Phenylalanine-24 in the N-terminal region of ammodytoxins is important for both enzymic activity and presynaptic toxicity

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Ammodytoxins (Atxs) are group II phospholipases A₂ (PLA₂s) with presynaptic toxicity from venom of the snake *Vipera ammodytes ammodytes*. The molecular basis of their neurotoxicity, and that of similar PLA₂ toxins, is still to be explained. To address this problem, a surface-exposed aromatic residue, Phe²⁴, in the N-terminal region of the most potent Atx, AtxA, was replaced by other aromatic (tyrosine, tryptophan), hydrophobic (alanine) and polar uncharged (serine, asparagine) residues. The mutants were produced in the bacterial expression system, refolded *in vitro* and purified to homogeneity. All but the Trp²⁴ mutant, whose activity was similar to that of the wild type, showed a considerable decrease (40–80 %) in enzymic activity on a micellar phosphatidylcholine substrate. This result indicates an important role for the aromatic side chains of phenylalanine or tryptophan, but not tyrosine, in PLA₂ activity, very likely at a

stage of interfacial adsorption of the enzyme to zwitterionic aggregated substrates. The substitutions of Phe²⁴ also significantly decreased toxicity in mice, with the most prominent decrease, of 130-fold, observed in the case of the Asn²⁴ mutant. The results with the mutants show that there is no correlation between enzymic activity, lethality and binding affinity for three AtxA neuronal receptors (R180, R25 and calmodulin). Our results suggest a critical involvement of Phe²⁴ in the neurotoxicity of AtxA, apparently at a stage which does not involve the interaction with the known Atx-binding neuronal proteins and catalytic activity.

Key words: calmodulin, phospholipase A₂, receptor-binding affinity, site-directed mutagenesis, *Vipera ammodytes ammodytes*.

INTRODUCTION

The expanding superfamily of phospholipase A₂ (PLA₂; EC 3.1.1.4) enzymes currently comprises 12 groups and several subgroups of secreted and intracellular enzymes which share the common ability to catalyse the hydrolysis of the *sn*-2 ester bond of glycerophospholipids [1–3]. The secreted PLA₂s (sPLA₂s) are relatively small (13–18 kDa), Ca²⁺-dependent and highly stable enzymes, due to the presence of between five and eight disulphide bonds. They can be found as mediators of various physiological processes in a variety of mammalian tissues and as potent toxins in different animal venoms [4]. Snake venom PLA₂s, although structurally very similar, exhibit an array of pharmacological effects including myotoxicity, neurotoxicity, cardiotoxicity and anticoagulant activity [5]. Kini and Evans [6] have explained this multiplicity of pharmacological activities by the existence of specific high-affinity protein-binding sites for toxic PLA₂s on the surface of target cells in specific target tissues.

Presynaptically acting PLA₂ neurotoxins interfere with the release of acetylcholine from peripheral neurons into the synaptic cleft. They all induce similar electrophysiological and pathomorphological changes in the intoxicated tissue and produce an irreversible blockade of neuromuscular transmission, leading to the death of the envenomed animal due to paralysis of the respiratory muscles [7]. Despite their similar tertiary structure and neurotoxic action, different extra- and intra-cellular neuronal receptors have been identified for different presynaptically toxic PLA₂s [8,9]. It has been proposed that these diverse neuronal targets could be mediators of the same physiological process,

probably endocytosis. Different PLA₂ neurotoxins could reach the same site of action inside the nerve ending through different import systems, resulting in nearly identical final pathological effects [9]. On arrival in the synaptic vesicle, the combination of the toxin's phospholipolytic activity and the transmembrane pH gradient, generated by a vacuolar ATPase proton pump, could lead to a specific rearrangement of the hydrolytic products in the synaptic vesicle membrane, which promotes the fusogenic and impairs the endocytic properties of the vesicle. Consequently, the synaptic vesicle cycle may be blocked irreversibly at the ultimate stage of endocytosis, as the vesicle neck closure is interrupted [10]. Although a direct correlation between enzymic activity and lethal potency of neurotoxic PLA₂s has not been observed [11], it was shown a long time ago that enzymic activity is essential for full expression of the neurotoxic effect [12]. In spite of numerous attempts to identify the surface residues of different PLA₂ neurotoxins involved in the complex process of presynaptic neurotoxicity, the molecular basis of their toxicity has yet to be resolved [5].

