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Ammodytoxins efficiently release arachidonic acid and induce apoptosis in a motoneuronal cell line in an enzymatic activity-dependent manner

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ABSTRACT

Secreted phospholipases A_2 ($sPLA_2s$) are phospholipolytic enzymes and receptor ligands whose action affects cell death and survival. We have previously shown that ammodytoxin A (AtxA), a snake venom $sPLA_2$, is rapidly internalized into motoneuronal NSC34 cells, inducing characteristic neurotoxic $sPLA_2$ cell damage and apoptosis. In this study, we have analyzed the role of $sPLA_2$ enzymatic activity, including arachidonic acid (AA) release, in the induction of motoneuronal apoptosis by AtxA and homologous recombinant $sPLA_2s$ with different enzymatic properties: an AtxA mutant (V31W) with very high enzymatic activity, enzymatically inactive S49-$sPLA_2$ (ammodytin L, AtnL), its mutant (LW) with restored enzymatic activity, and non-toxic, enzymatically active $sPLA_2$ ($AtnI_2$). Addition of AA, AtxA, AtxA-V31W and AtnL-LW, but not AtnL and $AtnI_2$, to NSC34 cells resulted in caspase-3 activation, DNA fragmentation and disruption of mitochondrial membrane potential, leading to a significant and rapid decrease in motoneuronal cell viability that was not observed in C2C12 myoblasts and HEK293 cells. AtxA, AtxA-V31W and AtnL-LW, but not AtnL and $AtnI_2$, also liberated large amounts of AA specifically from motoneuronal cells, and this ability correlated well with the ability to induce apoptotic changes and decrease cell viability. The enzymatic activity of AtxA and similar $sPLA_2s$ is thus necessary, but not sufficient, for inducing motoneuronal apoptosis. This suggests that specific binding to the motoneuronal cell surface, followed by internalization and enzymatic activity-dependent induction of apoptosis, possibly as a consequence of extensive extra- and intracellular AA release, is necessary for Atx-induced motoneuronal cell death.

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1. Introduction

Snake venoms are complex mixtures of biologically active compounds, including secreted phospholipases A_2 ($sPLA_2s$), that possess a wide variety of pharmacological effects, including neurotoxicity, myotoxicity, cardiotoxicity, pro- or anticoagulant activity, and hemolytic action (Kini, 2003). Structurally similar and homologous $sPLA_2s$ are also found in various mammalian tissues. Their physiological and pathological roles are diverse and, despite numerous studies, still not completely understood (Murakami et al., 2011).

Secreted PLA_2s are members of the PLA_2 superfamily of enzymes that catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids, releasing free fatty acids and lysophospholipids. The family of $sPLA_2s$ consists of relatively small (13–18 kDa), disulphide-rich and Ca^{2+}-dependent enzymes that are secreted from cells, but can also function intracellularly, either during secretion or after their subsequent binding to the target cell membrane and/or internalization (Lambeau and Gelb, 2008). The PLA_2 reaction is the primary pathway through which arachidonic acid (AA), the precursor of the eicosanoid signalling molecules, is liberated from membrane phospholipids (Balsinde et al., 2002). Enhanced PLA_2 activity and increased levels of AA and its eicosanoid metabolites in neurons and glial cells have been associated with different neurodegenerative conditions and traumatic injury (Bazan et al., 2002). However, very little is known about the involvement of $sPLA_2s$ in motoneuronal apoptotic cell death.

Ammodytoxin A (AtxA), a presynaptically neurotoxic group IIA $sPLA_2$ from the venom of the nose-horned viper, *Vipera a. ammodytes*, that causes complete failure of synaptic (acetylcholine) transmission at the vertebrate neuromuscular junction (NMJ), induces apoptosis in a mouse motoneuronal cell line, NSC34 cells, through the mitochondrial pathway (Jenko Pražnikar et al., 2009). Furthermore, AtxA is rapidly internalized, not only into cultured NSC34 cells (Jenko Pražnikar et al., 2008) but also into mammalian motor nerve terminals *in vivo* (Logonder et al., 2009). The aim of the present study was to define the role of enzymatic activity and AA-releasing potency of ammodytoxins (Atxs) and homologous $sPLA_2s$ in the induction of apoptosis in

