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Ammodytotoxins, Potent Presynaptic Neurotoxins, Are Also Highly Efficient Phospholipase A₂ Enzymes[†]

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ABSTRACT: The enzymatic activity of ammodytotoxins (Atxs), secreted phospholipases A₂ (sPLA₂s) 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 sPLA₂s 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 phosphatidylcholine (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 I₂, 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 Ca²⁺. 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 A₂ (PLA₂)¹ enzymes catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids, releasing lysophospholipids and fatty acids (1). Secreted PLA₂s (sPLA₂s) found in mammals, animal venoms, and plants are relatively small (13–19 kDa), Ca²⁺-dependent, and, due to the presence of 5–8 disulfide bonds, highly stable enzymes. Mammalian sPLA₂s form a family of enzymes with various (patho)physiological roles (2), while snake venom sPLA₂s, although structurally very similar, exhibit a wide variety of pharmacological effects including myotoxicity, pre- and postsynaptic neurotoxicity, cardiotoxicity, and anticoagulant activity (3).

Ammodytotoxins (Atxs) A, B, and C are group IIA sPLA₂s with presynaptic neurotoxicity, isolated from the venom of the long-nosed viper (*Vipera ammodytes ammodytes*) (4).

Presynaptically acting sPLA₂ neurotoxins interfere specifically with the release of acetylcholine from motoneurons, and their PLA₂ activity is essential for the irreversible blockade of neuromuscular transmission (5, 6). It has been suggested that structurally different sPLA₂ 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 sPLA₂s (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).

sPLA₂s bind to membrane surfaces by their interfacial binding surface (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 sPLA₂s, 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-

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¹ Abbreviations: Atx, ammodytotoxin; DO₆PC and DO₆PS, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and -phosphoserine; DPLA₂, sPLA₂ from *Daboia russelli russelli*; FABP, fatty acid-binding protein; HEK, human embryonic kidney; IBS, interfacial binding surface; LD₅₀, lethal dose for 50% of the population tested; PC/G/S, phosphatidylcholine/glycerol/serine; PLA₂, phospholipase A₂; POPC/G/S, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/glycerol/serine; sPLA₂, secreted PLA₂.

ties of the membrane, nature of the IBS of the enzyme, and concentration of phospholipids that are accessible to the sPLA₂ (27), are crucial determinants of PLA₂ activity. The (sub)cellular location of the membrane, which is the target for the enzymatic action of presynaptically neurotoxic sPLA₂s, is not known. Given the multistep and yet unknown molecular events leading to presynaptic neurotoxicity of sPLA₂s, 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 sPLA₂s 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 AtnI₂ were purified from *V. ammodytes ammodytes* venom as described (29, 30). Preparation of recombinant AtxA, DPLA₂, and their mutants, AtxA-F24S, AtxA-F24N, AtxA-F24W, AtxA-F24A, AtxA-F24Y, AtxA-Y115K/I116K/R118M/N119L (AtxA-KKML), and DPLA₂-K115Y/K116I/M118R/L119N (DPLA₂-YIRN), has been described (10, 31–33). Recombinant AtxB and AtxC were prepared using previously constructed expression plasmids (Pungerčar, 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 DO₆PC were from Avanti Polar Lipids. 11-Dansylundecanoic acid was from Molecular Probes (Eugene, OR). DO₆PS 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 sPLA₂s. 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 GGT GGC AAA GG-3' into the amplification product. The outer sense oligonucleotide primer, 5'-TAA TAC GAC TCA 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 sPLA₂s 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 OD₆₀₀ 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 S-sulfonated, refolded, and activated by trypsin as described previously (31). The sPLA₂s 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 Protean 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 sPLA₂s 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 Ca²⁺ and 0.9 mM Mg²⁺ (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 sPLA₂ (typically 1–2 μL). All dilutions of sPLA₂s 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 sPLA₂s to Phospholipid Vesicles. The binding affinities of sPLA₂s to unilamellar diether phospholipid vesicles were determined using the centrifugation method (38). Sucrose-loaded vesicles composed of 10% and 30% DO₆PS in DO₆PC, 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 sPLA₂ were set up at room temperature in 5 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4, 0.1 M KCl, and 2 mM CaCl₂. The vesicles were pelleted by ultracentrifugation, and supernatants were

