Identification of the Neuronal Acceptor in Bovine Cortex for Ammodytoxin C, a Presynaptically Neurotoxic Phospholipase A_2[†]

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ABSTRACT: A specific, high-affinity binding site for ammodytoxin C in synaptic membranes from bovine cerebral cortex was detected and partially characterized. Equilibrium binding analysis revealed a single population of [^{125}I]ammodytoxin C acceptors with the following binding parameters: K_d = 6.0 nM and B_{max} = 5.7 pmol/mg membrane protein. Such binding was strongly inhibited by three ammodytoxins (A, B, and C) and by crotoxin B. *Vipera berus berus* phospholipase A_2 was a weaker inhibitor; nontoxic phospholipase A_2, ammodytin I_2, and the myotoxic phospholipase A_2 homologue, ammodytin L, both from *Vipera ammodytes ammodytes* venom, inhibited binding only at very high concentrations, whereas α-dendrotoxin, β-bungarotoxin, and crotoxin A had no influence on the [^{125}I]ammodytoxin C-specific binding. The ammodytoxin C neuronal binding site therefore overlaps, at least partially, with the neuronal acceptors for some of the related presynaptically neurotoxic phospholipases A_2 (β-neurotoxins). [^{125}I]-Ammodytoxin C was covalently attached to its acceptor by chemical cross-linking. Subsequent SDS−PAGE analysis followed by autoradiography revealed saturably labeled membrane components with apparent M_r values of 51 000 (weaker band) and 53 000−56 000 (stronger band). Pretreatment of synaptic membranes with *Staphylococcus aureus* V-8 proteinase and proteinase K, heat, or low pH decreased the [^{125}I]ammodytoxin C-specific binding to various extents, but never abolished it completely. Membrane protein and certain phospholipids residing in its vicinity are therefore most likely involved in the binding of ammodytoxin C to bovine synaptic membranes.

Phospholipases A_2 (PLA_2,[1] EC 3.1.1.4) are enzymes that catalyze the hydrolysis of the 2-acyl ester bond of 1,2-diacyl-3-*sn*-phosphoglycerol lipids and are primarily involved in phospholipid turnover. Some of them, mostly found in snake venoms, have, however, acquired different pharmacological activities during evolution (Rosenberg, 1990). PLA_2s with neurotoxic activity are a particularly interesting group of toxins as they are potential tools for research on neurotransmission.

From the venom of the long-nosed viper (*Vipera ammodytes ammodytes*), three neurotoxic PLA_2s, ammodytoxins A, B, and C (ATXA, -B, and -C), have been isolated and characterized (Gubenšek et al., 1980; Ritonja & Gubenšek, 1985; Ritonja et al., 1986; Križaj et al., 1989). They are presynaptically neurotoxic enzymes that are able to block the release of neurotransmitter, acetylcholine (ACh), into the synaptic cleft and thus hinder the neuronal transmission. Their action on ACh release at the neuromuscular junction is triphasic, similar to most presynaptically neurotoxic PLA_2s. After an initial, transient blockade of ACh release, augmentation follows, which is terminated by the total and irreversible inhibition of neurotransmitter release (Lee et al., 1984).

Numerous attempts to clarify the phenomenon of the neurotoxicity of PLA_2s on the molecular level, mostly on localization of the so-called toxic site that is responsible for the neurotoxic action, have not given an unequivocal and generally valid answer (Dufton & Hider, 1983; Kini & Iwanaga, 1986; Tsai et al., 1987; Kondo et al., 1989; Wang et al., 1992; Chu et al., 1993). In ammodytoxin, the toxic site has been proposed to be near the C-terminus of the molecule (Ritonja et al., 1986; Križaj et al., 1989; Čurin-Šerbec et al., 1991). However, the mechanism of action of PLA_2 neurotoxins at the nerve ending is still poorly understood. Since the high-affinity binding of some PLA_2 neurotoxins was detected in brain preparations (Tzeng, 1993), it is very likely that PLA_2 neurotoxicity is an acceptor-mediated process rather than merely the result of specific PLA_2 activity. The latter is, indeed, a necessary but not sufficient prerequisite for the neuromuscular blocking effect. Not all presynaptically neurotoxic PLA_2s share the same neuronal binding site, although their action on ACh release is virtually the same (Chang, 1985). Until now, only the

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[1] Abbreviations: ACh, acetylcholine; AMI_2, ammodytin I_2; AML, ammodytin L; ATX, ammodytoxin; B_{max}, maximal binding capacity; BSA, bovine serum albumin; β-BuTX, β-bungarotoxin; CA, crotoxin A; CB, crotoxin B; α-DTX, α-dendrotoxin; DMS, dimethyl suberimidate; DTT, dithiothreitol; IC_{50}, concentration of an inhibitor at which the inhibition is 50 % of the maximum; K_d, dissociation constant; K_I, inhibition constant; LD_{50}, concentration of a toxin at which the lethality is 50%; MP, membrane protein; PBS, phosphate-buffered saline; PNGase F, peptide-*N*-glycosidase F; PLA_2, phospholipase A_2; PMSF, phenylmethanesulfonyl fluoride; OS_2, *Oxyuranus scutellatus* phospholipase A_2; SDS−PAGE, sodium dodecyl sulfate−polyacrilamide gel electrophoresis; STI, soybean trypsin inhibitor; *Vbb*PLA_2, *Vipera berus berus* phospholipase A_2.

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nature of β-bungarotoxin (β-BuTX) acceptor has been clarified to any extent. This presynaptic neurotoxin from Taiwan banded krait venom (*Bungarus multicinctus*) binds to the subpopulation of α-dendrotoxin (α-DTX) neuronal binding sites that have been successfully purified (Parcej & Dolly, 1989), sequenced, and shown to represent a subtype of voltage-dependent K^+ channels (Scott et al., 1990, 1994; Reid et al., 1992).

