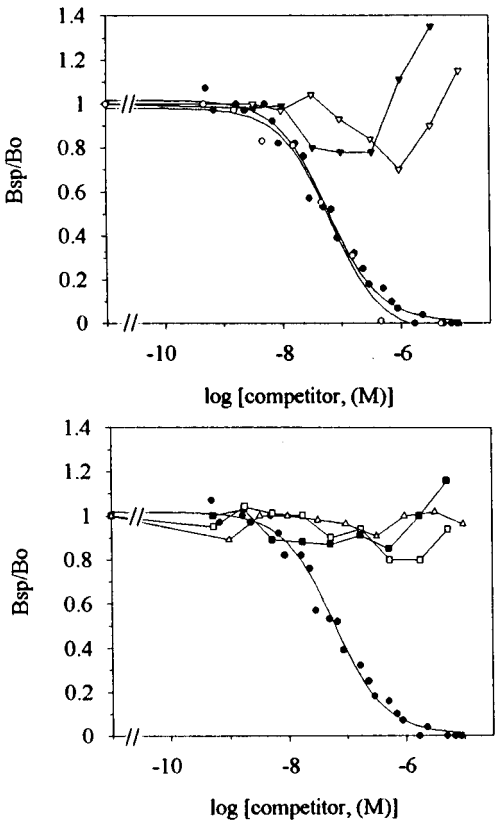

FIGURE 4: Effect of different molecules on [^{125}I]crotoxin binding to *Torpedo* synaptic membranes. Synaptic membranes were incubated with 0.67 nM of [^{125}I]crotoxin in the presence of crotoxin (●), CA (○), CB (▼), AtxC (▽), Agtx (□), $AtnI_2$ (■), and β-Butx (△). Bt and Bsp were determined as in Figure 1.

RESULTS

Specific Binding of [^{125}I]AtxC and [^{125}I]Crotoxin to Torpedo Synaptic Membranes. [^{125}I]AtxC binds to *Torpedo* synaptic membranes in a saturable manner (Figure 1). The corresponding Scatchard analysis (Figure 1, inset) indicates the existence of more than one population of binding sites. The data were fitted by assuming two independent binding sites, a high-affinity site (Kd_1 = 7.6 ± 2.1 nM; Bo_1 = 3.8 ± 0.6 pmol/mg of membrane proteins) and a low-affinity site (Kd_2 = 165 ± 20 nM; Bo_2 = 40 ± 2 pmol/mg of membrane proteins). Specific binding of AtxC was insensitive to neuraminidase treatment. However, specific binding was reduced to about 30% of the original value after

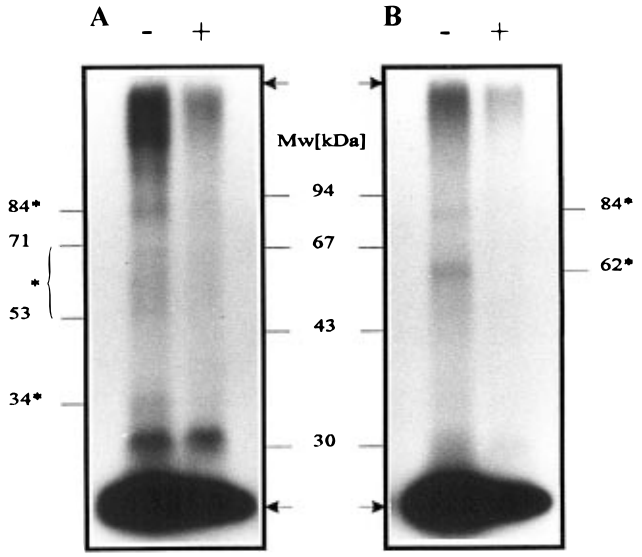

FIGURE 5: Affinity labeling of [^{125}I]AtxC and [^{125}I]crotoxin binding proteins on *Torpedo marmorata* synaptic membranes. *Torpedo* synaptic membranes were prelabeled with either [^{125}I]AtxC (A) or [^{125}I]crotoxin (B) before addition of the cross-linking reagent (DSS). Membrane pellets were solubilized and analyzed by SDS−PAGE under reducing conditions. Gels were autoradiographed (for experimental details see Experimental Procedures). Incubations were performed without (−) or with (+) a larger excess of unlabeled toxin. The positions of protein molecular mass standards (Pharmacia) are indicated: phosphorylase b (94 000 Da), albumin (67 000 Da), ovalbumin (43 000 Da), and carbonic anhydrase (30 000 Da). Specific conjugates are designated by an asterisk (*). Arrows indicate the top and the front of the gels.

