Ammodytoxins, Potent Presynaptic Neurotoxins, Are Also Highly Efficient Phospholipase A$_2$ Enzymes†

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ABSTRACT: The enzymatic activity of ammodytoxins (Atxs), secreted phospholipases A$_2$ (sPLA$_2$s) in snake venom, is essential for expression of their presynaptic neurotoxicity, but its exact role in the process is unknown. We have analyzed in detail the enzymatic properties of Atxs, their mutants, and homologues. The apparent rates of phospholipid hydrolysis by the sPLA$_2$s tested vary by up to 4 orders of magnitude, and all enzymes display a strong preference for vesicles containing anionic phospholipids, phosphatidylglycerol or phosphatidylserine (PS), over those containing zwitterionic phosphatidylycholine (PC). Nevertheless, Atxs are quite efficient in hydrolyzing pure PC vesicles as well as PC-rich plasma membranes of intact HEK293 cells. The presence of anionic phospholipids in PC vesicles dramatically increases the interfacial binding affinity and catalytic activity of Atxs, but not of their nontoxic homologue ammodytin I$_2$, that displays unusually low binding affinity and enzymatic activity on PS-containing vesicles and HEK293 plasma membranes. Aromatic and hydrophobic residues on the interfacial binding surface of Atxs are important for productive binding to both zwitterionic and anionic vesicles, while basic and polar residues have a negative impact on binding to zwitterionic vesicles. When tightly bound to the membrane interface, Atxs can reach full enzymatic activity at low micromolar concentrations of Ca$^{2+}$. Although Atxs have evolved to function as potent neurotoxins that specifically target presynaptic nerve terminals, they display a high degree of phospholipolytic efficiency on various phospholipid membranes.

Phospholipase A$_2$ (PLA$_2$) enzymes catalyze the hydrolysis of the sn-2 ester bond of glycerophospholipids, releasing lysophospholipids and fatty acids (1). Secreted PLA$_2$s (sPLA$_2$s) found in mammals, animal venoms, and plants are relatively small (13–19 kDa), Ca$^{2+}$-dependent, and, due to the presence of 5–8 disulfide bonds, highly stable enzymes. Mammalian sPLA$_2$s form a family of enzymes with various (patho)physiological roles (2), while snake venom sPLA$_2$s, although structurally very similar, exhibit a wide variety of pharmacological effects including myotoxicity, pre- and postsynaptic neurotoxicity, cardiotoxicity, and anticoagulant activity (3).

Ammodytoxins (Atxs) A, B, and C are group IIA sPLA$_2$s with presynaptic neurotoxicity, isolated from the venom of the long-nosed viper (Vipera ammodytes ammodytes) (4).

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¶ Abbreviations: Atx, ammodytoxin; DO$_4$PC and DO$_4$PS, 1,2-diOleyl-sn-glycero-3-phosphocholine and -phosphoserine; PLA$_2$s, sPLA$_2$s from Daboia russelli russelli; FABP, fatty acid-binding protein; HEK, human embryonic kidney; IBS, interfacial binding surface; LD$_{50}$, lethal dose for 50% of the population tested; PC/G/S, phosphatidylycholine/glycerol/serine; PLA$_2$s, phospholipase A$_2$s; POPC/G/S, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine/glycerol/serine; sPLA$_2$s, secreted PLA$_2$s.

Presynaptically acting sPLA$_2$s neurotoxins interfere specifically with the release of acetylcholine from motoneurons, and their PLA$_2$ activity is essential for the irreversible blockade of neuromuscular transmission (5, 6). It has been suggested that structurally different sPLA$_2$ neurotoxins from different snake venoms bind to different receptors on the presynaptic membrane and enter the nerve ending through different import systems (7). In the nerve cell, they may impair the cycling of synaptic vesicles by phospholipid hydrolysis (8) and by binding to specific protein targets such as calmodulin (9, 10) and 14-3-3 proteins (11) in the cytosol and R25 (12, 13) in mitochondria. Electron microscopy studies of neuromuscular junctions treated with different presynaptically neurotoxic sPLA$_2$s (14, 15) as well as their triphasic effect on acetylcholine release (6) suggest that these neurotoxins promote synaptic vesicle exocytosis but inhibit their retrieval from the presynaptic membrane (8, 16).

sPLA$_2$s bind to membrane surfaces by their interfacial binding site (IBS) (17–19), which is located on a flat exposed region surrounding the entrance to the active site pocket. The process is structurally and kinetically distinct from the subsequent binding and hydrolysis of a single phospholipid molecule in the active site (20, 21). The physiological functions of sPLA$_2$s, especially of the human group IIA (hGIIA), human group V (hGV), and human group X (hGX) enzymes, are determined by their different interfacial binding specificities and not by the specificity of their catalytic sites (22–26). Therefore, factors influencing interfacial binding, such as the composition and physical proper-
ties of the membrane, nature of the IBS of the enzyme, and concentration of phospholipids that are accessible to the sPLA$_2$ (27), are crucial determinants of PLA$_2$ activity. The (sub)cellular location of the membrane, which is the target for the enzymatic action of presynaptically neurotoxic sPLA$_2$s, is not known. Given the multistep and yet unknown molecular events leading to presynaptic neurotoxicity of sPLA$_2$s, as well as the complexity of interfacial enzymology (21), it is not surprising that numerous studies have failed in attempts to find a simple correlation between enzymatic activity of neurotoxic sPLA$_2$s and their lethal potency (28).

Therefore, to elucidate the relation between enzymatic activity and presynaptic neurotoxicity of Atxs, it is of prime importance to investigate their enzymatic properties in detail. In the present study, we addressed this issue for the first time and, without attempting to draw any correlations at this stage, investigated the interfacial binding and kinetic properties of Atxs and a number of their mutants and homologues.

