A High Affinity Acceptor for Phospholipase A$_2$ with Neurotoxic Activity Is a Calmodulin*

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One of the high affinity binding proteins for ammodytoxin C, a snake venom presynaptically neurotoxic phospholipase A$_2$, has been purified from porcine cerebral cortex and characterized. After extraction from the membranes, the toxin-binding protein was isolated in a homogenous form using wheat germ lectin-Sepharose, Q-Sepharose, and ammodytoxin-CH-Sepharose chromatography. The specific binding of $^{125}$I-ammodytoxin C to the isolated acceptor was inhibited to different extents by some neurotoxic phospholipases A$_2$, ammodytoxins, bee venom phospholipase A$_2$, agkistrodotoxin, and crotoxin; but not by nontoxic phospholipases A$_2$, ammodytin I$_2$, porcine pancreatic phospholipase A$_2$, and human type IIA phospholipase A$_2$, suggesting the significance of the acceptor in the mechanism of phospholipase A$_2$ neurotoxicity. The isolated acceptor was identified as calmodulin by tandem mass spectrometry. Since calmodulin is generally considered as an intracellular protein, the identity of this acceptor supports the view that secretory phospholipase A$_2$ neurotoxins have to be internalized to exert their toxic effect. Moreover, since ammodytoxin is known to block synaptic transmission, its interaction with calmodulin as an acceptor may constitute a valuable probe for further investigation of the role of the latter in this Ca$^{2+}$-regulated process.

Phospholipases A$_2$ (PLA$_{2}$, EC 3.1.1.4) form an expanding superfamily of enzymes, which catalyze hydrolysis of the ester bond at the sn-2 position of 1,2-diacyl-sn-3-phosphoglycerides. Intracellular and secretory PLA$_{2}$s (sPLA$_{2}$s) are currently classified under 12 structurally different groups (1, 2). Secretory PLA$_{2}$s are enzymes of 13–18 kDa containing five to eight disulfide bonds. They show much higher affinity for aggregated substrates (interfacial catalysis), and millimolar Ca$^{2+}$ is essential for their catalytic activity (reviewed in Ref. 3). Secretory PLA$_{2}$s have been associated with many physiological and pathophysiological processes, in certain cases not only the enzymatic activity of sPLA$_{2}$ but also its interaction with a specific target protein is required (reviewed in Refs. 4–6). Different membrane and soluble proteins have been identified as selective and high affinity acceptors for sPLA$_{2}$s. Secretory PLA$_{2}$s have been shown to bind to voltage-dependent K$^{+}$ channels (7), pentraxins (8, 9), reticulocalbins (10, 11), C-type multilectins (12–14), factor Xa (15), and proteoglycan glypican (16). Inhibitors for sPLA$_{2}$s, which belong to three distinct structural types, C-type lectins, three-finger proteins, and proteins containing leucine-rich repeats, have also been identified in sera of various animals (reviewed in Ref. 5). The increasing number of endogenous sPLA$_{2}$s identified in mammals and the versatility of their acceptors suggest that many biological roles for the different sPLA$_{2}$s are yet to be discovered.

The inhibition of neurotransmission by some sPLA$_{2}$s from snake venoms has also been found to depend on the interaction of toxic sPLA$_{2}$ with specific receptor(s) in the nerve terminal of the victim (reviewed in Ref. 17). Despite numerous studies, the molecular basis of this process is still largely unknown. To learn more about the molecular mechanism of PLA$_{2}$ neurotoxicity and about the physiological processes that are affected by these toxins, we have used the presynaptically neurotoxic group IIA PLA$_{2}$ ammodytoxin C (AtxC) from Vipera ammodytes ammodytes venom (18, 19). Two high affinity binding proteins for AtxC have been detected in porcine cerebral cortex, which are potentially implicated in the neurotoxicity of this PLA$_{2}$ (20, 21), and the purification and characterization of the high molecular mass AtxC-binding protein has been described previously (21). In this communication we report the purification of the other, 16-kDa, AtxC-binding protein (R16). This protein is identified as calmodulin (CaM), a very important and highly conserved EF-hand Ca$^{2+}$-binding protein that participates in signaling pathways that regulate many physiological processes (reviewed in Ref. 22).

