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PRIORITY PAPER

The C-terminal region of ammodytoxins is important but not sufficient for neurotoxicity

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Ammodytoxins (Atxs) are presynaptically acting snake venom phospholipase A_2 (PLA_2) toxins the molecular mechanism of whose neurotoxicity is not completely understood. Two chimeric PLA_2s were prepared by replacing the C-terminal part of a nontoxic venom PLA_2, ammodytin I_2, with that of AtxA(K108N). The chimeras were not toxic, but were able to bind strongly to an Atxs-specific neuronal receptor, R25. They also showed an increased affinity for calmodulin, a recently identified high-affinity binding protein for Atxs, whereas affinity for a neuronal M-type PLA_2 receptor remained largely unchanged. The results show that the C-terminal region of Atxs, which is known to be involved in neurotoxicity, is critical for their interaction with specific binding proteins, but that some other part of the molecule also contributes to toxicity.

Keywords: calmodulin; neuronal receptor; phospholipase A_2; snake venom; toxicity.

Phospholipases A_2 (PLA_2s, EC 3.1.1.4) constitute a diverse superfamily of enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of phospholipids. They are divided into intracellular (cytosolic) and extracellular (secreted) PLA_2s. Secreted PLA_2s ($sPLA_2$) are low molecular mass (13–18 kDa), disulfide cross-linked (5–8 bonds) and Ca^{2+}-dependent enzymes [1–3]. They are typical interfacial enzymes that access the substrate directly from the phospholipid–water interface. In addition to enzymatic activity, those that are found in animal venoms may also exhibit a variety of pharmacological effects including neurotoxicity, myotoxicity, cardiotoxicity, and anticoagulant and edema-inducing activities [4].

Presynaptically neurotoxic $sPLA_2$s of groups I and II are the most potent toxins found in snake venoms, but the molecular basis of their toxicity is not completely understood [5]. It was shown that they first bind to several specific binding sites (receptors) on the presynaptic membrane [6], after which they are presumably endocytosed. In the nerve cell, they may inhibit the recycling of synaptic vesicles by binding to certain target proteins [7] and hydrolyzing certain phospholipids [8], although no apparent correlation between enzymatic activity and toxicity has been found [9]. In the final stage of neurotoxicity, an irreversible blockade of acetylcholine release at neuromuscular junctions is observed [10].

Venom of the long-nosed viper (*Vipera ammodytes ammodytes*) contains several group IIA $sPLA_2$s. Ammodytoxins (Atxs) are presynaptic $sPLA_2$ neurotoxins, ammodytins (Atns) I_1 and I_2 are nontoxic $sPLA_2$s, and AtnL is a myotoxic but enzymatically inactive $sPLA_2$ homolog [11–15]. Two receptors for Atxs with apparent molecular masses of 25 kDa (R25) and 180 kDa (R180) have been found in porcine cerebral cortex. R180 is an M-type $sPLA_2$ receptor located in the plasma membrane, that binds both toxic and nontoxic $sPLA_2$s of groups I and II [16,17]. R25 is an intracellular receptor, specific for Atxs, whose identity is still unknown [18]. During purification of R25, another high-affinity binding protein for Atxs was isolated and identified as calmodulin (CaM) [19], indicating that this highly conserved, Ca^{2+}-sensing regulatory molecule [20] may play a role in the $sPLA_2$-neurotoxicity.

In our previous studies, we demonstrated that certain residues in the C-terminal region of Atxs are involved in both neurotoxicity and binding to neuronal receptors R25 and R180 [21–23]. Here we report a further investigation of this relationship by preparing two chimeric proteins, where the C-terminal region of nontoxic $AtnI_2$ was substituted with that of highly neurotoxic AtxA. One of the chimeras had an additional N24 → F substitution in the N-terminal region, as F24 is also found in AtxA. The critical role of the C-terminal region of Atxs in binding to R25 and CaM was confirmed. The two recombinant proteins, however, remained nontoxic, indicating that this region alone is not sufficient for the neurotoxic effect of Atxs.

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Abbreviations: Atn, ammodytin; Atx, ammodytoxin; CaM, calmodulin; PLA_2, phospholipase A_2; R180, an M-type PLA_2 receptor of 180 kDa; R25, Atxs-specific neuronal receptor of 25 kDa; $sPLA_2$, secreted PLA_2.

Enzyme: phospholipase A_2 (EC 3.1.1.4).