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motoneuronal NSC34 cells. We used a set of structurally similar recombinant $sPLA_2$s differing significantly in their enzymatic activity and toxicity, including, in addition to AtxA, a non-toxic but enzymatically active $sPLA_2$, ammodytin I_2 ($AtnI_2$), and a myotoxic but enzymatically inactive S49-$sPLA_2$ homologue, ammodytin L (AtnL). In addition, two mutants were prepared and tested for their ability to induce motoneuronal cell death: a single-site AtxA mutant (V31W) with a substantially higher enzymatic activity than the wild-type AtxA (Petan et al., 2005), and a four-site AtnL mutant (H28Y/L31W/N33G/S49D, named LW) in which the enzymatic activity, absent in the wild-type AtnL, was completely restored (Petan et al., 2007). Our results clearly show that ammodytoxins efficiently release AA from motoneuronal cell membranes, and thus induce apoptotic cell death in motoneuronal cells in an enzymatic activity-dependent manner.

2. Materials and methods

2.1. Materials

Recombinant AtxA, AtnL, $AtnI_2$, AtnL-LW and AtxA-V31W were prepared in *Escherichia coli* as described (Pungerčar et al., 1999; Petan et al., 2005, 2007). The AtxA(N79C)-Texas Red conjugate was prepared as reported (Jenko Pražnikar et al., 2008). Recombinant human group V (hGV) and group X (hGX) $sPLA_2$s, and the $sPLA_2$ inhibitor methyl-indoxam (Me-Indoxam) were provided by Prof. Michael H. Gelb (University of Washington, Seattle, USA). Purity of all recombinant $sPLA_2$s, used in the experiments, exceeded 95%. The expression plasmid encoding rat liver fatty acid-binding protein (FABP) was provided by Prof. David C. Wilton (University of Southampton, UK) and the recombinant protein was prepared as described (Worrall et al., 1991). The mouse myoblast C2C12 and mouse motoneuronal NSC34 cell lines were provided by Prof. Angelo Poletti (University of Milan, Italy). Dulbecco's modified Eagle's medium (DMEM) and heat-inactivated foetal bovine serum (FBS) were obtained from Invitrogen. Defined FBS was from HyClone and Invitrogen. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolocarbocyanine iodide (JC-1), propidium iodide (PI), acetyl-L-Asp-L-Glu-L-Val-L-Asp-α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA) and MitoFluor Green were from Invitrogen. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) assay kit was from Promega Biosciences. [^{3}H]-labelled AA (5,6,8,9,11,12,14,15-^{3}H; specific activity 7.77 TBq/mmol) was from Amersham GE Healthcare. AA, arachidonyl trifluoromethyl ketone ($AACOCF_3$) and methyl arachidonyl fluorophosphonate (MAFP) were from Calbiochem. Fatty acid-free bovine serum albumin (BSA) was from Sigma. All other chemicals were of at least analytical grade, and purchased from Sigma and Serva.

2.2. Analytical methods

SDS-PAGE was performed on a Mini Protean III electrophoresis system (Bio-Rad) in the presence of 150 mM dithiothreitol on 15% (w/v) polyacrylamide gels, with Coomassie Brilliant Blue R250 staining. $sPLA_2$ enzymatic activity was routinely assayed using a sensitive fluorometric $sPLA_2$ assay with small unilamellar phospholipid vesicles composed of 1-palmitoyl-2-(10-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol (Pyr-PG; Invitrogen, USA) on a Safire2 microplate reader (Tecan, Switzerland) as described (Kovačič et al., 2009). In inhibition assays, Me-indoxam (0–3000 nM) was pre-incubated with 10 nM $sPLA_2$s for 30 min before the addition of the phospholipid substrate (20 μM Pyr-PG).

