An aromatic, but not a basic, residue is involved in the toxicity of group-II phospholipase A₂ neurotoxins

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Ammodytotoxins (Atxs) A, B and C are basic phospholipase A₂s from *Vipera ammodytes ammodytes* snake venom, and they exhibit presynaptic toxicity. The most toxic is AtxA, followed by AtxC, its naturally occurring F124 → I/K128 → E mutant, which is 17 times less toxic. Two mutants of AtxA have been produced in bacteria and characterized. The specific enzymic activity of the K128 → E mutant on mixed phosphatidylcholine/Triton X-100 micelles is similar to that of the wild type. The K108 → N/K111 → N mutant, however, possesses 160 % of the wild-type activity. Replacement of the two basic residues by uncharged, polar residues on the opposite side of the protein to the enzyme active site and interfacial adsorption surface results in increased enzymic activity at the water/lipid aggregate interface, due to a redistribution of electrostatic charge. The binding affinity of the double mutant for the specific acceptor in bovine brain was

similar to that of AtxA, whereas the affinity of the single mutant was similar to that of AtxC, which was slightly weaker than that of AtxA. Interestingly, the substitution of any of these three basic surface residues did not significantly change the lethal potency of AtxA. Since the single mutant AtxA(K128 → E) is equivalent to the AtxC(I124 → F) mutant, this indicates that the residue at position 124 is important for presynaptic toxicity of Atxs. The more than 10-fold lower toxicity of AtxC, compared with AtxA, is a consequence of the substitution of Phe-124 (aromatic ring) with Ile (aliphatic chain). Exposed aromatic residues in the C-terminal region may also be important for the neurotoxicity of other similar toxins.

Key words: acceptor-binding affinity, ammodytotoxin, enzyme activity, protein engineering, *Vipera ammodytes ammodytes*.

INTRODUCTION

Ammodytotoxin (Atx) A is the most presynaptically toxic phospholipase A₂ (PLA₂) from the venom of the long-nosed viper, *Vipera ammodytes ammodytes*. Two other isoenzymes, AtxB and AtxC, from the same subspecies, are 28 and 17 times less toxic than AtxA, respectively [1]. AtxA is a group-IIA PLA₂ of 122 amino acid residues that is one of a number of monomeric β -neurotoxins, such as notexin and *Oxyuranus scutellatus* PLA₂-2 (group-I PLA₂s) and agkistrodotoxin (group-II PLA₂). The presynaptic toxicity of different β -neurotoxins results from a complex process leading to the irreversible blockade of the release of the neurotransmitter acetylcholine. One of the first events in this process is binding to a specific acceptor on the presynaptic nerve membrane. It has been shown that the binding sites for different β -neurotoxins are not the same [2], having been identified in the case of β -bungarotoxin as a K⁺ channel [3], and in the case of taipoxin as a neuronal pentraxin [4] and neuronal pentraxin receptor [5]. The enzymic activity of β -neurotoxins shows no significant correlation with their toxicity [6], but it is clearly obligatory for the full expression of neurotoxic activity since its inhibition prevents lethality. It has been shown that selective alkylation of His-48 in the enzyme active site of crotoxin B by *p*-bromophenacylbromide inhibits PLA₂ activity irreversibly and leads to loss of its lethality [7].

Both toxic and non-toxic secretory PLA₂s have highly similar three-dimensional structures. Most of the amino acid residues involved in calcium binding, catalytic activity and disulphide bonds are conserved. The other residues, however, enable the PLA₂ molecule to express a broad spectrum of pharmacological activities, including neurotoxicity. Several approaches towards understanding structure–function relationships in toxic PLA₂s

have been used, from comparison of their primary and tertiary structures, chemical modification and the use of specific site-directed antibodies to acceptor-binding studies. Despite numerous attempts, toxic PLA₂s still remain a complex multifunctional protein puzzle, yet to be solved [8]. After the first successful expression of snake-venom PLA₂s in *Escherichia coli* [9–12], this problem can now also be approached by protein engineering.