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EXPERIMENTAL PROCEDURES

Materials

$AtnI_2$ was isolated from *Vipera a. ammodytes* venom as described [14]. Restriction enzymes were from MBI Fermentas (Vilnius, Lithuania) and New England BioLabs. Vent DNA polymerase, T4 polynucleotide kinase and *Taq* DNA ligase were purchased from New England BioLabs. T4 DNA ligase was obtained from Boehringer Mannheim. Hog brain CaM was from Roche Molecular Biochemicals and oligonucleotides from MWG-Biotech (Ebersberg, Germany). Radioisotopes were obtained from PerkinElmer Life Sciences, and disuccinimidyl suberate from Pierce (Rockford, IL). All other chemicals were of analytical grade.

Construction of expression vectors

The coding sequences for both $AtnI_2$/AtxA(K108N) chimeric proteins were prepared by PCR using Vent DNA polymerase. The N-terminal fragment, encoding the $AtnI_2$ part of the chimeras (N1–F106), was obtained by amplifying $AtnI_2$ cDNA in pUC9 [14], using the sense oligonucleotide 5′-ca gga tcc atc gaa ggt cGG AAC CTT TAC CAG TTC GGG-3′ and the antisense oligonucleotide 5′-cg taa aac tgc agt tcg AAA GCA GAT TGC CGC GAC CC-3′ (sequences complementary to the template are in capital letters; restriction sites *Bam*HI, *Pst*I and *Bst*BI used for cloning are underlined). The PCR product (325 bp) was excised from a 1.7% (w/v) agarose gel, purified with GeneClean II (BIO101, Vista, CA), digested with *Bam*HI and *Pst*I, and ligated into pUC19. The *Bam*HI/*Bst*BI fragment (coding for N1–F106 of $AtnI_2$) was excised from this cloning vector and inserted, together with the *Bst*BI/*Hin*dIII fragment (coding for Arg107–Cys133 of the mutant AtxA(K108N) (J. Pungerčar, unpublished results), into the *Bam*HI/*Hin*dIII-linearized T7 promoter-based expression vector [21]. The coding sequence for the N24F mutant of $AtnI_2$/AtxA(K108N) was obtained by PCR-directed mutagenesis using a known method [24]. The outer sense primer 5′-TAA TAC GAC TCA CTA TAG-3′, the outer antisense primer 5′-GTT TAC TCA TAT ATA CTT TAG-3′ (both complementary to plasmid DNA) and the inner sense primer introducing the mutation, 5′-TT TCC TAC AGC TTT TAC GGA TGC-3′ (the two nucleotides introducing mutation are underlined), were used to amplify the $AtnI_2$/AtxA(K108N)-encoding expression plasmid. Two PCR products (391 bp and 565 bp) were detected on a 1.7% (w/v) agarose gel. The larger DNA fragment was purified from the gel, cleaved with *Bam*HI and *Hin*dIII, and the restriction fragment (399 bp) inserted into the expression vector as above. The nucleotide sequences of both constructs were confirmed using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). In both cases, the expression vectors enabled production of the two $AtnI_2$/AtxA(K108N) chimeric $sPLA_2$s as fusion proteins with the N-terminal fusion peptide of 13 amino acid residues (MARIRARGSIEGR).

Production and purification of recombinant proteins

Each of the two expression vectors was used to transform the *E. coli* BL21(DE3) strain (Novagen, Madison, WI), and the cells were grown at 37 °C in 5 × 450 mL of LB-enriched medium. When the optical density at 600 nm reached 2.0, production of the recombinant proteins was induced by isopropyl thio-β-D-galactoside and the incubation continued for an additional 3 h. Recombinant $sPLA_2$s were isolated as inclusion bodies, refolded *in vitro*, activated with acetylated trypsin and purified by FPLC on a Mono S column (HR 5/5; Pharmacia) as described [21,22]. The $AtnI_2$(N24F)/AtxA(K108N) mutant was additionally purified by reverse-phase HPLC.

Analytical methods

Protein samples were analyzed by SDS/PAGE in the presence of 150 mM dithiothreitol on 15% polyacrylamide gels. Reverse-phase HPLC was performed using an HP1100 system (Hewlett-Packard, Waldbronn, Germany) and an Aquapore 300 BU column (30 × 4.6 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid and eluted with a linear gradient of 0–80% (v/v) acetonitrile. The N-terminal sequence was determined by Edman degradation on an Applied Biosystems Procise 492A protein sequencing system (Foster City, CA). Electrospray ionization mass spectrometry was performed on a high-resolution magnetic-sector AutospecQ mass spectrometer (Micromass, Manchester, UK).