In the present work, a high-affinity binding site for ATXC, the second most toxic neurotoxin from the long-nosed-viper venom, has been detected and characterized in bovine cortex. The acceptor's subunit composition was analyzed by chemical cross-linking.

EXPERIMENTAL PROCEDURES

Materials. ATXA, -B, -C, and ammodytins I_2 (AMI_2) and L (AML) were isolated from the venom of *Vipera ammodytes ammodytes* (Gubenšek et al., 1980; Križaj et al., 1992). *Vipera berus berus* PLA_2 (*Vbb*PLA_2) was a gift from Dr. J. Siigur (Institute of Chemical Physics and Biophysics, Tallinn). α-DTX and β-BuTX were isolated from the crude venoms of *Dendroaspis angusticeps* and *Bungarus multicinctus*, respectively, as previously described (Black et al., 1986; Spokes & Dolly, 1980). A and B subunits of crotoxin (*Crotalus durissus terrificus*) were kindly provided by Dr. C. Bon (Institute Pasteur, Paris). $Na^{125}I$ (carrier-free) was obtained from DuPont-New England Nuclear. Proteinases and glycosidases were from Sigma or Boehringer Mannheim. Dinonyl phthalate was purchased from Fluka, and Dow-Corning MS-550 silicone fluid was from BDH. CM-Sepharose CL-6B was obtained from Pharmacia, and dimethyl suberimidate (DMS) was from Pierce. All other chemicals used were of analytical grade.

Preparation of Synaptic Membranes. The method of Bennett et al. (1986) was used to prepare a demyelinated P2 fraction of bovine cortex, referred to herein as synaptic membranes. During the isolation procedure, the following proteinase inhibitors were always present: EDTA (2 mM), bacitracin (25 μg/mL), soybean trypsin inhibitor (STI) (10 μg/mL), benzamidine (0.2 mM), and phenylmethanesulfonyl fluoride (PMSF) (0.1 mM). All manipulations were carried out at 4 °C and membranes were stored at −70 °C. For the determination of protein content in synaptic membrane preparations, a modification of the Folin−Lowry protein assay of Markwell et al. (1978) was used. Bovine serum albumin (BSA) was taken as a standard.

Radioiodination of ATXC. A modified chloramine T method of Greenwood et al. (1963) was used for the radioiodination of ATXC. To 52 μg of ATXC in 10 μL of didistilled water were added 10 μL of iodination buffer [100 mM HEPES (pH 7.4), 5 mM $CaCl_2$, and 50% (v/v) glycerol] and 20 μL of aqueous solution of $Na^{125}I$ (~2 mCi). The iodination was initiated by the addition of 10 μL of a freshly prepared solution of chloramine T in iodination buffer to a final concentration of 0.15 mM. After 20−40 s of intensive vortexing, the reaction was terminated by dilution to 1 mL with 10 mM HEPES (pH 7.4), 0.02% (v/v) Triton X-100, and 0.005% (w/v) NaN_3 (termination buffer). Specific radioactivity of the [^{125}I]ATXC was determined as specified by Black et al. (1986). [^{125}I]Toxin was separated from the remaining unreacted $Na^{125}I$ by ion exchange chromatography on a CM-Sepharose CL-6B column (2 mL) equilibrated with the termination buffer. Bound material was eluted from the column by 0.6 M NaCl in the termination buffer. Fractions (0.5 mL) containing [^{125}I]ATXC were stored at 4 °C and diluted immediately prior to use. The biological activity of [^{125}I]ATXC was tested by the determination of its LD_{50} (ip) for mice (Reed & Muench, 1938), the measurement of its PLA_2 activity (DeHaas et al., 1968), and the determination of its immunological properties in an ELISA test (Čurin-Šerbec et al., 1991) in comparison to native ATXC. The biological activity of [^{125}I]ATXC was stable when used within 4 weeks.

Binding Studies. Under the binding conditions used, the plateau of association of [^{125}I]ATXC to synaptic membranes was reached at 20 °C after 1 h and was stable for at least 30 min. The standard incubation buffer used in binding experiments was 20 mM Tris-HCl (pH 7.4), 75 mM NaCl, 1 mM $CaCl_2$, 0.1% (w/v) BSA, and 0.02% (w/v) Triton X-100 (buffer A). After a 1 h incubation of membranes and ligands, bound and free [^{125}I]ATXC were separated by rapid centrifugation of the membranes through a silicone-based oil mixture (Black et al., 1986). Samples were counted in a γ-counter with an efficiency of 78%. In order to minimize the adsorption of proteins to plasticware, particularly at low concentrations, tubes and pipet tips were silanized with a 2% (v/v) solution of dimethyldichlorosilane (BDH) in heptane before use. Saturation curves for [^{125}I]ATXC binding to synaptic membranes were determined by the incubation of membranes (800 μg/mL membrane protein) with increasing concentrations of [^{125}I]ATXC (0−34 nM) in buffer A for 1 h. The total incubation volume was 250 μL. Nonsaturable binding to membranes was determined in parallel incubations containing a 100-fold excess of unlabeled ATXC over the labeled toxin. In competition studies, the concentration of synaptic membranes was also 800 μg of membrane protein (MP)/mL of buffer A, and the [^{125}I]ATXC concentration was always 3.4 nM. Increasing concentrations of competitors were prepared by diluting stock solutions in buffer A. Data from saturation and competition experiments were analyzed using the nonlinear curve-fitting programs LIGAND (Munson & Rodbard, 1980) and GRAFIT (Leatherbarrow, 1990).