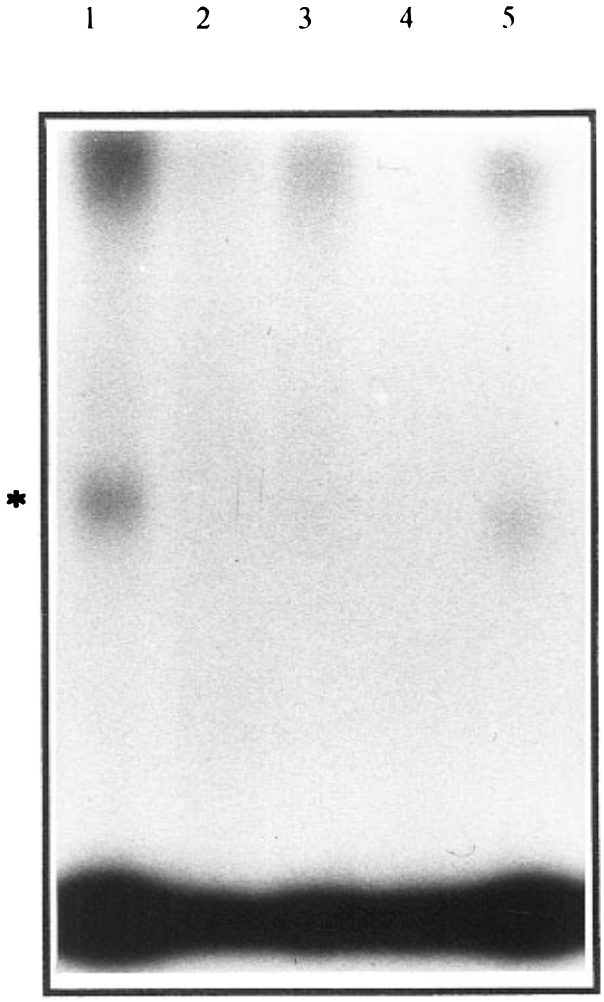

FIGURE 6: Effect of different pretreatments of *Torpedo* synaptic membranes on [^{125}I] crotoxin cross-linking. Before affinity-labeling with [^{125}I]crotoxin as in Figure 5, *Torpedo* synaptic membranes were exposed to various treatments, as follows: none (lane 1), proteinase K (lane 2), trypsin (lane 3), heating (lane 4), and neuraminidase (lane 5). An asterisk (*) indicates the specific conjugate.

treatment with either proteinase K or trypsin, or after heating. Furthermore, the Kd of [^{125}I]AtxC for heated synaptic *Torpedo* membranes (Kd = 165 nM), as determined by homologous competition experiments, exactly corresponded to Kd_2 of AtxC for native membranes (165 nM), indicating that only the high-affinity binding component was inactivated by heating.

In the case of [^{125}I]crotoxin, a saturable binding to *Torpedo* synaptic membranes was also detected. It was, however, not possible to determine precisely the concentration of free [^{125}I]crotoxin at equilibrium because of the dissociation of the toxin into its CA and CB subunits and therefore to calculate Kd values from the equilibrium binding experiment. Two types of crotoxin binding sites, high and low-affinity, were observed. An estimation of the maximal number of binding sites was $Bo_1 \approx 10$ pmol/mg of membrane proteins and $Bo_2 \approx 50$ pmol/mg of membrane proteins, respectively.

