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Biochimica et Biophysica Acta 1702 (2004) 217–225

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Basic amino acid residues in the β-structure region contribute, but not critically, to presynaptic neurotoxicity of ammodytoxin A

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Received 6 May 2004; received in revised form 30 August 2004; accepted 1 September 2004
Available online 15 September 2004

Abstract

The molecular mechanism of action of presynaptically toxic secreted phospholipases A_2 (sPLA$_2$s) isolated from snake venoms is not completely understood. It has been proposed that the positive charge in the β-structure region is important for their toxic activity. To test this hypothesis, we characterised several mutants of ammodytoxin A (AtxA) possessing substitution of all five basic residues in this region. The mutations had relatively little influence on the catalytic activity of AtxA, either on charge-neutral or anionic phospholipid vesicles. An exception was R72 when replaced by a hydrophobic (higher activity) or an acidic (lower activity) residue. Lethal potencies of the eight single site mutants were up to four times lower than that of the wild-type, whereas the triple mutant (K74S/H76S/R77L) was 13-fold less toxic. The substitutions also lowered the affinity of the toxin, slightly to moderately, for the neuronal receptors R25 and R180. Interaction with calmodulin was only slightly affected by substitutions of K86, more by those of the K74/H76/R77 cluster and most by those of R72 (up to 11-fold lower binding affinity). The results clearly indicate that the basic amino acid residues in the β-region of AtxA contribute to, but are not necessary for, its neurotoxic effect.

Keywords: Enzymatic activity; Phospholipase A_2; Neurotoxicity; Receptor-binding affinity; Site-directed mutagenesis; *Vipera ammodytes ammodytes*

1. Introduction

Phospholipases A_2 (PLA$_2$s, EC 3.1.1.4) are structurally diverse enzymes that hydrolyse the *sn*-2 ester bond of glycerophospholipids, releasing fatty acids and lysophospholipids. Secreted PLA$_2$s (sPLA$_2$s) are low molecular mass (13–19 kDa) and Ca^{2+}-dependent enzymes, possessing five to eight disulfide bonds. They have a catalytic His/Asp dyad and typically act at the water/lipid membrane interface [1]. sPLA$_2$s are present in different mammalian tissues and plants, and are especially abundant in the venom of reptiles and insects [2]. Snake venom sPLA$_2$s are involved in digestion of the prey and participate in venom toxicity. Many of them, despite a high degree of structural similarity, exhibit diverse (patho)physiological effects, including pre- and postsynaptic neurotoxicity, myotoxicity, anticoagulant activity and cardiotoxicity [3].

Presynaptically toxic snake venom sPLA$_2$s block the release of acetylcholine from motoneurons irreversibly, leading to death of the prey due to paralysis of the respiratory muscles. The molecular mechanism of presynaptic neurotoxicity, in which the enzymatic activity of an sPLA$_2$-toxin appears to be essential, is still largely

Abbreviations: Atx, ammodytoxin; CaM, calmodulin; FABP, fatty acid-binding protein; IBS, interfacial binding surface; LD_{50}, lethal dose for 50% of the population tested; PLA$_2$, phospholipase A_2; POPC/G, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/glycerol; R25 and R180, receptors for Atxs in porcine cerebral cortex of 25 kDa and 180 kDa, respectively; sPLA$_2$, secreted PLA$_2$

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doi:10.1016/j.bbapap.2004.09.002

unknown. Presumably, the $sPLA_2$-neurotoxin binds to a receptor in the presynaptic membrane and enters the nerve terminal where it impairs the cycling of synaptic vesicles, possibly by binding to intracellular protein targets [4] and/or by catalysing phospholipid hydrolysis [5].

Ammodytoxins (Atxs) A, B and C, isolated from *Vipera ammodytes ammodytes* venom, are basic $sPLA_2$s of group II A exhibiting presynaptic neurotoxicity. Their primary structures are very similar, but they differ considerably in toxicity, with AtxA being the most potent [6,7]. In the last few years, we have identified several Atx-binding proteins in porcine cerebral cortex: R180, which is an M-type plasma membrane $sPLA_2$ receptor [8,9], the intracellular membrane receptor R25 [10], most likely located in mitochondria [11], calmodulin (CaM) [12], and 14–3–3 protein γ and ε isoforms [13]. They bind Atxs in the nano- to low micromolar concentration range and may be involved in the process of presynaptic neurotoxicity. The surface residues of Atxs responsible for the neurotoxic effect have not been completely identified, but our previous studies indicate an important role of the C-terminal aromatic and hydrophobic residues Y115, I116 [14] and F124 [15], as well as F24 in the N-terminal part [16]. Recently, we have shown that the C-terminal region of Atxs, which is very important for neurotoxicity, is also critical for their interaction with the specific binding proteins, R25 and CaM [17]. From our studies, we have concluded that, besides the C- and N-terminal regions of Atxs, some other part of the molecule should be involved in neurotoxicity. On the basis of previous chemical modification [18] and structural studies [19–22], it has been proposed that the β-structure region rich in basic residues might be one of the important constituents of the 'neurotoxic site' of presynaptic $sPLA_2$ neurotoxins. In order to test this hypothesis, we studied the involvement of the β-structure basic residue cluster in the neurotoxicity, enzymatic activity and interaction of Atxs with neuronal binding proteins.