Enzyme, Acid, and Heat Treatments of Synaptic Membranes. Proteolytic treatment of synaptic membranes (10.9 mg MP/mL) by trypsin (0.2 mg/mL) and proteinase K (0.2 mg/mL or 2.5 units/mL) was accomplished in buffer A, whereas *Staphylococcus aureus* V-8 proteinase (1 mg/mL) treatment was performed in PBS (pH 7.8) and 0.02% (v/v) Triton X-100. Reaction mixtures were incubated at 37 °C for 1 h. Reactions were terminated by the addition of proteinase inhibitors: 1−5 mg/mL STI in the case of trypsin and 5 mM PMSF in the case of proteinase K. The reaction with *Staphylococcus aureus* V-8 proteinase was stopped by extensive washing of the membranes with buffer A (three times: resuspension, followed by centrifugation at 12000*g* for 2 min). In control experiments, proteinase inhibitors were present during the incubation, using the same concentrations as for the termination of reactions, or the membranes were incubated without the proteinase (*Staphylococcus aureus* V-8 proteinase control). Treatments of the membranes (10.9 mg MP/mL) with neuraminidase (0.1 unit/mL), peptide-*N*-glycosidase F (PNGase F) (2.5 units/mL), and both glycosidases at the same time were also performed in buffer A. Reaction mixtures were incubated at room temperature for

3 or 2 h, respectively. Reactions were stopped by extensive washing of the membranes with buffer A. Controls did not contain enzymes; otherwise they were treated in the same way. Heat treatment was performed by incubating synaptic membranes (10.9 mg MP/mL) in boiling water for 6−30 min. The control experiment was conducted at room temperature. Also, synaptic membranes (10.9 mg MP/mL) were equilibrated with 1 M HCl for 40 min at 37 °C, followed by extensive washing with buffer A and assay of toxin binding activity. In control experiment, buffer A was used instead of HCl.

Cross-Linking of [^{125}I]ATXC to Its Neuronal Acceptors. Bovine synaptic membranes (4 mg MP/mL) were incubated in 250 μL of buffer A with 6.8 nM [^{125}I]ATXC in the absence or presence of 500 nM ATXC. After a 1-h incubation at room temperature with occasional vortex mixing, the membranes were pelleted by centrifugation in an Eppendorf centrifuge (12000*g* for 10 min at 4 °C). Membranes were washed with 1 mL of ice-cold cross-linking buffer [0.25 M triethanolamine (pH 8.5), 75 mM NaCl, 5 mM $CaCl_2$, and 0.02% (v/v) Triton X-100]. Following centrifugation, the membrane pellet was resuspended in 500 μL of the cross-linking buffer containing 1 mg/mL dimethyl suberimidate (DMS), a cross-linking reagent, dissolved in the buffer just before the resuspension. The reaction, which proceeded at room temperature (20 °C), was stopped after 1 h by the addition of 500 μL of 0.5 M Tris-HCl (pH 6.8) and subsequent centrifugation (12000*g* for 10 min). The resulting pellets were solubilized in SDS−PAGE sample buffer (Laemmli, 1970) under reducing conditions (50 mM DTT) and analyzed by SDS−PAGE (10% homogeneous slab gels). Gels were stained with Coomassie Brilliant Blue R-250, dried, and autoradiographed at −70 °C using Kodak X-Omat S film and an intensifying screen.

Circular Dichroism. A Dichrograph III (Jobin Yvon, France) was used to measure circular dichroism in the near-UV region (255−310 nm). Samples were dialyzed overnight at 4 °C against 20 mM Tris-HCl buffer (pH 7.4) and 75 mM NaCl, containing 1 mM either $CaCl_2$ or $SrCl_2$, before measurement, which was performed at room temperature. ATXC concentration used in the experiments was 0.738 or 0.851 mg/mL. For calculation of the mean residue ellipticity ($[\theta]^{\lambda}_{M_{rw}}$) in deg·cm^2/dmol, a mean residue weight of 112.6 (M_{rw}) was used.

Determination of Protein Stock Concentrations. The concentrations of stock solutions of toxins and other proteins used in the experiments were determined spectrophotometrically by measurement of their absorbance at 280 nm. The molar absorption coefficient at 280 nm for each protein was calculated according to the method of Perkins (1986).

RESULTS

Preparation of [^{125}I]ATXC. As ATXC contains 10 Tyr residues, to limit the iodination to no more than 1 per molecule, all iodination procedures were carried out at a molar ratio of 3:1 toxin to iodine. The specific radioactivity routinely obtained for preparations was 600 Ci/mmol, corresponding to a mean incorporation of slightly less than 0.3 atom of ^{125}I per molecule. The biological activity of [^{125}I]ATXC was tested in comparison to native ATXC by the determination of its immunological properties, ip LD_{50} for mice and PLA_2 activity. The immunological properties

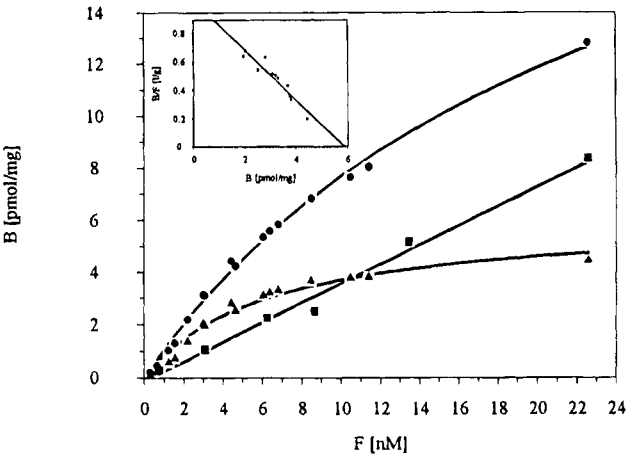

FIGURE 1: Equilibrium binding of [^{125}I]ATXC to bovine synaptic membranes. Membranes were incubated in Tris-HCl buffer with increasing concentrations of [^{125}I]ATXC. After 1 h at room temperature, aliquots were removed for the determination of bound (*B*) and free (*F*) labeled toxin concentrations as described in the Experimental Procedures. Specific binding (▲) is the difference between total (●) and nonsaturable binding (■), determined in the presence of a 100-fold excess of native ATXC. Average values for triplicate determinations are plotted. The insert shows a Scatchard plot for the same experiment.