As in the case of [^{125}I]AtxC, the high-affinity binding site was sensitive to proteolytic digestion and to heating (Figure 6), while the low-affinity binding site remained unaffected by these treatments. From homologous competition experiment on heated membranes, a low-affinity binding site dissociation constant (Kd_2) of 110 nM was obtained (Figure 2A). As it was established that [^{125}I]toxins form specific adducts exclusively with their high-affinity binding sites in synaptic membranes, a crotoxin high-affinity binding site dissociation constant (Kd_1) was estimated to be about 40 nM from the concentration of unlabeled crotoxin in the incubation mixture which caused half-disappearance of its specific conjugate with the membrane (Figure 2B).

Competition in Specific Binding Experiments. We analyzed the specific binding of [^{125}I]AtxC to *Torpedo* synaptic membranes in the presence of different neurotoxic and nonneurotoxic secretory PLA_2s, as well as PLA_2 analogues, belonging to the two structural groups (Figure 3). The type II PLA_2s used included monomeric PLA_2 neurotoxins (AtxC, AtxA, CB, and Agtx), dimeric toxins such as crotoxin, as well as the nontoxic PLA_2 $AtnI_2$, and the CA subunit of crotoxin which derives from a PLA_2 precursor after limited proteolysis. The type I PLA_2s were represented by two monomeric neurotoxins (OS_2 and notexin), dimeric (β-Butx) and trimeric (taipoxin) neurotoxins, and the nontoxic porcine pancreatic PLA_2. In our experimental conditions, the high-affinity binding of [^{125}I]AtxC represented about 25% of the total specific binding. Only native AtxC, AtxA, and CB were able to completely prevent the specific binding of [^{125}I]-AtxC. As in the case of homologous competition, the inhibition curves corresponded to a Hill coefficient lower than 1, suggesting the existence of more than one binding site corresponding to the high-affinity and low-affinity binding sites. AtxA was a slightly stronger inhibitor than AtxC, in good agreement with its higher lethal potency, while β-Butx and CA had no effect (Table 1). Crotoxin, taipoxin, $AtnI_2$, and porcine pancreatic PLA_2 inhibited the specific binding of AtxC by about 75%, suggesting that they interfere