EXPERIMENTAL PROCEDURES

Materials—Ammodytoxins, ammodytin I$_2$ (AtnI$_2$) and AtnL$_2$, were purified from V. ammodytes ammodytes venom as described previously (18, 23). Crotoxin (from Crotalus durissus terrificus) and agkistrodotoxin (from Agkistrodon bilineatus brevicaudus) were gifts from Dr. Cassian Bon, Institut Pasteur, Paris, France. OS$_{2}$ (from Oxyuranus scutellatus scutellatus) was a gift from Dr. Gerard Lambeau, Institut de Pharmacologie Moleculaire et Cellulaire, CNRS, Valbonne, France. Taipoxin (O. scutellatus scutellatus) and $\delta$-bunatorxin (Bungarus multicinctus) were from Sigma. Porcine pancreatic PLA$_{2}$, bee venom PLA$_{2}$, hog brain CaM, and Triton X-100 were from Roche Molecular Biochemicals. Mouse monoclonal anti-CaM antibodies were from Upstate Biotechnology. Na$^{125}$I (carrier-free) was from PerkinElmer Life Sciences. Disuccinimidyl suberate (DSS) was from Pierce. Affi-Gel 10 porcine cerebral cortex of 16, 25, and 180 kDa, respectively; sPLA$_{2}$, secretory PLA$_{2}$; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid; PVDF, polyvinylidene difluoride.
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and protein molecular mass standards were from Bio-Rad. Q-Sepharose and wheat germ lectin-Sepharose 6MB were from Amersham Pharmacia Biotech. All other reagents and chemicals were of analytical grade. Radioiodination of AtxC—AtxC was radioiodinated as described previously (24) to specific radioactivity around 300 Ci/nmol. 125I-AtxC was identical to the native AtxC in enzymatic, neurotoxic, and immunological properties.

Membrane Preparation from Porcine Cerebral Cortex—A demyelinated P2 fraction of porcine cerebral cortex was prepared and the protein content in the membrane preparation determined as described previously (21).

Solubilization of AtxC-binding Proteins—Membranes from porcine cerebral cortex (7.2 mg of membrane protein/ml) were extracted for 1 h by gentle agitation at 4 °C in 75 mM Hepes, pH 8.2, containing 150 mM NaCl, 2.5 mM CaCl₂, and 2.5% (w/v) Triton X-100. The extract was centrifuged at 106,000 × g for 1 h and cold deionized water added to the supernatant to give a final detergent concentration of 2.0% (w/v).

Cross-linking of 125I-AtxC to the Solubilized AtxC-binding Proteins—Samples were incubated for 30 min at room temperature with 125I-AtxC in the presence of an inert detergent. Disulfide bonds (DSS) was added (100 μM final concentration), and after 5 min the cross-linking reaction was stopped by the addition of SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE and gels dried and autoradiographed at ~70 °C using Kodak X-Omat AR films (21).

Coupling of AtxC to CH-Sepharose 4B—CH-Sepharose 4B was swollen according to the manufacturer’s recommendation. AtxC (2.4 mg/ml gel) was dissolved in 100 mM MES, pH 6.5, 5 mM CaCl₂, 0.5 mM NaCl; added to the activated Sepharose to a final concentration of 0.8 mg/ml; and incubated with agitation at 4 °C. After 4 h the gel was washed and its remaining active groups blocked with 1 M ethanolamine, pH 8.0, for 1 h. Routinely about 90% of AtxC was bound to the matrix. The resin was washed as recommended by the producer; equilibrated in 75 mM Hepes, pH 8.2, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% (w/v) Triton X-100; and stored at 4 °C.

Chromatography on Wheat Germ Lectin-Sepharose 6MB—9 ml of wheat germ lectin-Sepharose 6MB was equilibrated with 50 mM Hepes, pH 8.2, containing 140 mM NaCl and 2 mM CaCl₂. The detergent extract was incubated with the gel for 4 h at 4 °C with moderate agitation. The supernatant was separated from the gel on a sintered glass funnel.

Ion Exchange Chromatography—5 ml of Q-Sepharose was equilibrated in 75 mM Hepes, pH 8.2, containing 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% (w/v) Triton X-100. The resin was equilibrated in the presence of an active gelatin-Sepharose. Dispersed gelatin-Sepharose was incubated with the gel for 1 h at 4 °C with slight agitation. The gel was extensively washed with the equilibrium buffer. The bound material was eluted with 20 ml of the equilibrium buffer supplemented with 0.5 mM NaCl.