2. Materials and methods

2.1. Materials

Natural AtxC was purified from *V. a. ammodytes* venom [23]. Recombinant AtxA was prepared as described [15]. All basic chemicals were of analytical grade and were purchased from Sigma and Serva. Restriction endonucleases were from New England BioLabs and MBI Fermentas (Vilnius, Lithuania) and Vent DNA polymerase, T4 polynucleotide kinase and *Taq* DNA ligase from New England BioLabs. T4 DNA ligase was from Boehringer Mannheim. Oligonucleotides were from MWG-Biotech (Ebersberg, Germany), radiochemical $Na^{125}I$ from Perkin-Elmer Life Sciences and disuccinimidyl suberate from Pierce (Rockford, IL, USA). Triton X-100 and hog brain CaM were from Roche Molecular Biochemicals. Protein molecular mass standards were from Amersham Pharmacia Biotech. The expression plasmid encoding rat liver fatty acid-binding protein (FABP) was the generous gift from Dr. David C. Wilton (University of Southampton, UK). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-glycerol (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Site-directed mutagenesis

The mutations R72S, R72E, R72K and R72I were introduced into AtxA cDNA by a double-primer PCR method [24] using AmpliTaq DNA polymerase (Applied Biosystems). The T7 RNA polymerase-based expression plasmid encoding AtxA preceded by a 13-amino acid fusion peptide was used as a template [15]. Reactions were carried out with the corresponding mutagenic sense oligonucleotides 5′-GAAAC TGC AGC CCC AAA ACG GAC AKC TAC AAA TAC-3′ (K=G or T; degenerated primer for the R72I and R72S mutants) or 5′-GAAAC TGC AGC CCC AAA ACG GAC RAA TAC AAA TAC C-3′ (R=G or A; degenerated primer for the R72K and R72E mutants), both containing the *Pst*I restriction site (underlined), and the antisense oligonucleotide 5′-GGGCAAGCTTAAGC TTA GCA TTT CTC TGA C-3′ containing the *Hin*dIII restriction site (underlined). Reaction mixtures (100 μl) consisted of 2 ng of template plasmid DNA, PCRII reaction buffer (Perkin-Elmer/Roche), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer and 2.5 U AmpliTaq DNA polymerase. Amplification included 30 cycles of 45 s at 94 °C, 45 s at 66 °C and 45 s at 72 °C, with a final 7 min extension at 72 °C. The PCR amplified fragments encoding the second half of AtxA (Cys61–Cys133), with the mutations introduced and including the stop codon of AtxA cDNA, were digested with *Pst*I/*Hin*dIII, inserted into the *Pst*I/*Hin*dIII-linearised AtxA expression plasmid encoding the first half of AtxA [15] and sequenced using an ABI Prism 310 Genetic Analyser (Perkin-Elmer Applied Biosystems).

The mutations K86A, K86E, K86G and K86R were introduced into AtxA cDNA by a triple-primer PCR [25] using two outer oligonucleotides and an inner mutagenic oligonucleotide corresponding to each mutation. The same expression plasmid as above encoding a short fusion peptide-AtxA was used as a template. The outer sense oligonucleotide, 5′-TAATACGACTCACTATAG-3′, was complementary to the T7 RNA polymerase promotor site on the plasmid, and the outer antisense oligonucleotide, 5′-GTTTACTCATATATACTTTGA-3′, was complementary to a region on the plasmid downstream of the stop codon of AtxA cDNA. The mutagenic primers, 5′-GTC TGT GGA GCA GGC ACC TCG-3′ (for the K86A mutant), 5′-GTC TGT GGA GAA GGC ACC TCG-3′ (for the K86E mutant), 5′-GTC TGT GGA GGA GGC ACC TCG-3′ (for the K86G mutant), 5′-GTC TGT GGA AGA GGC ACC TCG-3′ (for the K86R mutant), were first phosphorylated at their 5′ ends

with T4 polynucleotide kinase according to the manufacturer's instructions. In addition to the mutagenic primer (50 pmol), each reaction mixture contained 10 ng of template plasmid DNA, 0.4 mM of each dNTP, *Taq* DNA ligase buffer, 50 pmol of each of the outer primers, supplemented with 5 U Vent DNA polymerase and 10 U *Taq* DNA ligase. The reaction conditions were 30 cycles of 30 s at 94 °C, 1 min at 48 °C and 4 min at 72 °C, with a final 7 min extension at 72 °C. After digestion with *Bam*HI/*Hin*dIII, the PCR-amplified fragments encoding the last six C-terminal residues (GSIEGR) of the fusion peptide and the entire AtxA, including the stop codon, were inserted into the *Bam*HI/*Hin*dIII-linearised AtxA expression plasmid and sequenced as above.