In this study, we describe the bacterial expression and site-directed mutagenesis of AtxA. In comparison with our first attempts [12], the expression and renaturation procedures have been modified to yield more recombinant toxin. Two mutants with the substitution of basic residues at the C-terminal part of the molecule, K128 → E and K108 → N/K111 → N (the common PLA₂ numbering of Renetseder et al. [13] is used), have been prepared and characterized. It has been demonstrated that Lys residues at these positions are not involved in neurotoxicity. However, our results suggest that an aromatic residue, Phe-124, plays an important role in the toxicity of Atxs.

MATERIALS AND METHODS

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer, Pharmacia and New England BioLabs. All other chemicals of at least analytical grade were from Sigma or Serva, unless stated otherwise. Radioisotopes used for nucleotide sequencing were from Amersham and those for binding studies from New England BioLabs. Oligonucleotides were purchased from Pharmacia or synthesized on an Applied Biosystems 381A DNA synthesizer and subsequently purified by

Abbreviations used: Atx, ammodytotoxin; PLA₂, phospholipase A₂.

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PAGE followed by a double precipitation with sodium acetate and ethanol. The *E. coli* DH5 α strain (Gibco-BRL) was routinely used as a host for cloning vectors.

Construction of expression plasmid

The AtxA cDNA [14] was used to construct and produce recombinant Atxs. The 5'-untranslated, signal peptide and 3'-untranslated regions were first trimmed off by exploiting internal restriction sites. A single *EcoRI* site was present at nucleotide position 58 at the codon encoding Glu-4. Additionally, two *PstI* sites existed at positions 216 and 422. The first *PstI* site was at the codon for Ser-67 and the second was just after the stop codon. The plasmid pUC9 containing AtxA cDNA was therefore digested by *EcoRI* and *PstI*, and the resulting *EcoRI*-*PstI* and *PstI*-*PstI* fragments of the appropriate sizes were inserted into the *EcoRI*/*PstI*-linearized pUC19. The correct orientation of the *PstI*-*PstI* fragment, encoding the second half of mature AtxA, was confirmed by nucleotide sequencing.

In order to produce AtxA as a non-toxic fusion protein, a short *BamHI*-*EcoRI* cassette was constructed encoding the first three amino acid residues of AtxA, Ser-1-Leu-3, preceded by a tetrapeptide, Ile-Glu-Gly-Arg, which is a potential factor-Xa cleavage site. The oligonucleotides used were 5'-GA TCC ATC GAA GGT CGT AGC TTG CTC G-3' (sense) and 5'-AA TTC GAG CAA GCT ACG ACC TTC GAT G-3' (antisense). The AtxA-coding region (Glu-4-Cys-133) was excised from the pUC19 with *EcoRI*/*HindIII* and ligated together with the cassette into the *BamHI*/*HindIII*-cut expression plasmid pT7-7 [15]. Plasmids for expressing the mutants, which were both only distinguishable from AtxA by the C-terminal region, were constructed by a simple exchange of the *PstI*-*HindIII* fragment.

Site-directed mutagenesis

The double mutant AtxA(K108N/K111N) was prepared by the gapped-duplex method, using amber selection in the bacteriophage M13mp18 [16]. The mutagenic antisense oligonucleotide 5'-ATA GTT GTA TGT ATT CAG ATT GTT TCG GAA GCA GAT TG-3' was used to introduce both mutations (underlined). After mutagenesis, the *PstI*-*HindIII* fragment was subcloned into pUC19, sequenced and inserted into the expression vector as described above.

The single mutation AtxA(K128E) was prepared by the double-primer method [17,18] using the Altered Sites *in vitro* mutagenesis system of Promega. The mutagenic sense oligonucleotide 5'-TG TGC AAG GAG GAG TCA G-3' introduced the point mutation. The expected substitution was confirmed by nucleotide sequencing on the pALTER-1 vector.

Sequencing

Nucleotide sequences were determined by the dideoxy chain-termination method [19] on a double-stranded template using a T7 sequencing kit and manual sequencing apparatus of Pharmacia. N-terminal sequencing was performed by Edman degradation using an Applied Biosystems 475A protein sequencer, connected online to a phenylthiohydantoin-amino acid 120A analyser from the same manufacturer. Prior to sequencing, the purities of recombinant toxins were confirmed by HPLC on a ChromSpher C₈ column (3 mm $\times$ 100 mm; Chrompack).