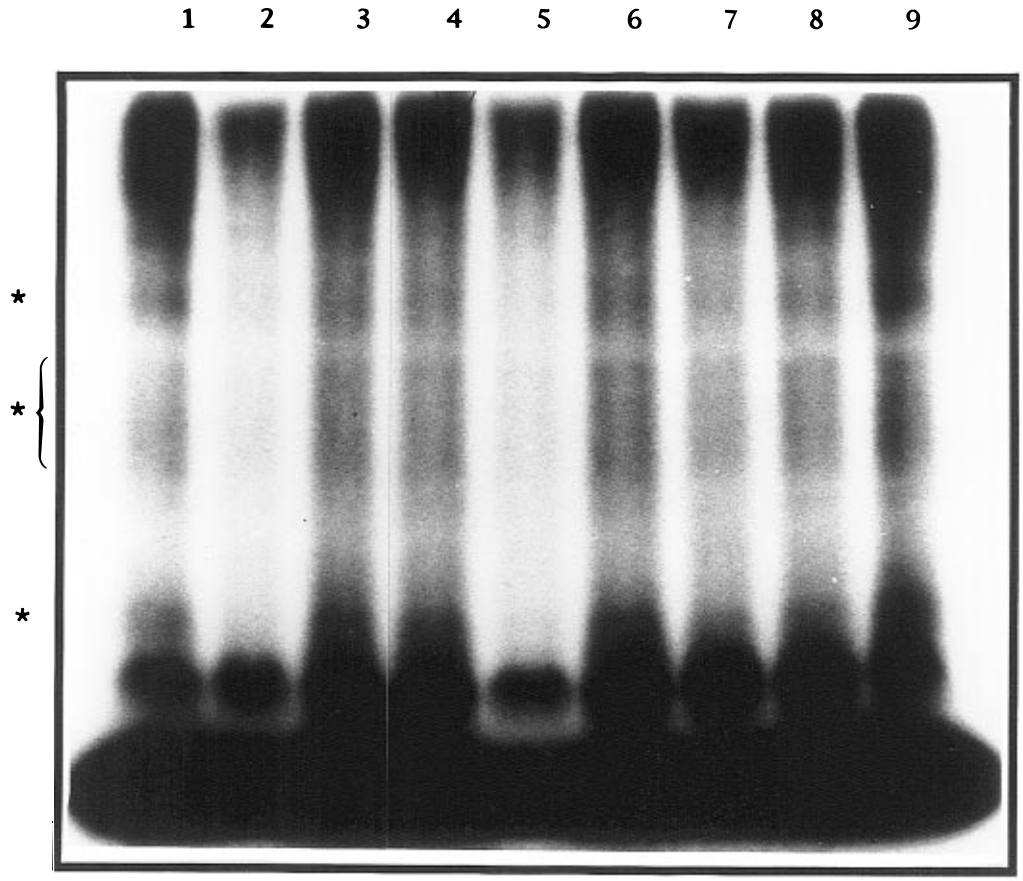

FIGURE 7: Effect of various PLA_2 on the formation of [^{125}I]AtxC-specific conjugates with *Torpedo* synaptic membranes. *Torpedo* synaptic membranes were cross-linked with 10.8 nM [^{125}I]AtxC in the absence (lane 1) or in the presence of different PLA_2 at a concentration of 10^{-6} M (lanes 2−9), as described in Experimental Procedures. (lane 2) AtxC; (lane 3) crotoxin; (lane 4) $AtnI_2$; (lane 5) AtxA; (lane 6) notexin; (lane 7) OS_2; (lane 8) taipoxin; (lane 9) β- Butx. Asterisks (*) indicate the positions of specific adducts.

with the low-affinity binding site. In the case of Agtx, notexin, and OS_2, which blocked about 80% of the specific binding of [^{125}I]AtxC (Table 1), the inhibition curves shown in Figure 3 suggest that these toxins interfere with low-affinity [^{125}I]AtxC binding sites as well as partially with its high-affinity binding site. In the case of the specific binding of [^{125}I]crotoxin, complete inhibition was obtained only with native crotoxin and with its acidic subunit, CA (Figure 4). β-Butx did not inhibit the specific binding of crotoxin. Other competitors (CB, Agtx, AtxC, and $Atnl_2$) had a paradoxical effect: they display some inhibition of crotoxin specific binding only in an intermediate range of concentrations, above which they induced an increase in [^{125}I]crotoxin "specific binding" (Figure 4).

Affinity Labeling of AtxC and Crotoxin to Their Membrane Acceptors. The bifunctional reagent DSS was used to cross-link the radioiodinated toxins to their membrane acceptors. An optimal concentration of cross-linker (100 μM) was first determined for which the protein patterns of *Torpedo* membranes and of the toxins incubated separately were unaffected, as revealed after Coomassie blue staining of the gels. Figure 5 shows the SDS−PAGE patterns, under reducing conditions, observed by autoradiography after cross-linking [^{125}I]AtxC and [^{125}I]crotoxin with *Torpedo* membranes. In the case of AtxC, the most intensively labeled bands have apparent molecular mass of 84 000 and 34 000 Da, indicating that [^{125}I]AtxC specifically cross-linked membrane components of 70 000 and 20 000 Da, respectively, if we assume the covalent binding of one molecule of AtxC (14 000 Da) per molecule of acceptor. Several other membrane components in the range from 57 000 to 39 000 Da were also labeled, as represented on Figure 5A by the bands from 71 000 to 53 000 Da. Higher molecular mass material was also labeled (Figure 5A). This probably resulted from excessive cross-linking, as the intensity of respective bands increased with the concentration of cross-linker, or aggregation.

In the case of crotoxin, we observed only one heavily labeled adduct at 62 000 Da (Figure 5B), which corresponds to a 48 000 Da membrane component, again assuming that this complex contains a single [^{125}I]CB molecule. When SDS−PAGE was performed under nonreducing conditions, the mobility of the adduct was slightly faster. In some experiments, we also observed a weaker adduct at 84 000 Da corresponding to the labeling of a 70 000 Da membrane component. High molecular mass labeled material due to excessive cross-linking was also observed, as in the case of [^{125}I]AtxC.