2.3. Cell cultures

NSC34 cells were maintained in DMEM supplemented with 5% (v/v) defined FBS, 1 mM glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin), and grown at 37 °C in a humidified atmosphere of 5% CO_2 in 25 cm^2 flasks. The medium was changed every 3 days. Cells were dissociated mechanically and seeded into new culture flasks on a weekly basis at a density of 5×10^5 cells/flask. The NSC34 cultures contain two populations of cells: small, undifferentiated cells that are able to undergo cell division and larger, differentiated multinucleate cells. Before each experiment, cytosine-β-D-arabinofuranoside hydrochloride (Ara-C) was added and the cells incubated for an additional 24 h in order to increase the number of differentiated cells and thus attain a well-defined and controlled cell population. C2C12 and HEK293 cells were maintained in DMEM with 20% (v/v) FBS and 10% (v/v) FBS, respectively, 1 mM glutamine and antibiotics (as above), and were grown at 37 °C in a humidified atmosphere of 5% CO_2 in 25 cm^2 flasks.

2.4. $sPLA_2$ enzymatic activity on intact cells and phospholipid vesicles

The NSC34, C2C12 and HEK293 cells were grown to 70–90% confluence, the culture medium was removed and cells dislodged. An equal volume of complete medium was added, and the cells pelleted and washed twice with Hanks' balanced salt solution with 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+} (HBSS). Unilamellar phospholipid vesicles, with a diameter of 0.1 μm and containing 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), were prepared by extrusion. Fatty acid release from intact cells and POPC vesicles by exogenously added $sPLA_2$s was determined using the fluorescence displacement assay (Bezzine et al., 2000; Petan et al., 2005). The initial velocity of phospholipid hydrolysis was measured by monitoring the displacement of a fluorescent fatty acid analogue from FABP. Assays were performed at 37 °C in HBSS containing 30 μM phospholipid or 5×10^5 cells, 1 μM 11-dansylundecanoic acid and 10 μg recombinant FABP, using a Perkin-Elmer LS50B fluorometer. Excitation was at 350 nm and emission at 500 nm, with 10 nm slit widths. Reactions were started by adding 0.5–2000 ng $sPLA_2$ (typically in 1 μl). We chose the amounts that resulted in a slope of approximately 45 degrees, i.e. for POPC: AtxA, 10 ng; AtxA-V31W, 1 ng; AtnL, 2000 ng; AtnL-LW, 300 ng; $AtnI_2$, 10 ng; hGV, 15 ng; hGX, 3 ng; for NSC34 cells: AtxA, 2 ng; AtxA-V31W, 0.3 ng; AtnL, 2000 ng; AtnL-LW, 30 ng; $AtnI_2$, 10 ng; hGV, 2 ng; hGX, 1 ng; for C2C12 cells: AtxA, 4 ng; AtxA-V31W, 0.5 ng; AtnL, 2000 ng; AtnL-LW, 40 ng; $AtnI_2$, 10 ng; hGV, 4 ng; hGX, 3 ng; and for HEK293 cells: AtxA, 8 ng; AtxA-V31W, 1 ng; AtnL, 2000 ng; AtnL-LW, 100 ng; $AtnI_2$, 20 ng; hGV, 8 ng; hGX, 3 ng. All dilutions of $sPLA_2$s were prepared in buffer containing 1 mg/ml fatty acid-free BSA to prevent loss of enzyme due to adsorption to the walls of the tube. Initial rates of hydrolysis were determined from data obtained in the first 5–10 s after addition of the enzymes. Assays were calibrated with a known amount of methanol solution of oleic acid (Sigma). Cell viability was assessed by hemocytometer counting and trypan blue exclusion before and after each assay, but no significant changes in cell viability were observed during the short time of cell exposure to the toxins.

2.5. Cell viability assay

Cells were seeded in 96-well cell-culture plates, in a volume of 100 μl of the appropriate supplemented medium at a density of 1×10^4 cells per well, and incubated at 37 °C for 24 h. Before each experiment, Ara-C was added and the cells were incubated at 37 °C for 24 h. Cell viability was assessed using the MTS assay (Promega Biosciences). At specified times following continuous exposure of

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