Binding to the high-affinity M-type receptor for secreted phospholipases A(2) is not obligatory for the presynaptic neurotoxicity of ammodytoxin A

ARTICLE *in* BIOCHIMIE · NOVEMBER 2006

Impact Factor: 2.96 · DOI: 10.1016/j.biochi.2006.06.008 · Source: PubMed

5 AUTHORS, INCLUDING:

Petra Prijatelj Znidaršič
University of Ljubljana
17 PUBLICATIONS **441** CITATIONS
SEE PROFILE

Nina Vardjan
University of Ljubljana
39 PUBLICATIONS **416** CITATIONS
SEE PROFILE

Igor Krizaj
Jožef Stefan Institute
146 PUBLICATIONS **2,971** CITATIONS
SEE PROFILE

Joze Pungercar
Jožef Stefan Institute
79 PUBLICATIONS **1,930** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
ScienceDirect
Biochimie 88 (2006) 1425–1433
BIOCHIMIE
www.elsevier.com/locate/biochi

Binding to the high-affinity M-type receptor for secreted phospholipases A_2 is not obligatory for the presynaptic neurotoxicity of ammodytoxin A

Petra Prijatelj[a,1], Nina Vardjan[b,1], Edward G. Rowan[c], Igor Križaj[b], Jože Pungerčar[b,*]

[a] *Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Aškerčeva 5, SI-1000 Ljubljana, Slovenia*
[b] *Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Jamova 39, SI-1000 Ljubljana, Slovenia*
[c] *Department of Physiology and Pharmacology, Strathclyde Institute for Biomedical Sciences, University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, UK*

Received 13 December 2005; accepted 5 June 2006
Available online 21 June 2006

Abstract

R180, isolated from porcine brain cortex, is a high-affinity membrane receptor for ammodytoxin A (AtxA), a secreted phospholipase A_2 ($sPLA_2$) and presynaptically active neurotoxin from venom of the long-nosed viper (*Vipera ammodytes ammodytes*). As a member of the M-type $sPLA_2$ receptors, present on the mammalian plasma membrane, R180 has been proposed to be responsible for one of the first events in the process of presynaptic neurotoxicity, the binding of the toxin to the nerve cell. To test this hypothesis, we prepared and analyzed three N-terminal fusion proteins of AtxA possessing a 12 or 5 amino acid residue peptide. The presence of such an additional "propeptide" prevented interaction of the toxin with the M-type receptor but not its lethality in mouse and neurotoxic effects on a mouse phrenic nerve-hemidiaphragm preparation. In addition, antibodies raised against the $sPLA_2$-binding C-type lectin-like domain 5 of the M-type $sPLA_2$ receptor were unable to abolish the neurotoxic action of AtxA on the neuromuscular preparation. The specific enymatic activities of the fusion AtxAs were two to three orders of magnitude lower from that of the wild type, yet resulting in a similar but less pronounced neurotoxic profile on the neuromuscular junction. This is in accordance with other data showing that a minimal enzymatic activity suffices for presynaptic toxicity of $sPLA_2$s to occur. Our results indicate that the interaction of AtxA with the M-type $sPLA_2$ receptor at the plasma membrane is not essential for presynaptic activity of the toxin. Interaction of AtxA with two intracellular proteins, calmodulin and the R25 receptor, was affected but not prevented by the presence of the N-terminal fusion peptides, implying that these proteins may play a role in the $sPLA_2$ neurotoxicity.

Keywords: Phospholipase A_2; Snake venom; *Vipera ammodytes ammodytes*; Neurotoxicity; M-type phospholipase A_2 receptor

1. Introduction

Phospholipases A_2 (PLA_2s, EC 3.1.1.4) constitute a diverse superfamily of enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of phospholipids. Several structurally related groups of PLA_2s have been found in animal venoms and secretions of various mammalian tissues, also designated as secreted PLA_2s ($sPLA_2$s). $sPLA_2$s are Ca^{2+}-dependent and disulfide-rich proteins, with molecular masses in the range of 13-18 kDa [1,2]. Ammodytoxin A (AtxA, also being the most toxic), AtxB and AtxC are monomeric $sPLA_2$s of group IIA, isolated from venom of the long-nosed viper, *Vipera ammodytes ammodytes*. They belong to presynaptically acting $sPLA_2$ neurotoxins that block the neuromuscular (NM) transmission in vertebrate skeletal muscles [3,4]. Atxs and similar $sPLA_2$ neurotoxins produce a characteristic triphasic response on the isolated mouse phrenic nerve-diaphragm preparation which ends with irreversible blockade of neuromuscular transmission [4]. The molecular mechanism of presynaptic $sPLA_2$ toxicity is still not completely understood. Both enzymatic activity of $sPLA_2$ toxins [5,6] and their binding to specific neuronal receptors [7] are believed to be involved in the process.

Two membrane receptors for Atxs, of 25 kDa (R25) and 180 kDa (R180), with K_ds in the nanomolar range, have been found in porcine nerve tissue. R25, which colocalizes with

Abbreviations: Abs, antibodies; Atx, ammodytoxin; 12-AtxA, ARIRARGSIEGR-AtxA; CaM, calmodulin; CTLD, C-type lectin-like domain; DAB, 3,3′-diaminobenzidine; FABP, fatty acid-binding protein; HP, horseradish peroxidase; I-AtxA, ASIGQ-AtxA; NM, neuromuscular; P-AtxA, ASPGQ-AtxA; PLA_2, phospholipase A_2; PC, egg-yolk phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; R25 and R180, receptors for Atxs in porcine cerebral cortex of 25 kDa and 180 kDa, respectively; $sPLA_2$, secreted PLA_2; $sPLA_2R$, $sPLA_2$ receptor.

* Corresponding author.
E-mail address: joze.pungercar@ijs.si (J. Pungerčar).

[1] Both authors contributed equally to this work.

0300-9084/$ - see front matter © 2006 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2006.06.008

mitochondria [8], binds only Atxs [9], whereas R180, identified as a plasma membrane M-type $sPLA_2$ receptor ($sPLA_2R$), binds both toxic and nontoxic $sPLA_2$s of groups IB and IIA [10,11]. Another high-affinity binding protein for Atxs was isolated from porcine nerve tissue and identified as a soluble intracellular protein, calmodulin (CaM) [12]. A few other intracellular proteins, 14-3-3γ and ε isoforms [13], and protein disulfide isomerase [14], have also been identified, which may play a role in the toxic action of Atxs and similar $sPLA_2$s.

Among the Atxs-binding proteins, only the M-type $sPLA_2R$ is located on the plasma membrane and could be responsible for specific targeting of $sPLA_2$ neurotoxins to presynaptic nerve terminals. It is a type I transmembrane glycoprotein and member of the Ca^{2+}-dependent (C-type) multilectin mannose receptor family [15,16]. Most of the extracellular part of $sPLA_2R$ comprises eight C-type lectin-like domains (CTLDs), involved in $sPLA_2$ binding. CTLD5 is the most important domain for $sPLA_2$ binding [17] and shows a high level of amino acid identity between the M-type $sPLA_2R$s from different vertebrate species [18]. Depending on the cell type and particular $sPLA_2$, binding of an active form of $sPLA_2$ to the M-type receptor on the cell surface can induce a variety of biological responses such as cell growth, cell proliferation, cell migration, lipid mediator production, hormone release, and cytokine production [19–21]. After binding to the receptor, $sPLA_2$ may undergo clathrin dependent receptor-mediated endocytosis [22–26]. The M-type $sPLA_2R$ can also act, after it is released from the plasma membrane, as a circulating endogenous inhibitor of $sPLA_2$, since the enzymatic activity of $sPLA_2$ is suppressed upon its binding to the receptor [27].