of both samples were identical. LD_{50} values were 70 and 80 μg/kg, respectively, for unlabeled and iodinated ATXC. PLA_2 activities for native and [^{125}I]ATXC were 285 and 290 units/mg. The slight difference in the ip LD_{50}s between the native and iodinated ATXCs results, in our opinion, from minor experimental error rather than from the loss of biological activity upon iodination since previous studies of the influence of iodination on ATXs have shown that they are quite resistant to it (Gubenšek et al., 1982; Koželj et al., 1986). By all of these criteria, it was concluded that [^{125}I]-ATXC retained virtually 100% of its original biological activity.

Specific Binding of [^{125}I]ATXC to Bovine Brain Synaptic Membranes. Saturable binding of [^{125}I]ATXC to bovine brain synaptic membranes in Tris-HCl buffer containing 1 mM Ca^{2+} was observed (Figure 1). The larger part of the total [^{125}I]ATXC binding was displaceable by a 100-fold excess of unlabeled ATXC, so that nonsaturable binding represented 30−50% of the total binding; the latter was relatively invariant over 3−4 weeks after iodination. The Scatchard plot of the specific binding appeared linear (inset Figure 1) and the experimental data could be easily fit to a one-binding-site model. Two independent measurements gave us the following binding constants: $K_d = 5.99 \pm 0.46$ nM and $B_{max} = 5.71 \pm 0.37$ pmol/mg MP. When higher concentrations of [^{125}I]ATXC were used, the resulting Scatchard plots were distinctly concave (not shown). Since computer analysis of the experimental data assuming a two-site model yielded only poorly defined parameter estimates for the putative low-affinity binding site, which was not well resolved from the nonspecific binding, the significance of this observation remained unclear.

The nature of the ATXC binding neuronal components was explored by pretreatment of the bovine synaptic membranes with some proteinases, glycosidases, heat, or acid, followed by binding experiments. The results are summarized in Table 1. Pretreatment of the synaptic membranes with proteinase K, *S. aureus* V-8 proteinase, heat,

Table 1: Effect of Various Treatments of Bovine Synaptic Membranes on [^{125}I]ATXC Acceptors[a]

pretreatment	relative specific binding of [^{125}I]ATXC (% ± SEM)
trypsin (0.2 mg/mL)	101 ± 18
S. aureus proteinase (1 mg/mL)	36 ± 9
proteinase K (0.2 mg/mL)	52 ± 30
neuraminidase (0.1 unit/mL)	100 ± 5
PNGase F (2.5 units/mL)	100 ± 4
neuraminidase and PNGase F	84 ± 16
acidification	58 ± 9
heating	39 ± 9

[a] Specific binding of [^{125}I]ATXC, quantified as detailed in the Experimental Procedures, is expressed relative to the appropriate control values. Data from triplicate samples from at least two independent experiments are given.

Table 2: Effect of Divalent Cations on [^{125}I]ATXC Saturable Binding[a]

addition	relative specific binding of [^{125}I]ATXC (% ± SEM)
Ca^{2+}	100 ± 3
Sr^{2+}	0
Ba^{2+}	0
Cu^{2+}	0
Mg^{2+}	22 ± 5
Mn^{2+}	0
Co^{2+}	0

[a] Determination of [^{125}I]ATXC specific binding to synaptic membranes was carried out as detailed in the Experimental Procedures, except that the indicated divalent cations, at a final concentration of 1 mM (all chloride salts except Cu^{2+}, which was added as the sulfate), were included. Data from duplicate samples from two independent experiments are given. All the data are expressed relative to the value where Ca^{2+} was present in the incubation.

or acid reduced the saturable binding of [^{125}I]ATXC to various extents, demonstrating the proteinaceous nature of the ATXC acceptor. Interestingly, the saturable binding of [^{125}I]ATXC could not be completely destroyed by any of these treatments.

Pharmacology of the [^{125}I]ATXC Binding Sites. The results of inhibition of [^{125}I]ATXC binding to the neuronal acceptor by neurotoxic and nonneurotoxic proteins are illustrated in Figure 2. Under the conditions employed in the competition experiments (see Experimental Procedures), the K_I, calculated from the IC_{50} (Black et al., 1986), for ATXC was 17 nM. Other presynaptically neurotoxic PLA_2s inhibited [^{125}I]ATXC binding at very low concentrations, the only exception being β-BuTX. K_I values for ATXA and -B were 6 and 20 nM, respectively. The most potent competitor of [^{125}I]ATXC was, however, crotoxin B (CB), a PLA_2 subunit of crotoxin with a K_I of 4 nM. Interestingly, *Vbb*PLA_2, which was considered nonneurotoxic (A.L. Harvey, personal communication), competed with [^{125}I]ATXC binding at relatively low concentrations (K_I = 77 nM). Nontoxic AMI_2 and myotoxic, enzymatically nonactive AML displaced [^{125}I]ATXC from its membrane acceptor only at very high concentrations (K_I > 640 nM). α-DTX, β-BuTX, and the chaperon subunit of crotoxin, crotoxin A (CA), had no influence on the [^{125}I]ATXC specific binding. The relative potencies of the homologues, isolated from the *Vipera ammodytes ammodytes* venom, for inhibition of [^{125}I]-ATXC-specific binding (ATXA > ATXC > ATXB ≫ AMI_2, AML) parallel their toxicities in mice (Gubenšek et al., 1980; Thouin et al., 1982), which emphasizes the functionality of the acceptor being studied.

