BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 209–212 (1998)
ARTICLE NO. RC989427

# Identification of a New High-Affinity Binding Protein for Neurotoxic Phospholipases $A_2$

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Received September 3, 1998

**Ammodytoxin C is a neurotoxic phospholipase $A_2$ which blocks the release of neurotransmitter from the nerve terminal. Using a radioiodinated derivative of the toxin, we located its specific high-affinity binding site in the demyelinated P2 fraction of porcine cerebral cortex ($K_d$ = 15 nM; $B_{max}$ = 1.5 pmol/mg membrane protein). In cross-linking experiments on a membrane preparation, $^{125}$I-ammodytoxin C labeled a protein of 25 kDa. The formation of a specific adduct was not inhibited by nontoxic phospholipases $A_2$ or even by neurotoxic phospholipases $A_2$ which have practically identical pathophysiological activities to ammodytoxin C: agkistrodotoxin, *Oxyuranus scutellatus* 2 phospholipase $A_2$, taipoxin, β-bungarotoxin, notexin, and crotoxin. $^{125}$I-ammodytoxin C specific cross-linking was inhibited, however, by mannosylated BSA, suggesting the presence of a carbohydrate-recognition domain in the acceptor structure. According to the pharmacological and structural properties, the ammodytoxin acceptor from porcine cerebral cortex differs from other so far identified as phospholipase $A_2$ acceptors and represents a new type of a high-affinity binding protein for neurotoxic phospholipases $A_2$.**


Phospholipases $A_2$ (EC 3.1.1.4) ($PLA_2$), which exhibit a variety of pharmacological effects, are abundant components of many snake venoms [1]. One of the most interesting activities of $PLA_2$s is presynaptic neurotoxicity where these toxins block the release of acetylcholine from the nerve terminal. It is widely accepted that the enzymatic activity of these $PLA_2$s plays an important role in their neurotoxic action, however, it is not sufficient *per se* to induce the neuro-muscular blockade (reviewed in [2]). Membrane acceptors, which seem to mediate the pharmacological effect, were found in presynaptic terminals of different animals (reviewed in [3] and [4]). The discovery of neuronal acceptors for neurotoxic $PLA_2$s has made these molecules especially interesting as potential tools for investigating neurotransmission.

$PLA_2$ neurotoxins have been classified into three groups on the basis of their quaternary structure: single-chain and multi-chain forms and β-bungarotoxins, in which two chains are covalently linked by a disulfide bridge [5]. Another classification distinguishes $PLA_2$s according to the number and position of intramolecular disulfide bonds (reviewed in [6]). Although pathomorphological and electrophysiological actions of the neurotoxic $PLA_2$s which belong to different structural classes are practically identical (reviewed in [5]), their neuronal targets are, surprisingly, different. Three distinct types of proteins have been identified to date as $PLA_2$-acceptors in various neuronal tissues: (1) the α-subunit of voltage-dependent $K^+$ channels [7], (2) proteins of the pentraxin family, neuronal pentraxin (NP) and its receptor (NPR), which are homologous to the acute phase proteins, serum amyloid P protein and C-reactive protein [8, 9], and (3) proteins homologous to calcium-binding proteins of the reticulocalbin family, namely taipoxin-associated calcium binding protein 49 (TCBP-49) [10] and crotoxin binding protein 50 (CBP-50) [11]. In addition to these three types of acceptors for neurotoxic $PLA_2$s, another type has been found in rabbit skeletal muscle, which is a homologue of macrophage mannose receptor [12].

In the present paper we report the discovery of an acceptor for ammodytoxin C, a monomeric $PLA_2$ neurotoxin from *Vipera ammodytes* venom, in porcine cerebral cortex. The toxin binding characteristics of the acceptor, its pharmacology and structural features are

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Abbreviations used: Agtx, agkistrodotoxin; $AtnI_2$, ammodytin $I_2$; Atx, ammodytoxin; β-Butx, β-bungarotoxin; CBP-50; crotoxin binding protein 50; CRD, carbohydrate-recognition domain; DSS, disuccinimidyl suberate, man-BSA, mannosylated bovine serum albumin; $OS_2$, *Oxyuranus scutellatus* 2 phospholipase $A_2$; $PLA_2$, phospholipase $A_2$; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCBP-49, taipoxin-associated calcium binding protein 49.



0006-291X/98 $25.00

shown to be different from those already known for neurotoxic $PLA_2$ acceptors, suggesting the existence of a new, fifth type of acceptor for presynaptically neurotoxic $PLA_2$s.