Table 1: Apparent Rates of Hydrolysis of Phospholipid Vesicles by sPLA₂s^a

sPLA ₂	rate of hydrolysis [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]				
	POPG	POPS	POPC	10% POPS/POPC	30% POPS/POPC
AtxA	1042 \pm 160	1251 \pm 188	3.8 \pm 0.5	56 \pm 6	450 \pm 21
AtxB	1149 \pm 34	1189 \pm 153	14 \pm 2	240 \pm 22	1133 \pm 150
AtxC	1116 \pm 91	477 \pm 101	1.9 \pm 0.2	14.0 \pm 1.3	166 \pm 21
AtnI ₂	1070 \pm 150	57 \pm 5	12.3 \pm 1.7	49 \pm 2	159 \pm 16
DPLA ₂	1191 \pm 149	1195 \pm 47	1.8 \pm 0.2	88 \pm 9	442 \pm 31
DPLA ₂ -YIRN	1207 \pm 74	996 \pm 129	0.29 \pm 0.04	4.2 \pm 0.4	141 \pm 20
AtxA-KKML	1148 \pm 126	1252 \pm 40	19 \pm 1	400 \pm 22	1322 \pm 42
AtxA-V31W	2102 \pm 88	1964 \pm 154	102 \pm 7	525 \pm 28	1957 \pm 36
AtxA-F24W	914 \pm 43	906 \pm 56	4.4 \pm 0.5	50 \pm 1	209 \pm 6
AtxA-F24Y	822 \pm 41	1304 \pm 102	2.1 \pm 0.1	9.5 \pm 0.5	163 \pm 11
AtxA-F24N	780 \pm 29	535 \pm 43	1.09 \pm 0.03	5.1 \pm 0.5	68 \pm 3
AtxA-F24A	813 \pm 107	1291 \pm 65	1.2 \pm 0.1	4.4 \pm 0.3	92 \pm 5
AtxA-F24S	400 \pm 73	430 \pm 42	0.71 \pm 0.06	4.4 \pm 0.1	24 \pm 4
hGIIA ^b	220 \pm 90	40 \pm 18	lag, 0.7 \pm 0.2	no data	no data
hGX ^b	14 \pm 0.8	4 \pm 2	30 \pm 0.2	no data	no data

^a The rate value for each sPLA₂ is the mean \pm 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.

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, $100(E/E_t) = K_d/(L + K_d)$, where E is the concentration of sPLA₂ in the supernatant, E_t is the total concentration of sPLA₂ (free and vesicle-bound), and L is the total phospholipid concentration (expressed as total moles of DO₆PS and DO₆PC 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 sPLA₂s was determined using the fluorescence displacement assay described above. Assays were performed with approximately 8×10^5 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 sPLA₂s on the concentration of free Ca²⁺ was determined using the fluorescence assay with FABP described above (25). Buffered solutions containing less than 20 μM Ca²⁺ and unbuf-

fered solutions with higher concentrations of free Ca²⁺ were prepared in Hanks' balanced salt solution without Ca²⁺ and Mg²⁺ (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 Ca²⁺ 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 LD₅₀ 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 (13861.8 Da), indicating that all seven disulfide bonds were formed and that no posttranslational modifications occurred after synthesis. The N-terminal sequence, SLLEFG..., which 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, POPS, 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

Table 2: Interfacial Binding Affinity of sPLA₂s for Nonhydrolyzable Sucrose-Loaded Vesicles^a

sPLA ₂	interfacial binding affinity, K_d	
	10% DO _{et} PS/DO _{et} PC (μ M)	30% DO _{et} PS/DO _{et} PC (μ M)
AtxA	820 \pm 60	80 \pm 2
AtxA-F24W	420 \pm 50	64 \pm 4
AtxA-F24S	ND ^b	700 \pm 200
DPLA ₂	170 \pm 20	28 \pm 3
DPLA ₂ -YIRN	ND	220 \pm 20
AtnI ₂	~2600	800 \pm 100
hGIIA ^c	no binding at 2 mM	23
hGX ^c	130 \pm 25	36 \pm 4