Ammodytoxin (Atx) A is the most potent of three presynaptically toxic PLA₂s from the venom of the western sand viper (*Vipera ammodytes ammodytes*) [13]. Three Atx-binding proteins, R25 [14], R180 [15] and calmodulin [16], have been identified recently in porcine cerebral cortex, and may have a potential role in presynaptic toxicity. Our previous studies have indicated that certain aromatic residues in the C-terminal part of AtxA are important for toxicity [17,18]. In the present study, we have investigated the possible involvement of another surface-exposed aromatic residue (Phe²⁴), located in the N-terminal part

Abbreviations used: Atx, ammodytoxin; IBS, interfacial binding surface; PLA₂, phospholipase A₂; sPLA₂, secreted PLA₂.

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but in the vicinity of those C-terminal residues. The results show that Phe²⁴ is important for both presynaptic toxicity and enzymic activity of the toxin, but not for interaction with the Atx-binding proteins, further underlining the subtle differences in the protein sites responsible for the different physiological activities.

MATERIALS AND METHODS

Materials

AtxA and AtxC were purified from *V. a. ammodytes* venom as described previously [19–21]. Restriction enzymes were obtained from MBI Fermentas and New England BioLabs, T4 DNA ligase was from Boehringer Mannheim and T4 polynucleotide kinase was from MBI Fermentas. *Taq* DNA polymerase and RNase One were purchased from Promega, and *Taq* DNA ligase and *Vent* DNA polymerase were from New England BioLabs. Oligonucleotides were from The Great American Gene Company (Ramona, CA, U.S.A.) and MWG-Biotech (Ebersberg, Germany). Radioisotopes were purchased from DuPont-NEN Life Science Products, disuccinimidyl suberate was from Pierce and protein molecular-mass standards were from Amersham Bioscience. Porcine brain calmodulin and Triton X-100 were from Roche Molecular Biochemicals. All other chemicals were of at least analytical grade and were purchased from Sigma and Serva.

Site-directed mutagenesis

Site-directed mutagenesis was performed by PCR using the method of incorporating a mutagenic oligonucleotide into the amplification product [22]. The previously constructed expression plasmid encoding wild-type AtxA preceded by a short fusion peptide of 13 amino acids was used as a template [18]. Each PCR reaction was performed using two outer oligonucleotide primers and an inner mutagenic primer corresponding to each mutant. The outer sense oligonucleotide, 5'-TAA TAC GAC TCA CTA TAG-3', was complementary to part of the T7 RNA polymerase promoter site on the plasmid, and the outer antisense oligonucleotide, 5'-GTT TAC TCA TAT ATA CTT TAG-3', was complementary to a region on the plasmid downstream from the stop codon of AtxA cDNA. The mutagenic primers 5'-CTC CTA CTC CTG GTA CGG ATG CT-3' (for the F24W mutant), 5'-CTC CTA CTC CTM TTA CGG ATG CT-3' (a degenerate primer for the F24S and F24Y mutants), 5'-TC CTA CTC CGC TTA CGG ATG CT-3' (for the F24A mutant) and 5'-CTC CTA CTC CAA TTA CGG ATG-3' (for the F24N mutant) were phosphorylated using T4 polynucleotide kinase prior to the mutagenesis reaction. Each PCR reaction mixture (100 µl) contained 100 ng of the plasmid template, 400 µM of each of the four deoxynucleoside triphosphates, *Taq* DNA ligase buffer [20 mM Tris/HCl/25 mM potassium acetate/10 mM magnesium acetate/10 mM dithiothreitol/1 mM NAD⁺/0.1 % (w/v) Triton X-100], 50 pmol of each of the three oligonucleotides (two outer and one mutagenic), 5 units of *Vent* DNA polymerase and 80 units of *Taq* DNA ligase. After 30 cycles of PCR amplification (94 °C for 30 s, 48 °C for 60 s and 4 min at 72 °C per cycle, with a 5 min final elongation at 72 °C), the PCR fragments of approx. 560 bp were purified with GeneClean II (BIO101) and digested with *Bam*HI and *Pst*I. The restriction fragments of 189 bp, which corresponded to the N-terminal half of AtxA, were sequenced using an ABI Prism 310 Genetic Analyser (Perkin-Elmer Applied Biosystems). After the desired mutations were confirmed, the restriction fragments were inserted into the *Bam*HI/*Pst*I-linearized expression plasmids.