Expression in *E. coli*

The *E. coli* strain BL21(DE3) (Novagen) was transformed with the expression plasmid containing the Atx gene. For large-scale

production, a single fresh bacterial colony was used to inoculate 5 ml of Luria-Bertani medium supplemented with 100 μ g/ml ampicillin and the cells were grown until the culture became turbid. The culture was added to 500 ml of rich medium containing 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 7.5 g/l Na₂HPO₄, 3 g/l KH₂PO₄, 1 g/l NH₄Cl, 4 g/l glucose and 1 mM MgSO₄ (pH 7.0), in the presence of 100 mg/l ampicillin. The pre-culture was grown overnight and used to inoculate 7 l of the same medium in a 10-l fermentor (New Brunswick). Bacteria were grown under vigorous aeration and agitation at 37 °C. Production of recombinant toxin was induced in mid-log phase ($D_{600} = 2.4$) by adding isopropyl β -D-thiogalactoside to a final concentration of 0.3 mM. Approx. 3 h after induction, when the culture became saturated ($D_{600} = 5-6$), the cells were harvested by centrifugation at 4 °C. Typically, about 10 g of wet bacterial cells per l of fermentation broth were obtained.

Isolation of inclusion bodies and refolding of proteins

The bacterial pellet from a 7-l culture was resuspended in 300 ml of ice-cold TE buffer [50 mM Tris/HCl/40 mM EDTA (pH 8.0)] containing 25 % (w/v) sucrose and homogenized. After adding of 50 mg of lysozyme, the suspension was incubated for 60 min on ice with occasional homogenization. TE buffer (300 ml) was then added and the suspension allowed to stand for 30 min on ice with periodic homogenization. The cells were sonicated three times on ice in 100-ml portions for 1.5 min each. In cases where the lysate still remained too viscous, 1 mg of DNase I was added and the sample was incubated for about 30 min at room temperature. After reduction of viscosity, 0.1 % (w/v) Triton X-100 was added and the lysed cells were homogenized for a few seconds and sonicated three times for 1 min each. The lysate was centrifuged for 30 min at 4000 g, the pellet thoroughly washed with 100 ml of buffer containing 0.5 M urea, 50 mM Tris/HCl and 5 mM EDTA (pH 8), and the inclusion bodies recovered by centrifugation using the same conditions.

The inclusion bodies were dissolved in 150 ml of a freshly prepared solution of 7 M guanidine hydrochloride/0.35 M Na₂SO₃ (pH 8.3). The fusion protein was S-sulphonated as described by Thannhauser and Scheraga [20] and precipitated subsequently by adding ice-cold glacial acetic acid to a final concentration of 1 % (v/v). The sulphonated protein was pelleted by centrifugation for 30 min at 5000 g, dissolved in 8 M urea or 5 M guanidine hydrochloride (pH 8.0), and stored at -20 °C until use. Refolding was performed for 16-20 h at 4 °C in 25 mM boric acid (pH 8.0) containing 10 mM CaCl₂, 8 mM cysteine, 1 mM cystine and 1 mM EDTA, in the presence of 0.5-2 M denaturant; either guanidine hydrochloride (0.5-1 M) or urea (1-2 M). Typically, the protein concentration was about 75 μ g/ml in 2 l of renaturation solution.

Activation and purification

After overnight refolding, the renaturation solution was centrifuged for 30 min at 5000 g to remove insoluble protein. Trypsin was added in an amount of about 10 % (w/w) of the recombinant PLA₂ toxin, and the solution was stirred gently at room temperature for a few hours. PLA₂ activity was measured by a pH-stat method every 15 min to monitor the process of activation. When the activity reached maximum, usually after 3 h, the pH was adjusted to 4.5 and the mixture dialysed against 10 mM sodium acetate, pH 4.5. The sample was applied to a CM-cellulose column (100 ml of CM-52; Whatman), washed with 5 mM sodium acetate buffer, pH 4.5, containing 0.25 M NaCl, and eluted batchwise with the same buffer containing 2 M NaCl. The eluate was dialysed against 5 mM acetic acid and the

