Charge reversal of ammodytoxin A, a phospholipase A₂-toxin, does not abolish its neurotoxicity

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The positive charge concentrated at the C-terminal region of ammodytoxin (Atx) A, which is involved in presynaptic toxicity, has been reversed. A six-site mutant of AtxA (K108N/K111N/K127T/K128E/E129T/K132E, where K108N = Lys¹⁰⁸ → Asn etc.) was prepared, in which five out of seven C-terminal basic amino acid residues were substituted with neutral or acidic ones. The mutant was approximately 30-fold less lethal, but still neurotoxic. Consistent with this, its binding affinity for the neuronal receptors decreased by only a factor of five. Ad-

ditionally, a single-site mutant of AtxA was prepared, with substitution at only one position (K127T) out of six mutated in the six-site mutant. Its toxicity indicated that most, if not all, of the six mutated residues partially contribute to the decreased lethality of the multiple-site mutant.

Key words: enzyme activity, receptor-binding affinity, site-directed mutagenesis, *Vipera ammodytes ammodytes*.

INTRODUCTION

Secreted phospholipases A₂ (PLA₂s; EC 3.1.1.4) are present in mammalian tissues and the venoms of different organisms. These enzymes are relatively small (approx. 14 kDa), require Ca²⁺ for their catalytic activity and contain 6–8 disulphide bonds [1]. Although they are structurally similar and have low selectivity for phospholipids with different polar-head groups and different degrees of saturation of fatty acyl chains [2], snake venom PLA₂s, in particular, exhibit a wide variety of pharmacological effects, including neurotoxicity, myotoxicity and anticoagulant activity [3]. It is believed that the diverse pharmacological effects are largely mediated by high-affinity membrane proteins in specific target tissues [4].

Presynaptically neurotoxic PLA₂s are the most potent toxins in snake venoms. Although different snake PLA₂ neurotoxins bind to different membrane proteins (receptors) in the nerve terminal, they all produce a final, irreversible blockade of acetylcholine release at the neuromuscular junction following binding to the neuronal receptor [5]. The enzymic activity of PLA₂ neurotoxins is believed to be important in the later stage(s) of the process of presynaptic toxicity, although it does not correlate directly with their lethal potency [6]. Two receptors (R25 and R180, with apparent molecular masses of 25 and 180 kDa respectively), identified in porcine brain cortex, may be important for the neurotoxic action of ammodytoxins (Atx), which are presynaptically neurotoxic group IIA PLA₂s from *Vipera ammodytes ammodytes* venom. They differ from other PLA₂-binding proteins identified so far. The smaller, R25, binds only Atxs (naturally occurring isotoxins AtxA, AtxB and AtxC) with high affinity [7], whereas R180 binds both toxic and non-toxic PLA₂s of either group I or II [8].

The amino acid residues involved in neurotoxicity of PLA₂s are located at different positions in different PLA₂ molecules, but have not yet been individually identified. Sequencing of the three presynaptically neurotoxic isotoxins from *V. ammodytes ammo-*

dytes venom showed that the C-terminal amino acid residues Tyr¹¹⁵–Lys¹²⁸ in Atxs are involved in neurotoxicity [9]. The most toxic AtxA is 17 times more toxic than AtxC, a natural AtxA(F124I/K128E) mutant, and 28 times more toxic than AtxB, a natural AtxA(Y115H/R118M/N119Y) mutant [10]. This was later confirmed by site-specific antibodies directed toward the C-terminal-region epitopes, which neutralized the lethality of Atxs [11,12], and by site-directed mutagenesis [13].

Most presynaptically neurotoxic PLA₂s are highly basic proteins (pI > 9). In AtxA in particular there are seven basic amino acid residues concentrated at the C-terminal region (Cys¹⁰⁵–Cys¹³³), which bends over the top of the molecule. As the C-terminal region is involved in neurotoxicity, it is reasonable to speculate that the basic clusters in this region may play an important role in toxicity. In order to test this hypothesis, we prepared and characterized a six-site mutant of AtxA, where an acidic C-terminal was generated similar to the C-termini present in non-toxic PLA₂s. We also generated a single-site mutation of AtxA in this region and compared its effect with that of the six-site mutation.

MATERIALS AND METHODS

Materials

AtxC and ammodytin (Atn) I₂ were isolated from *V. ammodytes ammodytes* venom as described previously [14,15]. AtxA and the AtxA(K108N/K111N) mutant were produced in *Escherichia coli* and purified as described in [13]. Restriction enzymes, *Taq* polymerase and T7 DNA ligase were obtained from Boehringer Mannheim and Pharmacia. Oligonucleotides were purchased from The Great American Gene Company (Ramona, CA, U.S.A.) and MWG-Biotech (Ebersberg, Germany), and used without further purification. Radioisotopes were obtained from DuPont–NEN Life Science Products, and disuccinimidyl suberate was from Pierce. All other chemicals were of analytical grade.