It has been proposed that AtxA and similar $sPLA_2$ neurotoxins enter the nerve cell using at least one of several potential pathways, including the M-type $sPLA_2R$-mediated endocytosis, and act inside the cell [7]. This hypothesis has been strengthened by the discovery of several intracellular binding proteins for AtxA (see above) and by our recent demonstration of the internalization of AtxA into rat hippocampal neurons [28]. In the present study, we were interested in elucidating the potential role of the M-type $sPLA_2R$ in the presynaptic toxicity of AtxA. Studies on the interaction of homologous mammalian $sPLA_2$s of groups IB and X showed that their high-affinity binding to the M-type $sPLA_2R$ requires cleavage of the N-terminal propeptide from the enzymatically inactive proenzyme [29–31]. On the basis of this finding, we prepared three N-terminal fusion proteins of AtxA, and studied their protein-protein interactions and biological properties.

2. Materials and methods

2.1. Materials

Recombinant AtxA was produced in *Escherichia coli* and purified as described [32]. Restriction enzymes were from MBI Fermentas (Vilnius, Lithuania) and New England BioLabs. T4 DNA ligase was obtained from Boehringer Mannheim. Hog brain CaM was from Roche Molecular Biochemicals and oligonucleotides from MWG-Biotech (Ebersberg, Germany). Radioisotopes were obtained from Perkin-Elmer Life Sciences, and disuccinimidyl suberate from Pierce (Rockford, IL, USA). The expression plasmid encoding rat liver FABP was a kind gift from Dr. David C. Wilton (University of Southampton, UK). Recombinant FABP was prepared as described previously [33]. POPG was from Avanti Polar Lipids (Alabaster, AL, USA), Hanks' balanced salt solution from Invitrogen (Carlsbad, CA, USA) and 11-dansylundecanoic acid from Molecular Probes (Eugene, OR, USA). Protein standards were from BioRad (Hercules, CA, USA). Nitrocellulose membrane was from Serva (Heidelberg, Germany). Horseradish peroxidase (HP)-conjugated secondary anti-rabbit/mouse IgG Abs were from ECL detection kit (BM Chemiluminescence Western Blotting Kit (Mouse/Rabbit); Roche Diagnostics; Mannheim, Germany) and goat HP-conjugated anti-rabbit secondary Abs were from Jackson ImmunoResearch Labs. (West Grove, PA, USA). 3,3′-diaminobenzidine (DAB) was from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of fusion AtxAs

The nucleotide sequence coding for AtxA fused with the peptide MARIRARGSIEGR on its N-terminus has been inserted into the pT7-7 plasmid and its expression performed essentially as described previously [32]. The only difference was that after the isolation of inclusion bodies and in vitro renaturation of the recombinant fusion protein, the tryptic activation was omitted. The fusion peptide (MARIRARGSIEGR) coding region was exchanged by cassette mutagenesis following the *Nde*I/*Eco*RI digestion of the expression vector, to obtain two other N-terminal fusions, MASIGQ and MASPGQ. The complementary oligonucleotides 5′-*T ATG* GCT TCA ATC GGT CAA AGC CTG TTG *G*-3′ (sense) and 5′-*AA TTC* CAA CAG GCT TTG ACC GAT TGA AGC *CA*-3′ (antisense; italics are the partial restriction sites *Nde*I and *Eco*RI at both ends of the cassette) introduced the sequence coding for the fusion peptide MASIGQ followed by SLLE of mature AtxA. The coding sequence for MASPGQ followed by SLLE was introduced by the oligonucleotides 5′-*T ATG* GCT TCA CCT GGT CAA AGC CTG TTG *G*-3′ (sense) and 5′-*AA TTC* CAA CAG GCT TTG ACC AGG TGA AGC *CA*-3′ (antisense; partial restriction sites italics as above). For preparation of the cassettes, 300 pmol of the sense and antisense oligonucleotides were mixed in a final volume of 20 μl of distilled water, incubated at 95 °C for 1 min, and then the mixture was left to cool slowly to room temperature. The constructions were verified by nucleotide sequencing using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

2.3. Analytical methods

After purification the protein samples were analyzed by SDS/PAGE and reverse-phase HPLC using a HP1100 system (Hewlett-Packard, Waldbronn, Germany) [34]. The N-terminal sequence was determined by Edman degradation on an

Applied Biosystems Procise 492A protein sequencing system (Foster City, CA, USA). Electrospray ionization mass spectrometry was performed using a high-resolution magnetic-sector AutospecQ mass spectrometer (Micromass, Manchester, UK). Circular dichroism (CD) spectra were recorded from 250–200 nm at 25 °C on an Aviv 62A DS CD spectrometer with a bandwidth set to 2 nm, step size 1 nm and averaging time 2 s.

2.4. Toxicity

Lethality was determined by intraperitoneal injection of 0.5 ml of a toxin in 0.9% (w/v) NaCl into BALB/c albino mice. After 48 h observation of neurotoxic effects on experimental animals, LD_{50} was determined using a standard method [35]. All experimental procedures on mice were performed in accordance with the EC Council Directive regarding animal experimentation.

2.5. Neuromuscular activity

The neuromuscular activity of AtxA and the three fusion AtxA proteins was studied on isolated mouse phrenic nerve-hemidiaphragm preparations. The preparations were dissected from male mice (BALB/c, 20–25 g) that were killed by exposure to CO_2. Each isolated preparation was mounted in a 10 ml tissue bath, containing Krebs solution (118.4 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.4 mM $MgSO_4$ and 2.5 mM $CaCl_2$), maintained at 37 °C and oxygenated with a mixture 95% O_2 and 5% CO_2. The phrenic nerve was stimulated with supramaximal rectangular pulses of 0.2 ms duration at a frequency of 0.2 Hz. Muscle was connected to a Grass force displacement transducer and muscle contractions recorded on a Grass polygraph model 79 (Grass Instruments; Quincy, MA, USA). In order to reveal any facilitation of neuromuscular transmission, the preparation was partly paralyzed by reducing the concentration of Ca^{2+} to 0.38–0.50 mM. Prior to the start of experiment, the tissues were allowed to equilibrate for 20–40 min in a low Ca^{2+} Krebs solution. In certain experiments, antibodies against the M-type $sPLA_2R$ were added to the bath 1 h before adding the toxin.

2.6. Protein binding studies

AtxC was radioiodinated (^{125}I-AtxC) [36] and membranes extracted from porcine cerebral cortex as described [12]. The membrane extract or CaM solution, 10 nM concentration of ^{125}I-AtxC and increasing concentrations of each unlabeled recombinant toxin were incubated at room temperature for 30 min with occasional vortexing. Cross-linking of proteins was achieved by adding freshly prepared disuccinimidyl suberate solution to a final concentration of 100 μM, and the reaction was stopped by adding SDS/PAGE sample buffer containing dithiothreitol. Following electrophoresis and autoradiography, the intensities of the specific adducts were quantified and the data analyzed as described [37].

2.7. Enzymatic assays

Specific activity of recombinant toxins on mixed micelles with phosphatidylcholine was determined by a slightly modified pH-stat method [38] in an 8 ml reaction mixture consisted of egg-yolk phosphatidylcholine, 1% (v/v) Triton X-100 and 15 mM $CaCl_2$, at pH 8.0 and 40 °C. The fatty acids released were titrated with 10 mM NaOH using a 718 STAT Titrino pH-stat (Metrohm; Herisau, Switzerland).