^a 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 PLA₂ activity in the supernatant at each phospholipid concentration. ^b Not determined. ^c The interfacial binding affinities of the human group IIA (hGIIA) and human group X (hGX) enzymes were determined previously by Bezzine et al. (23).

displayed the highest rises in activity. The activities of AtxA-V31W, AtxA-KKML, and AtxB on 30% POPS/POPC vesicles were already in the range of those determined on pure POPS 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 POPS 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 POPS 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 over a range of appropriate concentrations of phospholipid. Given that products of sPLA₂ hydrolysis can modulate the interfacial binding affinity of the enzyme (21), nonhydrolyzable DO_{et}PC vesicles containing 10% and 30% of anionic DO_{et}PS were used. The presence of DO_{et}PS was necessary to enable effective sedimentation, as pure DO_{et}PC vesicles were shown not to pellet well (25). The method has proved to be suitable for quantifying relatively weak interfacial binding of sPLA₂s to PC-rich vesicles and was successfully applied to the full set of mammalian sPLA₂s (23, 25, 26). The use of the sedimentation method to study interfacial binding of sPLA₂s has been discussed in detail (23).

The values of K_d determined for the subset of sPLA₂s used in this study are presented in Table 2, along with our

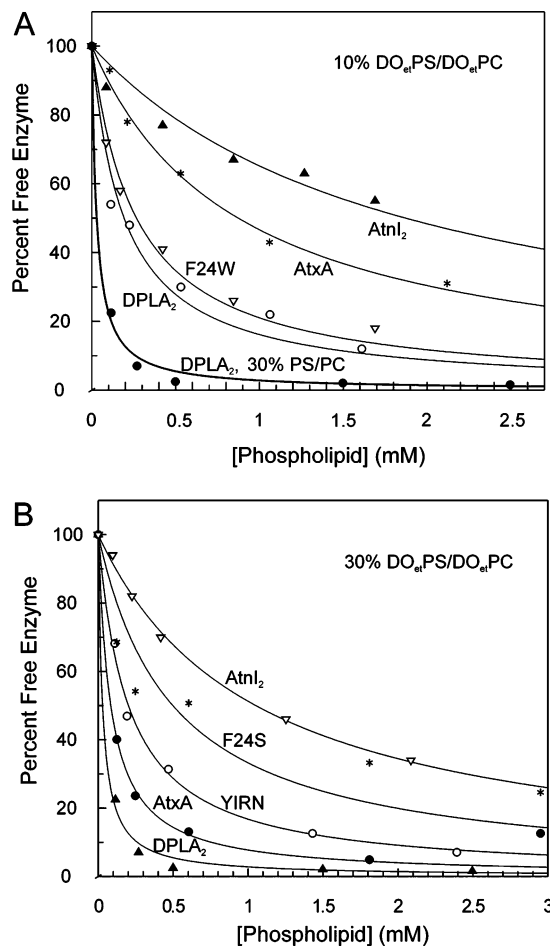


FIGURE 2: Interfacial binding of sPLA₂s to nonhydrolyzable phospholipid vesicles. Sucrose-loaded vesicles of (A) 10% and (B) 30% of DO_{et}PS in DO_{et}PC were pelleted by ultracentrifugation, and the percentage of sPLA₂ activity remaining in the supernatant was plotted as a function of the concentration of total phospholipid in the binding mixture. (A) DPLA₂ at 10% DO_{et}PS/DO_{et}PC (○); DPLA₂ at 30% DO_{et}PS/DO_{et}PC (●) (given for comparison); AtxA-F24W (▽); AtxA (*) ; AtnI₂ (▲). (B) AtxA (●); DPLA₂ (▲); DPLA₂-YIRN (○); AtxA-F24S (*); AtnI₂ (▽). Independent binding studies were carried out at least three times for each sPLA₂.