Figure 1 Expression-plasmid construct

The grey box represents the N-terminal fusion peptide of 13 amino acid residues, including the *Bam*HI–*Eco*RI oligonucleotide cassette (underlined in the nucleotide sequence) encoding the Ile-Glu-Gly-Arg factor-Xa/trypsin-cleavage site (denoted by the arrowhead below the sequence), and the first three amino acid residues of AtxA (Ser¹–Leu³). The black box shows the Glu⁴–Cys¹³³ coding region of AtxA. P_{T7}, T7 promoter; Amp^R, β -lactamase region; ColEI ori, origin of replication; rbs, ribosome-binding site.

Figure 2 Ribbon diagram of a three-dimensional model of AtxA

The enzyme is oriented with its putative interfacial binding site and the N-terminal α -helix pointing towards the viewer. The amino acid residues that were mutated (Lys-108, -111 and -128), Phe-124 involved in toxicity and active-site His-48 are shown in space-filling representations.

protein recovered by lyophilization. The toxin was dissolved in buffer A [20 mM sodium acetate (pH 5.0)], and purified by FPLC on a Mono S column (HR 5/5; Pharmacia). The recombinant protein was eluted routinely with a linear salt gradient of 0–1 M NaCl in buffer A at a flow rate of 1 ml/min.

SDS/PAGE and isoelectric focusing

Protein electrophoresis was performed according to Laemmli [21] on a Mini Protean II (Bio-Rad) using a 15% (w/v) polyacrylamide gel and staining with Fast Green (Sigma) or a PhastSystem (Pharmacia) using pre-cast 8–25% gradient gels. Electrophoresis and staining with Coomassie Brilliant Blue 250 were conducted according to the manufacturer's instructions. Prior to application to a gel, samples were reduced by boiling in an SDS buffer containing 150 mM dithiothreitol.

Isoelectric focusing was carried out using a 5% (w/v) polyacrylamide gel supplemented with 10% (v/v) glycerol according to the instructions of Pharmacia.

Molecular-mass analysis

Electrospray ionization MS analysis was performed using a high-resolution magnetic-sector AutospecQ mass spectrometer (Micromass). The protein samples were introduced into an electrospray nebulizer at a flow rate of 10 μ l/min with a syringe pump. The spectra were obtained by scanning from m/z 2500 to 200 at 10 s/scan. Calibration was performed by sodium iodide cluster ions.

CD

The far-UV CD spectra of proteins were obtained using an Aviv 62A DS CD spectrometer. The spectra were measured at 25 °C over a wavelength range of 200–250 nm. The protein concentrations of native AtxA and recombinant toxins AtxA(K128E) and AtxA(K108N/K111N) were 11.6, 9.4 and 10.1 μ M in water, respectively. Each spectrum is the smoothed average of five repeat scans, measured with a bandwidth of 1 nm, at a recording rate of 0.5 nm/s and using a cell pathlength of 1 mm.

Enzymic activity

PLA₂ activity was measured titrimetrically on mixed micelles in an egg-yolk assay [22], using a pH-stat apparatus equipped with a TTT60 titrator (Radiometer, Copenhagen, Denmark). The reaction mixture contained egg-yolk phosphatidylcholine as substrate in the presence of 1% (w/v) Triton X-100/10 mM CaCl₂. All measurements were made at 40 °C and pH 8.0. One enzyme unit is defined as the amount of enzyme required to hydrolyse 1 μ mol of phospholipid per min. Concentrations of the toxins were calculated from their absorbance at 280 nm using the method of Perkins [23].

Lethality

Lethal potency was assessed by intraperitoneal injection of 0.2–2 μ g of each mutant in 0.5 ml of 0.9% (w/v) NaCl into NMRI albino mice. LD₅₀ was determined using the method of Reed and Muench [24].