The known K^+ channel blockers, tetraethylammonium chloride (≤10 mM) and 4-aminopyridine (≤10 mM), had no effect on the [^{125}I]ATXC-specific binding to synaptic membranes.

Effects of Cations on [^{125}I]ATXC Binding. The monovalent ions K^+ and Na^+ had no influence on [^{125}I]ATXC-specific binding up to a concentration of 75 mM. However, specific binding of [^{125}I]ATXC to the bovine synaptic membranes showed a distinct dependence on the presence of Ca^{2+}. In the presence of EDTA, saturable binding and cross-linking of [^{125}I]ATXC disappeared; also, if Ca^{2+} was substituted by Sr^{2+}, Ba^{2+}, Mn^{2+}, Co^{2+}, or Cu^{2+}, the binding and cross-linking were lost. The substitution of Ca^{2+} for Mg^{2+} also diminished specific binding and cross-linking of [^{125}I]ATXC to synaptic membranes (Table 2). The structural consequences of the exchange of Ca^{2+} for Sr^{2+} in the molecule of ATXC are reflected in circular dichroism (CD) spectra in the near-UV region (255−310 nm). The differences between CD spectra recorded in Ca^{2+} and Sr^{2+} buffers show the main discrepancy to occur in the region between 280 and 285 nm, where the conformation of Tyr residues is reflected (Figure 3).

Cross-Linking of [^{125}I]ATXC to Its Synaptic Membrane Binding Proteins. Identification of the bovine synaptic membrane components exhibiting high affinity toward [^{125}I]-ATXC was achieved by cross-linking. Carbodiimide DMS was used to cross-link [^{125}I]ATXC with its synaptic membrane acceptor. Different concentrations of DMS were tested, and the best results were obtained with a cross-linker concentration of 1 mg/mL. After SDS−PAGE under reducing conditions followed by autoradiography, two adducts were detected. The predominant band was a broad band at M_r 67 000−70 000. A less intense band at M_r 65 000 was sometimes barely visible due to the intensity of the major adduct (Figure 4). The autoradiogram pattern was the same in the presence or absence of proteinase inhibitors during the preparation of synaptic membranes and in the cross-linking procedure (data not shown). For that reason, it is unlikely that the lower M_r [^{125}I]ATXC binding component is the result of the proteolytic degradation of the larger one. The adducts were not seen when either the cross-linker or the synaptic membranes were omitted from the incubations or when unlabeled ATXC was added in excess to the incubation mixtures, confirming that both cross-linked bands resulted from saturable [^{125}I]ATXC binding. An appreciable amount of specifically bound radioactivity failed to enter the gel (Figure 4). As its intensity paralleled the concentration of cross-linker used, this high molecular mass material probably arose from extensive cross-linking or aggregation. Radioactivity at the ion front, however, represented free [^{125}I]-ATXC and, perhaps, its conjugates with lower molecular mass membrane components (e.g., lipids).

Several other neurotoxic and nonneurotoxic proteins were also tested for their ability to suppress the formation of specific adducts of [^{125}I]ATXC with the bovine synaptic membrane components. The results are shown in Figure 5. Among those examined, ATXA, ATXB, CB, and *Vbb*PLA_2 were effective inhibitors, whereas AML was only weakly inhibitory. In contrast, β-BuTX and α-DTX showed no inhibition at all. These results confirmed that the adducts resulted from the interaction of [^{125}I]ATXC with the same