Specific activity on POPG vesicles was measured by monitoring the displacement of a fluorescent fatty acid analogue (11-dansylundecanoic acid) from FABP [39,40]. 13 μl of vesicles (3 mM phospholipid in water, diameter 0.1 μm) were added to 1.28 ml of Hanks' balanced salt solution with 0.9 mM Ca^{2+}. The reaction mixture was supplemented with 11-dansylundecanoic acid to a final concentration of 1 μM and approximately 10 μg of FABP, and assayed in plastic fluorometric cuvettes at 37 °C with magnetic stirring. Excitation on a Perkin-Elmer LS50B fluorimeter was at 350 nm, emission at 500 nm, slits were 10 nm and pathlength was 10 mm. The amount of $sPLA_2$ added was chosen so that approximately the same signal for all samples was achieved. The assay was calibrated by successive addition of 200 pmol of oleic acid to the reaction mixture containing all the components except $sPLA_2$.

In both assays, one enzyme unit (U) corresponds to 1 μmol of hydrolyzed phospholipid per minute.

2.8. Design and production of antibodies

Nucleotide sequence encoding the CTLD5 domain of the mouse M-type $sPLA_2R$ was amplified from the DNA clone IMAGp998F2211909 (RZPD Deutsches Ressourcenzentrum für Genomforschung; Berlin, Germany) by PCR using the upstream primer 5′-GC*G GAT CC*G GCG AGT ACC TCT TCC ACA CCC-3′, with the *Bam*HI restriction site (italics), and the antisense primer 5′-GGG C*AA GCT T*GC TTA CAG ATG CTG GGC AA-3′, with the *Hin*dIII restriction site (italics). The amplification mixture (20 μl) consisted of 20 ng of clone DNA, PCR II reaction buffer (Perkin-Elmer Life Sciences, USA), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.4 μM each primer and 0.5 U AmpliTaq DNA polymerase (Perkin-Elmer Life Sciences, USA). After the amplification (30 cycles of 94 °C for 30 s, 50 °C for 30 s, 74 °C for 30 s, and final extension 74 °C for 5 min), the PCR product of 370 bp was purified by QIAquick Gel extraction kit (Qiagen, Hilden, Germany), digested with *Bam*HI and *Hin*dIII and inserted into the *Bam*HI/*Hin*dIII-linearized expression vector pT7-7 [41]. The expected sequence was confirmed using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems).

The *E. coli* strain BL21(DE3) (Novagen, USA) was transformed with the expression plasmid encoding the CTLD5 domain of the mouse M-type $sPLA_2R$. A fresh overnight culture was used to inoculate 2 × 400 ml of enriched Luria–Bertani medium in 2-liter Erlenmeyer shaking flasks. The culture

was grown to an OD_{600} of ~2.0, and then expression of the CTLD5 domain was induced by adding isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. Cells were incubated for additional 4 h and harvested by centrifugation. Inclusion bodies (~2.6 mg of recombinant protein) were isolated as described [32] and the amino acid sequence of the CTLD5 domain determined by N-terminal sequencing using an Applied Biosystems Procise 492A protein sequencing system (Foster City, CA, USA).

Inclusion bodies (~350 μg of protein) with complete Freund's adjuvant were injected subcutaneously into a rabbit. In an interval of three weeks, the rabbit received two booster doses, containing ~420 and ~560 μg of proteins, respectively, without Freund's adjuvant. Two weeks after the last booster dose the serum was collected and antibodies purified on a Protein A-Sepharose column (final protein concentration ~2 mg/ml). Specificity of the antibodies raised against the CTLD5 domain of the mouse M-type $sPLA_2R$ was tested by Western blot analysis of the bacterial inclusion bodies, and synaptic membrane fractions of porcine and mouse cerebral cortexes [42].

3. Results

3.1. Production of recombinant fusion AtxAs

Three N-terminal fusion proteins of AtxA were prepared and characterized with the aim of observing the effects of the additional peptide. These peptides are similar to the N-terminal propeptides present in precursors of mammalian group IB (e.g. the heptapeptide DSGISPR in human pancreatic $proPLA_2$ [43]) and group X $sPLA_2$s (e.g. the undecapeptide EASRILRVHRR in human proenzyme [44]). N-terminal amino acid sequencing showed that the initial methinonine residue followed by an alanine in all the three fusion AtxAs has been efficiently removed in vivo in bacterial cells as expected [45]. The resulting fusion proteins of AtxA thus possessed an additional N-terminal "propeptide" of 12 (ARIRARGSIEGR) or 5 amino acid residues (ASIGQ or ASPGQ), and were named 12-AtxA, I-AtxA and P-AtxA, respectively. Their structure was confirmed by the molecular masses of fusion proteins, as well as that of wild-type recombinant AtxA, determined by electrospray ionization mass spectrometry which essentially matched the calculated values (Table 1). The far-UV CD spectra of the fusion AtxAs were similar to that of recombinant AtxA (Fig. 1) indicating that the additional peptide did not induce significant conformational changes in the protein structure.

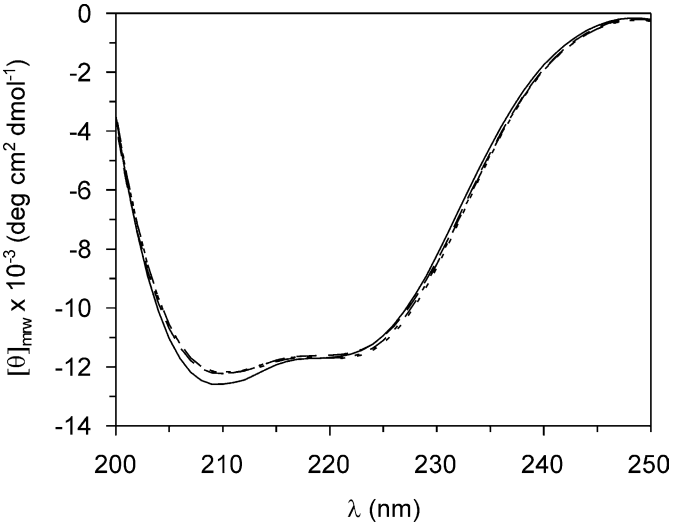

Fig. 1. CD-spectra of AtxA (full line) and fusion AtxAs (broken lines). The concentrations of proteins were: 13.9 μM, AtxA; 18.5 μM, 12-AtxA; 11.8 μM, I-AtxA and 18.2 μM, P-AtxA, all in 20 mM Tris–HCl, pH 7.0.

3.2. Binding affinity and enzymatic activity of fusion AtxAs

Binding of fusion AtxAs (12-AtxA, I-AtxA and P-AtxA) to the M-type $sPLA_2R$ was not detected at the concentrations (up to 10 mM) used in our experiments (Fig. 2). This indicates that the presence of the N-terminal "propeptides" (at least those of 5 amino acid residues or more) practically prevents the interaction of AtxA with this plasma membrane receptor. The binding affinities to CaM were considerably lower (12- to 63-fold) in the case of all three fusion AtxAs compared to native AtxA. Interestingly, the fusion AtxAs showed higher binding affinities (twofold to threefold) for the Atxs receptor (R25), the shorter fusion AtxA (I- and P-AtxAs) having a slightly higher affinity to this receptor (Table 1).

The fusion AtxAs were still enzymatically active (Fig. 3), although their activity was much lower than that of the wild type (less than 0.4% of wild-type activity). Among the fusion AtxAs, P-AtxA showed the lowest specific enzymatic activity on both zwitterionic PC/Triton-X 100 mixed micelles and anionic POPG vesicles, approximately 0.05% and 0.1% of wild-type activity, respectively (Table 2).