Preparation of synaptic membranes

A modification of the method of Bennett et al. [25] was used to prepare de-myelinated P2 fraction of bovine cerebral cortex. To determine protein content in synaptic membrane preparations, a

Table 1 Alignment of AtxA with other presynaptically toxic group-II PLA₂s

The common numbering of PLA₂s by Renetseder et al. [13] is used. A dot indicates identical amino residues, and gaps introduced to optimize the alignment are shown by dashes. The mutated residues are doubly underlined. Elements of the secondary structure (α -helices and β -sheets) taken from the crystal structure of agkistrotoxin [29] are shown below the sequences. Intravenous LD₅₀ values are shown for most β -neurotoxins. In some cases, intraperitoneal LD₅₀ (*) or LD₁₀₀ (**) is given. Abbreviations and references for PLA₂ toxins: Atx, ammodytoxins from *Vipera a. ammodytes* [1,30-32]; VrVIIIa, the most basic, myonecrotic and neurotoxic PLA₂ from *Daboia (Vipera) russelli* [33,34]; Tmtx, trimucrotoxin from *Trimeresurus mucrosquamatus* [35]; Aghtx, agkistrotoxin from *Agkistrodon blomhoffi brevicaudus* [36,37]; CB1 and CB2, basic-isoform subunits of crotoxin from *Crotalus durissus terrificus* [38,39]; the sequence of CB2 is identical to that of basic subunit of Mojave toxin from *Crotalus s. scutulatus*; Bicaud, caudoxin from *Bitis caudalis* [40,41]; Vipox, vipoxin from *V. a. meridionalis* [42,43]; PfcblI, basic subunit CblI from *Pseudocerastes fieldi* [44,45]; Rv4, basic subunit of Russtoxin from *D. (V.) r. formosensis* [46].

	1	10	20	30	40	50	60	70	
AtxA	SLLEFGMMILGETG	-KNPLTSYSFYGCYCGVGGK	GTPKDATDRCCFVHDCCYGNLP	--D-C----	SPKT				
AtxC		-.....							
AtxB		-.....							
VrVIIIa	K...E...-	LAIP...S.....	W.....						N..S
Tmtx	N..Q.NK..KIM.K-	AIPF..S.....	W..Q.K.....	K.T--	-----	S			
Agktx	N..Q.NK..KE....-	AIPF.A.....	W..Q.K..G.....	R.V--	N-----	NT.S			
CB1	H..Q.NK..KF..R-	AIPF.A.....	W..R.R.....	K.A-K-	-----	NT.W			
CB2	...Q.NK..KF..R-	AVPF.A.....	W..Q.R.....	K.A-K-	-----	NT.W			
Bicaud	N.IQ..N..SAMT.-	S-SLA.AS.....	W...Q...D.....	KAD-K-	-----	M			
Vipox	N.FQ.AK..N.KL-	AFSVWN.IS.....	W..Q.....	RVR-G-	-----	N..L			
PfcbII	N.FQ.TK..N.KL-	AFAVLN.IS.....	W..Q.....	RVK-G-	-----	N..L			
Rv4	N.FQ.AR..N.KL-	AFSVWN.IS.....	W..Q.....	GVK-G-	-----	N..L			
	αA				αC				
	80	90	100	110	120	130	LD ₅₀ (μ g/kg)		
AtxA	DRYKYHRENGAIVCGK	-GTSCENRICECDRAAA	ICFR <u>KNLK</u> TYN	YIYRNYPDFLC	-K <u>KE</u> SEKC		21		
AtxC		-.....			I..-E.....		360		
AtxB		-.....			H..MY.....		580		
VrVIIIa	K.V.....	E.....	K.....	Q..N..SKK	ML.....	-G..L..	5300*		
Tmtx	.I.S.SWKT.I.I..E-	E..KK.....	V.LGH..R..KKR	MF.....	-TDP....		1200		
Agktx	.I.S.SLKE.Y.T....	N..EQ.....	V..E..R..D..NG	MF.R.SK.-	TET..E.		55		
CB1	.I.P.SLKS.Y.T....	W..EQ.....	V..E.L.RS.S..K	G.MF...SR-	RGP..T.		480		
CB2	.I.R.SLKS.Y.T....	W.KEQ.....	V..E.L.RS.S..K	NE.MF...SR-	REP..T.		700		
Bicaud	IL.S.KFH..N....	D-KNA.KKKV....	V.....AASKHS..	KNLWR..SSK	-TGTA...		180*		
Vipox	AI.S.SFKK.N....	-NNG.LRD.....	V..N..HQ.KN...	KN.KFLSSSR-	RQT..Q.		100**		
PfcbII	AI.S.SFQK.N....	-NNG.LRD.....	V..N..HQ.KN...	RN..FLSSSR-	RQT..Q.		1000		
Rv4	AI.S.SFQR.N....	R-NNG.LRT.....	V..N..HQ.KN...	KE.KFLSSSK-	RQR..Q.		320		
	β				αE				