The triple mutant AtxA(K74S/H76S/R77L) was prepared by a double-primer mutant method [26] using the Altered Sites in vitro mutagenesis system of Promega. The expected substitutions, introduced by the mutagenic sense primer, 5′-C AAA ACG GAC AGA TAC AGT TAC AGC CTG GAG AAC GGG GTA TC-3′, were confirmed by nucleotide sequencing. The selected positive clone was digested with *Pst*I/*Hin*dIII and the fragment encoding the second half of AtxA, from Cys61 up to Cys133 and including the stop codon, was subcloned into the *Pst*I/*Hin*dIII-linearised AtxA expression plasmid.

2.3. Production and purification of mutants

The expression plasmids encoding all nine mutants were transformed into the *E. coli* BL21(DE3) strain (Novagen). An overnight bacterial culture in Luria–Bertani medium supplemented with 100 μg/ml ampicillin (LBA medium) was used to inoculate 8×450 ml of enriched M9-LBA medium in 2-l Erlenmeyer shaking flasks for each mutant. The cells were grown to an OD_{600} of 2.5, when expression of the mutants was induced by adding IPTG to a final concentration of 0.4 mM. Three hours after induction, cells were harvested by centrifugation at 4000×*g*, for 10 min at 4 °C, and stored at −20 °C until used. Isolation of inclusion bodies, refolding in vitro, activation with acetylated trypsin and purification of recombinant proteins were performed essentially as described for AtxA and its C-terminal mutants [14,15].

2.4. Analytical and spectrometrical methods

Native and SDS/PAGE were performed on a Mini Protean III apparatus (Bio-Rad) using 15% (w/v) and 12% (w/v) polyacrylamide gels, respectively. Electrophoresis and staining with Coomassie Brilliant Blue R-250 (Serva) were conducted according to the manufacturer's instructions. Native electrophoresis was performed at pH 4.0 as described [27]. The N-terminal sequence was determined on an Applied Biosystems Procise 492A protein sequencing system (Foster City, CA, USA). Electrospray ionisation mass spectrometry analysis was performed on a high-resolution magnetic-sector AutospecQ mass spectrometer (Micromass, Manchester, UK) as described [15]. CD spectra were recorded in the range 250–200 nm at 25 °C on an Aviv 62A DS CD spectrometer, with a bandwidth of 2 nm, a step size of 0.5 nm and an averaging time of 3 s. Toxin concentrations in 0.1 M Tris–HCl, pH 7.0, were: 13.9 μM for recombinant AtxA, 12.8 μM for R72S, 11.0 μM for R72E, 10.1 μM for R72K, 16.1 μM for R72I, 14.3 μM for K86A, 14.0 μM for K86E, 22.2 μM for K86G, 15.8 μM for K86R, 22.4 μM for K74S/H76S/R77L. Concentrations of the mutants were calculated from their A_{280} using the method of Perkins [28]. The protein samples were scanned in a cell with a pathlength of 1 mm, and the spectra were averaged and smoothed.