Circular dichroism spectroscopy

CD spectra were recorded in the range of 250–200 nm at 25 °C on an Aviv 62A DS CD spectrometer. A bandwidth of 2 nm, a stepsize of 1 nm, and an averaging time of 2 s were used. Protein concentrations were 15.2 μM for $AtnI_2$/AtxA(K108N), 16.5 μM for $AtnI_2$(N24F)/AtxA(K108N) and 33.3 μM for wild-type $AtnI_2$. Protein water solutions and water were scanned three times in a cell of 1 mm pathlength, the spectra were then averaged and smoothed.

Binding studies

AtxC was radioiodinated [25] and membranes were extracted from a demyelinated crude mitochondrial-synaptosomal fraction of porcine cerebral cortex as described [19]. The membrane extract or CaM solution, 10 nM ^{125}I-labeled AtxC and increasing concentrations of unlabeled competitor (mutant or wild-type $sPLA_2$) were incubated at room temperature for 30 min with occasional vortexing. Cross-linking of $sPLA_2$s to their binding proteins was achieved by adding disuccinimidyl suberate dissolved in dimethylsulfoxide just before use, to a final concentration of 100 μM. The reaction mixture was mixed vigorously for 5 min at room temperature, and the cross-linking reaction stopped by adding SDS/PAGE sample buffer containing dithiothreitol [19]. Following electrophoresis and autoradiography, the intensities of the specific adducts on autoradiographs were analyzed by QuantiScan (Biosoft, Cambridge, UK) and the nonlinear curve fitting program GRAFIT, Version 3.0 (Erithacus Software, Staines, UK).

PLA_2 activity

Enzymatic activity was determined by a slightly modified standard method using a micellar substrate [26]. Hydrolysis of egg-yolk PtdCho was measured in a reaction mixture (8 mL) supplemented with 1% (v/v) Triton X-100 and

15 mM $CaCl_2$, at pH 8.0 and 40 °C. The fatty acids released were titrated with 10 mM NaOH using a 718 STAT Titrino pH-stat (Metrohm, Herisau, Switzerland). One enzyme unit (U) corresponds to 1 μmol of hydrolyzed phospholipid per minute.

Toxicity

Lethality was determined by intraperitoneal injection of each $sPLA_2$ into NMRI albino mice. Just prior to application, different doses of $sPLA_2$s (2–360 μg) were prepared in 0.5 mL of 0.9% (w/v) NaCl. LD_{50} was determined after 24 h using a standard method [27]. The experiments on mice were carried out in accordance with the EC Council Directive regarding animal experimentation.

RESULTS

Construction of chimeric proteins

Both chimeric $sPLA_2$s (Fig. 1A) were constructed to substitute the 25 C-terminal amino acid residues in nontoxic $AtnI_2$ with the corresponding 26 residues of neurotoxic AtxA ($AtnI_2$ and AtxA are composed of 121 and 122 amino acid residues, respectively). To ease the construction, the AtxA-encoding fragment (R107-C133; amino acid numbering according to [28]) was obtained from the plasmid aimed for expression of the AtxA(K108N) mutant, where, in addition to this substitution, a silent mutation in the vicinity introduced a unique *Bst*BI restriction site at the F106 and R107 codons suitable for cloning (J. Pungerčar, unpublished result). As a result, the two chimeric $sPLA_2$s that we prepared possess a single mutation (K108N) in the C-terminal, AtxA-like region. As shown by the studies of the AtxA double mutant (K108N/K111N) [21,22], the influence of K108N substitution on neurotoxicity and protein-binding properties of AtxA was expected to be relatively small. In one of the two chimeric proteins, N24 in the $AtnI_2$-part was substituted with F which is also present in neurotoxic AtxA and plays an important role in the presynaptic neurotoxicity of the toxin [29].