Abbreviations used: Atn, ammodytin; Atx, ammodytoxin; K108N, Lys¹⁰⁸ → Asn etc.; LD₅₀, lethal dose for 50% of the population tested; PLA₂, phospholipase A₂.

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Site-directed mutagenesis

Mutagenesis was performed by PCR using the expression plasmid encoding either wild-type AtxA or the AtxA(K108N/K111N) mutant as a template [13]. The amplification mixture (100 μ l) consisted of 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μ M each of four deoxynucleoside triphosphates, 50 ng of the plasmid, 400 nM each primer and 2.5 units of *Taq* polymerase. The sense oligonucleotide, 5'-GA TCC ATC GAA GGT CGT AGC TTG CTC G-3', corresponded to the end of the fusion peptide followed by the first three residues of AtxA (SIEGR↓SLL). C-terminal mutations were introduced with the antisense oligonucleotide, either with 5'-GGGCAAGC TTA GCA TTC CTC TGA CGT CTC CGT GCA CAA AAA GTC CGG GTA ATT CC-3' (for the six-site mutant, using the AtxA(K108N/K111N)-coding plasmid) or with 5'-GGGCAAGC TTA GCA TTT CTC TGA CTC CTT CGT GCA C-3' (for the single-site mutant, using the AtxA-coding plasmid). After PCR (67 °C for 45 s, 72 °C for 60 s and 95 °C for 45 s for 30 cycles), the PCR products of 390 bp were purified by GeneClean II (BIO101). Following digestion with *Pst*I and *Hind*III, the restriction fragments coding for the C-terminal half of AtxA with the generated mutations were respectively inserted into the *Pst*I-*Hind*III opened expression plasmid, which still contained the coding sequence for the N-terminal half of AtxA [13]. The sequences of the two constructs were confirmed by dideoxynucleotide sequencing [16].

Expression and purification of AtxA mutants

The expression plasmids harbouring the AtxA(K108N/K111N/K127T/K128E/E129T/K132E) and AtxA(K127T) mutants were used to transform *E. coli* strain BL21(DE3) (Novagen). The overnight cultures were used to inoculate 2 × 450 ml of a Luria-Bertani enriched medium in 2 litre Erlenmeyer shaking flasks. Approx. 6.5 g of wet bacterial cells/l of fermentation broth was obtained. Inclusion bodies were isolated and the proteins were refolded and activated as described previously [13]. The mixtures containing activated proteins were dialysed against 20 mM sodium acetate, pH 4.8, and the mutants purified by FPLC on a Mono S column (HR 5/5; Pharmacia). The toxins were eluted with 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. Pooled fractions were dialysed against distilled water and stored at –20 °C. Protein concentrations were calculated from the A_{280} using the method of Perkins [17].

Analytical methods

Protein samples were analysed by SDS/PAGE in the presence of 150 mM dithiothreitol using 15% (w/v) polyacrylamide gels and Coomassie Brilliant Blue R250 staining. A PhastSystem apparatus (Pharmacia) was used for isoelectric focusing according to manufacturer's instructions.

Reverse-phase HPLC was performed using a Milton Roy system. The samples were loaded on to an Aquapore 300 BU column (30 mm × 4.6 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid and eluted with a linear gradient of 0–80% (v/v) acetonitrile at a flow rate of 1 ml/min. The N-terminal sequence was determined by Edman degradation on an Applied Biosystems Procise 492A protein sequencing system.

CD

CD spectra were recorded from 250–200 nm at 25 °C on an Aviv 62A DS CD spectrometer, using a bandwidth of 2 nm, a step size

of 1 nm, and an averaging time of 2 s. Protein concentrations were 15.0 μ M for recombinant AtxA, 6.8 μ M for AtxA(K127T) and 10.2 μ M for the sextuple AtxA mutant, all in water in a cell of 1 mm path length. Protein solutions and water were scanned three times each, the spectra were then averaged and smoothed.

Toxicity

Lethal potency was determined by intraperitoneal injection of each mutant into NMRI albino mice. Before application, different amounts of the recombinant toxins (from 0.2–70 μ g) were dissolved in 0.5 ml of 0.9% (w/v) NaCl. Neurotoxic effects on experimental animals were observed within 24 h, and the LD₅₀ (lethal dose for 50% of the population tested) was determined using a standard method [18].