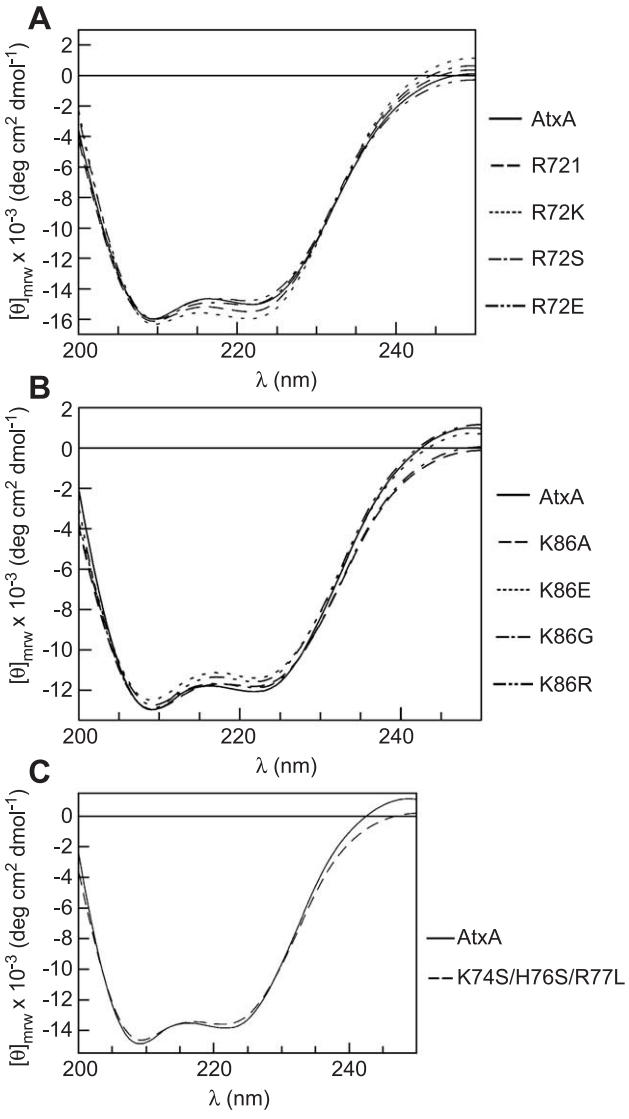

Fig. 1. CD spectra of AtxA and its mutants. (A) CD spectra of the R72 mutants compared with that of AtxA. (B) CD spectra of the K86 mutants compared with that of AtxA. (C) CD spectra of the K74S/H76S/R77L mutant and AtxA. Measuring conditions are given in Materials and methods.

2.5. Enzymatic activity

During renaturation and purification, PLA_2 activity was measured on a micellar substrate using a 718 STAT Titrino pH-stat (Metrohm, Herisau, Switzerland). Hydrolysis of egg yolk phosphatidylcholine was measured in an 8 ml reaction mixture 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.

The specific enzymatic activity of purified mutants was determined by a fluorescence displacement assay using FABP [29]. In this assay, hydrolysis of phospholipid vesicles by $sPLA_2$ is monitored in real-time by following the decrease in fluorescence intensity resulting from displacement of a fluorescent fatty acid analogue bound to FABP by the free fatty acids released during the hydrolysis reaction. The reaction mixture contained Hank's balanced salt solution (Invitrogen, Carlsbad, CA, USA) with 1.26 mM Ca^{2+}, 0.9 mM Mg^{2+}, 10 μg FABP, 1 μM 11-dansylundecanoic acid (Molecular Probes, Eugene, OR, USA) and 30 μM extruded phospholipid (POPG or POPC) vesicles [30] in a final volume of 1.3 ml. Different amounts of AtxA or its mutants (from 0.7 ng to 1.1 μg) were added to the reaction mixture, and the decrease in fluorescence corresponding to phospholipid hydrolysis was followed. $sPLA_2$ dilutions with concentrations below 100 μg/ml were prepared in reaction buffer supplemented with 1 mg/ml of fatty acid-free BSA (Sigma). All measurements were performed in plastic fluorometric cuvettes with a magnetic stirring bar at 37 °C using a Perkin-Elmer luminescence spectrometer LS50B (excitation at 350 nm, emission at 500 nm, slits open at 10 nm). Assays were calibrated by adding a known amount of oleic acid and monitoring the decrease in fluorescence.

2.6. Toxicity

Lethal potency of each mutant was determined using the method of Reed and Muench [31]. Recombinant proteins (4–200 μg) dissolved in 0.5 ml of 0.9% (m/v) NaCl were injected intraperitoneally into BALB/c albino mice. Three to four mice (20–30 g each) were injected with a given dose of each recombinant toxin. The experiments on mice were performed in accordance with the European Commission Council Directive regarding animal experimentation.

2.7. Receptor binding studies

Radioiodination of AtxC and preparation of the porcine cerebral cortex membrane extract were performed as described [8]. The Atx-binding proteins, isolated R25 and R180, and CaM [12], were incubated for 30 min at room temperature with ^{125}I-AtxC (10 nM final concentration), each in the presence or absence of unlabelled competitor (wild-type AtxA and mutants). Disuccinimidyl suberate, dissolved in dimethyl sulfoxide, was added to a final concentration of 100 μM. The cross-linking reaction was stopped by adding SDS sample buffer. Samples were analysed by SDS/PAGE under reducing conditions. Gels were dried and autoradiographed at −70 °C using Kodak X-Omat AR films. The intensities of the specific bands on autoradiographs were quantified using QuantiScan (Biosoft, Cambridge, UK). The data were analysed by the non-linear curve-fitting program GraFit, Version 3.0 (Erithacus Software, Staines, UK).

3. Results

3.1. Expression and basic characterisation of recombinant toxins

Nine mutants with various substitutions of basic residues in the β-structure region of AtxA (i.e. in the β-sheet and its close proximity: R72E, R72I, R72K, R72S, K74S/H76S/R77L, K86A, K86E, K86G and K86R) were produced as N-terminal fusion proteins in the bacterial expression system, refolded, correctly activated by trypsin (as confirmed by N-terminal sequencing) and purified to homogeneity (as observed by SDS and native PAGE). The average yield of purified mutants was ~3 mg per litre of bacterial culture.