In the cases of both [^{125}I]toxins, the formation of specific adducts was restrained by pretreatment of the presynaptic membranes with proteinase K, or, less effectively, with trypsin. The specific labeling was also suppressed after heating the membranes at 100 °C for 60 min before the cross-linking experiment, as illustrated in Figure 6. Pretreatment of membranes with neuraminidase did not affect the formation of adducts.

Competition in Cross-Linking Experiments. We also examined the effect of competitors on the formation of cross-

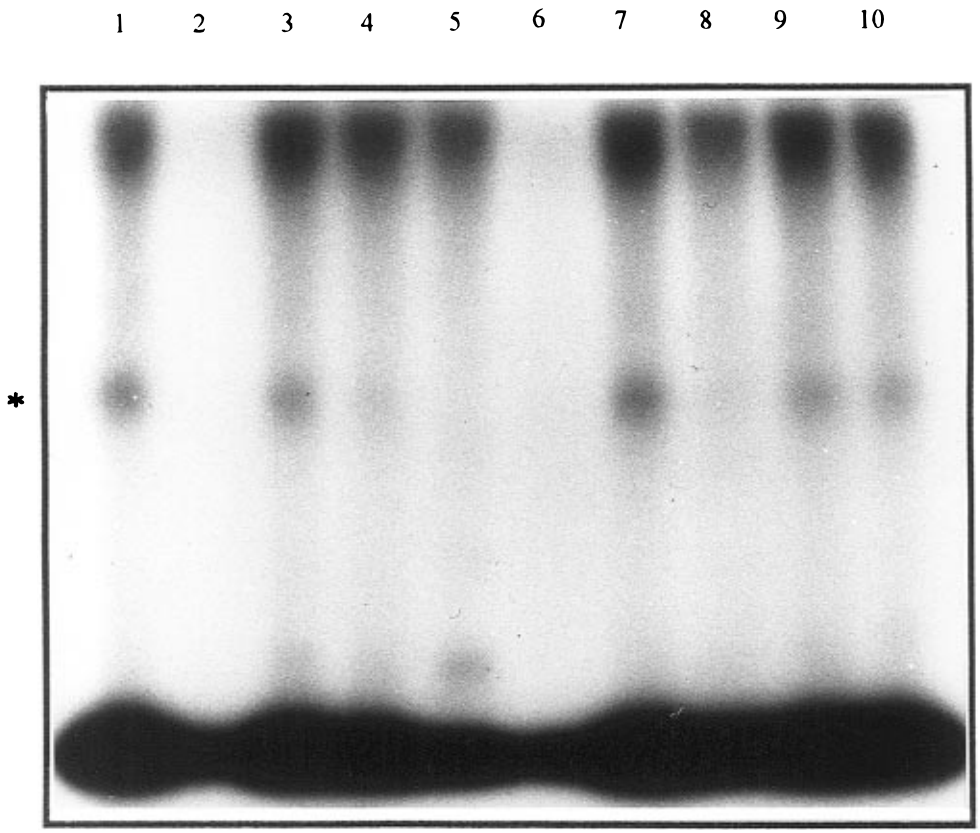

FIGURE 8: Effect of various PLA_2 on the specific cross-linking of [^{125}I]crotoxin with *Torpedo* synaptic membranes. *Torpedo* synaptic membranes were cross-linked with 3.4 nM [^{125}I]crotoxin in the absence (lane 1) or in the presence of different PLA_2 at a concentration of 10^{-6} M: (lane 2) crotoxin; (lane 3) AtxC; (lane 4) Agtx; (lane 5) CB; (lane 6) CA; (lane 7) β-Butx; (lane 8) notexin; (lane 9) taipoxin; and (lane 10) OS_2. An asterisk (*) indicates the 62 000 Da specific conjugate.

linked adducts (Figure 7). AtxA and AtxC completely prevented the cross-linking of [^{125}I]AtxC to all membrane components. OS_2 weakly reduced [^{125}I]AtxC cross-linking, which was not readily apparent in the case of notexin. Nontoxic PLA_2 $AtnI_2$, as well as crotoxin, β-bungarotoxin, and taipoxin, had no inhibitory effect.