**EXPERIMENTAL PROCEDURES**

**Materials.** AtxA, AtxC, and AtnI$_2$ were purified from *V. ammodytes ammodytes* venom as described (29, 30). Preparation of recombinant AtxA, DPLA$_2$, and their mutants, AtxA-F24S, AtxA-F24N, AtxA-F24W, AtxA-F24A, AtxA-F24Y, AtxA-Y115K/I116K/R118M/N119L (AtxA-KKML), and DPLA$_2$-K115Y/K116I/M118R/L119N (DPLA$_2$-YIRN), has been described (10, 31–33). Recombinant AtxB and AtxC were prepared using previously constructed expression plasmids (Pungèrac, unpublished data). The expression plasmid encoding rat liver FABP was a generous gift from Dr. David C. Wilton (University of Southampton, U.K.), and the recombinant protein was prepared as described previously (34). Restriction endonucleases were obtained from MBI Fermentas and New England BioLabs. T4 polynucleotide kinase was from MBI Fermentas and T4 DNA ligase from Boehringer Mannheim. Taq DNA polymerase and RNase One were from Promega, and Vent DNA polymerase and Taq DNA ligase were from New England BioLabs. Oligonucleotides were from MWG-Biotech (Ebersberg, Germany). Triton X-100 was from Roche Molecular Biochemicals. POPG, POPS, POPC, and DO$_2$PC were from Avanti Polar Lipids. 11-Dansylundecanoic acid was from Molecular Probes (Eugene, OR). DO$_2$PS was synthesized as described (35). All other chemicals were of at least analytical grade and were from Sigma and Serva.

Production and Purification of Mutant and Wild-Type Recombinant sPLA$_2$s. The expression plasmid for the AtxA-V31W mutant was prepared as described for the Phe-24 mutants (33). Site-directed mutagenesis using PCR was performed by incorporating the mutagenic oligonucleotide 5’-TAC TGC GGC TGG GTG GGC AAA GG-3’ into the amplification product. The outer sense oligonucleotide primer, 5’-TAA TAG TAC CTA CTA TAG-3’, was complementary to part of the T7 RNA polymerase promoter site on the plasmid encoding wild-type AtxA (31), and the outer antisense oligonucleotide, 5’-GTT TAC TCA TAT ATA CTT TAG-3’, was complementary to a region on the plasmid downstream of the stop codon of AtxA cDNA. The mutagenic oligonucleotide was phosphorylated at the 5’ end using T4 polynucleotide kinase prior to the mutagenesis reaction. PCR reactions were performed as described (33), and fragments were purified with a QIAquick PCR purification kit (QIAGEN), digested with *Bam*HI and *Hind*III, inserted into the linearized expression plasmid, and sequenced using the ABI Prism 310 genetic analyzer (Perkin-Elmer Applied Biosystems).

The T7 RNA polymerase-based expression plasmids encoding recombinant sPLA$_2$s were used to transform the *Escherichia coli* strain BL21(DE3) (Novagen). Overnight bacterial cultures of each protein were used to inoculate 8 × 400 mL of enriched Luria–Bertani medium in 2 L Erlenmeyer shaking flasks. Expression of proteins was induced at OD$_{600}$ of 1.5–2.0 by adding isopropyl β-d-thiogalactoside to a final concentration of 0.4 mM. Three hours after induction, the cells were harvested by centrifugation. Inclusion bodies were isolated and the proteins sulfonated, refolded, and activated by trypsin as described previously (31). The sPLA$_2$s were concentrated by ultrafiltration and purified by FPLC and reverse-phase HPLC as described for AtxA and its Phe-24 mutants (31, 33).

**Analytical Methods.** SDS–PAGE was performed on Mini Protein II and III systems (Bio-Rad) in the presence of 150 mM dithiothreitol on 15% (w/v) polyacrylamide gels using Coomassie Brilliant Blue R250 staining. Electrospray ionization mass spectrometry (ESI-MS) analysis of the proteins was performed using a high-resolution magnetic sensor AutospecQ mass spectrometer (Micromass, U.K.) (31). N-Terminal sequencing was performed on an Applied Biosystems Procise 492A protein sequencing system.

**Interfacial Kinetic Studies with Phospholipid Vesicles.** The initial rate of hydrolysis of phospholipid vesicles by sPLA$_2$s was measured by monitoring the displacement of a fluorescent fatty acid analogue from fatty acid-binding protein (FABP) (22, 36). Unilamellar phospholipid vesicles, with a diameter of 0.1 μm and containing POPC, POPS, POPG, 10% POPS/POPC, or 30% POPS/POPC, were prepared by extrusion (37). Assays were performed in Hanks’ balanced salt solution with 1.26 mM Ca$^{2+}$ and 0.9 mM Mg$^{2+}$ (Invitrogen, Carlsbad, CA) containing 30 μM phospholipid, 1 μM 11-dansylundecanoic acid, and 10 μg of recombinant FABP (22). Solutions with a final volume of 1.3 mL were assayed in plastic fluorometric cuvettes at 37 °C with magnetic stirring, using a Perkin-Elmer LS50B fluorometer. Excitation was at 350 nm and emission at 500 nm, with 10 nm slit widths. Reactions were started by adding 0.5–2000 ng of sPLA$_2$s (typically 1–2 μL). All dilutions of sPLA$_2$s were prepared in buffer containing 1 mg/mL fatty acid-free bovine serum albumin (Sigma) to prevent loss of enzyme due to adsorption to the walls of the tube. Assays were calibrated by adding a known amount of methanol solution of oleic acid (Sigma) and monitoring the decrease in fluorescence.