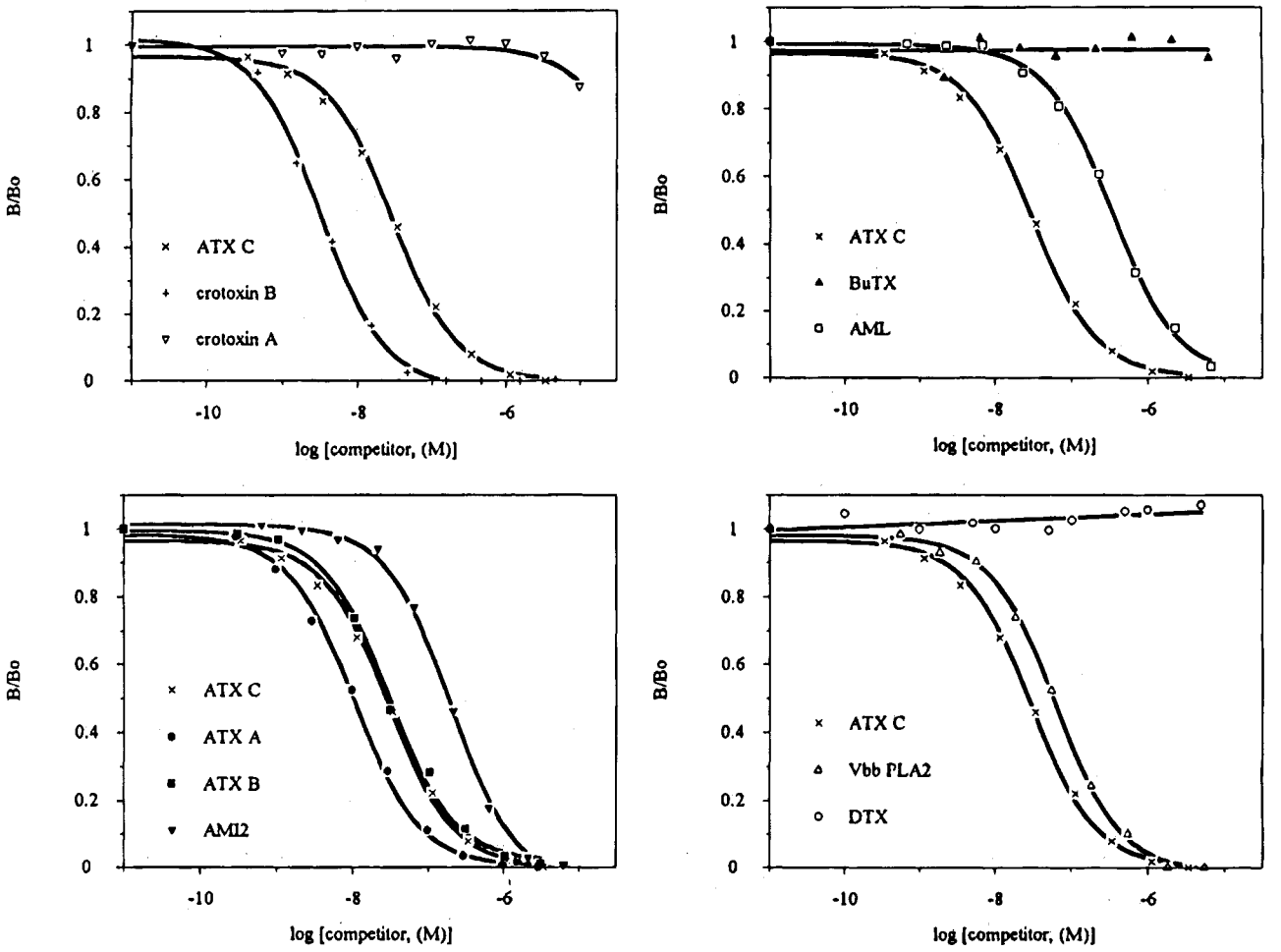

FIGURE 2: Effect of increasing concentrations of some presynaptically neurotoxic PLA_2s and other molecules on [^{125}I]-ATXC equilibrium binding to bovine brain synaptic membranes. Membranes were incubated in Tris-HCl buffer at room temperature for 1 h in the presence of 3.4 nM [^{125}I]ATXC and increasing concentrations of ATXA (●), ATXB (■), ATXC (×), CA (▽), CB (+), *Vbb*PLA_2 (△), β-BuTX (▲), α-DTX (○), AML (□), and AMI_2 (▼). Nonsaturable binding, determined in the presence of 340 nM ATXC in the incubation mixture, was subtracted from the total binding measured as detailed in the Experimental Procedures. The data (*B*, average of duplicates) are shown relative to the specific binding observed in the absence of competing ligand (B_o).

binding components that were detected in the binding experiments.

DISCUSSION

ATXC, a presynaptically neurotoxic PLA_2 from the long-nosed-viper venom, was radioiodinated to a high level and yet retained its toxicity, consequently allowing the measurement of its specific binding to bovine synaptic membranes. Under the experimental conditions employed in the binding studies, the total enzymatic activity of ATXC was negligible since the Ca^{2+} concentration was kept at 1 mM instead of 10 mM, which is required for full enzymatic activity. It was found that [^{125}I]ATXC saturably binds to synaptic membranes (Figure 1). The observed [^{125}I]ATXC high-affinity binding to bovine synaptic membranes can be described with the equation for one distinct binding site. K_d (5.99 nM) and B_{max} (5.71 pmol/mg MP) are in the usual range found for receptors of neurotransmitters, toxins, or drugs in synaptic membranes. At higher concentrations of [^{125}I]ATXC in the incubation mixtures the Scatchard plot became curvilinear, indicating the existence of another, low-affinity binding site, which was, however, not characterized further. This is probably also the reason for the slightly higher K_I of 17 nM calculated from the IC_{50} value obtained in a homologous displacement experiment. The binding characteristics of [^{125}I]crotoxin to the guinea pig brain acceptors are similar to those of [^{125}I]ATXC in bovine brain synaptic membranes. [^{125}I]crotoxin binds to two distinct binding sites with K_d = 4 and 87 nM and maximal binding capacities of 2 and 10 pmol/mg MP (Degn et al., 1991). OS_2, the single-chain PLA_2 neurotoxin from the taipan venom (*Oxyuranus scutellatus scutellatus*), whose rat brain acceptors have been studied, displays, however, much higher affinity toward its neuronal acceptors (K_{d1} = 1.5 pM and K_{d2} = 45 pM) but comparable binding site densities to ATXC (B_{max1} = 1 pmol/mg MP and B_{max2} = 3 pmol/mg MP) (Lambeau et al., 1989). The most studied PLA_2 neurotoxin, β-BuTX, which is a two-chain toxin, revealed only one binding site in the chick and rat brain preparations, with K_d = 0.47 and 0.6 nM and B_{max} = 50 and 135−160 fmol/mg MP, respectively (Othman et al., 1982; Rehm & Betz, 1982). The binding site density in the case of β-BuTX is apparently much lower than those of ATXC and other PLA_2 presynaptic neurotoxins. β-BuTX and OS_2 are elapid neurotoxins and structurally not as close to ATXC as crotoxin (Heinrikson et al., 1977), which is apparently also reflected in their different binding characteristics.

The probable protein nature of [^{125}I]ATXC binding components in bovine synaptic membranes was suggested