Table 1
Enzymatic activity of AtxA and its mutants[a]

$sPLA_2$	Specific activity on POPG (μmol/(min×mg))	Relative activity on POPG as a percentage of AtxA	Specific activity on POPC (μmol/(min×mg))	Relative activity on POPC as a percentage of AtxA
AtxA	1102±203	100	2.0±0.4	100
R72E	438±26	40	0.33±0.07	16
R72I	1655±108	150	6.9±0.8	345
R72K	686±102	62	1.2±0.1	60
R72S	800±82	73	1.6±0.3	80
K74S/H76S/R77L	895±270	81	1.2±0.2	60
K86A	872±85	79	1.40±0.05	70
K86E	962±236	87	1.7±0.3	85
K86G	991±105	90	1.2±0.2	60
K86R	1156±139	105	1.4±0.1	70

[a] The enzymatic activity value for each $sPLA_2$ is the mean±S.D. of at least five independent measurements.

Table 2
Lethal potency and receptor-binding affinity of AtxA and its variants

SPLA$_2$	LD$_{50}$ (μg/kg)[a]	IC$_{50}$ for CaM (nM)[b]	IC$_{50}$ for R25 (nM)[b]	IC$_{50}$ for R180 (nM)[b]
AtxA	21	6±2	10±3	18±2
R72E	84	71±3	14±1	78±7
R72I	32	17±2	18.0±0.4	28±3
R72K	50	46±2	11.7±0.4	83±9
R72S	55	24±2	16±1	35±1
K74S/H76S/R77L	276	18±1	18±1	107±8
K86A	24	8.1±0.3	15±1	20±3
K86E	32	7±1	18±1	42±4
K86G	34	7.5±0.2	12.1±0.1	65±4
K86R	31	8.8±0.5	16.3±0.5	36±1

[a] The LD$_{50}$ values are accurate to within ±5–10%.
[b] The IC$_{50}$ values are means±S.D. of at least three independent measurements.

The single N-terminal sequence in all the mutants (SLLEF…), which was identical to that of AtxA, showed that no internal cleavages occurred due to trypsin activation. The relative molecular masses determined by mass spectroscopy (the calculated mass for each mutant is shown in parentheses) were as follows: 13,747.5 (13,747.7) for R72E, 13,730.4 (13,731.7) for R72I, 13,745.7 (13746.0) for R72K, 13,705.2 (13,705.6) for R72S, 13,639.9 (13,640.6) for K74S/H76S/R77L, 13,717.3 (13,716.9) for K86A, 13,774.3 (13,774.9) for K86E, 13,703.1 (13,702.9) for K86G and 13,802.2 (13,802.0) for K86R. The observed masses confirmed the absence of any post-translational modification after synthesis of the recombinant proteins in *E. coli*. The far-UV CD spectra of wild-type AtxA and all nine mutants (Fig. 1) confirmed that the conformations of the latter do not differ significantly from that of AtxA.

3.2. Enzymatic and biological activity of AtxA mutants

Specific enzymatic activity (Table 1), toxicity and binding affinity for three neuronal Atx-binding proteins, R180, R25 and CaM (Table 2), were determined for all mutants. In general, AtxA and its β-structure mutants

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           1       10        20        30        40        50        60
AtxA       SLLEFGMMILGETG-KNPLTSYSFYGCYCGVGGKGTPKDATDRCCFVHDCCYGNLP--D-
DPLA2      ......K...E...-.LAIP...S......W..........................--.-
BPLA2      H..Q.RK..KKM..-.E.VV..A.......S..R.K................EKVT--G-
APLA2      ..IQ.ETL.MKVAK-.SGMFW..N......W..Q.R.Q..............KVT--G-
Vipox_B    N.FQ.AK..N.KL.-AFSVWN.IS......W..Q..................RVR--G-
RV-4       N.FQ.AR..N.KL.-AFSVWN.IS......W..Q..................GVK--G-
Agtx       N..Q.NK..KE...-..AIPF.A.......W..Q.K...G............R.V--N-
CB1        H..Q.NK..KF..R-..AIPF.A.......W..R.R................K.A--K-