**Interfacial Binding of sPLA$_2$s to Phospholipid Vesicles.** The binding affinities of sPLA$_2$s to unilamellar diether phospholipid vesicles were determined using the centrifugation method (38). Sucrose-loaded vesicles composed of 10% and 30% DO$_2$PS in DO$_2$PC, with a diameter of 100 nm, were prepared and analyzed as described (23). Binding reactions containing different concentrations of phospholipid and a constant concentration of sPLA$_2$s were set up at room temperature in 5 mM 4-morpholinopropansulfonic acid (MOPS), pH 7.4, 0.1 M KCl, and 2 mM CaCl$_2$. The vesicles were pelleted by ultracentrifugation, and supernatants were
carefully removed and diluted with 3% fatty acid-free bovine serum albumin in water. The amount of enzyme remaining in the supernatant was determined relative to the binding mixture without vesicles using a sensitive fluorometric sPLA2 assay (39) with 1-palmitoyl-2-pyrenedecanoyl-sn-glycero-3-phosphoglycerol (Molecular Probes, Eugene, OR) on a Perkin-Elmer LS50B fluorometer equipped with a plate reader. The background rate of hydrolysis was accounted for using a minus-sPLA2 control. The percentage of enzyme remaining in the supernatant was plotted versus total phospholipid present in the binding reaction, and the equilibrium constant for dissociation of vesicle-bound enzyme into the aqueous phase, $K_d$, was calculated using the standard equation for equilibrium dissociation, $100(E/E_0) = K_d/(L + K_d)$, where $E$ is the concentration of sPLA2 in the supernatant, $E_0$ is the total concentration of sPLA2 (free and vesicle-bound), and $L$ is the total phospholipid concentration (expressed as total moles of DOPEPS and DOPEPC divided by the volume of the reaction sample) (23).

**Cell Studies.** Human embryonic kidney 293 (HEK293) cells (DSMZ ACC 305) (40) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL), streptomycin (100 mg/mL), and 2 mM glutamine at 37 °C in a humidified atmosphere of 5% CO2. Cells were grown to 70–90% confluence, cell culture medium was removed, and cells were dislodged with TrypLE Select (Invitrogen). An equal volume of complete medium was added, and cells were pelleted and washed twice with Hanks’ balanced salt solution with calcium and magnesium. Cell counts were determined using a hemocytometer in the presence of Trypan blue to measure viability. Fatty acid release from intact HEK293 cells by exogenously added sPLA2 was determined using the fluorescence displacement assay described above. Assays were performed with approximately $8 \times 10^5$ cells in Hanks’ balanced salt solution at 37 °C as described above for the vesicle studies.

**Calcium Affinity Studies.** The dependence of the initial rate of hydrolysis of phospholipid vesicles by sPLA2 on the concentration of free $\text{Ca}^{2+}$ was determined using the fluorescence assay with FABP described above (25). Buffered solutions containing less than 20 μM $\text{Ca}^{2+}$ and unbuffered solutions with higher concentrations of free $\text{Ca}^{2+}$ were prepared in Hanks’ balanced salt solution without $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ (Invitrogen) as described (35). The apparent calcium dissociation constant ($K_{\text{Ca(app)}}$) of each sPLA2 was obtained by fitting the initial rates of hydrolysis determined at increasing concentrations of free $\text{Ca}^{2+}$ to the simple hyperbolic equation (25).

**Toxicity.** Lethal potency of the toxins was determined by intraperitoneal injection into BALB/c albino mice. Five dose levels and nine mice per dose were used for each toxin. The samples of recombinant toxins (1–250 μg), dissolved in water, were diluted to a final volume of 0.5 mL in 0.9% (w/v) NaCl just prior to application. Animals were observed after 24 h and LD$_{50}$ values determined using a standard method (41).

**RESULTS**

**Properties of Recombinant Atxs, Their Mutants, and Homologues.** Recombinant wild-type and mutant sPLA2s, including the newly constructed mutant AtxA-V31W, were prepared, and their structural integrity was confirmed as described previously (10, 31–33). Homogeneity was demonstrated by SDS–PAGE, analytical HPLC, ESI-MS, and N-terminal sequencing. The relative molecular mass of the V31W mutant (13861.0 Da) was within one mass unit of that expected (13861.0 Da), indicating that all seven disulfide bonds were formed and that no posttranslational modifications occurred after synthesis. The N-terminal sequence, SLLEFG..., was identical to that of AtxA, confirmed the proper removal of the fusion peptide and absence of internal cleavages due to trypsin activation. All proteins had high and similar catalytic activity on POPG vesicles (Table 1), indicating no significant conformational changes in the structure or perturbation of the active site due to the mutations introduced. The enzymatic activities of natural and recombinant toxins, either AtxA or AtxC, on POPG, POPC, and POPC vesicles were identical within experimental error (not shown), confirming the absence of any structural perturbations in the recombinant proteins. Additionally, the calcium binding affinity of recombinant AtxA coincided with that of the natural enzyme (see below), providing further evidence for proper folding and structural integrity of the

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**Table 1: Apparent Rates of Hydrolysis of Phospholipid Vesicles by sPLA2s**