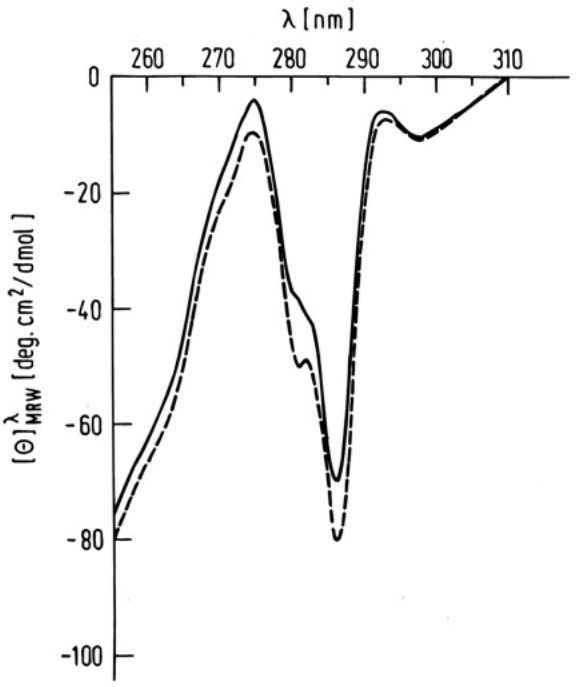

FIGURE 3: Comparative CD spectra of ATXC in the near UV in the presence of Ca^{2+} (—) and Sr^{2+} (– –). Samples were dialyzed overnight against Tris-HCl buffer (pH 7.4) containing 1 mM either Ca^{2+} or Sr^{2+} before measurements. M_{rw}, mean residue weight; $[\theta]^{\lambda}_{M_{rw}}$, mean residue ellipticity.

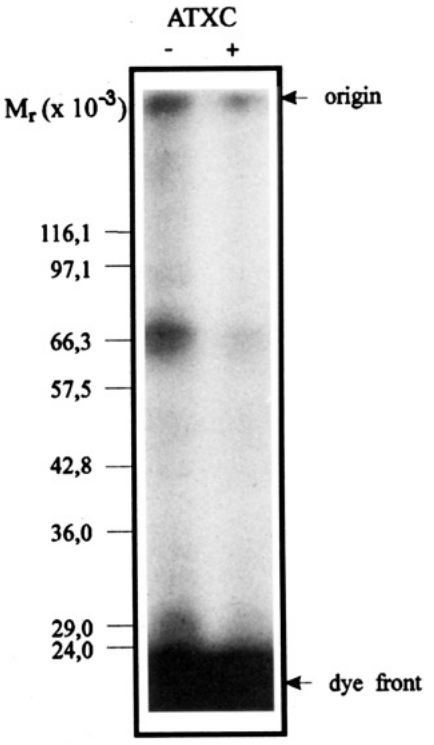

FIGURE 4: Autoradiogram patterns of [^{125}I]ATXC binding components in the bovine synaptic membrane covalently labeled using dimethyl suberimidate (DMS). After the membranes were prelabeled with [^{125}I]ATXC, DMS was added. Membrane pellets were solubilized and then analyzed on SDS–PAGE under reducing conditions. The SDS–PAGE gel was autoradiographed (see Experimental Procedures). – and + signs on the top of each lane indicate the absence or presence of a 50-fold excess of native ATXC in the incubation over [^{125}I]ATXC. Arrows indicate the positions of protein M_r standards: β-galactosidase (116 100), phosphorylase *b* (97 100), bovine serum albumin (66 300), catalase (57 500), ovalbumin (42 800), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), chymotrypsinogen (24 000). A diffuse band with M_r 67 000–70 000 and another, weaker band with M_r 65 000 were specifically labeled.

by their sensitivity to *Staphylococcus aureus* V-8 proteinase and proteinase K, to heating, and to acid treatment of membranes, which all partially decreased the specific binding of [^{125}I]ATXC. Interestingly, none of the membrane pretreatments employed could completely abolish the saturable binding of [^{125}I]ATXC, which may also imply the involvement of other membrane components resistant to these pretreatments in the binding. Negatively charged lipids have already been proposed as a part of the neuronal binding site in the cases of crotoxin and β-BuTX (Radvanyi et al., 1987, 1989). It might also be expected that in the case of ATXC certain phospholipid environment could play a role in the neurotoxin binding or that the ATXC neuronal binding site is formed in part by resistant proteins.

The specific binding of [^{125}I]ATXC to the membrane preparation was strongly influenced by divalent ions, but only when Ca^{2+} ion, which is an essential cofactor for the catalytic activity of PLA_2 (Verheij et al., 1980), was present in the incubation buffer was the specific binding considerable. The structural consequences of Ca^{2+}–Sr^{2+} exchange in the molecule of ATXC were evaluated by CD measurements, and indeed, conformational changes associated with Tyr side chains were indicated (Figure 3), which may have perturbed the interaction of ATXC with its neuronal acceptor. Similar conformational changes upon divalent ion substitution have already been observed in the case of some PLA_2s (Abe et al., 1977; Teshima et al., 1989). Other divalent ions that were tested in the binding experiments (Ba^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, and Mg^{2+}) also probably induce conformational changes in the molecule of ATXC, which then lower the affinity of the neurotoxin for its membrane acceptor or even render it incapable of binding to it. On the contrary, Delot and Bon (1993) obtained very high specific binding of [^{125}I]-crotoxin on *Torpedo* presynaptic membranes in the presence of Ba^{2+} and Zn^{2+}. OS_2 also bound specifically to rat synaptic membranes in the presence of Zn^{2+} (Lambeau et al., 1989).

The ATXC acceptor was further characterized by covalent cross-linking to [^{125}I]ATXC. Affinity labeling experiments provided an additional indication of the proteinaceous nature of [^{125}I]ATXC high-affinity neuronal binding components. Chemical cross-linking using the diimido ester, DMS, revealed the formation of specific adducts with apparent M_r values of 65 000 and 67 000–70 000, respectively. By assuming the covalent cross-linking of one molecule of [^{125}I]-ATXC (M_r = 14 000) with one molecule of the acceptor protein, the M_r values for the latter of 51 000 and 53 000–56 000 are deduced. The M_rs of the ATXC acceptors are different from those reported for the other presynaptically neurotoxic PLA_2s. Two crotoxin binding proteins were identified in a guinea pig brain synaptosomal membrane preparation in two different experiments by the group of Tzeng, one having M_r 85 000 (Hseu et al., 1990) and the other M_r 45 000 (Yen & Tzeng, 1991). Taipoxin, a three-chain PLA_2 neurotoxin from the taipan venom (*Oxyuranus scutellatus scutellatus*), binds to a protein in guinea pig brains whose M_r is also 45 000 (Tzeng et al., 1989). The rat brain binding sites for OS_2 were demonstrated to be located on 85 000–88 000 and 36 000–51 000 proteins (Lambeau et al., 1989). It must be emphasized that high M_r membrane