## MATERIALS AND METHODS

*Materials.* Ammodytoxins and ammodytin $I_2$ ($AtnI_2$) were purified from the venom of *Vipera ammodytes ammodytes* [13, 14]. Crotoxin (*Crotalus durissus terrificus*) and agkistrodotoxin (*Agkistrodon blomhoffii brevicaudus*) (Agtx) were a kind gift from Dr. Cassian Bon, Institut Pasteur, Paris, France, notexin (*Notechis scutatus scutatus*) from Dr. S. Gasparini, CEA, Saclay, France and *Oxyuranus scutellatus* 2 $PLA_2$ ($OS_2$) from Dr. G. Lambeau, Institut de Pharmacologie Moleculaire et Cellulaire, CNRS, Valbonne, France. Taipoxin (*Oxyuranus scutellatus scutellatus*), β-Butx (*Bungarus multicinctus*), porcine pancreatic $PLA_2$ and bee venom $PLA_2$ (*Apis meliffera*) were from Sigma. $Na^{125}I$ (carrier-free) was from New England Nuclear and disuccinimidyl suberate (DSS) from Pierce.

*Membrane preparation.* A slightly modified method of Bennett *et al.* [15] was used to prepare a demyelinated P2 fraction of porcine cerebral cortex. To minimize proteolytic degradation samples were kept on ice or at 4°C throughout the isolation and the following proteinase inhibitors were added: 1 mM EDTA, 25 μg/ml bacitracin, 10 μg/ml soybean trypsin inhibitor, 0.2 mM benzamidin, 0.1 mM phenylmethylsulphonyl fluoride. Protein content in the final preparation was determined by the method of Markwell *et al.* [16], using bovine serum albumin as a standard protein.

*Preparation of radioiodinated toxins.* The modified chloramine T method of Greenwood *et al.* [17] and the purification protocols as described by Križaj *et al.* [18, 19] were used to prepare $^{125}$I-AtxC and $^{125}$I-crotoxin, which were identical in their biological activities to the respective unlabeled toxins. Specific radioactivity of different $^{125}$I-AtxC preparations was always around 300 Ci/mmol and of $^{125}$I-crotoxin, 670 Ci/mmol.

*Binding studies.* Binding experiments were performed at room temperature. Various incubation conditions were tested where the effects of mono and divalent cations, pH, ionic strength and incubation time on the $^{125}$I-AtxC specific binding were examined. The final composition of the incubation buffer in which specific binding of the toxin was high and its $PLA_2$ activity negligible was: 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. Membranes (1 mg membrane protein/ml) were incubated with $^{125}$I-AtxC (6.4 nM) for 30 min in the presence or absence of an excess of unlabeled competitor (2–4 μM). Separation of membrane bound and free radioligand was made by rapid centrifugation of the membranes through a silicone-based oil mixture [20]. The non-linear curve fitting program GraFit 3.0 [21] was used to analyse the data obtained from γ-counting of the samples (80% efficiency).

*Cross-linking experiments.* After the membranes were prelabeled as described under *Binding studies*, they were pelleted by centrifugation (14,000 × *g* for 10 min). The supernatant was aspirated and the membranes resuspended in 490 μl of cross-linking buffer (0.5 M triethanolamine, pH 8.2, 150 mM NaCl). DSS, dissolved in 10 μl of dimethyl sulfoxide was added to a final concentration of 100 μM. After 5 min at room temperature the reaction was stopped by adding of 500 μl 0.5 M Tris–HCl, pH 6.8. The membranes were pelleted (14,000 × *g* for 10 min), solubilized in SDS–PAGE sample buffer (50 mM DTT) and analysed on 10% polyacrylamide gels [22]. Gels were stained with Coomassie Brilliant Blue R-250, dried, and autoradiographed at −70°C using Kodak XAR films and two intensifying screens.

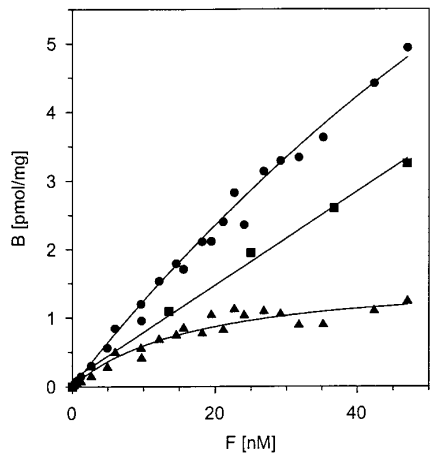


**FIG. 1.** Equilibrium binding of $^{125}$I-AtxC to porcine cerebral cortex membrane preparation. The membranes were incubated at pH 7.4 with increasing concentrations of $^{125}$I-AtxC. At equilibrium, aliquots were removed for determination of the membrane bound (B) and free (F) $^{125}$I-AtxC. Specific binding (▲) is the difference between total (●) and non-saturable binding (■), which was determined in the presence of a 200-fold excess of unlabeled over labeled toxin. Average values of triplicate determinations are plotted.