previously published data on hGIIA and hGX sPLA₂s (23). Some examples of the interfacial binding profiles are presented in Figure 2. AtxA, AtxA-F24W, and DPLA₂ were able to bind relatively well to PC-rich vesicles, displaying much higher binding affinity for 10% DO_{et}PS/DO_{et}PC than hGIIA and only up to 6-fold lower binding affinity than hGX. In the presence of 30% DO_{et}PS phospholipids the interfacial binding affinities of the above-mentioned venom and human sPLA₂s were very similar, with hGIIA displaying more than a 100-fold greater binding affinity than that determined on 10% DO_{et}PS/DO_{et}PC vesicles. Overall, increasing the concentration of DO_{et}PS in DO_{et}PC vesicles from 10% to 30% caused a 6–10-fold increase in interfacial binding affinity of the venom sPLA₂s, which correlates well with the 3–8-fold increase of activity observed for the same subset of sPLA₂s on the corresponding hydrolyzable POPS/POPC vesicles (Table 1). Additionally, DPLA₂, AtxA-F24W, and AtxA, that showed the highest binding affinities, also had the highest enzymatic activities on the PS-containing PC vesicles, while the low activity of DPLA₂-YIRN and AtxA-F24S can be attributed in large part to their poor binding affinity for such vesicles. The interfacial binding affinities

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

sPLA ₂	rate of hydrolysis [$\mu\text{mol}/(\text{min}\cdot\text{mg})$]		
	10% POPG/POPC	30% POPG/POPC	HEK293 cells
AtxA	54 \pm 3	754 \pm 71	5.8 \pm 0.4
AtxB	256 \pm 13	982 \pm 66	13.9 \pm 0.8
AtxA-V31W	579 \pm 27	1210 \pm 30	80 \pm 8
AtnI ₂	89 \pm 8	556 \pm 52	0.61 \pm 0.04

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

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 for PS-containing vesicles cannot be correlated with its high activity on pure PC vesicles. Furthermore, AtnI₂ displayed a more than 10-fold lower binding affinity for 30% DO_{et}PS/DO_{et}PC vesicles than AtxA, although both enzymes had similar activities on the corresponding hydrolyzable vesicles. Evidently, the presence of PS in the membrane has a more complex effect on interfacial binding and overall activity of AtnI₂ than of Atxs.

Hydrolysis of Cell Membranes by Snake Venom sPLA₂s. Upon entering the blood circulation of the snake-bite victim, presynaptically neurotoxic sPLA₂s are exposed to the Ca²⁺-rich extracellular environment and theoretically could bind and hydrolyze the PC-rich plasma membranes of a variety of cells. Nevertheless, these sPLA₂s bind specifically to presynaptic membranes, most probably to a high-affinity acceptor residing in the membrane (3). Additionally, it has been suggested that sPLA₂ neurotoxins may hydrolyze the membrane phospholipids in the vicinity of their receptor, thereby modifying its function (16). It was of interest, therefore, to determine whether Atxs can hydrolyze plasma membranes of intact mammalian cells. HEK293 cells have been used successfully in a number of studies on mammalian sPLA₂s, and a strong correlation exists between the ability of these enzymes to hydrolyze PC vesicles and HEK293 plasma membranes (22, 23, 25, 26). Similarly, Atxs were observed (Table 3) to hydrolyze intact HEK293 cells, and a clear correlation exists between the ability of AtxA, AtxB, and the AtxA-V31W mutant to release fatty acids from the PC-rich plasma membranes and from pure POPC vesicles (Figure 3). In a control experiment (not shown), hGIIA sPLA₂ exhibited negligible activity on these cells, and the rate of hydrolysis [$\sim 0.04 \mu\text{mol}/(\text{min}\cdot\text{mg})$] was similar to that recently reported (26). Therefore, the rates of hydrolysis of HEK293 cells, together with the data on pure POPC vesicles, indicate that Atxs are similar to hGV and hGX sPLA₂s in their ability to hydrolyze PC-rich membranes (23, 25, 26). Nontoxic AtnI₂, on the other hand, displayed a surprisingly low activity (almost 10-fold lower than AtxA) on these cells, despite its high activity on pure POPC vesicles. Given that AtnI₂ displays low activity and binding affinity for all PS-containing vesicles, it is possible that during displacement the cells are damaged (22), leading to exposure of PS which normally resides in the intracellular layer of mammalian