Enzymic activity

PLA₂ activity was determined by a slight modification of the method of Dennis [19]. Hydrolysis of egg-yolk phosphatidylcholine was measured in a reaction mixture supplemented with 1% (v/v) Triton X-100 and 15 mM $CaCl_2$, at pH 8.0 and 40 °C. Released fatty acids in 8 ml of reaction mixture were titrated with 10 mM NaOH on a pH-stat (718 STAT Titrino; Metrohm, Herisau, Switzerland). One enzyme unit corresponds to 1 μ mol of hydrolysed phospholipid/min.

Binding studies

AtxC was radioiodinated [20] and a membrane extract of porcine cerebral cortex, containing solubilized AtxC-binding proteins, was prepared as described previously [8]. The membrane extract, ¹²⁵I-labelled AtxC (10 nM final concentration) and unlabelled recombinant or native toxin (final concentrations from 0–2 μ M) were incubated at room temperature for 30 min with occasional vortex mixing. Toxins and their receptors were cross-linked by adding the bifunctional reagent disuccinimidyl suberate, dissolved in DMSO, to a final concentration of 100 μ M. The reaction mixture was vigorously mixed for 5 min at room temperature. The cross-linking reaction was stopped by the addition of SDS/PAGE sample buffer containing dithiothreitol. Following electrophoresis and autoradiography, the intensity of the specific adducts on autoradiographs was quantified by QuantiScan (Biosoft) and the data obtained were analysed using the non-linear curve-fitting program GraFit, Version 3.0 (Erithacus Software).

RESULTS AND DISCUSSION

The six-site mutant of AtxA (K108N/K111N/K127T/K128E/E129T/K132E), possessing an acidic C-terminus, and the AtxA(K127T) mutant (Figure 1) were produced in *E. coli*, renatured from inclusion bodies, purified to homogeneity and characterized. The mutations K127T, K128E and E129T were designed on the basis of the sequence of a non-toxic PLA₂ from *V. ammodytes ammodytes* venom, AtnI₂. They were added to the previously prepared double AtxA mutant, K108N/K111N [13]. As this double mutation had little effect on neurotoxicity, we decided not to mutate the basic arginine residue (R107) in the vicinity. However, K132 was replaced with glutamic acid (and not with glutamine residue, which is present in AtnI₂), to lower the pI of the sextuple AtxA mutant.

Figure 1 Sequence alignment of basic, neurotoxic ammodytoxin A with neutral, non-toxic AtnI₂

The common numbering of PLA₂ residues is used [27]. The substitutions in the sextuple and single mutants of AtxA are shown by arrows. Amino acid residues identical in both PLA₂s are in black boxes. Broken lines indicate gaps introduced to optimize alignment with other homologous PLA₂s. AtxA, ammodytoxin A [28]; AtnI₂, ammodytin I₂ [15].

Table 1 Characteristics of the six-site and single-site AtxA mutants in comparison with other PLA₂s

The pI of the AtxA(K127T) mutant was not determined (n.d.).

PLA ₂	pI	Net charge	Specific activity (units/mg of protein)	LD ₅₀ (μg/kg)	IC ₅₀ for R180 (nM)	IC ₅₀ for R25 (nM)
AtxA	10.2	+6	280 ^a	21 ^a	15 ^b	3
AtxA(K108N/K111N)	9.1	+4	450 ^c	67 ^c	68	38
AtxA sextuple mutant	6.2	0	960	660	100	16
AtxA(K127T)	n.d.	+5	210	35	300	22
AtxC	9.1	+4	288 ^a	360 ^a	155 ^b	50
AtnI ₂	6.8	0	880	> 2.5 × 10 ⁴	approx. 1000	> 2 × 10 ⁴

^a[10], ^b[8], ^c[13].

Figure 2 CD spectra of the AtxA(K127T) and six-site mutants

The CD spectra of the AtxA(K127T) (long dashes) and AtxA (K108N/K111N/K127T/K128E/E129T/K132E) (broken line) mutants are compared with that of recombinant wild-type AtxA (solid line). For details, see the Materials and methods section.

Some of biochemical and biological properties of the two mutants were compared with those of three naturally occurring PLA₂s from *V. ammodytes ammodytes* venom and the AtxA(K108N/K111N) mutant (Table 1). The N-terminal sequences of the first 12 amino acid residues (SLLEFGMMILGE...), and molecular masses of 13 693 Da for the six-site and 13 748 Da for the single-site mutants, determined by electrospray-ionization MS, confirmed the correct primary structure and

purity of the mutants. The specific enzymic activity, as well as the far-UV CD spectra, of the mutated proteins, which were similar to the CD spectrum of wild-type AtxA (Figure 2), gave further assurance that the mutations did not induce any significant conformational change in the molecule. This was expected, as all the mutated residues are exposed on the surface of the PLA₂ molecule (Figure 3), and thus should not substantially interfere with refolding.