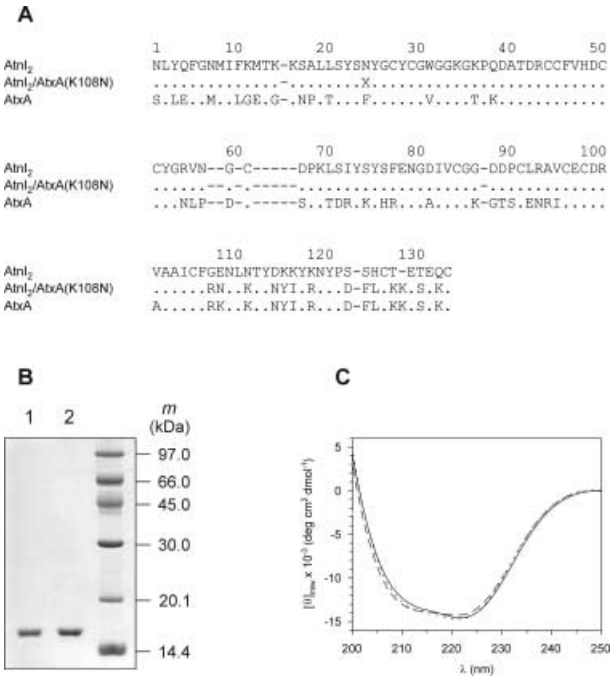

Fig. 1. Alignment, SDS/PAGE and CD spectra of chimeric $sPLA_2$s. (A) Amino acid sequence alignment of chimeric $sPLA_2$s with nontoxic ammodytin I_2 ($AtnI_2$) and neurotoxic ammodytoxin A (AtxA). The common numbering of $sPLA_2$ residues is used [28] and gaps, shown by dashes, are introduced to optimize alignment. Identical residues are shown by dots. X represents N in $AtnI_2$/AtxA(K108N) and F in $AtnI_2$(N24F)/AtxA(K108N). (B) SDS/PAGE of recombinant $sPLA_2$s after trypsin activation and purification. Lane 1, $AtnI_2$/AtxA(K108N); lane 2, its N24F mutant. Proteins (2 μg) were reduced by dithiothreitol and stained with Coomassie Brilliant Blue R250. (C) CD spectra of mutant and natural $sPLA_2$s. The far-UV CD spectra of $AtnI_2$/AtxA(K108N) (short-dashed line) and $AtnI_2$(N24F)/AtxA(K108N) (long-dashed line) are compared with that of wild-type $AtnI_2$ (solid line).

Bacterial production and characterization of recombinant PLA_2s

Recombinant $sPLA_2$s produced in *E. coli* as N-terminal fusion proteins were successfully activated by mild trypsinolysis and purified to homogeneity as judged by SDS/PAGE (Fig. 1B) and reverse-phase HPLC. The final yield was approximately 2.6 mg and 0.7 mg per litre of bacterial culture of $AtnI_2$/AtxA(K108N) and its N24F mutant, respectively. The single N-terminal amino acid sequence, NLYQF…, of each recombinant chimera confirmed the specific cleavage of the fusion protein during activation just after the last R of the fusion peptide. The molecular masses of chimeric proteins, determined by electrospray ionization mass spectroscopy, 13.904 kDa for $AtnI_2$/AtxA(K108N) and 13.937 kDa for $AtnI_2$(N24F)/AtxA(K108N), perfectly match their theoretical values, assuming formation of all seven disulfide bonds in the $sPLA_2$ molecule.

Influence of the mutations on the secondary structure and overall conformation of the $AtnI_2$ molecule was analyzed by CD spectroscopy. The far-UV CD spectra of both chimeric $sPLA_2$s and natural $AtnI_2$ were very similar (Fig. 1C) indicating that the C-terminal region of AtxA, with the 14 residues differing from the corresponding $AtnI_2$ region distributed mainly on the molecular surface (Fig. 2), did not induce any substantial conformational changes in the protein fold.

In contrast to wild-type $AtnI_2$, both chimeric proteins strongly inhibited binding of radiolabeled AtxC to the Atxs-specific receptor, R25, with IC_{50} values in the range of 20–24 nM, which is close to that of AtxA (Fig. 3A, Table 1). The introduced C-terminal AtxA residues also enabled the chimeric $sPLA_2$s to bind to CaM. However, the interaction of the chimeras with this high-affinity binding protein for AtxA was considerably weaker than with AtxA (Fig. 3B, Table 1). Binding of both chimeric proteins to R180 was similar to that of $AtnI_2$. No substantial difference was observed between the two chimeras in regard to their interaction with R25, R180 and CaM.

$AtnI_2$/AtxA(K108N) showed only 50% of the wild-type $sPLA_2$ activity on PtdCho–Triton X-100 mixed micelles. When N24 was substituted by F, the specific enzymatic activity of the chimera almost recovered to that of $AtnI_2$. The chimeric $sPLA_2$s, at intraperitoneal doses 5–10 $mg \cdot kg^{-1}$, were not lethal to mice (Table 1).