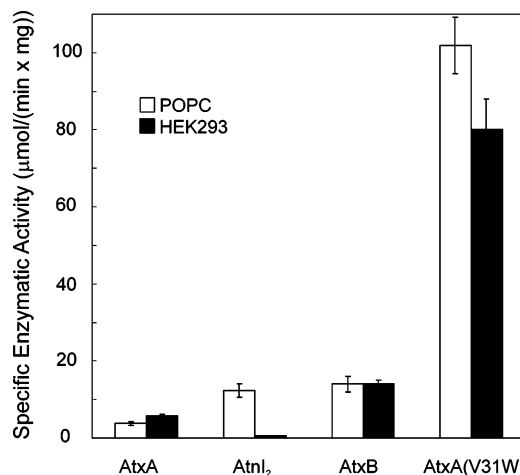


FIGURE 3: Enzymatic activity of sPLA₂s on intact HEK293 cells and POPC vesicles. The rate of hydrolysis for each sPLA₂ was determined with the fluorometric assay using fatty acid-binding protein, and the values are given in Tables 1 and 3. Assays with a final volume of 1.3 mL were performed at 37 °C in Hanks' balanced salt solution with 1.26 mM Ca²⁺ and 0.9 mM Mg²⁺ containing 30 μM phospholipid vesicles or approximately 8×10^5 HEK293 cells. Error bars are the standard deviation from at least five independent measurements.

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

sPLA ₂	calcium affinity, $K_{\text{Ca}(\text{app})}$	
	POPG (μM)	POPC (μM)
AtxA	31 \pm 8, ^b 25 \pm 4 ^c	1480 \pm 200
DPLA ₂	13 \pm 2	1290 \pm 280
AtxA-F24W	33 \pm 7	1950 \pm 270
AtxA-F24N	190 \pm 30	1430 \pm 180
AtnI ₂	360 \pm 70	590 \pm 80

^a $K_{\text{Ca}(\text{app})}$ values were determined from at least two independent calcium rate profiles, and each point (representing the average initial velocity of hydrolysis of the indicated vesicles at a given concentration of free calcium) in the rate profile was determined by at least three independent measurements. ^b Determined with AtxA isolated from *V. ammodytes ammodytes* snake venom. ^c Determined with recombinant AtxA.

plasma membranes (43, 44), but this was not investigated further.

Calcium Affinity Studies. Apparent calcium dissociation constants ($K_{\text{Ca}(\text{app})}$) 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 of an sPLA₂ is synergistic with calcium binding and occurs only when the enzyme is bound to the phospholipid surface (21). $K_{\text{Ca}(\text{app})}$ therefore not only depends on the calcium and phospholipid binding affinity of the enzyme's 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 $K_{\text{Ca}(\text{app})}$ 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

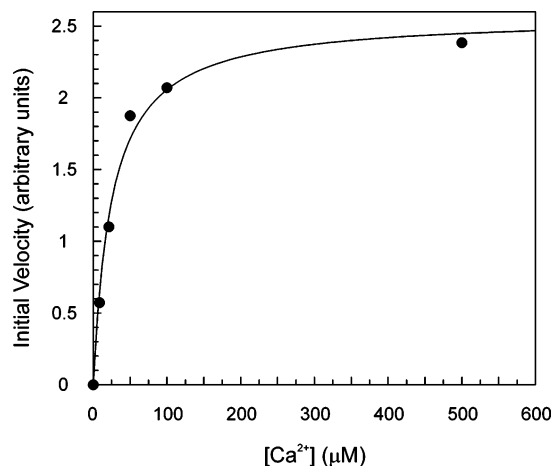


FIGURE 4: Calcium affinity profile of AtxA on POPG vesicles. Initial velocities (measured with the fatty acid-binding protein assay) for the hydrolysis of 30 μ M POPG vesicles by AtxA are shown as a function of free calcium concentration. The regression fit to the standard hyperbolic binding equation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