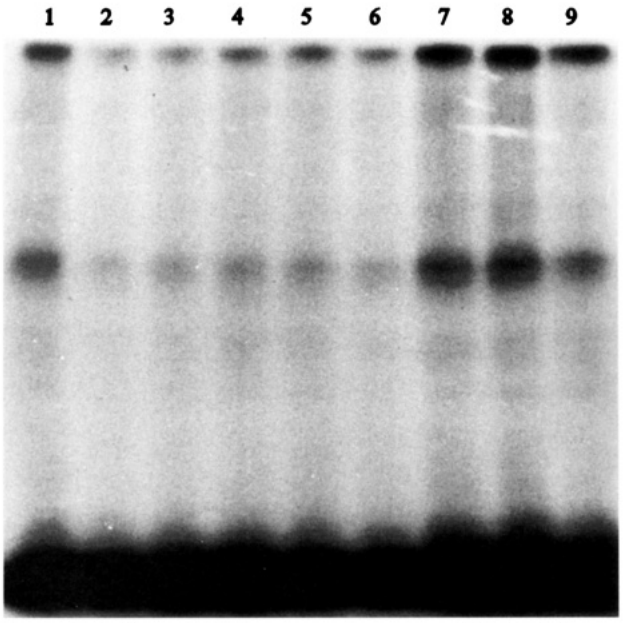

FIGURE 5: Effect of different proteins on the [^{125}I]ATXC cross-linking labeling pattern on the bovine synaptic membranes. Competitors were added to incubations in 50-fold molar excess over [^{125}I]ATXC as follows: none (lane 1); ATXC (lane 2); ATXA (lane 3); ATXB (lane 4); CB (lane 5); *Vbb*PLA$_2$ (lane 6); β-BuTX (lane 7); α-DTX (lane 8); and AML (lane 9). For details see the Experimental Procedures.

components in the cases of crotoxin and OS_2 were detected only when the photoaffinity labeling technique was used rather than chemical cross-linking. Interestingly, another high-affinity binding protein for neurotoxic OS_2 was found in rabbit skeletal muscle. Its M_r (180 000) is, however, much higher (Lambeau et al., 1990, 1991, 1994). β-BuTX formed specific adducts with proteins with M_rs of about 75 000 and 28 000 in chick brain synaptosomal membranes (Schmidt & Betz, 1989). By assuming the species variation, e.g., different extents of glycosylation of receptors for α-DTX in different species is well-known, and the differences in analytical protocols (Rehm, 1991), the M_r of the ATXC neuronal binding components is most similar to the M_r of low M_r neuronal binding components for crotoxin, taipoxin, and OS_2, but not similar to the M_r of the neuronal binding components for β-BuTX.

PLA_2 neurotoxins bind to neuronal membrane components that must have a physiological function. β-BuTX, which interacts with a subpopulation of α-DTX binding sites (Black & Dolly, 1986), almost certainly binds to one of the voltage-gated K^+ channels (Parcej & Dolly, 1989; Scott et al., 1990; Reid et al., 1992). It was shown by electrophysiological measurements that crotoxin, taipoxin, and notexin [the latter is a PLA_2 neurotoxin from the venom of the tiger snake (*Notechis scutatus scutatus*)], similar to β-BuTX, inhibit a fraction of the total outward K^+ current in the motor nerve terminals (Dreyer & Penner, 1987; Rowan & Harvey, 1988), which causes the second, facilitatory phase in the usually triphasic action of PLA_2 presynaptic neurotoxins. β-BuTX and α-DTX showed, however, no inhibition of [^{125}I]ATXC binding or cross-linking to the membrane acceptor, thus providing evidence that [^{125}I]ATXC binding sites are different from those previously described for [^{125}I]-β-BuTX. On the contrary, the crotoxin basic subunit strongly inhibited [^{125}I]-ATXC-specific binding and adduct formation. It is interesting that CB did not at all influence the specific binding of [^{125}I]crotoxin to guinea pig and *Torpedo* synaptic membranes and that ATXA was only a moderate inhibitor of [^{125}I]-crotoxin binding (Degn et al., 1991; Delot & Bon, 1993). These results suggest the existence of distinct neuronal binding sites for crotoxin and its basic subunit in these species. In the bovine brain synaptic membrane preparation, ATXC primarily binds to the CB neuronal binding site. K^+ channel blockers, 4-aminopyridine and tetraethylammonium, did not interfere with [^{125}I]ATXC binding to synaptic membranes. K^+ ions also did not affect the binding. The same was observed in the case of [^{125}I]crotoxin (Yen & Tzeng, 1991). Contrary to this, Lambeau et al. (1989) reported the inhibition of [^{125}I]OS_2 binding to rat brain synaptic membranes by aminopyridines, quinine, and quinidine, as well as by K^+ and Rb^+. In addition, the striking difference between [^{125}I]OS_2 and [^{125}I]ATXC in affinity toward specific brain acceptors may suggest that these two snake PLA_2 neurotoxins do not interact with the same neuronal binding sites.

One may speculate, therefore, that [^{125}I]ATXC acts directly on a subtype of the K^+ channel that is different from those sensitive to β-BuTX, α-DTX, or OS_2 by binding to it or acts indirectly by affecting some neuronal membrane component connected to it. The clear answer about the exact nature and physiological role of the ATXC neuronal acceptors is still to come; therefore, experiments are underway to isolate them for structural and functional studies.

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