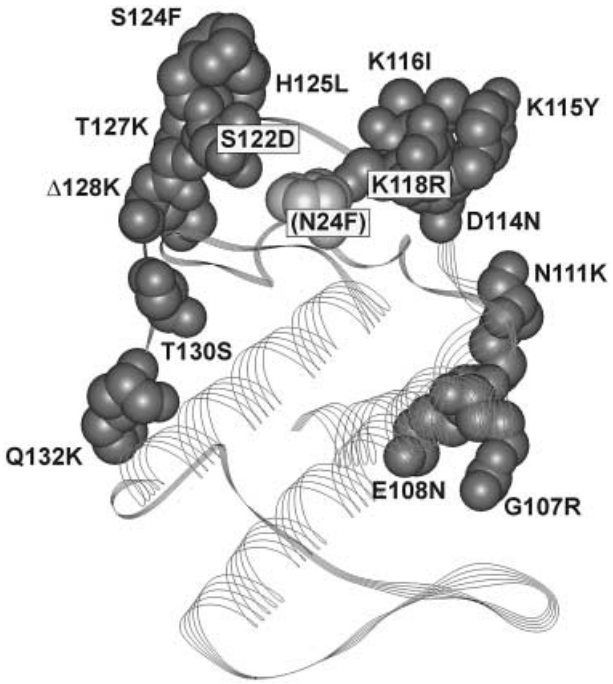

Fig. 2. Location of the mutated residues in chimeric $sPLA_2$s. The polypeptide backbone is shown in line ribbon representation and the residues introduced into $AtnI_2$ resulting in the chimeric proteins in CPK (spacefilling) representation. The figure was generated using WebLab VIEWERLITE software (Molecular Simulations, Cambridge, UK).

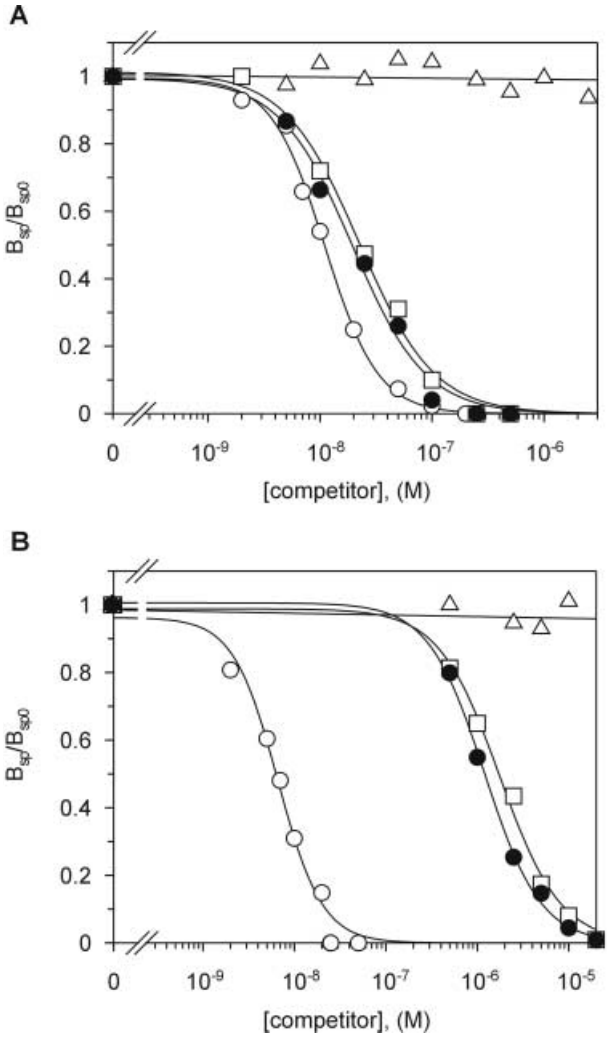

Fig. 3. Competition of different $sPLA_2$s for the binding of ^{125}I-labeled AtxC to high-affinity binding proteins. R25 (A) or CaM (B) were incubated with labeled AtxC in the presence of increasing concentrations of AtxA (○), $AtnI_2$/AtxA(K108N) (●), $AtnI_2$(N24F)/AtxA(K108N) (□) and $AtnI_2$ (△) to inhibit affinity labeling. The radioactivity of the ^{125}I-labeled AtxC-binding protein adduct is shown relative to that in the absence of any competitor.