concentrations. The weakly toxic AtxA-F24N mutant (33) and the nontoxic AtnI₂ 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 DPLA₂ 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 AtnI₂ for calcium decreased only 1.6-fold on POPC vesicles and was 2.5 times higher than that of AtxA. The most likely explanation is that AtnI₂ 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 AtnI₂) 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 sPLA₂s are very efficient enzymes in comparison to groups I, II, V, X, and XII of mammalian sPLA₂ enzymes (23, 25, 26). The enzymatic activities of Atxs on anionic PG vesicles are comparable to those displayed by the most potent mammalian sPLA₂s (pancreatic group IB sPLA₂s) 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 AtnI₂ on POPS vesicles determined in this study is in the range of activities displayed by the mammalian sPLA₂s that display the highest activities on these vesicles, group IB and IIA sPLA₂s (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 sPLA₂ (Figure 1), which is well-known for its preference for anionic phospholipid substrates and negligible

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 sPLA₂s, which are by far the most potent among the mammalian sPLA₂s 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 sPLA₂ do not contain a Trp on their IBS, while DPLA₂, 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 sPLA₂s to bind well to such membrane surfaces, with affinities comparable to those of mammalian group V and X sPLA₂s (23, 25). Additionally, there is a good correlation between the interfacial binding affinity of Atxs, but not of nontoxic AtnI₂ (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 hGX (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 sPLA₂s. 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 sPLA₂s has been highlighted in the case of the human group V (45) and X enzymes (22, 23), the acidic sPLA₂ from *Naja naja atra* snake venom (46), a range of mutants of hGIIA (26, 47), and the pancreatic group IB sPLA₂s (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 hGX and in the range of that of cobra venom sPLA₂, which is well-known for its very high activity on PC membrane surfaces (26, 46). Hydrolysis of PC-rich

plasma membranes is supposed to be the (patho)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 sPLA₂s 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 sPLA₂s 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-

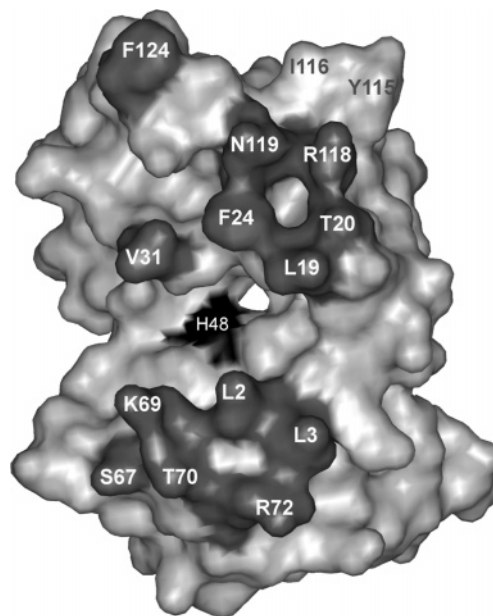


FIGURE 5: The putative IBS of AtxA. The presumed IBS amino acid residues (Leu-2, Leu-3, Leu-19, Thr-20, Phe-24, Val-31, Ser-67, Lys-69, Thr-70, Arg-72, Arg-118, Asn-119, and Phe-124; shaded dark gray) surround the active site pocket with His-48 (shown in black) and face the viewer. Residues Tyr-115 and Ile-116, which are on the upper back side of the molecule, away from the presumed IBS, are presented in dark gray type and are not shaded. The IBS residues were predicted on the basis of the homologous three-dimensional structures of hGIIA and bovine pancreatic group IB sPLA₂s and their putative IBS (52–54). The figure was generated using WebLab Viewer (Accelrys, Cambridge, U.K.).

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 DPLA₂ and its YIRN mutant, despite the presence of Trp-31, have lower or similar activities on PC-rich vesicles than AtxA. DPLA₂ 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 DPLA₂ antagonizes the positive effects of Trp or modifies the orientation of DPLA₂ 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 DPLA₂ 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 DPLA₂ 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 sPLA₂s (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 sPLA₂s.

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 zwitterionic 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 crotoxin (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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