Chromatography on AtxC-CH-Sepharose 4B—The gel was equilibrated with 75 mM Hepes, pH 8.2, containing 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% (w/v) Triton X-100. The eluate from Q-Sepharose was incubated with 5 ml of gel at 4 °C for 4 h with gentle agitation. The resin was washed as recommended by the producer; equilibrated in 75 mM Hepes, pH 8.2, 150 mM NaCl, 2.5 mM CaCl₂, and 0.1% (w/v) Triton X-100; and stored at 4 °C.

Electroblotting and Immunochemiluminescence Detection—Samples were run on SDS-PAGE (12.5% acrylamide gels) and transferred (90 min at 250 mA) to a PVDF membrane (Bio-Rad). The transfer buffer was 0.1 M KH₂PO₄/K₂HPO₄, pH 7.0. After transfer, the membrane was incubated with mouse monoclonal anti-CaM antibodies at the concentration of 1 μg/ml. Immunodetection was performed by the BM chemiluminescence Western blotting detection system (Roche Molecular Biochemicals) following the manufacturer’s instructions.

Mass Spectrometry—The sample was analyzed by mass spectrometry as described previously (27). Briefly, 1 μg of the R16 sample was separated on a 12% acrylamide gel (12% acrylamide gel, 2% SDS–PAGE gels). The gel was stained with silver and the protein band excised and transferred to a siliconized tube. The gel piece was destained overnight then reduced with dithiothreitol, alkylated with iodoacetamide, and digested with Promega modified trypsin for 16 h. The peptides were extracted from the gel with 50% acetonitrile/5% formic acid and concentrated for LC-MS and MS/MS analysis on a Finnigan LCQ ion trap mass spectrometer. The spectra obtained were analyzed by data base searching using the Sequest algorithm against the NCBI nonredundant data base. Those peptides not matched were interpreted manually.

RESULTS AND DISCUSSION

AtxC-binding proteins, solubilized from the demyelinated P2 fraction of porcine cerebral cortex with Triton X-100, retained their toxin binding activity (Fig. 1B, lane 1). Two specific adducts were clearly observed after affinity labeling of the extract with 125I-AtxC as described (Fig. 1A, lane 6, eluate from AtxC-CH-Sepharose). The positions of specific adducts are indicated by arrowheads.

In contrast to R180, R25 is not retained by concanavalin A, wheat germ lectin, or lentil lectin-Sepharose (20, 21). In addition to R180 and R25, AtxC was retained by wheat germ lectin-Sepharose 6MB was equilibrated with 50 mM Hepes, pH 6.5, 5 mM CaCl₂, and 2.5% (w/v) Triton X-100. The extract was centrifuged at 106,000 × g for 1 h and cold deionized water added to the supernatant to give a final detergent concentration of 2.0% (w/v).

We devised a strategy for purifying R25 based on the following observations. 1) The interaction between AtxC and R25 depends on Ca²⁺ ions. 2) R25 loses affinity for AtxC below pH 5.5 and regains it completely when the pH is returned to 7.4. 3. In contrast to R180, R25 is not retained by concanavalin A, wheat germ lectin, or lentil lectin-Sepharose (20, 21). In addition to R180 and R25, AtxC was retained by wheat germ lectin-Sepharose to remove R180 before being applied to Q-Sepharose at pH 7.4. The Q-Sepharose-retained AtxC-binding protein was eluted batchwise with a high concentration of 0.5 M NaCl.
NaCl. This step was important to reduce the concentration of Triton X-100 in the preparation and so enable efficient subsequent purification steps. 125I-AtxC affinity labeling of the eluate from ion exchange chromatography revealed, however, not the expected 39-kDa adduct but only a specific adduct of about 30 kDa (Fig. 1B, lane 3). Such a specific adduct was sometimes also visible in the crude membrane extract after storage for a longer time at −20 °C. Since the specific adduct at 39 kDa disappeared at the same time as the specific adduct at 30 kDa appeared following the Q-Sepharose step, it appears that R25 is an oligomeric protein in which the 16-kDa subunit (R16) carries the toxin-binding site. AtxC-Affi-Gel 10, successfully used in purification of R180, did not give satisfactory results in the purification of R16 (21). Using activated CH-Sepharose, we prepared an AtxC-affinity resin, which was much more efficient. Analysis of the sample after the toxin-affinity chromatography is shown in lanes 6 of Fig. 1, A and B. From 72 mg of membrane protein in the starting preparation we obtained about 2 μg of pure R16, as judged by semiquantitative densitometric analysis of the silver-stained SDS-PAGE band of the final product. The affinity of AtxC for the isolated R16 was estimated in the cross-linking competition experiment (21). Native AtxC displaced 125I-AtxC from R16 with a dissociation constant of 11 nM.