<table>
<thead>
<tr>
<th>sPLA2</th>
<th>POPG</th>
<th>POPS</th>
<th>POPC</th>
<th>10% POPS/POPC</th>
<th>30% POPS/POPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtxA</td>
<td>1042 ± 160</td>
<td>1251 ± 188</td>
<td>3.8 ± 0.5</td>
<td>56 ± 6</td>
<td>450 ± 21</td>
</tr>
<tr>
<td>AtxB</td>
<td>1149 ± 34</td>
<td>1189 ± 153</td>
<td>14 ± 2</td>
<td>240 ± 22</td>
<td>113 ± 150</td>
</tr>
<tr>
<td>AtxC</td>
<td>1116 ± 91</td>
<td>477 ± 101</td>
<td>1.9 ± 0.2</td>
<td>14.0 ± 1.3</td>
<td>166 ± 21</td>
</tr>
<tr>
<td>AtmA</td>
<td>1070 ± 150</td>
<td>57 ± 5</td>
<td>12.3 ± 1.7</td>
<td>49 ± 2</td>
<td>159 ± 16</td>
</tr>
<tr>
<td>DPLA2</td>
<td>1191 ± 149</td>
<td>1195 ± 47</td>
<td>1.8 ± 0.2</td>
<td>88 ± 9</td>
<td>442 ± 31</td>
</tr>
<tr>
<td>DPLA2-YIRN</td>
<td>1207 ± 74</td>
<td>996 ± 129</td>
<td>0.29 ± 0.04</td>
<td>4.2 ± 0.4</td>
<td>141 ± 20</td>
</tr>
<tr>
<td>AtxA-KKML</td>
<td>1148 ± 126</td>
<td>1252 ± 40</td>
<td>19 ± 1</td>
<td>400 ± 22</td>
<td>1322 ± 42</td>
</tr>
<tr>
<td>AtxA-V31W</td>
<td>2102 ± 88</td>
<td>1964 ± 154</td>
<td>102 ± 7</td>
<td>525 ± 28</td>
<td>1957 ± 36</td>
</tr>
<tr>
<td>AtxA-F24W</td>
<td>914 ± 34</td>
<td>906 ± 56</td>
<td>4.4 ± 0.5</td>
<td>50 ± 1</td>
<td>209 ± 6</td>
</tr>
<tr>
<td>AtxA-F24Y</td>
<td>822 ± 41</td>
<td>1304 ± 102</td>
<td>2.1 ± 0.1</td>
<td>9.5 ± 0.5</td>
<td>163 ± 11</td>
</tr>
<tr>
<td>AtxA-F24N</td>
<td>780 ± 29</td>
<td>535 ± 43</td>
<td>1.09 ± 0.03</td>
<td>5.1 ± 0.5</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>AtxA-F24A</td>
<td>813 ± 107</td>
<td>1291 ± 65</td>
<td>1.2 ± 0.1</td>
<td>4.4 ± 0.3</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>AtxA-F24S</td>
<td>400 ± 73</td>
<td>430 ± 42</td>
<td>0.71 ± 0.06</td>
<td>4.4 ± 0.1</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>hGIIA</td>
<td>220 ± 90</td>
<td>40 ± 18</td>
<td>lag, 0.7 ± 0.2</td>
<td>no data</td>
<td>no data</td>
</tr>
<tr>
<td>hGX6</td>
<td>14 ± 8</td>
<td>4 ± 2</td>
<td>30 ± 0.2</td>
<td>no data</td>
<td>no data</td>
</tr>
</tbody>
</table>

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a The rate value for each sPLA2 is the mean ± SD of at least five independent measurements. b The activities of the human sPLA2s, hGIIA and hGX, were determined in our previous study (25). See text for additional information.
Figure 1: Amino acid alignment of group IIA sPLA2s from snake venom and some mammalian sPLA2s. Ammodytoxins (Atxs) A, B, and C are basic group IIA sPLA2s and have a high degree of amino acid identity (48%) with both human group IIA (hGIIA) and human group V (hGV) sPLA2s. The neutral ammodytin I2 (AtmI2) is a nontoxic homologue of Atxs from the same venom, with 58% amino acid identity to AtxA. The weakly neurotoxic sPLA2 from Russell’s viper, D. russelli russelli, DPLA2, differs from AtxA in only 22 residues (82% identity). The human group X (hGX) sPLA2 shares about 41% of amino acid residues with AtxA. Numbering of amino acid residues is according to Renetseder et al. (42). Gaps, represented by dashes, were used to align the homologous sPLA2s. Identical amino acid residues in sPLA2s are shown by dots. Residues that were replaced in mutants of AtxA and DPLA2 used in this study are underlined. Residues that are present on the putative IBS of AtxA are presented in bold type. The amino acid sequences were obtained from the publicly available database at the NCBI, Bethesda, MD.
displayed the highest rises in activity. The activities of AtxA-V31W, AtxA-KKML, and AtxB on 30% POPG vesicles were already in the range of those determined on pure POPC vesicles. Therefore, in the presence of 30% PS these three sPLA₂s are already fully bound to vesicles, and increasing the PS concentration further would have no effect on their activity. The activities of the rest of the enzymes on 30% PS/PC vesicles were still 3–18-fold lower than their respective activities on pure POPC vesicles. The influence of adding 10% and 30% anionic POPG phospholipids to POPC vesicles on the activities of AtxA, AtxA-V31W, and AtxB (Table 3) was similar to the effect of adding POPG phospholipids as described above, confirming the expected nonspecific nature of interactions between anionic membrane phospholipids and sPLA₂s (43). On the other hand, the nontoxic AtnI₂ displayed an increase in activity on the PG-containing vesicles similar to that of AtxA, in contrast to its relatively low level of activation on PS containing PC-rich vesicles. This indicates that it is not the negative charge of PS per se that interferes with interfacial binding (see below) and activity of AtnI₂ but that a more specific effect of the PS headgroup must be involved.