                   70 ↓ ↓ ↓↓ 80     ↓ 90       100       110    a   120
AtxA       C-----SPKTDRYKYHRENGAIVCGK-GTSCENRICECDRAAAICFRKNLKTYN-YIYRNY
DPLA2      .-----N..S.....K.V......E.-...........K.......Q..N..S-KK.ML.
BPLA2      .-----D..W.D.T.SWK..T....G-DDP.KKEV....K.......D.....-KKR.MA.
APLA2      .-----D..M.V.SFSE...D....G-DDP.KKE............D..TL..DKK.WAF
Vipox_B    .-----N..LAI.S.SFKK.N.....-NNG.LRD......V..N..HQ.KN...-KN.KFL
RV-4       .-----N..LAI.S.SFQR.N....R-NNG.LRT......V..N..HQ.KN...-KE.KFL
Agtx       .-----NT.S.I.S.SLKE.Y.T...-..N..EQ......V..E...R..D...-NG.MF.
CB1        .-----NT.W.I.P.SLKS.Y.T...-..W..EQ......V..E.L.RS.S..K-.G.MF.
                            ============
                            β-structure
                                                  LD50
                 a 130                           (mg/kg)
AtxA       PD-FLC-KKESEKC                         0.021
DPLA2      ..-...-.G.-L..                         3.1
BPLA2      ..-I..-SSK....                        20.0
APLA2      GA-KN.PQE...P.                       300.0
Vipox_B    SS-SR.-RQT..Q.                         0.1
RV-4       SS-SK.-RQR..Q.                         0.32
Agtx       R.-SK.-TET..E.                         0.055
CB1        ..-SR.-RGP..T.                         0.48
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Fig. 2. Amino acid alignment of AtxA with some other group IIA sPLA$_2$s. Numbering of amino acid residues is according to Renetseder et al. [32]. Gaps, shown by dashes, and insertions, indicated by a's, were used to permit the alignment with homologous sPLA$_2$s. Identical amino acid residues in sPLA$_2$s are shown by dots. Mutated residues in the β-structure region of AtxA are indicated by arrows. Abbreviations of sPLA$_2$s, with references to their intravenous lethalities (unless indicated otherwise): AtxA, ammodytoxin A from *V. a. ammodytes* [6], whose lethality was recently confirmed by intraperitoneal LD$_{50}$ of recombinant protein [16]; DPLA$_2$, weakly neurotoxic sPLA$_2$ from *D. r. russelli* (former *D. r. pulchella*) with intraperitoneal LD$_{50}$ of recombinant protein [33]; BPLA$_2$, basic non-neurotoxic sPLA$_2$s from *G. b. brevicaudus* (former *Agkistrodon halys* Pallas) [34]; APLA$_2$, acidic non-neurotoxic sPLA$_2$s from *G. b. brevicaudus* [34]; Vipox_B, basic sPLA$_2$ subunit of vipoxin from *V. a. meridionalis* with LD$_{100}$ shown [35]; RV-4, basic sPLA$_2$ subunit of viperotoxin F from *D. r. formosensis* [20]; Agtx, agkistrodotoxin from *G. b. brevicaudus* [34]; CB1, basic iso-subunit of crotoxin from *Crotalus durissus terrificus* [36]. The amino acid sequences were obtained from the publicly available database at the NCBI, Bethesda, MD, USA.

greatly prefer anionic phosphatidylglycerol (POPG) vesicles, the specific enzymatic activities on zwitterionic (charge-neutral) phosphatidylcholine (POPC) vesicles being lower by two to three orders of magnitude than those on anionic vesicles. The substitution of basic residues in the β-region of AtxA had relatively limited influence on the enzymatic activity of AtxA, either on POPG or POPC vesicles, with the exception of R72 when replaced by a hydrophobic or an acidic residue. The R72I mutant was 1.5- and 3.5-fold more active than AtxA, and the R72E mutant 2.5- and 6-fold less active on POPG and POPC vesicles, respectively. Interestingly, even the replacement of R72 with another basic residue (R72K) reduced the enzymatic activity to approx. 60% (on both substrates) of the wild-type activity. In all other cases, the difference between the PLA_2 activities of a particular mutant and AtxA was less than 2-fold.

The lethalities of the mutants were up to four times lower than that of wild-type AtxA, with the exception of the triple K74S/H76S/R77L mutant (substitutions were designed on the basis of the primary structure of a presynaptically neurotoxic neutral $sPLA_2$, agkistrodotoxin, in that region; see Fig. 2), which was 13-fold less toxic (Table 2). In the case of the triple mutant, the substitution of basic residues in the β-structure also moderately lowered the binding affinities of AtxA for the porcine cerebral cortex receptors R25 and R180, by up to 2- and 7-fold, respectively. Interaction with CaM was only slightly affected by the substitutions of K86, more by those of the K74/H76/R77 cluster (three times lower binding affinity) and most of all by those at position 72 (3, 4, 8 and 12 times lower affinity of the R72I, R72S, R72K and R72E mutants, respectively).

4. Discussion

The specific enzymatic activities determined in this study clearly show that AtxA and its β-structure mutants prefer anionic (POPG) over zwitterionic (POPC) phospholipid vesicles. POPG has also been found to be the preferred substrate for most of the mammalian $sPLA_2$s [39], which share a relatively high degree of identity with AtxA (up to 48%, in the case of the human group IIA homologue). $sPLA_2$s bind to aggregated phospholipid surfaces, such as micelles, vesicles and cell membranes, with their interfacial binding surface (IBS) located on a flat exposed region surrounding the entrance to the active site pocket. Interfacial binding of $sPLA_2$s is structurally and kinetically distinct from the subsequent binding of a phospholipid molecule in the active site and, under conditions of high affinity binding, the bound enzyme can perform successive cycles of catalysis without leaving the membrane surface [40]. Most $sPLA_2$s bind more tightly to anionic than to zwitterionic interfaces [1], but do not show significant phospholipid headgroup or *sn*-2 acyl chain specificity when bound to the interface [39,41]. Therefore, the difference in enzymatic activity on POPG and POPC vesicles observed in our study, is most probably due to the much higher affinity of AtxA and its mutants towards anionic POPG vesicles.