3.3. Lethality and presynaptic neurotoxicity

Peptides fused to the N-terminus of AtxA reduced but did not prevent the lethality of the toxins. The LD_{50}s of the three

Table 1
Properties of recombinant AtxA and the N-terminal fusion mutants. The IC_{50} values are mean ± S.D. of at least three independent measurements. The LD_{50} values are accurate to within ± 10% [a] [37]

$sPLA_2$	Mr measured	Mr calculated	LD_{50} (μg/kg)	IC_{50} (nM)		
				R180	CaM	R25
AtxA	13774.3	13774.8	20	16 ± 5	6 ± 2[a]	10 ± 5
12-AtxA	15097.5	15098.3	280	$> 10^4$	72 ± 15	5 ± 2
I-AtxA	14230.9	14231.3	500	$> 10^4$	250 ± 60	3.4 ± 0.3
P-AtxA	14214.6	14215.2	420	$> 10^4$	380 ± 85	3.5 ± 0.5

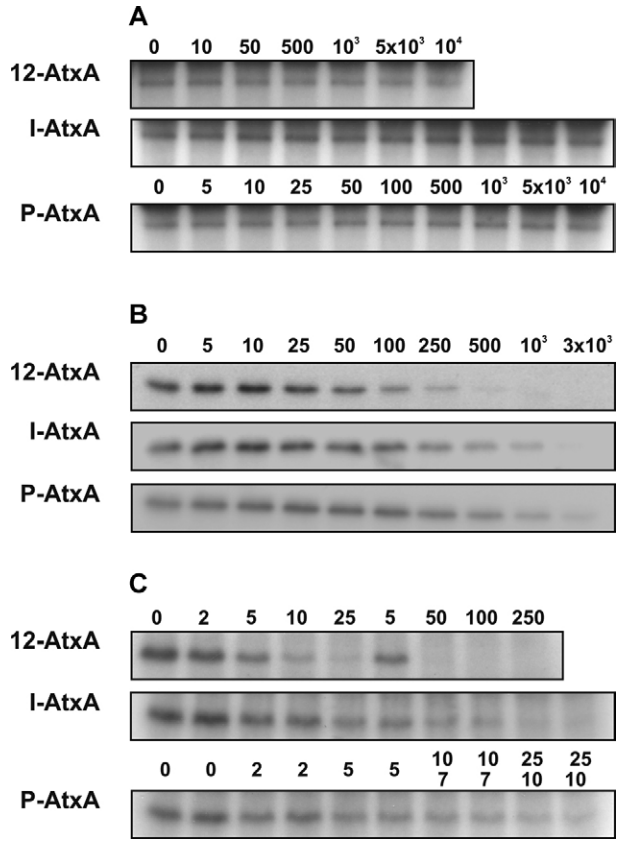

Fig. 2. Competitive binding assay. Cross-linking of radioactively labeled AtxC with R180 (A), CaM (B) and R25 (C) was inhibited by adding the fusion AtxA mutants at nM concentrations indicated.

fusions were 14- to 25-fold higher than that of AtxA (Table 1). In other words, the fusion AtxAs were roughly an order of magnitude less potent in mice than the wild type.

We were also interested if the reduced lethality of the fusion AtxAs was due to lower presynaptic neurotoxicity of the constructs. On mouse neuromuscular preparations, recombinant AtxA (10 μg/ml) showed typical triphasic modulation of twitch tension, which is indicative of changes to acetylcholine release. Upon addition of the toxin to the organ bath there was an initial depression of muscle contraction, followed by a transient increase after which there was a progressive decline in muscle contractility leading to muscle paralysis approximately 1 h after application of the toxin (Fig. 4A). In the case of the fusion AtxAs no effect on twitch tension was observed at 10 μg/ml, therefore, the concentration of fusion AtxAs was increased threefold. At 30 μg/ml all the three fusion AtxAs caused the first inhibitory phase which was then followed by the enhancement of twitch tension. However, the late inhibitory phase was

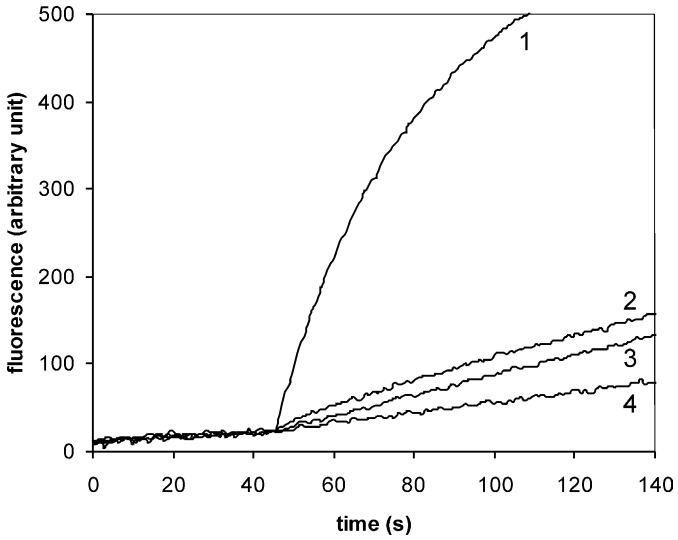

Fig. 3. Enzymatic activity curves on POPG large unilamellar vesicles. Curve 1, 2.0 ng AtxA; curve 2, 12-AtxA; curve 3, I-AtxA; curve 4, P-AtxA (all 50 ng). One fluorescence unit corresponds to 3.3 pmol of oleic acid.

delayed in those preparations exposed to 12-AtxA and I-AtxA and absent in tissues exposed to P-AtxA (Fig. 4B–D).

3.4. Characterization and use of the M-type $sPLA_2R$ antibodies

Antibodies raised against the CTLD5 domain of the mouse M-type $sPLA_2R$ (anti-CTLD5 Abs) recognized recombinant CTLD5 domain (~15 kDa) in inclusion bodies isolated from *E. coli* cells transformed with the expression plasmid encoding the CTLD5 domain of the mouse M-type $sPLA_2R$. No band with an apparent molecular mass of 15 kDa was observed in the control experiment with *E. coli* proteins only (Fig. 5A, panel a). Anti-CTLD5 Abs also labeled a 180 kDa protein from synaptic membranes of both mouse and porcine cerebral cortexes (Fig. 5B, panel a). The apparent molecular mass of 180 kDa corresponds to that of the M-type $sPLA_2R$ that was previously shown to be present in synaptic membranes of porcine brain cortex [10,11]. No bands were observed in negative control experiments on the same protein samples using rabbit preimmune serum (Fig. 5A, B, panels b). Preincubating the neuromuscular preparations with anti-CTLD5 Abs did not affect the activity of a subsequent addition of AtxA (Fig. 5C).

4. Discussion

In the present study, we investigated the potential role of a plasma membrane $sPLA_2R$, the so called M-type receptor, on

Table 2
Specific activities of recombinant AtxA and its fusion mutants. The values are mean ± S.D. of at least three independent measurements

$sPLA_2$	Mixed micelles with PC		POPG vesicles	
	(U/mg)	%	(U/mg)	%
AtxA	280 ± 28	100.0	1380 ± 60	100.0
12-AtxA	0.76 ± 0.06	0.27	5.2 ± 0.7	0.38
I-AtxA	0.96 ± 0.04	0.34	4.0 ± 0.2	0.29
P-AtxA	0.13 ± 0.03	0.046	1.29 ± 0.04	0.093