modification of the Folin–Lowry protein assay of Markwell et al. [26] was used. BSA was taken as a standard.

Radioiodination of AtxC

¹²⁵I-AtxC was prepared using the modified chloramine-T method [27]. ¹²⁵I-AtxC was separated from unreacted Na¹²⁵I by ion-exchange chromatography on a CM–Sephacrose CL-6B column. The specific radioactivity of preparations was routinely about 500 Ci/mmol, its enzymic activity remaining unchanged.

Binding studies

Binding experiments were carried out at room temperature in incubation buffer [20 mM Tris/HCl/75 mM NaCl/1 mM CaCl₂/0.1 % (w/v) BSA/0.02 % (w/v) Triton X-100 (pH 7.4)]. Presynaptic membranes (0.8 mg of membrane protein/ml) were incubated with the radiolabelled ligand (3.4 nM final concentration) in the presence or absence of unlabelled competitor for 60 min in a final volume of 250 μ l [27]. Membrane-bound and

free radioligands were separated by rapid centrifugation of the membranes through a silicone-based oil mixture [28].

RESULTS

Bacterial production of toxins

The T7 RNA polymerase-based expression plasmid used in this study for production of recombinant Atxs is represented in Figure 1. The N-terminal fusion peptide of 13 amino acid residues includes, as in our previous expression system [12], a tetrapeptide recognition site (Ile-Glu-Gly-Arg) for potential factor-Xa cleavage of the fusion protein, just preceding the initial Ser residue of AtxA.

Mutant Atxs (see Figure 2 and Table 1 for the positions of the mutations), produced in insoluble form as inclusion bodies to a level of 10–15 % of total bacterial protein, were dissolved in guanidine hydrochloride and sulphonated prior to refolding. Renaturation was performed in the presence of low concentrations of denaturant, either guanidine hydrochloride (0.5–1 M)

Figure 3 CD spectra of AtxA and its mutants

The solid line (—) represents the spectrum of native wild-type AtxA, the broken line (---) is the Atx(K128E) mutant and the 'hyphenated' line (----) is the Atx(K108N/K111N) mutant. Experimental details are given in the Materials and methods section.

or urea (1–2 M). Refolding in guanidine hydrochloride was more efficient than that in urea by a factor of 2.5. The presence of Ca^{2+} ions greatly improved the yield of refolding, as did lower temperatures. Due to the high adsorption of Atxs to surfaces, its concentration was kept at 50–100 $\mu\text{g/ml}$. Concentrations of Atx above 100 $\mu\text{g/ml}$ significantly reduced the percentage of re-naturation due to increased aggregation. Under optimal conditions, the yield of refolding was up to 25%.

The refolded proteins could be activated with factor Xa. However, we decided instead to cleave off the N-terminal fusion peptide by mild trypsinolysis, which could be performed directly on a refolding mixture and proved to be more efficient. Using trypsin cleavage, incorrectly folded molecules were also degraded, making subsequent purification easier. Following activation, toxins were purified to homogeneity by two-step cation-exchange chromatography. The overall yield of Atx mutants was 2–5 mg per l of bacterial culture.

Characterization of recombinant proteins

Purity of recombinant Atxs was confirmed by SDS/PAGE, isoelectric focusing, analytical HPLC and N-terminal amino acid sequencing. In both mutants, the N-terminal sequences were identical to that of AtxA. No additional internal cleavages, arising during trypsin activation, were observed.