From our results it is also evident that four of the exchanged basic residues in the β-structure region, K74, H76, R77 and K86, have no significant influence on enzymatic activity of AtxA, either on anionic or charge-neutral phospholipid vesicles. Additionally, the fact that the K86 mutants show very similar initial velocities of hydrolysis of POPC vesicles (when conditions of low affinity binding apply) indicates that the nature of residue 86 does not influence interfacial binding. Given that the above mentioned four residues are quite distant from the enzyme active site and obviously do not contribute to interfacial binding and activity, our results confirm that they do not belong to the presumed IBS of AtxA (Fig. 3). On the other hand, substitution of R72, which is a part of the putative IBS of AtxA, had a more pronounced, but still modest, effect on the enzymatic activity of AtxA. This result is in accordance with other studies, especially those performed on bee venom (group III) [42,43] and human group IIA enzymes [44,45], that conclusively showed that interfacial binding of $sPLA_2$s to both anionic and zwitterionic phospholipid surfaces has a significant nonelectrostatic component and that individual basic residues account for only a small portion of the total interfacial binding

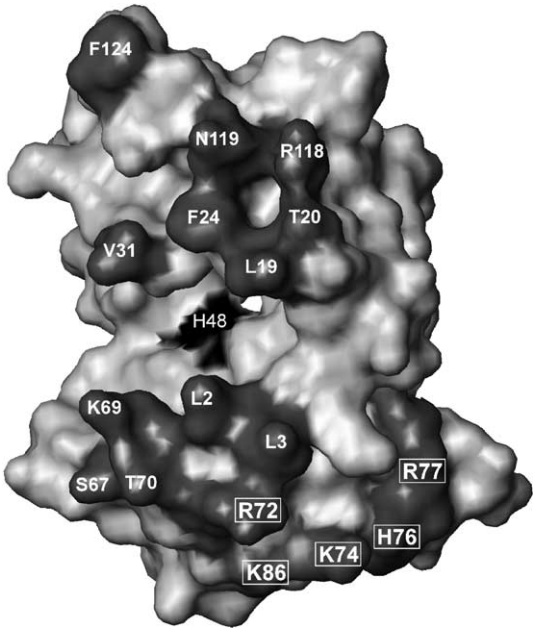

Fig. 3. Basic residues in the β-structure region and the putative IBS of AtxA. The presumed IBS amino acid residues (L2, L3, L19, T20, F24, V31, S67, K69, T70, R72, R118, N119 and F124; shaded dark grey) surrounding the active site pocket with H48 (shown in black) faces the viewer and the β-structure is in the lower right-hand corner. The IBS residues were predicted on the basis of the homologous three-dimensional structure of human IIA $sPLA_2$ [37] and its putative IBS [38]. The mutated residues in the β-structure of AtxA, R72, K74, H76, R77 and K86, are indicated. The figure, based on a three-dimensional model of AtxA [15], was generated using the WebLab Viewer (Accelrys, Cambridge, UK).

energy. In line with this view is the increased activity of the AtxA(R72I) mutant on both anionic and zwitterionic vesicles, most probably due to increased hydrophobic interaction of the IBS with the interface which contributes to more efficient enzyme–membrane association. This positive effect of hydrophobic Ile, as well as the negative impact of anionic Glu (R72E mutant), on interfacial binding and activity is more pronounced in the case of zwitterionic POPC vesicles because of the absence of strong nonspecific electrostatic interactions which would be expected to predominate when a basic $sPLA_2$ such as AtxA (p*I* 10.2, net charge +6) acts on anionic POPG vesicles [45,46]. It also appears that the specific character of a (basic) residue at position 72 is important for interfacial binding of AtxA, thus favouring an arginine over a lysine residue (lower enzymatic activity of the R72K mutant).

The protein binding experiments indicate that the β-structure region of AtxA is not a part of the R25 binding site. This is in accordance with our previous study where the C-terminal region of neurotoxic Atxs was shown to be sufficient for binding a non-toxic $sPLA_2$ to this intracellular membrane receptor [17]. The β-structure region, however, appears to be partially involved in the interaction of the toxin with the larger $sPLA_2$ receptor, R180, and with CaM. For most mutants, binding affinity decreased by less than 10-fold in comparison to wild-type AtxA, suggesting that this region of the toxin is not critical for interaction with either of these two binding proteins. The residue at position 72 may be slightly more important in interaction of Atxs with the highly flexible structure of CaM. This is also in agreement with a novel binding site for CaM which we recently identified in the region 107–125 in the C-terminal part of AtxA [33]. R72, situated on the IBS of AtxA, is not far from this binding site and it is possible that it interacts with CaM. The influence of more distant basic residues in the β-structure region (at positions 74, 76, 77 and 86) on CaM binding is negligible, indicating that they do not participate in this interaction.