Expression and purification of the mutants

The T7 RNA polymerase-based expression plasmids encoding the five mutants were used to transform *Escherichia coli* strain BL21(DE3) (Novagen). The bacterial cultures of each mutant grown overnight were used to inoculate 6 × 450 ml of enriched Luria–Bertani medium in 2 litre Erlenmeyer shaking flasks. When the cells were grown to a D_{600} of 2.5, expression of the mutants was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. Then, 3 h after induction, the cells were harvested by centrifugation. The inclusion bodies were isolated and the mutated proteins were S-sulphonated, refolded and activated by tryptic cleavage of the fusion peptide as described previously [18]. The renaturation buffer was removed by dialysis against 20 mM sodium acetate, pH 4.8, the samples concentrated by ultrafiltration and the mutant proteins purified by FPLC on a Mono S column (HR 5/5; Amersham Biosciences) using a linear gradient of 0–2 M NaCl in 20 mM sodium acetate buffer, pH 4.8, at a flow rate of 1 ml/min. Proteins were purified to homogeneity by reversed-phase HPLC performed on a Hewlett Packard series 1100 system, using an Aquapore BU 300 column (30 mm × 4.6 mm) equilibrated with 0.1 % (v/v) trifluoroacetic acid. The mutants were eluted with a linear gradient of 0–80 % (v/v) acetonitrile at a flow rate of 1 ml/min. The HPLC peaks were collected, vacuum-dried, dissolved in distilled water and stored at –20 °C.

Analytical methods

Proteins were analysed by SDS/PAGE using the Mini Protean II and III systems (Bio-Rad) in the presence of 150 mM dithiothreitol on 15 % (w/v) polyacrylamide gels and stained using Coomassie Brilliant Blue R250. Molecular-mass analysis of the mutants was performed using a high-resolution magnetic sensor AutospecQ mass spectrometer with an electrospray ionization chamber (Micromass) [18]. N-terminal sequencing was performed by Edman degradation on an Applied Biosystems Procise 492A protein-sequencing system. Purity was also confirmed by HPLC as described above. Protein concentrations were calculated from the A_{280} using the method of Perkins [23].

The far-UV CD spectra of the mutants were recorded at 25 °C from 250 to 200 nm on an Aviv 62A DS CD spectrometer, with a bandwidth of 2 nm, a step size of 1 nm and an averaging time of 2 s. Following HPLC purification of the mutant proteins and vacuum drying of the collected peaks as described above, the protein samples used for CD measurements were dissolved in 0.1 M Tris/HCl, pH 7.0. Protein concentrations were as follows: 13.9 µM for AtxA, and 15.6, 11.8, 27.3, 31.3 and 17.5 µM for the F24S, F24Y, F24W, F24A and F24N mutants, respectively. The final spectra were smoothed averages of three repeated scans, measured in a cell with a 1 mm pathlength.

Enzymic activity

PLA₂ activity was measured on phosphatidylcholine/Triton X-100 mixed micelles by a slight modification of a standard method [24]. Hydrolysis of egg yolk phosphatidylcholine was measured titrimetrically in the presence of 1 % (v/v) Triton X-100 and 15 mM CaCl₂ in an 8 ml reaction mixture, at pH 8.0 and 40 °C. The fatty acids released were titrated with 10 mM NaOH using a 718 STAT Titrimetric pH-stat (Metrohm, Herisau, Switzerland). One enzyme unit corresponded to the amount of enzyme required to hydrolyse 1 µmol of phospholipid/min.

Figure 1 CD spectra of the Phe²⁴ mutants of AtxA

The CD spectra of the F24W (long-dashed line), F24A (thin solid line), F24N (long- and short-dashed line), F24S (short-dashed line) and F24Y (long- and double-short-dashed line) mutants, dissolved in 0.1 M Tris/HCl, pH 7.0, are compared with that of the wild-type AtxA (thick solid line). The spectra are smoothed averages of three scans.

Toxicity

Lethal potency of the toxins was determined by intraperitoneal injection into NMRI albino mice. Five dose levels and nine mice/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. After 24 h observation of the animals, the LD₅₀ values were determined using a standard method [25].