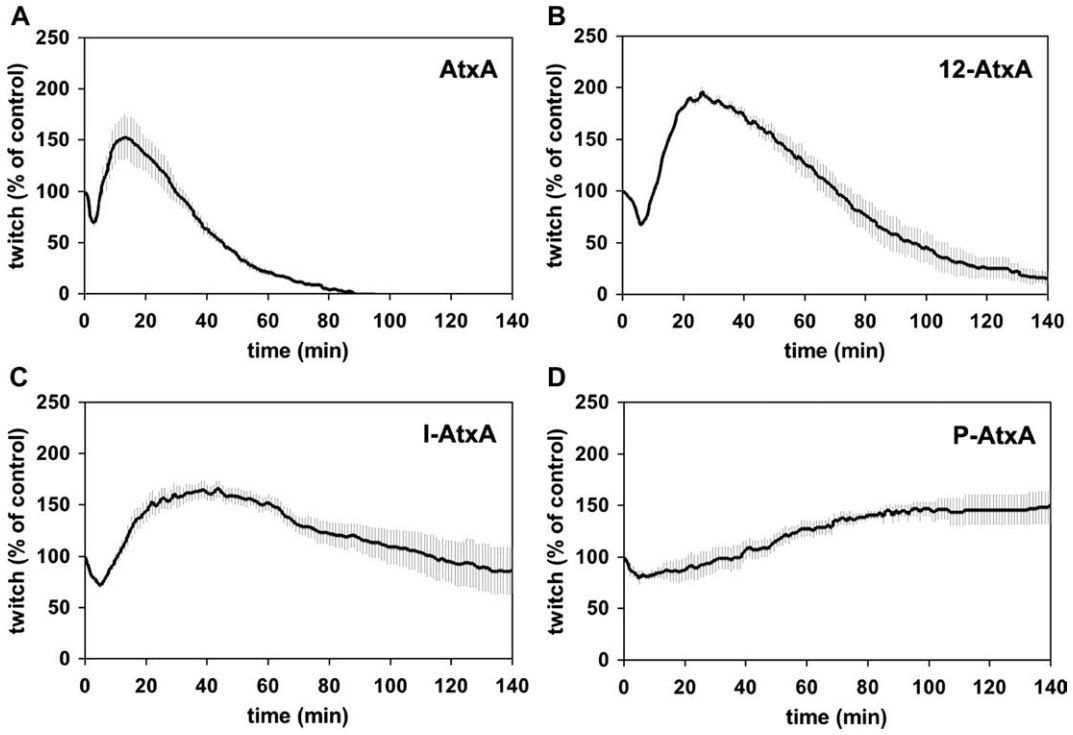

Fig. 4. Effect of AtxA (10 μg/ml) and fusion AtxA mutants (30 μg/ml) on twitch tension of mouse phrenic nerve-diaphragm preparation at 37 °C in low Ca^{2+} (0.38–0.50 mM) Krebs solution. Preparations were stimulated indirectly as described in Section 2. Each point on the diagram represents the mean ± S.E. of 5 (AtxA) or 3 (fusion AtxA mutants) experiments. Standard errors are indicated by bars unless smaller then symbols.

the presynaptic neurotoxicity of Atxs, potent snake venom toxins of group IIA $sPLA_2$s, by using N-terminal fusion mutants of AtxA. We showed by a competitive binding assay that the longer fusion AtxA (ARIRARGSIEGR-AtxA, named 12-AtxA), in contrast to wild-type AtxA, did not bind to the porcine M-type $sPLA_2$ receptor, R180. This was in accordance with our expectations as the proenzymes of homologous mammalian $sPLA_2$s of groups IB and X have to be activated by proteolytic removal of their hepta- and undecapropeptides, respectively, before they can efficiently bind to the M-type $sPLA_2$R [29,31]. To our surprise, however, the fusion protein 12-AtxA was quite toxic to mice, although its lethality was reduced by approximately an order of magnitude, relative to AtxA. Indeed, lethality of 12-AtxA was close to that of AtxC (with an LD_{50} of 360 μg/kg [3]), the second most (neuro)toxic component of *Vipera a. ammodytes* venom.

Toxicity of the longer fusion AtxA could be, in principle, also a consequence of proteolytic in vivo cleavage of the N-terminal fusion peptide of 12 residues, which includes 4 Arg residues, the last of them just preceding the first residue (Ser1) of mature toxin. In fact, we routinely activate the fusion AtxA by in vitro proteolytic removal of such a peptide using factor Xa or, preferentially, trypsin (mild trypsinolysis) to cleave recombinant protein after the last Arg of the "proregion" during bacterial expression and purification of recombinant AtxA [32]. Furthermore, it has been reported that certain serine proteinases, secreted from cells or present on the cell surfaces, including trypsin and plasmin that cut polypeptides after basic amino acid residues, are involved in in vivo cleavage of groups IB and X pro-$sPLA_2$s [46–48,29,31]. To exclude this possibility, we prepared two additional, shorter N-terminal fusion mutants of AtxA (I- and P-AtxA), which have no basic residues in the "propeptide" regions (ASIGQ and ASPGQ). They differ in only one position, at the P3 site, according to the nomenclature of the substrate cleaving sites of Schechter and Berger [49]. One of them possesses a Pro at the P3 site, thus further diminishing the possibility of a proteolytic cleavage.

The two shorter fusion AtxAs showed toxicity on mice in the range of the longer variant, although being somewhat less lethal, thus making the possibility of in vivo cleavage of the N-terminal "propetide" unlikely or very limited. Indeed, all the three fusion AtxAs were quite similar in their toxicity, protein binding affinities, enzymatic activity and biological action. They did not bind to the M-type $sPLA_2$R even at 10 μM concentration, but were considerably toxic and able to induce a specific (presynaptic) neurotoxic effect on the isolated mouse phrenic nerve-diaphragm preparation already at a threefold higher concentration compared to wild-type AtxA. Amino acid residues within or close to the Ca^{2+}-binding loop of pancreatic, group IB, $sPLA_2$ were identified as essential for binding to this receptor [30]. That part of the $sPLA_2$ molecule comprises together with certain residues at the N-terminal end of the mature protein a significant portion of the so-called "interfacial binding surface", a flat exposed region surrounding the entrance to the active site, essential for binding of the enzyme to the phospholipid membrane. We assume that, similar to pro-$sPLA_2$s of groups IB and X, in our fusion AtxAs the peptide attached to the N-terminus sterically hinders interaction of