The multiple mutations in the sextuple AtxA mutant led to an approx. 30-fold increase in LD₅₀ in mice, from 21 μg/kg (for AtxA) to 660 μg/kg, but the mutant still retained the neurotoxic effects of Atxs on mice. The toxicity of the six-site mutant was close to that of AtxB (LD₅₀ 580 μg/kg), a naturally occurring Atx isoform with pI 10.0 [10]. The substitutions drastically lowered the pI of the AtxA molecule from 10.2 to 6.2, but only moderately decreased the toxicity. In addition to particular basic residues, some polar, hydrophobic and even acidic residues have been suggested to be important for presynaptic toxicity [13,21–25]. The lower neurotoxic potency of the mutant could be, however, explained by the possibility that the positive charge plays an important role in the approach of the PLA₂ to a high-affinity protein receptor in the nerve membrane, which in turn triggers the process of neurotoxicity [20]. As the charge distribution of the sextuple mutant of AtxA is significantly changed, the orientation of the molecule approaching the specific receptor would also be different from that of wild-type AtxA, which may also affect the lethality. According to the lethal potencies of single and double mutants with mutations in this part of the molecule, it appears that each mutation slightly lowers the neurotoxicity and partially contributes to the low lethality of the six-site mutant. For example, the recently reported AtxA(K128E) and AtxA(K108N/K111N) mutants were approx. 2- and 3-fold respectively less toxic than wild-type AtxA [13]. Also, the

Figure 3 Location of the mutated residues in the C-terminal region of AtxA

The molecule shown (the back view) has been rotated 180° around the vertical y -axis, which is in the plane of the page, with respect to a typical 0° view of structurally similar phospholipases A_2 , where the N-terminus is pointed toward the viewer and the β -wing is at the bottom right corner. The mutated amino acid residues, which are all exposed on the surface of the protein, are coloured; basic residues are shown in blue and acidic residues in red. The surface view of a three-dimensional structural model of AtxA was generated using WebLab ViewerLite (Molecular Simulations).

mutation K127T used in the present study decreased the LD_{50} of AtxA only by approx. 2-fold.

Substitution of five basic residues in the C-terminal region of AtxA, which is apart from the interfacial binding surface region responsible for the interaction of the enzyme with aggregated substrates, resulted in a 3-fold higher specific enzymic activity of the six-site mutant against mixed phosphatidylcholine/Triton X-100 micelles (Table 1). The enzymic activity of AtxA was increased 1.6-fold with the double mutation K108N/K111N [13], whereas the increase in activity of the single AtxA(K128E) mutant was barely discernable [13], and, interestingly, the specific enzymic activity with the mutation K127T was even slightly lower. The electrostatic potentials calculated for some secretory PLA_2 s have shown a maximum positive value in the vicinity of the hydrophobic channel leading to the buried active site. The structurally bound cofactor, Ca^{2+} , additionally enhances the positive electrostatic potential in this area, which further accentuates the electrostatic asymmetry of the molecule [26]. The present mutations could therefore shift the maximum positive potential around the hydrophobic pocket, thus favouring interfacial binding to the micelles to a greater or lesser degree.

The correlation between the affinity of Atxs and $AtnI_2$ for porcine brain receptors and their lethal potency in mice (Table 1) indicates that similar receptors could also be involved in the process of presynaptic neurotoxicity in mice. The ability of the six-site mutant to inhibit the binding of ^{125}I -labelled AtxC to neuronal receptors R25 and R180 was approximately one order of magnitude lower than that of wild-type AtxA (Table 1). However, the degree of inhibition of the specific binding of radiolabelled AtxC by the mutant was similar to that of naturally occurring isotoxin AtxC, which is consistent with their toxicity in mice. It should be stressed that the correlation between binding and toxicity is not simple. For example, the AtxA(K108N/

K111N) and AtxA(K127T) mutants, with binding affinities for both receptors in the same range as those of the six-site mutant, are approx. 10–20 times more toxic than the six-site mutant. Obviously, there are steps in the process of neurotoxic action, other than the initial binding to the specific receptor(s), that contribute to the final outcome. Nevertheless, the results of the present study clearly indicate that the basic C-terminus of presynaptic PLA_2 neurotoxins, such as Atxs, is not an absolute prerequisite for neurotoxic activity.

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