The cross-linking of [^{125}I]crotoxin was completely inhibited by crotoxin, CA, and CB (Figure 8), more weakly by Agtx and notexin, and negligibly by taipoxin and OS_2. AtxC and β-Butx were completly inactive. In some cases on autoradiograms a labeled band appeared at about 28 kDa. It was ascribed to an [^{125}I]CB dimer formed after the liberation of [^{125}I]CB either from [^{125}I]crotoxin or from the specific binding sites on the membrane as detailed elsewhere (Križaj et al., 1996).

DISCUSSION

Radiolabeled AtxC and crotoxin bind specifically to *Torpedo marmorata* presynaptic membranes. Both toxins display high- and low-affinity binding sites. Similarly, two families of binding sites have been identified for AtxC and AtxA in a bovine brain synaptic membrane preparation (Križaj et al., 1994, 1995) and for crotoxin in guinea pig synaptosomal membranes (Degn et al., 1991), as first observed for OS_2 (Lambeau et al., 1989) and for β-Butx (Breeze & Dolly, 1989) in rat brain synaptic membranes. In the case of crotoxin, however, Délot and Bon (1993) identified only one binding site, with Kd of 0.7 μM, on *Torpedo marmorata* synaptic membranes. The high-affinity binding site was probably overlooked because of the low specific radioactivity of the [^{125}I]crotoxin used in these experiments.

The dissociation constants of AtxC (Kds: 8 and 165 nM) and crotoxin (Kds: ~40 and 110 nM) for *Torpedo* synaptic membranes, are comparable to the previously reported values in different membrane preparations (Tzeng et al., 1989; Hseu et al., 1990; Degn et al., 1991; Yen & Tzeng, 1991; Križaj et al., 1994, 1995). Moreover, the binding constants of crotoxin and AtxC correlate well with those determined from the influence of these β-neurotoxins on ACh release from *Torpedo* synaptosomes (Délot & Bon, 1992). On the other hand, the dissociation constants for crotoxin and AtxC were much lower than those measured for OS_2 in rat brain membranes (1.5 and 45 pM) by Lambeau et al. (1989). Surprisingly, the inhibition of [^{125}I]OS_2 binding by crotoxin performed by these authors would suggest a higher affinity of crotoxin for synaptic membranes than was found in our experiments. This may reflect differences between rat brain synaptosomes (Lambeau et al.,1989) and synaptic membranes from *Torpedo* electric organs.

Radioiodinated toxins were used as affinity probes to label their specific membrane acceptors. [^{125}I]AtxC was cross-linked mainly to 70 000 and 20 000 Da membrane components, and weaker bands were also observed in the range corresponding to 57 000 − 39 000 Da (Figure 5A). In bovine cortex synaptic membranes, [^{125}I]AtxC and [^{125}I]AtxA labeled membrane proteins of 51 000 and 53 000 − 56 000 Da (Križaj et al., 1994, 1995). The absence of high molecular mass complexes, corresponding to the labeling of membranes protein of 70 000 Da, was probably due to the

different cross-linking protocol using DMS instead of DSS, as observed in the case of OS_2 on rat brain membranes (Lambeau et al., 1989). The differences in the molecular mass of the labeled bands, might however be attributed to differences between animal species.

[^{125}I]Crotoxin produced a very different cross-linking pattern, with labeling restricted almost entirely to a single membrane protein of 48 000 Da (Figure 5B). Similarly, [^{125}I]-crotoxin and [^{125}I]taipoxin were previously shown to cross-link membrane proteins of about 45 000 Da in brain synaptosomes from guinea pig (Tzeng et al., 1989; Yen & Tzeng, 1991). Another crotoxin-binding protein of 85 000 Da molecular mass was detected by photoaffinity labeling on the same membrane preparation (Hseu et al., 1990). A weak band of about 84 000 Da, sometimes visible in our autoradiograms, may represent the *Torpedo* homologue of this high molecular mass acceptor from guinea pig brain synaptosomes. These results clearly show that AtxC and crotoxin cross-link different neuronal proteins and therefore possess distinct binding sites. It is not possible, however, to determine whether these sites belong to independent neuronal acceptors or to different subunits of a common target, as observed for example in the case of the various toxins which interact with independent subsites of voltage-sensitive Na^+ channels (Jover et al., 1988; Kirsch, 1994).