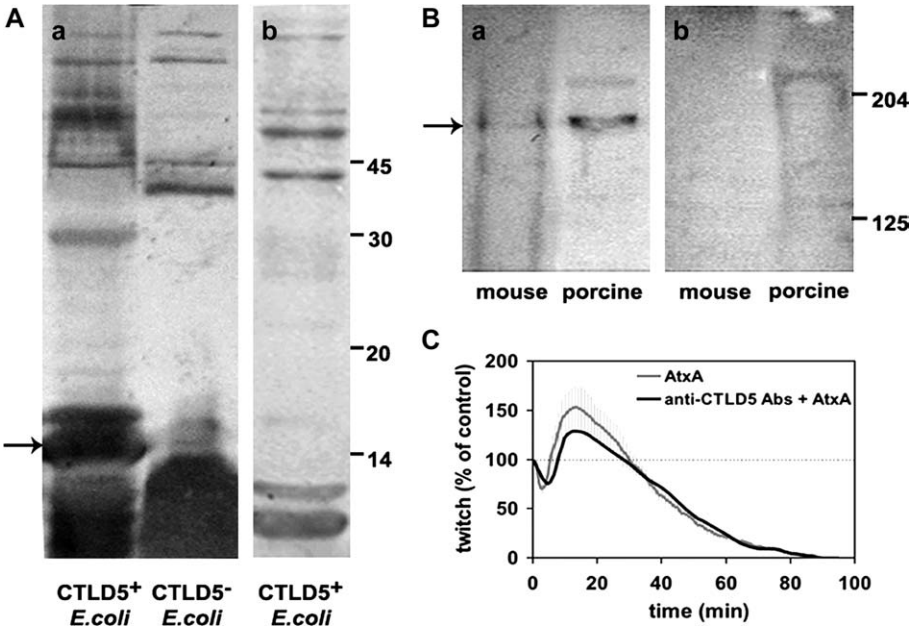

Fig. 5. Analysis of Abs raised against the CTLD5 domain of the M-type $sPLA_2R$. A) Western blot analysis of inclusion bodies isolated from the CTLD5 domain expressing bacteria ($CTLD5^+$ *E. coli*) and *E. coli* protein lysate only ($CTLD5^-$ *E. coli*; a negative control) by anti-CTLD5 Abs (panel a), and a control experiment with the preimmune serum (panel b). Proteins (~0.65 μg) were separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. The transferred proteins on blots were probed by either the anti-CTLD5 Abs (1:30; panel a) or the preimmune serum (1:30; panel b), and than by secondary goat HP-conjugated anti-rabbit Abs (1:100). Bound Abs were detected with DAB substrate. The arrow indicates a ~15 kDa protein corresponding to the apparent molecular mass of the CTLD5 domain. The molecular masses of protein standards (in kDa) are indicated on the right. B) Western blot analysis of synaptic membranes of mouse and porcine cerebral cortexes (~9 μg of proteins) by anti-CTLD5 Abs (panel a), and a control experiment with the preimmune serum (panel b). Proteins were separated on a 7% SDS-PAGE gel and transferred to a nitrocellulose membrane. The transferred proteins on blots were probed by either anti-CTLD5 Abs (1:100; panel a) or the preimmune serum (1:100; panel b), and than with secondary HP-conjugated anti-rabbit/mouse IgG Abs (1:10000). Bound Abs were detected by ECL-detection. The arrow indicates a ~180 kDa protein corresponding to the apparent molecular mass of the M-type $sPLA_2R$. The molecular masses of protein standards (in kDa) are indicated on the right. C) Effect of anti-CTLD5 Abs on the neurotoxicity of AtxA on a mouse phrenic-nerve diaphragm preparation (black line). The preparation was preincubated with anti-CRD5 Abs (1:20) for 1 h at 37 °C prior to the adding of AtxA (10 μg/ml). The gray line on the diagram represents the neurotoxic effect of AtxA on the mouse preparation without adding Abs.

the toxin with the M-type $sPLA_2R$, presumably mainly with its CTLD5 domain.

Binding of a $sPLA_2$ to the plasma membrane M-type $sPLA_2R$ can lead to different cellular responses, and also to clathrin dependent receptor-mediated endocytosis of $sPLA_2$ [2]. It has been shown that the M-type $sPLA_2R$ binds in addition to nontoxic also presynaptically toxic $sPLA_2$s, such Atxs, OS_2, taipoxin, and textilotoxin [10,50]. This finding suggests involvement of the M-type $sPLA_2R$ in the presynaptic neurotoxicity of $sPLA_2$s by their internalization into the axon terminals [6,7]. In the case of our N-terminal fusion AtxAs, which do not bind to the M-type $sPLA_2R$ (see Fig. 2), this way of entering the cell would be abolished and consequently also their neurotoxic action. However, the fusion AtxAs were lethal to mice and clearly showed presynaptic effects on the mammalian NM preparation, indicating that the M-type $sPLA_2R$ does not play a crucial role in the presynaptic neurotoxicity of AtxA and, likely, other similar $sPLA_2$ neurotoxins.

To further examine the potential involvement of the M-type $sPLA_2R$ in the presynaptic neurotoxicity of AtxA, we prepared polyclonal Abs against the CTLD5 domain, the most important $sPLA_2$-binding domain of this receptor [18]. Abs could recognize mouse and porcine M-type $sPLA_2R$s in neuronal tissue but were unable to neutralize the effect of AtxA on the mouse NM preparation. Inability of the used Abs to inhibit the presynaptic neurotoxic effect of AtxA supports our conclusion that the presence of the M-type $sPLA_2R$ is not obligatory for its neurotoxicity at the NM junction. Nevertheless, since the soluble form of the M-type $sPLA_2R$ was identified in mouse serum [27], there is still a possibility of its interaction with AtxA that would prevent nonspecific binding of the toxin before reaching the target presynaptic membrane. On the contrary, such a binding of the fusion AtxAs is abolished due to the presence of a short N-terminal “propeptide”.

The attached N-terminal peptides also reduced the binding affinity of AtxA for CaM (Table 1), with the shorter “propeptide” containing Pro, an amino acid residue with restricted flexibility, being most effective. This indicates that such a peptide, even of as little as five residues, sterically interfere with binding of the presumed CaM-binding site in the C-terminal region of the toxin [37] to CaM. AtxA with the longer "propeptide" attached (12-AtxA) had the highest binding affinity for CaM. Its 12 amino acid propeptide is positively charged, which may at least partially contribute to better electrostatic interaction with the canonical binding site on CaM, surrounded by several negative charged residues [51]. The binding affinities of all three AtxA fusion mutants for the R25 receptor were, surprisingly, even higher than that of wild-type AtxA (Table 1), whose R25-binding site is primarily located in its C-terminal part [52]. In this case, the attached peptide may serve as an

additional "anchor" to strengthen interaction of the toxin with R25. Interaction of the fusion AtxAs with both intracellular binding proteins, CaM and R25, but not with the M-type $sPLA_2R$, still points to their potential role in the presynaptic activity of AtxA after its presumed internalization into the motoneuron.

It has been known for a long time that enzymatic activity is necessary for the full expression of $sPLA_2$ neurotoxicity, since its inhibition prevents lethality [53] and NM blocking activity [54]. It appears from our study that only a minimal activity of fusion AtxAs is sufficient for lethality in mice and for the presynaptic effects observed on the mouse NM preparation. Namely, the additional peptide on the N-terminus of AtxA lowered its $sPLA_2$ activity by two to three orders of magnitude on both zwitterionic (electroneutral) and anionic substrates. Markedly reduced enzymatic activity has also been observed in studies on the N-terminal fusion $sPLA_2$s of different groups. For example, the addition of a single amino acid, Met or Ala, lowered enzymatic activity of a human group IIA $sPLA_2$ to approximately 1% relative to the wild type [55]. Similarly, at least a 100-fold decrease of catalytic activity was observed in a porcine group IB pro-$sPLA_2$ compared to its zymogen [56,57], whereas for a mouse group X pro-$sPLA_2$ about 8% of the mature enzyme activity was reported [31]. The three-dimensional structures of bovine and porcine group IB pro-$sPLA_2$s show that the N-terminal propeptide induces limited structural changes in the molecule that impair chemical steps in the catalytic process, presumably due to the absence of a critical active site water molecule when compared to the activated form [57,58].

In our experiments on the NM preparation, using the same concentration of any of the three fusion AtxAs, the second (facilitatory) and, especially, the third (late) inhibitory phases were clearly evident only in the case of 12-AtxA and I-AtxA but not in P-AtxA. As the latter fusion toxin had threefold to sevenfold lower enzymatic activity in comparison to the first two fusions, it appears that the catalytic activity of P-AtxA was already too low to induce a typical triphasic response at the same concentration. In addition, the P-AtxA mutant had the lowest binding affinity for CaM of all three fusions which may also be important at later stages of the neurotoxic process. Interestingly, lethality of this Pro variant was slightly higher than that of the Ile variant, indicating that other effects, such as nonspecific binding before the toxin reaches the target presynaptic membrane, may also contribute to the final outcome of intoxication.

In conclusion, by engineering a short peptide preceding the N-terminus of highly potent AtxA we have demonstrated that its interaction with the high-affinity M-type $sPLA_2R$ is not essential for the process of presynaptic neurotoxicity where at least a minimal phospholipase activity of the toxin is required.