Binding studies

Preparation of the porcine cerebral cortex membrane extract, containing Atx-binding proteins (R25 and R180), and radioiodination of AtxC were performed as described previously [15,26]. The solubilized Atx-binding proteins, as well as commercial porcine brain calmodulin, were incubated for 30 min at room temperature with ¹²⁵I-AtxC (10 nM final concentration) in the presence or absence of unlabelled wild-type or mutant toxins (final concentrations ranging from 0 to 2 µM). Cross-linking of the toxins to their receptor proteins was performed by adding the bifunctional reagent disuccinimidyl suberate, dissolved in DMSO, to a final concentration of 100 µM. The reaction mixture was mixed vigorously for 5 min at room temperature and the reaction was stopped by adding SDS sample buffer containing dithiothreitol. After electrophoresis, the gels were dried and autoradiographed for several days at –70 °C using Kodak X-Omat AR films. The intensities of the specific bands on autoradiographs were quantified by QuantiScan (Biosoft), and the data were analysed by the non-linear curve-fitting program GraFit (Erithacus Software).

RESULTS

In order to assess the potential involvement of the surface-exposed Phe²⁴ (numbering according to Renetseder et al. [27]) in the toxicity of AtxA, five mutants were prepared in which Phe²⁴ was replaced by an aromatic (F24Y, F24W), hydrophobic (F24A) or polar uncharged (F24S, F24N) residue. All the mutants were produced as inclusion bodies in the *E. coli* expression system,

Table 1 Enzymic activity, lethal potency and receptor-binding affinity of AtxA and the Phe²⁴ mutants

The enzymic activity values for each mutant are means ± S.E.M. from at least eight independent measurements. The IC₅₀ values were determined from three to six independent experiments and the values shown are also means ± S.E.M. The lethality of wild-type AtxA was the same as that reported previously [13]. We estimate that our reported LD₅₀ values are accurate to within ± 5–10%.

PLA ₂	Enzymic activity (units/mg)	LD ₅₀ (µg/kg)	IC ₅₀ (nM)		
			Calmodulin	R25	R180
AtxA	280 ± 10	21	6 ± 2	10 ± 3	16 ± 3
AtxA(F24N)	70 ± 4	2800	5.6 ± 0.7	14 ± 2	26 ± 1
AtxA(F24S)	75 ± 5	380	7.4 ± 0.2	14 ± 1	16 ± 2
AtxA(F24Y)	50 ± 3	330	9.2 ± 0.9	14 ± 3	19 ± 4
AtxA(F24W)	305 ± 10	175	13.6 ± 0.3	37 ± 5	26 ± 3
AtxA(F24A)	160 ± 8	90	7.0 ± 0.9	15 ± 1	17 ± 2

refolded, activated by cleavage of the fusion peptide and purified to homogeneity. The overall yield of purified AtxA mutants was 3–4 mg/l of bacterial culture.

The N-terminal sequence of each recombinant protein (SLLEFG ...), which was identical with that of AtxA, confirmed the absence of internal cleavage due to trypsin activation. Homogeneity was demonstrated by analytical HPLC and MS. The single molecular masses determined (13790, 13715, 13813, 13698 and 13741 Da for the F24Y, F24S, F24W, F24A and F24N mutants, respectively), were within one mass unit of those expected, indicating that all seven disulphide bonds had formed correctly and that no post-translational modification had occurred after synthesis. In order to confirm that the proteins were folded properly, we measured the far-UV CD spectra of native AtxA and the five mutants (Figure 1). The CD spectra of the proteins showed no significant differences, implying that the introduced single-site mutations did not induce any significant conformational changes in the secondary structure.

Specific enzymic activities of the mutants on mixed phosphatidylcholine/Triton X-100 micelles, their lethal potencies and binding affinities for the porcine neuronal receptors were determined (Figure 2 and Table 1). The mutants showed a significant (40–80 %) decrease in enzymic activity, with the exception of the F24W mutant, whose activity was similar to that of the wild-type enzyme. The F24N mutant displayed a substantial, 130-fold, decrease in lethal potency, whereas the LD₅₀ values of the F24A, F24W, F24Y and F24S mutants were 4-, 8-, 16- and 18-fold higher, respectively, than that of the wild type. We tested the ability of the five mutants and wild-type AtxA to inhibit specific binding of radio-iodinated AtxC to the neuronal receptors from porcine cerebral cortex (R25, R180 and calmodulin), and determined the IC₅₀ values. The binding affinity of the tryptophan mutant for R25 was 4-fold lower than that of the wild type, whereas the other four mutants showed smaller, approx. 1.5-fold, decreases. The IC₅₀ values of the mutant proteins for the R180 receptor did not differ significantly from that of the wild type, except for a slight increase in the case of the F24W and F24N mutants. The binding affinity of AtxA for porcine brain calmodulin did not change significantly, with the exception of the F24W mutant, which had a slightly reduced affinity (Table 1).