Several toxic and nontoxic sPLA2s were tested for their ability to inhibit the formation of the specific adduct under conditions between 125I-AtxC and the isolated acceptor. Only the neurotoxic ammodyotoxins and bee venom PLA2 were able to completely prevent the binding of 125I-AtxC to R16 under the experimental conditions used. Agkistrodonotoxins, crototoxins, and myotoxicanmodytoxin L were weaker inhibitors, while OS2n, taipoxin, and β-bungarotoxin as well as the nontoxic AtnL2, porcine pancreatic PLA2, and human type IIA PLA2 did not inhibit the binding (Fig. 2).

The isolated AtxC-binding protein was identified by tandem MS analysis. R16 was reduced, alkylated, and digested with trypsin in the gel. The resulting peptides were extracted from the polyacrylamide and their molecular weights and amino acid sequences determined using an LC-MS system. Analysis of the data gave the result shown in Fig. 3A. The partial sequence of R16 matches completely with that of CaM, a protein essential to many fundamental physiological processes (reviewed in Ref. 22). Comparing the sequence of CaM with the primary structures of other sPLA2-binding proteins, some similarity has been found only to TCBP-49 (taipoxin-associated calcium-binding protein of 49 kDa) (10) and crocolin (11). These two proteins belong to reticulocalbins (28) and are, like CaM, EF-hand Ca2+-binding proteins (29).

sPLA2 neurotoxins interfere with membrane trafficking (reviewed in Ref. 30). The implication of CaM in different modes of membrane trafficking: transcytosis (31), endosome fusion (32, 33), vacuole fusion (Refs. 34 and 35 and reviewed in Ref. 36), intra-Golgi membrane transport (37), and rapid endocytosis in adrenal chromaffin cells (38) is well documented. It may also be involved as one of the Ca2+-sensors in regulated exocytosis (39, 40). CaM would be therefore a perfect target for the toxin, which is known to block synaptic transmission.

To confirm that the target for neurotoxic PLA2 is indeed a CaM, the R16 preparation was incubated with 125I-AtxC in the absence and presence of 2 μM native AtxC. Following the cross-linking, reaction mixtures were separated on SDS-PAGE and electro-blotted onto PVDF membrane. Immunodetection with anti-CaM Ab revealed a positive band at 30 kDa (Fig. 3B). The same PVDF membrane was used as the chemiluminescence had been completely quenched, autoradiographed to visualize the 125I-AtxC. As is evident from Fig. 3B, 125I-AtxC was specifically present in the band having the same molecular mass as the band labeled with anti-CaM Ab. In addition, the interaction between 125I-AtxC and commercially available porcine brain CaM was demonstrated (not shown). CaM is a soluble protein, so the fact that a high concentration of the detergent was necessary to extract R16 from the P2 membranes suggests that R16 is part of an oligomeric membrane-anchored protein complex (R25).

An acceptor for sPLA2, ammodyotoxin, is shown to be a calmodulin. Since CaM is generally regarded as an intracellular protein, this finding strengthens the proposition that the neurotoxic sPLA2 has to enter the neuron to produce the arrest of synaptic vesicle cycling. In addition, the observed specific and high affinity interaction between CaM and neurotoxic AtxC could be used to investigate the role of CaM in this process.

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**FIG. 2. sPLA2 binding properties of R16.** The preparation of R16 was incubated with 10 nM 125I-AtxC in the absence and presence of the indicated sPLA2 at 2 nM final concentration, followed by cross-linking with DSS. Only the sPLA2 with neurotoxic activity were able to inhibit the adduct formation. Agtx, agkistrodotoxin; bv, bee venom; pp, porcine pancreatic; β-Butx, β-bungarotoxin; ha IIA, human type IIA.

**FIG. 3. Molecular identification of R16.** A, using tandem mass spectrometry, R16 was identified as CaM. Boldface amino acids indicate those sequenced. Underlined amino acids indicate modification of the amino acid. Specifically, Ala-1 was found to be acetylated at the N terminus and Lys-115 was found to be N3-trimethylated. B, the preparation of R16 was incubated with 10 nM 125I-AtxC in the absence (−) and the presence (+) of 2 μM AtxC, after which the cross-linker DSS was added. Western blotting (WB) analysis with monoclonal anti-CaM antibodies and 125I autoradiography (AR) of the same PVDF membrane confirmed CaM and AtxC as components of the specific adduct (arrowheads).
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