The molecular masses of the two recombinant mutants, determined by electrospray ionization MS, were 13775 Da for AtxA(K128E) and 13746 Da for AtxA(K108N/K111N), and were identical to those calculated from the amino acid sequences. The expected molecular masses prove that no post-translational modification occurs after synthesis of the recombinant proteins in the bacterial cytoplasm. Furthermore, the molecular masses, together with enzymic activity of the mutants, also provide evidence that all seven disulphide bonds are properly formed.

In order to exclude a possibility of a larger impact of point mutations on the protein conformation, we measured the CD spectra of native wild-type AtxA and mutant toxins (Figure 3). The spectra of the three proteins did not differ significantly, indicating that the mutations caused no significant change in the conformation of the polypeptide backbone. The observation of similar or even higher enzymic activities of the mutants, compared

Table 2 Enzymic activities, lethality and acceptor-binding activities of Atxs

Intravenous and intraperitoneal LD_{50} values are shown for native and mutant toxins, respectively. Values for enzymic activity and IC_{50} are means of triplicate measurements within 15% difference. The data on enzymic activities and toxicities of native AtxA and AtxC are from reference [1]. For details, see the Materials and methods section.

PLA ₂	pI	Specific activity (units/mg)	LD_{50} ($\mu\text{g/kg}$)	IC_{50} (nM)
AtxA	10.2	280	21	7.0
AtxC	9.4	288	360	18.9
AtxA(K128E)	9.1	300	45	19.4
AtxA(K108N/K111N)	9.1	450	67	14.2

with the wild-type, further supports the absence of significant conformational change.

The isoelectric points of the single (K128E) and double (K108N/K111N) AtxA mutants were similar, approx. 9.1. Specific enzymic activities and lethal potencies of the recombinant Atxs are shown in Table 2. The lethality of the AtxA mutants did not differ significantly from that of the wild-type toxin. The enzymic activity towards egg-yolk phosphatidylcholine of the single mutant was similar to that of AtxA, whereas the double mutant had 60% higher activity. AtxA(K108N/K111N) and AtxA(K128E) were tested for their ability to inhibit the specific binding of ^{125}I -AtxC on the bovine cerebral cortex membrane preparation (Table 2). The double mutant produced 50% maximal inhibition (IC_{50}) at 14.2 nM and the single mutant at 19.4 nM. At the same time, IC_{50} values obtained with native AtxA and AtxC were 7 and 18.9 nM, respectively.

DISCUSSION

The aim of the present study was to produce adequate amounts of recombinant Atxs to characterize residues involved in toxicity. Our previous expression system used a long 81-residue N-terminal fusion peptide followed by a tetrapeptide factor-Xa-recognition site, under the control of a *tac* promoter [12]. That system had two main disadvantages: the *tac* promoter was not tightly regulated and the refolding of such a long fusion protein was inefficient, leading to low final yields. The cysteine residue in the adenylate kinase fusion peptide could also interfere with cysteines in the Atx molecule during refolding.

In contrast to mammalian pancreatic PLA₂s that are expressed *in vivo* as pre-proenzymes with a 7 amino acid propeptide, Atxs are synthesized in venom glands of the viper only as pre-proteins. Although pancreatic and viperid enzymes, based on their primary structures, belong to different groups of PLA₂s, their three-dimensional structures are rather similar [13]. We therefore proposed that a shorter N-terminal fusion peptide would not interfere in the process of refolding *in vitro*. Shortening the N-terminal fusion peptide by up to 13 amino acid residues, together with use of a well-regulated T7 RNA polymerase-based promoter proved to be effective. The final yield was 5–10-fold higher than that from the previous system.

As most PLA₂ neurotoxins are basic proteins, it has been thought that their basic character is important for their pre-synaptic toxicity. On the basis of this assumption, we designed, expressed and characterized two mutants of AtxA in which several basic residues in the C-terminal region were mutated. The mutations caused no significant structural change in the AtxA molecule. As a good correlation exists between lethality (i.e.

peripheral neurotoxicity) and binding affinity to specific brain receptors in the case of other presynaptic PLA₂ toxins [6], this justifies the use of the bovine brain neuronal preparation as a model for neurotoxicity and structure–function studies of our toxins.