DISCUSSION

It has been demonstrated that Atxs, the presynaptically neurotoxic $sPLA_2$s from venom of the long-nosed viper, strongly and specifically bind to neuronal receptor R25 and CaM [18,19]. No binding to these proteins was observed with $AtnI_2$, a nontoxic $sPLA_2$ from the same venom, which differs from Atxs in more than 40% of amino acid residues. The two chimeric $sPLA_2$s ($AtnI_2$/AtxA(K108N) and $AtnI_2$(N24F)/AtxA(K108N)), prepared in this study on a nontoxic $AtnI_2$-scaffold, still differ from Atxs in about 30% of residues, but were able to interact with both Atx-binding proteins. The ability of the chimeric proteins to inhibit ^{125}I-labeled AtxC binding to R25 was practically at the level of wild-type AtxA, indicating the crucial role of the last 26 amino acid residues of AtxA for this interaction. This is in accordance with our previous results, which suggested that specifically distributed positively charged amino acid residues, and particular hydrophobic and aromatic residues on the surface (residues 115–124) in the C-terminal region of

Table 1. Binding properties, enzymatic activity and toxicity of chimeric $sPLA_2$s. IC_{50} values are mean ± S.E.M. of at least three independent measurements. The enzymatic activity values are accurate to within ± 10%.

$sPLA_2$	IC_{50} (nM)			Specific enzymatic activity ($U \cdot mg^{-1}$)	LD_{50} ($\mu g \cdot kg^{-1}$)
	R25	CaM	R180		
AtxA	10 ± 3[a]	6 ± 2[a]	16 ± 3[a]	280[b]	21[b]
$AtnI_2$	> 10^4	> 10^4	610 ± 100	880	> 10^4
$AtnI_2$/AtxA(K108N)	20 ± 6	1300 ± 200	490 ± 100	440	> 10^4
$AtnI_2$(N24F)/AtxA(K108N)	24 ± 6	1700 ± 300	850 ± 200	840	> 5000

[a] [29], [b] [12].

neurotoxic Atxs are involved in binding to this receptor [22,23]. The C-terminal region of Atxs, however, is not critically involved in binding to R180, as the binding affinities of $AtnI_2$ and both chimeras for this M-type $sPLA_2$ receptor were similar. This is also in line with the proposed structural elements of $sPLA_2$s, mainly located in or close to the Ca^{2+}-binding loop, that are involved in binding to M-type $sPLA_2$ receptors [30].

It appears that the binding site for R25, located in the C-terminal region of Atxs, at least partially overlaps with that for CaM. Since the binding affinities of the two chimeric $sPLA_2$s for CaM are still considerably lower than that of AtxA, we assume that amino acid residues from some other region of Atxs, which are spatially close to the C-terminal residues contribute to this interaction. Substitution of N24 by F in the second chimera did not significantly influence binding to each of the three Atxs-binding proteins. A similar, small effect on binding affinity for these proteins was also observed by the reverse mutation (F24N) of AtxA, although in that case lethality of the toxin was dramatically decreased [29]. However, the replacement of N24 by F in this study did not result in higher toxicity of the construct; only the enzymatic activity on PtdCho–Triton X-100 mixed micelles increased twofold. The increase in enzymatic activity of the N24F chimera was expected, since the residue at position 24 is a constitutive part of the interfacial binding surface, important for adsorption of $sPLA_2$s to aggregated phospholipid substrates, such as membranes, vesicles and micelles [31,32]. In certain cases, as shown by a study of human group IIA $sPLA_2$ [33], even the introduction of a single aromatic (F) residue to this surface may considerably increase binding, particularly to aggregated zwitterionic (e.g. PtdCho) substrates. The higher enzymatic activity of the N24F chimera is also in agreement with the behavior of the AtxA(F24N) mutant, where the reverse substitution (F24N) resulted in fourfold lower enzymatic activity [29].

Our previous studies have demonstrated the importance for neurotoxicity of the C-terminal region stretching over the top of the Atx molecule (Fig. 2), particularly certain hydrophobic/aromatic and basic residues [21–23]. The same region is also involved in the high-affinity binding of these toxins to R25 and CaM. In contrast to $AtnI_2$, both $AtnI_2$/AtxA(K108N) chimeric proteins were able to bind to these AtxA-binding proteins, but were not toxic. Our results show that F24 and the last 26 amino acid residues of potently neurotoxic AtxA are not enough to transform a nontoxic $sPLA_2$ into a neurotoxic one. It is evident that some other residues on the toxin molecule also contribute to produce the neurotoxic effect.

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