*Membrane treatments.* Synaptic membranes were treated before cross-linking experiments with proteinase K, trypsin or heat as described [18, 23].

## RESULTS AND DISCUSSION

$^{125}$I-AtxC bound saturably to demyelinated membrane fraction (P2 fraction) from porcine cerebral cortex (Fig. 1). The specific binding was equally high (about 40 % of the total binding) if $Sr^{2+}$ ions in the incubation buffer were substituted for $Ca^{2+}$ or $Ba^{2+}$ ions. On the contrary in the presence of $Mg^{2+}$ or $Cu^{2+}$ ions the specific binding was close to zero. Complete removal of free divalent ions by EDTA also substantially lowered the specific binding; however, it was not completely eliminated. In bovine cerebral cortex membrane preparation the specific binding of $^{125}$I-AtxC to depended much more on the type of divalent ion present during the incubation [18]. The specific binding of $^{125}$I-AtxC to porcine brain membranes was pH-dependent. From pH 7.4, where it was maximal, it drops to zero at pH 5.5 while in the pH interval from 7.4 to 9 it remained high. The addition of NaCl or KCl in concentrations up to 200 mM did not influence the specific binding. The specific binding reached its maximum after 30 min at room temperature and remained stable for at least an hour. Using the incubation buffer with 10 mM $Sr^{2+}$ and 0.5 mM EGTA, where the $PLA_2$ activity of the toxin is insignificant, the following binding parameters were obtained: $K_d$ = 15 nM; $B_{max}$ = 1.5 pmol/mg membrane protein (Fig. 1).

Disuccinimidyl suberate (DSS) was used to cross-link $^{125}$I-AtxC with its membrane acceptor. At 100 μM

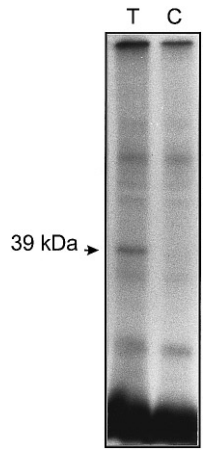


**FIG. 2.** Affinity labeling of the AtxC-binding component in porcine synaptic membranes. The membrane preparation was incubated with $^{125}$I-AtxC in the absence (T) or presence (C) of a 600-fold excess of unlabeled over labeled toxin. After cross-linking (DSS), membranes were solubilized and analyzed on SDS–PAGE under reducing conditions. The patterns shown are autoradiographs. The apparent molecular mass of the specific adduct was determined using Bio-Rad kaleidoscope prestained standards.

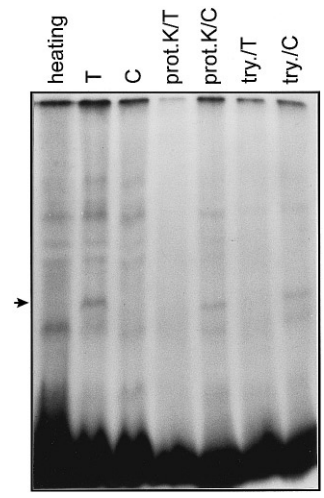


**FIG. 3.** Effect of proteinase and heat treatment of the membrane preparation on the $^{125}$I-AtxC cross-linking pattern. The membranes were either boiled for 5 min (heating), exposed to proteinase K (prot.K/T) or trypsin (try./T), before $^{125}$I-AtxC-labeling. In control experiments, proteinase inhibitors, PMSF or soybean trypsin inhibitor, were present during the membrane exposure to proteinase K (prot.K/C) or trypsin (try./C), respectively, before $^{125}$I-AtxC-labeling. (T) and (C) refer to $^{125}$I-AtxC-labeling of the untreated membranes in the absence or presence of a 600-fold excess of unlabeled over labeled toxin. An arrow indicates the position of the specific $^{125}$I-AtxC-acceptor complex.

DSS no effect on the membrane protein pattern was noticed on SDS–PAGE gels after Coomassie blue staining. Subsequent autoradiography of the gels revealed the formation of only one specific adduct (Fig. 2) whose apparent molecular mass was 39 kDa. Assuming that one molecule of $^{125}$I-AtxC (14 kDa) binds one molecule of the acceptor, the molecular mass of the latter is 25 kDa. Proteinase (trypsin or proteinase K) or heat treated membranes lost the ability to form the specific adduct with $^{125}$I-AtxC, indicating the proteinaceous nature of the AtxC membrane acceptor (Fig. 3). The ammodytoxin binding proteins found in bovine cerebral cortex [18, 23] and *Torpedo marmorata* electric organ [19] differ in their molecular weights from the one identified in porcine brain, as do the acceptors for other presynaptically neurotoxic $PLA_2$s identified to date [7, 24–28].