Pretreatment of the membranes by heating or incubation with proteinases completely abolished the high-affinity binding of [^{125}I]AtxC and [^{125}I] crotoxin and cross-linking proving the proteinaceous nature of the high-affinity binding sites for these two toxins. The high-affinity binding of [^{125}I]-AtxC was inhibited by AtxC, AtxA, CB, and weakly by Agtx, notexin, and OS_2, but not by crotoxin, taipoxin, $AtnI_2$, and porcine pancreatic PLA_2. The heterogeneity of AtxC-neuronal binding sites appears similar as in the case of α-Dtx neuronal receptors, of which a subpopulation binds exclusively α-Dtx, while another also binds β-Butx (Black & Dolly, 1986). In the present work, we performed binding experiments in a Sr^{2+}/EGTA buffer, which inhibits the PLA_2 activity of the toxins. This is probably the reason why we clearly observed a residual saturable binding of [^{125}I]AtxC in the presence of competitors, in contrast to previous studies where Ca^{2+} buffers were used (Lambeau et al., 1989, 1991; Degn et al., 1991; Délot & Bon, 1993; Križaj et al., 1994, 1995). The fact that low-affinity binding sites were heat-resistant and less specific than high-affinity sites suggests that they may be related to phospholipids, as proposed earlier (Radvanyi et al., 1987, 1989; Križaj et al., 1994). On the other hand, the low-affinity binding site of OS_2 in rat brain seems to be constituted of membrane proteins as it was sensitive to proteinase treatment as well as to heating (Lambeau et al., 1989).

CA was the only competitor which completely inhibited the specific binding of [^{125}I]crotoxin, as previously reported on the same membrane preparation (Délot & Bon, 1993) and on guinea pig brains (Degn et al., 1991). Several PLA_2s (CB, Agtx, AtxC, $AtnI_2$) which showed a slight inhibitory effect on the specific [^{125}I]crotoxin binding at low concentrations were found to apparently increase the binding at high concentrations. The results obtained in [^{125}I]crotoxin cross-linking competition experiments showed, however, a different picture. The specific labeling of a 48 000 Da membrane protein by [^{125}I]crotoxin was completely blocked by crotoxin, CA and CB, partially by notexin and Agtx, poorly by OS_2 and taipoxin, and not at all by AtxC, $AtnI_2$, and β-Butx. The surprising observation that CB prevented [^{125}I]crotoxin cross-linking while it did not inhibit its high-affinity binding, is probably due to an apparent inhibition of [^{125}I]crotoxin cross-linking. CB reduces [^{125}I]crotoxin cross-linking not by competition for its binding site but by exchange of the labeled for unlabeled CB subunit in the [^{125}I]crotoxin complex (Faure et al., 1993; Križaj et al., 1996). A similar explanation most likely applies to Agtx which was also shown to possess a high affinity for CA (Choumet et al., 1993). This is consistent with the fact that CB and crotoxin exert qualitatively different effects on ACh release from *Torpedo* synaptosomes (Délot & Bon, 1992). In addition, the fact that isolated basic subunit CB of crotoxin is able to abolish the high-affinity binding of [^{125}I]AtxC, in contrast to crotoxin (CB associated with CA), suggests that CA does not behave simply as a *chaperone* for CB, lowering its non-specific adsorption, but also confers a distinct binding specificity to the crotoxin.

Our competition and cross-linking studies of [^{125}I]AtxC and [^{125}I]crotoxin on *Torpedo* presynaptic membranes clearly show that β-neurotoxins bind to a variety of high-affinity neuronal acceptor sites, although the resulting pharmacological actions are practically identical. Some of these sites are completely different (as those of AtxC, crotoxin, and β-Butx), while others overlap to different extents (as those of AtxC and notexin). This paradox may be explained if different toxins bind to functionally related membrane components, e.g., voltage-sensitive K^+ channels, which represent a very heterogenous group of proteins.

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