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

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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; $sPLA_2$R, $sPLA_2$ receptor.

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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