Several toxic and non-toxic $PLA_2$s were tested for their ability to inhibit the formation of the specific adduct of $^{125}$I-AtxC with the porcine brain synaptic membranes. Only two iso-ammodytoxins, AtxA and AtxB, were able to compete with $^{125}$I-AtxC for its membrane acceptor while the neurotoxins agkistrodotoxin, $OS_2$, crotoxin, taipoxin and $\beta$-bungarotoxin as well as non-toxic $AtnI_2$ did not inhibit binding (Fig. 4A). Notexin, bee venom $PLA_2$ and porcine pancreatic $PLA_2$ also did not inhibit binding (results not shown).

Recently, neuronal acceptors for crotoxin were isolated from porcine brain [11]. Two proteins of 50 and 18 kDa were retained on a crotoxin-affinity column. Partial amino acid sequence of the 50 kDa crotoxin binding protein (CBP-50) show homology with a group of calcium binding proteins, namely reticulocalbin [29], TCBP-49 [10], calmodulin, troponin C and parvalbumin [30]. In the experiments performed in our laboratory, $^{125}$I-crotoxin affinity labeled only a 50-kDa protein in porcine brain membranes, and the labeling

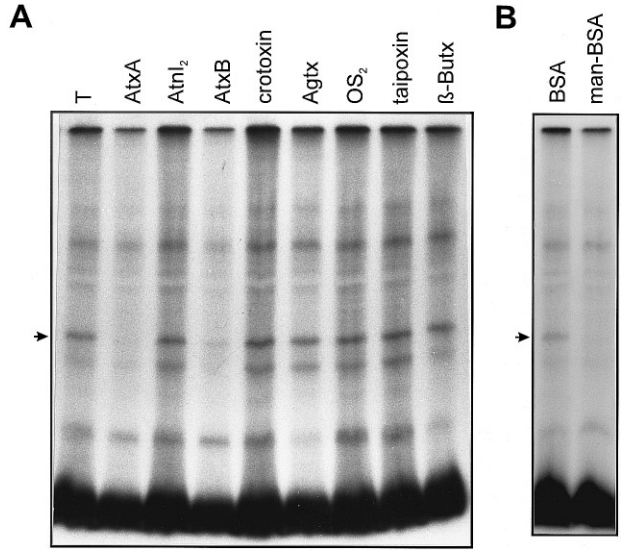


**FIG. 4.** The influence of various $PLA_2$s (A) and mannosylated BSA (B) on the $^{125}$I-AtxC-acceptor adduct formation. Porcine synaptic membranes were incubated with $^{125}$I-AtxC in the absence (T) or presence of the indicated competitors in excess, before the cross-linking procedure. An arrow points the position of the specific $^{125}$I-AtxC adduct.

could not be prevented by AtxC (data not shown). As already shown for the peripheral nervous system [19], in the central nervous system AtxC and crotoxin do not interact with the same binding proteins, indicating that their mechanisms of action are different at the molecular level. Besides ammodytoxins, the only substance able to inhibit the binding of $^{125}$I-AtxC to its membrane acceptor, was mannosylated BSA (Fig. 4B), suggesting the presence of a carbohydrate-recognition domain (CRD) in the acceptor's structure. It is known that domains related to CRDs of C-type lectins are present in the structures of muscle or M-type 180-kDa $PLA_2$ receptors, and that they are implicated in $PLA_2$ binding [12, 31]. It seems that the 25-kDa neuronal acceptor and M-type receptors [32] bind $PLA_2$s in a similar way but, considering their substantially different biochemical characteristics, they cannot be classified into the same group of $PLA_2$ acceptors. Voltage-dependent $K^+$ channels [7] and reticulocalbins (TCBP-49) [10] do not contain CRDs in their structures. However, the C-terminal parts of pentraxins (NP and NPR) [8, 9] are homologous to CRD5 of rabbit M-type $PLA_2$ receptor exhibiting about 20% identity. On the other hand, neuronal pentraxins differ from ammodytoxin binding protein in their molecular weight, which is almost two-times higher and by their high affinity for taipoxin, which is not recognized at all by ammodytoxin acceptor.

Based on the pharmacological and structural properties presented here, ammodytoxin acceptor from porcine cerebral cortex differs from all the types of $PLA_2$ acceptors so far identified and represents a new type of a high affinity binding protein for neurotoxic phospholipases $A_2$.

## ACKNOWLEDGMENTS

We thank Dr. Roger H. Pain for critical reading of the manuscript. This work was supported by the Ministry of Science and Technology of Slovenia (Grant J1-7261-0106).

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