DISCUSSION

The interfacial binding surface (IBS) of sPLA₂s towards aggregated phospholipid substrates, such as membranes, micelles and

Figure 2 Inhibition of cross-linking of ^{125}I -AtxC with the Atx-binding proteins by the Phe²⁴ mutants and wild-type AtxA

Atx-binding proteins R25 and R180, contained in a porcine cerebral cortex membrane extract, and commercial porcine brain calmodulin (CaM), were incubated for 30 min at room temperature with radio-iodinated AtxC (final concentration, 10 nM) in the presence of the indicated nanomolar concentrations of unlabelled wild-type AtxA (**A**) and its Phe²⁴ mutants, F24N (**B**), F24S (**C**), F24Y (**D**), F24W (**E**) and F24A (**F**). Cross-linking was performed using disuccinimidyl suberate (final concentration, 100 μM) [15,16]. The reaction was stopped by adding SDS sample buffer containing dithiothreitol. Electrophoresis was performed by SDS/PAGE with 15% (for the membrane extract) and 10% (for calmodulin) gels, which were then dried and autoradiographed at -70°C . The positions of the specific adducts between ^{125}I -AtxC and its receptor proteins are indicated by arrows.

vesicles, comprises the residues on the protein surface that surround the narrow hydrophobic pocket extending to the active site [28–30]. The entrance to the hydrophobic pocket is encircled by a ring of highly conserved hydrophobic residues, whereas around them and on the edges of the IBS some polar, basic and acidic residues can be found [31–33]. Since most sPLA₂s have high binding affinities for anionic membrane surfaces, it was thought that electrostatic forces between cationic residues of the enzyme and anionic membrane phospholipids are crucial for membrane binding and activity of sPLA₂s [34]. However, it has become evident that hydrophobic, aromatic and hydrogen-bonding interactions account for most of the binding energy, even to anionic surfaces [29,30,35,36]. Recently, it has been shown that electrostatic interactions are responsible for proper orientation and initial association of sPLA₂s with both anionic and zwitterionic membranes. Further stabilization of the binding is then achieved by hydrophobic interactions when aliphatic and aromatic residues on the IBS penetrate the membrane surface [37].

Tryptophan residues on the IBS of human group V [38], human group X [39] and cobra venom group I [40] PLA₂s play an important role in supporting high-affinity binding to zwitterionic membrane surfaces. Mutants of human group IIA [41] and pancreatic group I PLA₂s [42–44] have also shown the importance of tryptophan residues on the IBS for enhanced binding to both

neutral and anionic vesicles. Of the AtxA Phe²⁴ mutants studied in the present paper, F24W shows the highest enzymic activity, close to that of the wild type. This indicates that tryptophan plays a similar role to phenylalanine in the binding dynamics, presumably involving the hydrophobic part of tryptophan, since the polar residues (serine, asparagine and tyrosine) decrease the activity to an extent greater than does the weakly hydrophobic (alanine) residue. This also suggests a role for Phe²⁴ similar to those proposed for some other aromatic residues on the IBS of homologous sPLA₂s from groups I and V [37,40].

The lethal potencies of the Phe²⁴ mutants decreased by factors of 4–130 (Table 1), suggesting that the hydrophobic/aromatic property of a residue at position 24 is also important for neurotoxicity. However, this also shows some more subtle differences in the 'active site' for toxicity from that for enzymic activity. Our recent studies have suggested an important role of certain aromatic and hydrophobic residues in the toxicity of AtxA (Figure 3). AtxC, a naturally occurring isoenzyme of AtxA, is 17 times less toxic than the latter, although their primary structures differ by just two amino acid residues at the C-terminal part of the molecule [21]. It has been shown that Phe¹²⁴, which is replaced by isoleucine in AtxC, is responsible for the higher toxicity of AtxA [18]. Furthermore, an AtxA mutant in which Tyr¹¹⁵ and Ile¹¹⁶ were replaced by lysines showed a 240-fold decrease in toxicity [17]. In addition, a six-site charge-