Acknowledgements

We thank Dr. Tadej Malovrh for help in lethality measurements, Dr. Toni Petan for assistance in enzymatic activity assays and Dr. Bogdan Kralj for molecular mass analysis. This work was supported by grant P1-0207 from the Slovenian Ministry of Higher Education, Science and Technology, by NATO Collaborative Linkage Grant No. 980899 and by Collaborative Experimental Scholarship for Central and Eastern Europe from Federation of European Biochemical Societies.

References

[1] D.A. Six, E.A. Dennis, The expanding superfamily of phospholipase A_2 enzymes: classification and characterization, Biochim. Biophys. Acta – Mol. Cell. Biol. Lipids 1488 (2000) 1–19.
[2] I. Kudo, M. Murakami, Phospholipase A_2 enzymes, Prostaglandins Other Lipid Mediat. 68-69 (2002) 3–58.
[3] L.G. Thouin Jr., A. Ritonja, F. Gubenšek, F.E. Russell, Neuromuscular and lethal effects of phospholipase A from *Vipera ammodytes* venom, Toxicon 20 (1982) 1051–1058.
[4] C.Y. Lee, M.C. Tsai, Y.M. Chen, A. Ritonja, F. Gubenšek, Mode of neuromuscular blocking action of toxic phospholipases A_2 from *Vipera ammodytes* venom, Arch. Int. Pharmacodyn. Ther. 268 (1984) 313–324.
[5] B. Hawgood, C. Bon, Snake venom presynaptic toxins, in: A.T. Tu (Ed.), Handbook of Natural Toxins, Marcel Dekker, New York, USA, 1991, pp. 3–52 (Vol. 5).
[6] C. Montecucco, O. Rossetto, How do presynaptic PLA_2 neurotoxins block nerve terminals?, Trends Biochem. Sci. 25 (2000) 266–270.
[7] I. Križaj, F. Gubenšek, Neuronal receptors for phospholipases A_2 and β-neurotoxicity, Biochimie 82 (2000) 807–814.
[8] J. Šribar, A. Čopič, M. Poljšak-Prijatelj, J. Kuret, U. Logonder, F. Gubenšek, I. Križaj, R25 is an intracellular membrane receptor for a snake venom secretory phospholipase A_2, FEBS Lett. 553 (2003) 309–314.
[9] N. Vučemilo, A. Čopič, F. Gubenšek, I. Križaj, Identification of a new high-affinity binding protein for neurotoxic phospholipases A_2, Biochem. Biophys. Res. Commun. 251 (1998) 209–212.
[10] A. Čopič, N. Vučemilo, F. Gubenšek, I. Križaj, Identification and purification of a novel receptor for secretory phospholipase A_2 in porcine cerebral cortex, J. Biol. Chem. 274 (1999) 26315–26320.
[11] N. Vardjan, N.E. Sherman, J. Pungerčar, J.W. Fox, F. Gubenšek, I. Križaj, High-molecular-mass receptors for ammodytoxin in pig are tissue-specific isoforms of M-type phospholipase A_2 receptor, Biochem. Biophys. Res. Commun. 289 (2001) 143–149.
[12] J. Šribar, A. Čopič, A. Pariš, N.E. Sherman, F. Gubenšek, J.W. Fox, I. Križaj, A high affinity acceptor for phospholipase A_2 with neurotoxic activity is a calmodulin, J. Biol. Chem. 276 (2001) 12493–12496.
[13] J. Šribar, N.E. Sherman, P. Prijatelj, G. Faure, F. Gubenšek, J.W. Fox, A. Aitken, J. Pungerčar, I. Križaj, The neurotoxic phospholipase A_2 associates, through a non-phosphorylated binding motif, with 14-3-3 protein γ and ε isoforms, Biochem. Biophys. Res. Commun. 302 (2003) 691–696.
[14] J. Šribar, G. Anderluh, J.W. Fox, I. Križaj, Protein disulphide isomerase binds ammodytoxin strongly: possible implications for toxin trafficking, Biochem. Biophys. Res. Commun. 329 (2005) 733–737.
[15] G. Lambeau, P. Ancian, J. Barhanin, M. Lazdunski, Cloning and expression of a membrane receptor for secretory phospholipases A_2, J. Biol. Chem. 269 (1994) 1575–1578.
[16] J. Ishizaki, K. Hanasaki, K. Higashino, J. Kishino, N. Kikuchi, O. Ohara, H. Arita, Molecular cloning of pancreatic group I phospholipase A_2 receptor, J. Biol. Chem. 269 (1994) 5897–5904.
[17] J.P. Nicolas, G. Lambeau, M. Lazdunski, Identification of the binding domain for secretory phospholipases A_2 on their M-type 180-kDa membrane receptor, J. Biol. Chem. 270 (1995) 28869–28873.
[18] K. Higashino, J. Ishizaki, J. Kishino, O. Ohara, H. Arita, Structural comparison of phospholipase-A_2-binding regions in phospholipase-A_2 receptors from various mammals, Eur. J. Biochem. 225 (1994) 375–382.
[19] K. Hanasaki, H. Arita, Phospholipase A_2 receptor: a regulator of biological functions of secretory phospholipase A_2, Prostaglandins Other Lipid Mediat. 68-69 (2002) 71–82.