The positioning of the first mutation at the end of α -helix E was based on the results of immunological studies. It has been shown that antibodies and Fab fragment against L2 peptide (F¹⁰⁶RKNLKY¹¹³) completely block toxicity of the neurotoxin, whereas enzymic activity is only slightly reduced [47]. Basic residues located in that region of AtxA were suggested to comprise part of the toxic site. In the first mutant, we therefore replaced two basic residues (Lys-108 and Lys-111) with two uncharged polar residues (Asn). The lethality and acceptor-binding activity of the K108N/K111N mutant were only slightly affected, demonstrating that these two lysines were not important for neurotoxicity. Furthermore, the significantly increased enzymic activity of the double mutant (by 60 % compared with the wild-type), using phosphatidylcholine as a substrate, did not have any influence on toxicity. Similarly, AtxB is enzymically nearly twice as active as AtxA [1], whereas its lethality is almost 30-fold lower.

Increased enzymic activity of the double mutant against an electrically neutral, zwitterionic, substrate (phosphatidylcholine) is interesting, particularly because the two basic residues concerned are located on the opposite side of the enzyme to the active site and the presumed interfacial adsorption surface for aggregated substrate (Figure 2). A similar increase of enzymic activity on a micellar phosphatidylcholine substrate was observed in porcine pancreatic PLA₂ after acylation of Lys-108 [48]. It has been shown that, in the binding of secretory PLA₂s to membranes, both electrostatic and hydrophobic interactions are important [49], although the contribution of each can vary significantly in different PLA₂ enzymes. According to the analysis of the electrostatic potentials of different secretory PLA₂s [50], the reduction of the positive charge in this particular region of AtxA will lead to significant charge redistribution, affecting molecular asymmetry, orientation and kinetics of interfacial binding of the enzyme.

From our results, and since only a few of the other presynaptically toxic PLA₂s of group II possess basic residues at positions 108 and 111 (Figure 3), we conclude that basic residues in the mutated region are not essential for neurotoxicity, at least in those PLA₂ toxins which bind to the same neuronal acceptor(s) as AtxA (i.e. AtxC, AtxB and crotoxin B subunit).

The second mutation that we made was located at the C-terminal end of the molecule at one of the two positions where AtxA and AtxC differ. AtxC is a naturally occurring F124 → I/K128 → E mutant of AtxA and is 17 times less toxic. The single AtxA(K128E) mutant, which can be also regarded as the AtxC(I124F) mutant, showed lethality similar to that of AtxA. Its acceptor-binding activity was, however, closer to that of AtxC. Clearly, a basic residue at position 128 is involved in acceptor binding, although apparently it does not contribute to higher neurotoxicity. As in crotoxin CBd subunit [27], which is an isoform of CB1, the affinity of the specific binding of a β -neurotoxin does not always parallel its toxicity *in vivo*. Binding of a PLA₂ toxin to the specific neuronal acceptor is only one of the events in the multi-step process of neurotoxicity. In order to better understand the results obtained with different mutants, this complex process should be divided into several sequential events in which different regions of the toxin are probably involved.

The more than 10-fold-higher toxicity of AtxA compared with AtxC is, surprisingly, a consequence of the substitution of Ile-124

by Phe. The aromatic ring of Phe-124 is exposed, as predicted from a three-dimensional model of AtxA, and as such is obviously very important for toxicity of the molecule. In β -neurotoxins similar to AtxA, position 124 is often occupied by Ser. However, in most of these toxins, there is another exposed Phe residue in this region at position 119 (in AtxA, Asn-119). The potential role of Phe-119 in toxicity should be checked by site-directed mutagenesis of these toxins.

Native AtxA and AtxC, which differ slightly in their C-terminal regions, show a distinct difference in their toxicities. Our results show that the aromatic and not the basic amino acid residue is responsible for the difference. Furthermore, it appears that certain exposed aromatic residues in this region could be very important for the neurotoxicity of similar β -neurotoxins.

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