Figure 3 Location of the residues important for neurotoxicity of AtxA

The presumed IBS residues [51] of AtxA, surrounding the opening to the active-site pocket with His⁴⁸, are shaded dark grey and pointed towards the reader. The residues identified so far by site-directed mutagenesis as being important for neurotoxicity of AtxA are indicated, and are shown in black. The surface view of the three-dimensional model of AtxA was generated using WebLab ViewerPro (Molecular Simulations).

reversal mutant of AtxA has proved that the basic residues in the C-terminal region are not crucial for neurotoxicity [45]. Given the decrease in toxicity of the five Phe²⁴ mutants, and taking into account the importance of the C-terminal aromatic and hydrophobic residues (Phe¹²⁴, Tyr¹¹⁵ and Ile¹¹⁶), which are in the vicinity of Phe²⁴ (Figure 3), we infer that the exposed aromatic ring of Phe²⁴ also plays an important role in neurotoxicity. This conclusion is strengthened by the observation that ammodytin I₂ [46], a non-toxic PLA₂ homologous to AtxA from the same venom, has an asparagine residue at position 24, a serine residue at position 124, and lysine residues at positions 115 and 116. This is also in agreement with the observation that the F24N mutant had by far the lowest toxicity of the five AtxA Phe²⁴ mutants.

Some other presynaptically neurotoxic group II sPLA₂s have a serine residue in place of Phe²⁴ or Phe¹²⁴, but they usually possess an additional aromatic residue(s) in the vicinity. For example, agkistrodotoxin from the venom of *Gloydius halys* [47] has Phe²⁴, Phe¹¹⁹ and Ser¹²⁴, trimucrotoxin from *Trimeresurus mucrosquamatus* [48] has Phe²¹, Ser²⁴ and Phe¹²⁴, and vipoxin from *V. a. meridionalis* [49] and RV-4 from *Daboia russelli formosensis* [50] have Phe¹⁷, Trp²⁰, Ser²⁴, Phe¹¹⁹ and Ser¹²⁴. It seems, therefore, that certain exposed aromatic residues on the IBS from both C- and N-terminal regions play an important role in presynaptic neurotoxicity of snake venom PLA₂s, but that their location is not restricted to one particular site.

In comparison with wild-type AtxA, only the F24W mutant showed a slight decrease in binding affinity for the 25 kDa receptor, R25, and calmodulin, both of which specifically bind

Atxs with high affinity, but not some other neurotoxic (e.g. oligomeric taipoxin and monomeric OS₂; both group I PLA₂s) and non-neurotoxic (e.g. ammodytin I₂ and porcine pancreatic PLA₂) [14,16] sPLA₂s. It is possible that Phe²⁴ is in the vicinity of the receptor-binding sites, not critically involved in the binding, but that the bulky tryptophan side chain might be able to slightly perturb the toxin–receptor interaction. The receptor-binding studies were performed by measuring competition of the AtxA Phe²⁴ mutants with AtxC, rather than AtxA. AtxC, which is equivalent to an AtxA(F124I/K128E) mutant, is 17 times less toxic than AtxA and lacks Phe¹²⁴, which is located at the IBS. Phe¹²⁴, present in the Phe²⁴ mutants but not in AtxC, appears to be important for both toxicity [18] and receptor binding [45]. The lack of correlation between the affinity towards neuronal receptors and toxicity of the mutants *in vivo* does not necessarily mean that the binding to receptors is not important for the toxicity [9]. Presynaptic neurotoxicity is a complex and as yet incompletely understood process, involving protein–protein interactions and phospholipid hydrolysis. The residues involved in binding to one receptor may not be important for the binding to another at some different stage of the process.

In conclusion, the similar affinities of the Phe²⁴ mutants and wild-type AtxA for binding to the porcine neuronal receptors indicate that Phe²⁴ is not part of the binding sites for the three receptors. Nevertheless, our results indicate clearly that, in addition to certain C-terminal aromatic and hydrophobic residues [17,18], the N-terminal aromatic residue Phe²⁴, on the IBS is involved in the neurotoxicity of AtxA.

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