[20] M. Hernandez, L. Fuentes, F.J. Fernandez Aviles, M.S. Crespo, M.L. Nieto, Secretory phospholipase A_2 elicits proinflammatory changes and upregulates the surface expression of Fas ligand in monocytic cells: potential relevance for atherogenesis, Circ. Res. 90 (2002) 38–45.
[21] D.W. Park, J.R. Kim, S.Y. Kim, J.K. Sonn, O.S. Bang, S.S. Kang, J.H. Kim, S.H. Baek, Akt as a mediator of secretory phospholipase A_2 receptor-involved inducible nitric oxide synthase expression, J. Immunol. 170 (2003) 2093–2099.
[22] K. Hanasaki, H. Arita, Characterization of a high affinity binding site for pancreatic-type phospholipase A_2 in the rat. Its cellular and tissue distribution, J. Biol. Chem. 267 (1992) 6414–6420.
[23] P. Ancian, G. Lambeau, M. Lazdunski, Multifunctional activity of the extracellular domain of the M-type (180 kDa) membrane receptor for secretory phospholipases A_2, Biochemistry 34 (1995) 13146–13151.
[24] G.P. Rossini, J.M. Fayard, C. Tessier, C. Laugier, Binding and internalization of extracellular type-I phospholipase A_2 in uterine stromal cells, Biochem. J. 315 (1996) 1007–1014.
[25] E. Zvaritch, G. Lambeau, M. Lazdunski, Endocytic properties of the M-type 180-kDa receptor for secretory phospholipases A_2, J. Biol. Chem. 271 (1996) 250–257.
[26] Y. Yokota, M. Notoya, K. Higashino, Y. Ishimoto, K. Nakano, H. Arita, K. Hanasaki, Clearance of group X secretory phospholipase A_2 via mouse phospholipase A_2 receptor, FEBS Lett. 509 (2001) 250–254.
[27] K. Higashino, Y. Yokota, T. Ono, S. Kamitani, H. Arita, K. Hanasaki, Identification of a soluble form phospholipase A_2 receptor as a circulating endogenous inhibitor for secretory phospholipase A_2, J. Biol. Chem. 277 (2002) 13583–13588.
[28] U. Petrovič, J. Šribar, A. Pariš, M. Rupnik, M. Kržan, N. Vardjan, F. Gubenšek, R. Zorec, I. Križaj, Ammodytoxin, a neurotoxic secreted phospholipase A_2, can act in the cytosol of the nerve cell, Biochem. Biophys. Res. Commun. 324 (2004) 981–985.
[29] T. Nakano, H. Fujita, N. Kikuchi, H. Arita, Plasmin converts pro-form of group I phospholipase A_2 into receptor binding, active forms, Biochem. Biophys. Res. Commun. 198 (1994) 10–15.
[30] G. Lambeau, P. Ancian, J.P. Nicolas, S.H. Beiboer, D. Moinier, H. Verheij, M. Lazdunski, Structural elements of secretory phospholipases A_2 involved in the binding to M-type receptors, J. Biol. Chem. 270 (1995) 5534–5540.
[31] Y. Morioka, A. Saiga, Y. Yokota, N. Suzuki, M. Ikeda, T. Ono, K. Nakano, N. Fujii, J. Ishizaki, H. Arita, K. Hanasaki, Mouse group X secretory phospholipase A_2 induces a potent release of arachidonic acid from spleen cells and acts as a ligand for the phospholipase A_2 receptor, Arch. Biochem. Biophys. 381 (2000) 31–42.
[32] J. Pungerčar, I. Križaj, N.-S. Liang, F. Gubenšek, An aromatic, but not a basic, residue is involved in the toxicity of group-II phospholipase A_2 neurotoxins, Biochem. J. 341 (1999) 139–145.
[33] A.F. Worrall, C. Evans, D.C. Wilton, Synthesis of a gene for rat liver fatty-acid-binding protein and its expression in *Escherichia coli*, Biochem. J. 278 (1991) 365–368.
[34] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, Am. J. Hyg. 27 (1938) 493–497.
[35] P. Prijatelj, A. Čopič, I. Križaj, F. Gubenšek, J. Pungerčar, Charge reversal of ammodytoxin A, a phospholipase A_2-toxin, does not abolish its neurotoxicity, Biochem. J. 352 (2000) 251–255.
[36] I. Križaj, J.O. Dolly, F. Gubenšek, Identification of the neuronal acceptor in bovine cortex for ammodytoxin C, a presynaptically neurotoxic phospholipase A_2, Biochemistry 33 (1994) 13938–13945.
[37] P. Prijatelj, J. Šribar, G. Ivanovski, I. Križaj, F. Gubenšek, J. Pungerčar, Identification of a novel binding site for calmodulin in ammodytoxin A, a neurotoxic group IIA phospholipase A_2, Eur. J. Biochem. 270 (2003) 3018–3025.
[38] E.A. Dennis, Kinetic dependence of phospholipase A_2 activity on the detergent Triton X-100, J. Lipid Res. 14 (1973) 152–159.
[39] D.C. Wilton, A continuous fluorescence displacement assay for the measurement of phospholipase A_2 and other lipases that release long-chain fatty acids, Biochem. J. 266 (1990) 435–439.
[40] S. Bezzine, R.S. Koduri, E. Valentin, M. Murakami, I. Kudo, F. Ghomashchi, M. Sadilek, G. Lambeau, M.H. Gelb, Exogenously added human group X secreted phospholipase A_2 but not the group IB, IIA, and V enzymes efficiently release arachidonic acid from adherent mammalian cells, J. Biol. Chem. 275 (2000) 3179–3191.
[41] S. Tabor, Expression using the T7 RNA polymerase/promoter system, in: F.A. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Eds.), Current Protocols in Molecular Biology, Greene Publishing and Wiley-Interscience, New York, USA, 1990, pp. 16.2.1–16.2.11.
[42] V. Bennett, A.J. Baines, J. Davis, Purification of brain analogs of red blood cell membrane skeletal proteins: ankyrin, protein 4.1 (synapsin), spectrin, and spectrin subunits, Methods Enzymol. 134 (1986) 55–69.
[43] R. Grataroli, R. Dijkman, C.E. Dutilh, F. van der Ouderaa, G.H. de Haas, C. Figarella, Studies on prophospholipase A_2 and its enzyme from human pancreatic juice. Catalytic properties and sequence of the N-terminal region, Eur. J. Biochem. 122 (1982) 111–117.
[44] L. Cupillard, K. Koumanov, M.G. Mattei, M. Lazdunski, G. Lambeau, Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A_2, J. Biol. Chem. 272 (1997) 15745–15752.
[45] P.H. Hirel, M.J. Schmitter, P. Dessen, G. Fayat, S. Blanquet, Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side-chain length of the penultimate amino acid, Proc. Natl. Acad. Sci. USA 86 (1989) 8247–8251.
[46] G.H. de Haas, N.M. Postema, W. Nieuwenhuizen, L.L. van Deenen, Purification and properties of an anionic zymogen of phospholipase A from porcine pancreas, Biochim. Biophys. Acta 159 (1968) 118–129.
[47] D. Rae, N. Beechey-Newman, L. Burditt, N. Sumar, J. Hermon-Taylor, Activation of human granulocyte type 1-prophospholipase A_2, Scand. J. Gastroenterol. Suppl. 219 (1996) 24–27.
[48] S. Uchiyama, N. Iijama, Partial purification and characterization of prophospholipase A_2 activating proteases from gill membranes of the red sea bream, *Chrysophrys major*, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 141 (2005) 121–127.
[49] I. Schechter, A. Berger, On the size of the active site in proteases, Biochem. Biophys. Res. Commun. 27 (1967) 157–162.
[50] G. Lambeau, L. Cupillard, M. Lazdunski, Membrane receptors for venom phospholipases A_2, in: R.M. Kini (Ed.), Venom Phospholipases A_2 Enzymes, Structure, Function and Mechanism, Wiley & Sons, Chichester, UK, 1997, pp. 389–412.
[51] R. Chattopadhyaya, W.E. Meador, A.R. Means, F.A. Quiocho, Calmodulin structure refined at 1.7 Å resolution, J. Mol. Biol. 228 (1992) 1177–1192.
[52] P. Prijatelj, I. Križaj, B. Kralj, F. Gubenšek, J. Pungerčar, The C-terminal region of ammodytoxins is important but not sufficient for neurotoxicity, Eur. J. Biochem. 269 (2002) 5759–5764.
[53] T.W. Jeng, H. Fraenkel-Conrat, Chemical modification of histidine and lysine residues of crotoxin, FEBS Lett. 87 (1978) 291–296.
[54] C.C. Chang, M.J. Su, Presynaptic toxicity of the histidine-modified, phospholipase A_2-inactive, β-bungarotoxin, crotoxin and notexin, Toxicon 20 (1982) 895–905.
[55] S. Di Marco, F. Märki, H. Hofstetter, A. Schmitz, J. van Oostrum, M.G. Grütter, Purification, analysis, and enzymatic activity of recombinant human synovial fluid phospholipase A_2 and N-terminal variants, J. Biochem. (Tokyo) 112 (1992) 350–354.
[56] M.K. Jain, J. Rogers, G.H. De Haas, Kinetics of binding of phospholipase A_2 to lipid/water interfaces and its relationship to interfacial activation, Biochim. Biophys. Acta 940 (1988) 51–62.
[57] T.M. Epstein, B.Z. Yu, Y.H. Pan, S.P. Tutton, B.P. Maliwal, M.K. Jain, B.J. Bahnson, The basis for k^*_{cat} impairment in prophospholipase A_2 from the anion-assisted dimer structure, Biochemistry 40 (2001) 11411–11422.
[58] K. Tomoo, A. Yamane, T. Ishida, S. Fujii, K. Ikeda, S. Iwama, S. Katsumura, S. Sumiya, H. Miyagawa, K. Kitamura, X-ray crystal structure determination and molecular dynamics simulation of prophospholipase A_2 inhibited by amide-type substrate analogues, Biochim. Biophys. Acta 340 (1997) 178–186.