**Interfacial Binding of Snake Venom sPLA₂s to Phospholipid Vesicles.** The equilibrium constants for the dissociation of sPLA₂s from phospholipid vesicles into the aqueous phase were determined by the centrifugation method adopted from Buser et al. (38). Sucrose-loaded vesicles can be sedimented by ultracentrifugation, and the amount of sPLA₂ remaining in the supernatant relative to the total enzyme is determined by ultracentrifugation, and the percentage of sPLA₂ activity remaining in the supernatant was plotted as a function of the concentration of total phospholipid in the binding mixture. (A) DPLA₂ at 10% DO₆PS/DO₆PC (O); DPLA₂ at 30% DO₆PS/DO₆PC (●) (given for comparison); AtxA-F24W (▼); AtxA (■); AtnI₂ (▲). (B) AtxA (●); DPLA₂ (▲); DPLA₂-YIRN (○); AtxA-F24S (●). Independent binding studies were carried out at least three times for each sPLA₂.

![Figure 2: Interfacial binding of sPLA₂s to nonhydrolyzable phospholipid vesicles.](image)

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**Table 2: Interfacial Binding Affinity of sPLA₂ for Nonhydrolyzable Sucrose-Loaded Vesicles**

<table>
<thead>
<tr>
<th>sPLA₂</th>
<th>10% DO₆PS/DO₆PC (µM)</th>
<th>30% DO₆PS/DO₆PC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtxA</td>
<td>820 ± 60</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>AtxA-F24W</td>
<td>420 ± 50</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>AtxA-F24S</td>
<td>ND</td>
<td>700 ± 200</td>
</tr>
<tr>
<td>DPLA₂</td>
<td>170 ± 20</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>DPLA₂-YIRN</td>
<td>ND</td>
<td>220 ± 20</td>
</tr>
<tr>
<td>AtnI₂</td>
<td>~2600</td>
<td>800 ± 100</td>
</tr>
<tr>
<td>hGIIA</td>
<td>no binding at 2 mM</td>
<td>23</td>
</tr>
<tr>
<td>hGX</td>
<td>130 ± 25</td>
<td>36 ± 4</td>
</tr>
</tbody>
</table>

* The values of $K_d$ for each sPLA₂ were determined from at least two independent binding profiles. Each point of the binding curve is the average of at least five measurements of the remaining PL₂ activity in the supernatant at each phospholipid concentration. *  
* The interfacial binding affinities of the human group IIA (hGIIA) and human group X (hGX) enzymes were determined previously by Bezzine et al. (23).
of the venom sPLA₂s tested, with the exception of AtnI₂, also showed a good correlation with their enzymatic activities on pure POPC vesicles, indicating that the introduction of anionic PS phospholipids in PC vesicles increases interfacial binding affinity to a similar extent for this group of basic enzymes. In the case of the neutral AtnI₂, its weak interfacial binding affinity to a similar extent for this group of basic enzymes also showed a good correlation with their enzymatic activities of cells. Nevertheless, these sPLA₂s bind specifically to the plasma membranes (43, 44), but this was not investigated further.

Calcium Affinity Studies. Apparent calcium dissociation constants (K<sub>Ca(app)</sub>) for AtxA and selected mutants and homologous proteins were determined by measuring the initial rates of hydrolysis of vesicles over an appropriate range of free calcium concentrations (Table 4 and Figure 4). Binding of a single phospholipid molecule in the active site but also is a function of the fraction of sPLA₂ bound to vesicles. All enzymes tested had high and similar activities on POPG vesicles, indicating that they bind to these vesicles with high and similar affinities. Therefore, the K<sub>Ca(app)</sub> values determined under conditions of high-affinity binding are a good measure of the actual affinity of Atxs for calcium. In the absence of calcium in the buffer no hydrolysis was detected. The results in Table 4 clearly show that AtxA, AtxA-F24W, and DPLA₂ can achieve half-maximal activities on POPG vesicles in the presence of 13–33 μM calcium.

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**Table 3: Apparent Rates of Hydrolysis of Phospholipid Vesicles and Intact HEK293 Cells by Selected sPLA₂s**

<table>
<thead>
<tr>
<th>sPLA₂</th>
<th>10% POPG/POPC</th>
<th>30% POPG/POPC</th>
<th>HEK293 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtxA</td>
<td>54 ± 3</td>
<td>754 ± 71</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>AtxB</td>
<td>256 ± 13</td>
<td>982 ± 66</td>
<td>13.9 ± 0.8</td>
</tr>
<tr>
<td>AtxA-V31W</td>
<td>579 ± 27</td>
<td>1210 ± 30</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>AtnI₂</td>
<td>89 ± 8</td>
<td>556 ± 52</td>
<td>0.61 ± 0.04</td>
</tr>
</tbody>
</table>

*The rate values for each sPLA₂ were determined with the fluorometric assay using fatty acid-binding protein and are means ± SD of at least five independent measurements.

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**Table 4: Calcium Affinity of sPLA₂s Determined on POPG and POPC Vesicles**

<table>
<thead>
<tr>
<th>sPLA₂</th>
<th>POPG (μM)</th>
<th>POPC (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtxA</td>
<td>31 ± 8, 25 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1480 ± 200</td>
</tr>
<tr>
<td>DPLA₂</td>
<td>13 ± 2</td>
<td>1290 ± 280</td>
</tr>
<tr>
<td>AtxA-F24W</td>
<td>33 ± 7</td>
<td>1950 ± 270</td>
</tr>
<tr>
<td>AtxA-F24N</td>
<td>190 ± 30</td>
<td>1430 ± 180</td>
</tr>
<tr>
<td>AtnI₂</td>
<td>360 ± 70</td>
<td>590 ± 80</td>
</tr>
</tbody>
</table>