The substitution of five basic residues in the β-structure region of AtxA had only a moderate effect on neurotoxicity. All nine mutants were less toxic than the wild-type, although the least toxic triple mutant was still more toxic than, for example, AtxC. The latter is a natural F124I/K128E mutant of AtxA, where the phenylalanine to isoleucine substitution was shown to be primarily responsible for a 17-fold decrease in neurotoxicity [15]. Tang et al. [47] reported that two regions, turn 55–61 and stretch 85–91, in the vicinity of the β-structure (consisting of a single antiparallel β-sheet; residues 74–85) of a potent presynaptic $sPLA_2$ neurotoxin of group IIA, agkistrodotoxin (Agtx), isolated from the venom of *Gloydius* (*Agkistrodon*) *blomhoffi brevicaudus*, have a distinctly different conformation and electrostatic characteristics from those in two non-neurotoxic $sPLA_2$ isoforms from the same venom. They also found that the electrostatic properties of residues K86, E92 and E93 are highly conserved in presynaptic neurotoxins such as Agtx, a basic iso-subunit of crotoxin (CB1) and AtxA (only E93 is replaced with Asn), whereas the charge distribution in the non-neurotoxic isoforms is just the opposite, D88, D89, K92 and K93 (Fig. 2). The authors suggested that these differences might be important for neurotoxicity. Similar differences in local conformation and charge distribution in the same region of presynaptic neurotoxins, compared with non-neurotoxic and postsynaptically neurotoxic $sPLA_2$s, were observed in the three-dimensional structures of a group IIA $sPLA_2$ from *Daboia russelli russelli* ($DPLA_2$) [48] and even in a group IA $sPLA_2$ from *Bungarus caeruleus* [49]. Furthermore, on the basis of the recent crystal structures of homologous heterodimeric $sPLA_2$s, vipoxin from *V. ammodytes meridionalis* [50] and viperotoxin F from *V. russelli formosensis* [22], in both of which the basic subunits alone are presynaptically neurotoxic, the positively charged β-structure was designated as the 'neurotoxic region' of the toxins.

Results of the present study, together with our previous observations, however, do not support the above predictions of a distinct 'neurotoxic site' in the β-structure region. The charge of residue 86, at least in AtxA, does not appear to be very important for neurotoxicity. The substitution of K86 by another basic, an acidic or a neutral residue did not significantly lower the toxicity (up to 1.6-fold in the K86G mutant). Rather more important for neurotoxicity is the electrostatic character of residue 72 in the β-structure region, in addition to those at positions 74, 76 and 77. The introduction of an acidic residue at this position (R72E mutant) lead to 4-fold decrease in toxicity, whereas the exchange of basic residues K74, H76 and R77 for two polar uncharged Ser and a hydrophobic Leu lowered toxicity of AtxA by approx. one order of magnitude (the triple mutant, Table 2). The relatively high toxicity of Agtx, with the same S74/S76/L77 cluster, which is only 2.6-fold less lethal than AtxA [34], is also in line with this result.

It is therefore evident that basic residues at positions 72, 74, 76 and 77 contribute to the higher toxicity but that they are not crucial for the neurotoxic activity of Atxs, and perhaps also for that of other (similar) group IIA $sPLA_2$ toxins. In one of our previous studies, we showed that basic residues in the C-terminal part of AtxA, spread over a large area of the toxin, are not critical for the toxic action but that most, if not all, of them contribute partially to neurotoxicity [51]. It appears that, rather than basic residues, certain aromatic and hydrophobic surface residues, particularly those at positions 24, 115, 116 and 124 [14–16], play a more prominent role in presynaptic neurotoxicity of Atxs. Among them, those in the C-terminal part of the molecule are most important, but still not sufficient, for full expression of the presynaptic toxicity [17]. The residues involved in the neurotoxicity of snake venom $sPLA_2$s are obviously distributed over a relatively large surface of the toxin molecule and therefore, most probably, do not form a distinct 'neurotoxic site' in a certain region, such as the β-structure or some other region. The presynaptic $sPLA_2$

neurotoxicity is a complex, multi-step process where different parts of the toxin's surface are involved in protein–protein and protein–phospholipid interactions. This may also be the reason why there is no simple correlation between either affinity for neuronal binding proteins or enzymatic activity of Atxs and their neurotoxicity.

Acknowledgements

We thank Dr. Tadej Malovrh for help in lethality measurements, Dr. Bogdan Kralj for molecular mass analysis, Dr. Gregor Anderluh and Dr. Nika Lovšin for their help in the triple mutant construction, and Dr. Roger H. Pain for critical reading of the manuscript. This work was supported by grant P0-0501-0106 from the Slovenian Ministry of Education, Science and Sport, and in part by National Institutes of Health grant HL36235 to M.H.G.

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