* Determined with recombinant AtxA.
Ammodytoxins, Potent Neurotoxins and Efficient Enzymes

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The role of tryptophan in supporting interfacial binding of sPLA$_2$s has been highlighted in the case of the human group V (45) and X enzymes (22, 23). The role of tryptophan in supporting interfacial binding of sPLA$_2$s has been highlighted in the case of the human group V (45) and X enzymes (22, 23), the acidic sPLA$_2$ from Naja naja atra snake venom (46), a range of mutants of hGIIA (26, 47), and the pancreatic group IB sPLA$_2$s (48, 49). Despite the fact that Atxs already displayed relatively high activity on PC vesicles, the substitution of Val-31 with Trp led to a dramatic 27-fold rise in activity of AtxA. In fact, this mutant displayed by far the highest activity of all the enzymes tested on all phospholipid substrates used in this study. It reached a level of activity on PC-rich vesicles higher than that reported for hGV and hGIX and in the range of that of cobra venom sPLA$_2$, which is well-known for its very high activity on PC membrane surfaces (26, 46). Hydrolysis of PC-rich

activity on PC-rich membrane surfaces (23, 25, 26). Most importantly, Atxs display relatively high activities on zwitterionic PC vesicles, much higher than the hGIIA enzyme, but still lower than that displayed by group V and X sPLA$_2$s, which are by far the most potent among the mammalian sPLA$_2$s in hydrolyzing PC vesicles (22, 23, 25, 26). The presence of Trp on the IBS of the latter enzymes is very important for their ability to bind and hydrolyze PC-rich vesicles, crucially influencing their physiological role (23, 25, 26, 45). However, it is not only the presence or absence of Trp on the IBS of Atxs and hGIIA that is crucial for the significant difference between their activities on PC-rich membranes. For example, AtxA and hGIIA sPLA$_2$s do not contain a Trp on their IBS, while DPLA$_2$, which differs from AtxA in only 22 residues, has a Trp residue at position 31 and yet it displays interfacial binding and kinetic properties similar to that of AtxA (see below). The $K_d$ values presented in Table 2 confirm that the high activity of Atxs on PC-rich vesicles is a consequence of the ability of these venom sPLA$_2$s to bind well to such membrane surfaces, with affinities comparable to those of mammalian group V and X sPLA$_2$s (23, 25). Additionally, there is a good correlation between the interfacial binding affinity of Atxs, but not of nontoxic AtnI$_2$ (see below), and the rate of hydrolysis of PC-rich vesicles containing increasing amounts of anionic phospholipids. That is, the greater the content of PS or PG in PC vesicles, the greater the number of Atx molecules bound to vesicles and hence the rate of hydrolysis. Thus, unlike the neutral hGIX (24) and similarly to the highly cationic hGIIA enzyme (23), the presence of anionic phospholipids in the membrane surface can greatly enhance binding affinity of Atxs. On the other hand, since the interfacial binding affinities of Atxs for PC-rich membranes are much closer to those of the mammalian groups V and X enzymes than to that of the group IIA enzyme, Atxs would be expected to bind and hydrolyze plasma membranes of intact eukaryotic cells. Indeed, Atxs released fatty acids from plasma membranes of intact HEK293 cells at a rate that correlated well with their rate on PC vesicles (Figure 3). Thus, Atxs bind strongly to and hydrolyze rapidly both anionic and zwitterionic phospholipid substrates, including mammalian plasma membranes, presenting a combination of properties characteristic of mammalian group IIA, V, and X sPLA$_2$s. This degree of phospholipolytic activity appears to be at odds with the specific neurotoxic action of Atxs at presynaptic nerve terminals.

The Role of Different IBS Residues in Supporting Interfacial Binding and Activity of Atxs. The role of tryptophan in supporting interfacial binding of sPLA$_2$s has been highlighted in the case of the human group V (45) and X enzymes (22, 23). The acidic sPLA$_2$ from Naja naja atra snake venom (46), a range of mutants of hGIIA (26, 47), and the pancreatic group IB sPLA$_2$s (48, 49). Despite the fact that Atxs already displayed relatively high activity on PC vesicles, the substitution of Val-31 with Trp led to a dramatic 27-fold rise in activity of AtxA. In fact, this mutant displayed by far the highest activity of all the enzymes tested on all phospholipid substrates used in this study. It reached a level of activity on PC-rich vesicles higher than that reported for hGV and hGIX and in the range of that of cobra venom sPLA$_2$, which is well-known for its very high activity on PC membrane surfaces (26, 46). Hydrolysis of PC-rich

concentrations. The weakly toxic AtxA-F24N mutant (33) and the nontoxic AtnI$_2$ displayed the lowest calcium affinities on POPG vesicles, 8- and 14-fold lower than AtxA, respectively. The 60–100-fold lower calcium affinities of AtxA, AtxA-F24W, and DPLA$_2$ when acting on POPC vesicles are consistent with their much lower binding affinity for zwitterionic than anionic phospholipid vesicles. The AtxA-F24N mutant displayed only 8-fold lower calcium binding affinity when hydrolyzing POPC vesicles, but its value of $K_{ca(app)}$ was similar to that determined for AtxA. In contrast, the affinity of AtnI$_2$ for calcium decreased only 1.6-fold on POPG vesicles and was slightly higher than that of AtxA. The most likely explanation is that AtnI$_2$ has a higher binding affinity for POPC vesicles than AtxA and the rest of the enzymes in Table 4 (judged from the higher enzymatic activity of AtnI$_2$) and is therefore activated by lower calcium concentrations on these vesicles.

**DISCUSSION**

*Interfacial Kinetic and Binding Properties of Atxs.* The rates of phospholipid hydrolysis and membrane binding affinities of Atxs determined on different phospholipid vesicles clearly show that these presynaptically neurotoxic sPLA$_2$s are very efficient enzymes in comparison to groups I, II, V, X, and XII of mammalian sPLA$_2$s enzymes (23, 25, 26). The enzymatic activities of Atxs on anionic PG vesicles are comparable to those displayed by the most potent mammalian sPLA$_2$s (pancreatic group IB sPLA$_2$s) on these vesicles (25). Furthermore, Atxs show especially high activities on vesicles containing PS, the most abundant anionic phospholipid in eukaryotic membranes. In fact, the exceptionally low activity of nontoxic AtnI$_2$ on POPS vesicles determined in this study is in the range of activities displayed by the mammalian sPLA$_2$s that display the highest activities on these vesicles, group IB and IIA sPLA$_2$s (25). The high activity of Atxs on anionic vesicles was expected because Atxs are basic proteins (AtxA has a pI of 10.2, net charge +6) and share a relatively high degree of identity with hGIIA sPLA$_2$ (Figure 1), which is well-known for its preference for anionic phospholipid substrates and negligible

**FIGURE 4: Calcium affinity profile of AtxA on POPG vesicles.** Initial velocities (measured with the fatty acid-binding protein assay) for the hydrolysis of 30 µM POPG vesicles by AtxA are shown as a function of free calcium concentration. The regression fit to the standard hyperbolic binding equation is presented as a solid line. See Experimental Procedures for additional information.

Initial Velsity (arbitrary units)
plasma membranes is supposed to be the (path)physiological role of the latter three enzymes, while Atxs are specific neurotoxins and their ability to bind to and hydrolyze mammalian cell membranes would greatly reduce the number of toxin molecules that reach the target presynaptic membrane (3). However, despite its very high affinity for PC-rich surfaces the AtxA-V31W mutant does not show a major reduction of neurotoxic potency in vivo. Given the importance of the concentration of phospholipids available to sPLA$_2$s in their local environment (27), it is possible that this concentration is too low in the extracellular space in vivo to allow significant nonspecific binding of Atxs to cell membranes. Therefore, Trp at position 31 is able to increase enzymatic activity of Atxs significantly, most probably by promoting interfacial binding to both anionic and zwitterionic interfaces, but has little influence on neurotoxicity.

On the other hand, substitution of Phe-24 with Trp did not cause a substantial increase in enzymatic activity (Table 1) or interfacial binding affinity (Table 2) of AtxA, highlighting the fact that both Phe and Trp at position 24 have a similar role in interfacial binding, as we previously suggested (33). The role of aromatic residues in the interfacial binding of sPLA$_2$s depends on the nature of the residue itself, its position on the IBS, and the orientation of its side chain (46, 50). Trp-31 is obviously in a much better position in AtxA to influence interfacial binding than Trp-24, which is consistent with the results obtained in the case of the F24W and V31W mutants of hGIIA (26).

AtxA and its F24W mutant stand out among the Phe-24 mutants with their high activities on PC-rich vesicles containing different amounts of PS, emphasizing the ability of Phe and Trp to support interfacial binding to negatively charged membrane surfaces as well. The polar side chains of Ser, Asn, and even Tyr presumably interfere with productive binding to both anionic and zwitterionic phospholipid vesicles. Additionally, the absence of Phe-24 in the AtxA-F24A mutant and of Phe-124 in AtxC (Figure 1) is reflected in their low activity on PC-rich vesicles. The highly amphiphilic Trp (51), that favors partitioning in the interfacial phospholipid headgroup region of the bilayer, and the aromatic Phe, which is capable of penetrating deeper in the hydrophobic core of the phospholipid acyl chains (46, 50), are obviously both well suited to take advantage of the presence of anionic phospholipid in the interface, which, besides providing the basis for electrostatic interactions, also facilitates nonpolar interactions as a result of membrane perturbations (43). The enzymes in our study that hydrolyze PC-rich vesicles very well, AtxB, AtxA-KKML, and AtxA-V31W, have a more hydrophobic/aromatic IBS than AtxA (Figure 5) and are fully bound to PC-rich vesicles containing 30% anionic phospholipid, clearly pointing out the importance of nonpolar interactions in interfacial binding to anionic surfaces as well.

AtxB differs from AtxA only in three residues (Figure 1), Y115H, R118M, and N119Y. The residue at position 115 is on the upper back side of the molecule (Figure 5) and most probably does not contact the membrane surface (32). Substitution of a basic (Arg-118) and a polar (Asn-119) residue in AtxA with the hydrophobic Met and aromatic Tyr residue in AtxB, respectively, causes a significant enhancement of initial velocity of hydrolysis of PC-rich vesicles. If we consider the similar activities of AtxB and the AtxA-KKML mutant, in which both lysines are in the back of the molecule away from the presumed IBS (32), on POPC and PC-rich vesicles, it appears that the roles of Leu (KKML mutant) and Tyr (AtxB) at position 119 are in fact quite similar.

It is intriguing that DPLA$_2$ and its YIRN mutant, despite the presence of Trp-31, have lower or similar activities on PC-rich vesicles than AtxA. DPLA$_2$ displays a higher binding affinity (Table 2) and activity (Table 1) than AtxA on PC vesicles that contain PS, but it is obvious that the positive effect of Trp is far from that observed in the case of the AtxA-V31W mutant. Most probably the presence of some other residues on the IBS of DPLA$_2$ antagonizes the positive effects of Trp or modifies the orientation of DPLA$_2$ on the membrane, preventing a productive interaction of Trp-31 with the interface. Such residues could include Ser-24, which, in the case of the F24S mutant of AtxA, has a pronounced negative impact on both interfacial binding and enzymatic activity (Tables 1 and 2), as well as the charged Lys-7 and Glu-11, which are on the edges of the IBS of DPLA$_2$ and are replaced by Met and Gly in AtxA, respectively. Two basic residues at similar positions in the hGIIA enzyme, Arg-7 and Lys-10, were shown to influence interfacial binding (23, 53). There are four N-terminal residues left on the putative IBS of DPLA$_2$ that differ from those of AtxA (residues 17–20), including a Pro, which, given the suggested flexibility of the region preceding the calcium binding loop of sPLA$_2$s (54), could influence the local structure of the protein backbone and orientation of the Trp-31 side chain. Thus, the role of Trp-31 in interfacial binding can be
modified to a significant extent by the presence (or absence) of a relatively small number of residues on the IBS of sPLA2.

Enzymatic Properties of Nontoxic AtnI2. AtnI2 is a neutral protein (pI 6.8, net charge 0) (55) that contains Trp at position 31, and the overall nature of its IBS is more hydrophobic than that of AtxA (Figures 1 and 5). Therefore, the high activity of AtnI2 on zwiterion POPC vesicles was expected and is in accordance with its high activity on mixed micelles of egg yolk PC and Triton X-100 (55). AtnI2 also showed high activity on anionic POPG vesicles but surprisingly low affinity and activity on PS-containing vesicles and cell membranes. Nevertheless, its binding affinity as well as its activity on PS/PC vesicles was modestly higher as we increased the percentage of PS in vesicles, and this effect was more pronounced on the PG-containing PC vesicles. Therefore, the presence of anionic phospholipid in the membrane increases the interfacial binding affinity of AtnI2, albeit to a lower degree than Atxs. AtnI2 lacks the basic residues of Atxs that would enhance the nonspecific electrostatic interactions with anionic phospholipids, but the hydrophobic nature of its IBS and the presence of tryptophan could provide a network of nonpolar interactions with the hydrophobic core of the perturbed PC bilayer (43). Given the exceptionally low activity of AtnI2 on POPS and other PS-containing vesicles, the presence of PS in the PC-rich membrane probably has an additional negative impact on binding and activity of AtnI2. A possible explanation is that the PS phospholipid headgroup is not well accommodated in the active site of AtnI2, which might be one of the reasons for poor binding of AtnI2 to the PS-containing vesicles, because binding of a single phospholipid molecule and Ca2+ in the active site of an sPLA2 influences by mass action the equilibrium between free and membrane-bound enzyme (23). However, we cannot explain the discrepancy between the low activity of AtnI2 on HEK293 plasma membranes and its high activity on POPC vesicles. Both the phospholipid headgroup specificity of the active site of AtnI2 and its interaction with mammalian cells await further studies. Nevertheless, the lower activity of AtnI2 on PS-containing membrane surfaces and mammalian plasma membranes than Atxs is intriguing, given that the presumed role of this nontoxic snake venom sPLA2 is nonspecific membrane hydrolysis upon envenomation of the victim.

Neurotoxic Atxs May Be Enzymatically Active in Target Mammalian Cells. It is generally accepted that sPLA2s require submillimolar to millimolar calcium concentrations for full activity. However, our results show that, when conditions of high-affinity binding apply (i.e., when the enzyme in the reaction vessel is fully bound to vesicles), Atxs can reach their half-maximal initial rates of hydrolysis at low micromolar concentrations of calcium. Similar results on calcium requirements were recently obtained using the full set of mammalian sPLA2s (25) and the sPLA2 subunit of crototoxin (56). In light of the recent suggestions of a possible cytosolic site of action of these neurotoxins (13, 56, 57) and taking into account the relatively high degree of stability of AtxA under conditions resembling those in the cytosol of eukaryotic cells (57), the transient cytosolic microdomains of high local calcium concentrations (~100 μM) (58), and the presence of anionic phospholipids (especially PS) on the cytosolic face of the plasma membrane (44) and internal cellular organelles would probably enable the toxic sPLA2 to be enzymatically active, at least for a certain period of time. On the other hand, if the toxin acts in a membrane compartment, for example, synaptic vesicles or caveolae (7, 8), the concentration of phospholipids that the toxin “sees” (27) would be high enough to induce high-affinity binding and enable efficient hydrolysis, even at lower concentrations of calcium and in the absence of anionic phospholipids in the interface.

In this study, we have demonstrated that Atxs, snake venom sPLA2s with presynaptic neurotoxicity that share striking structural and functional similarities with the mammalian (nontoxic) sPLA2s, are very efficient in binding to and hydrolyzing different phospholipid membrane surfaces, despite the fact that they have evolved to be specific and potent neurotoxic molecules. Our results provide the first insight into the interfacial kinetic and binding properties of these sPLA2 neurotoxins and open the way for further studies that should elucidate the role and site of PLA2 activity in the process of their toxicity. Atxs bind well to PC-rich surfaces, but their membrane binding affinity increases dramatically in the presence of anionic phospholipids, which may have an important influence on both localization of the toxin to its target membrane and its enzymatic efficiency in vivo. When tightly bound to the membrane interface, the Ca2+ requirements of Atxs are in the micromolar range, opening up the possibility that these neurotoxins are enzymatically active in those subcellular compartments where Ca2+ concentrations are low. Although these suggestions remain to be confirmed, our results clearly show that the interfacial binding and kinetic properties of Atxs are an indication of their potential for high enzymatic activity, which may be of crucial importance at a certain highly localized step in the complex sequence of events leading to the irreversible effects of sPLA2 neurotoxin envenomation.

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