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Functional and structural studies of a Phospholipase A_2-like protein complexed to zinc ions: Insights on its myotoxicity and inhibition mechanism

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ABSTRACT

Background: One of the main challenges in snakebite envenomation treatment is the development of stable, versatile and efficient *anti*-venom therapies. Local myotoxicity in accidents involving snakes from the *Bothrops* genus is still a consequence of serum therapy inefficient neutralization that may lead to permanent sequelae in their victims. One of the classes of toxins that participate in muscle necrosis is the PLA_2-like proteins. The aim of this work was to investigate the role of zinc ions in the inhibition of PLA_2-like proteins and to advance the current knowledge of their action mechanism.
Methods: Myographic and electrophysiological techniques were used to evaluate the inhibitory effect of zinc ions, isothermal titration calorimetry assays were used to measure the affinity between zinc ions and the toxin and X-ray crystallography was used to reveal details of this interaction.
Results: We demonstrated that zinc ions can effectively inhibit the toxin by the interaction with two different sites, which are related to two different mechanism of inhibition: preventing membrane disruption and impairing the toxin state transition. Furthermore, structural study presented here included an additional step in the current myotoxic mechanism improving the comprehension of the allosteric transition that PLA_2-like proteins undergo to exert their function.
Conclusions: Our findings show that zinc ions are inhibitors of PLA_2-like proteins and suggest two different mechanisms of inhibition for these ions.
General significance: Zinc is a new candidate that can assist in *anti*-venom treatments and can promote the design of new and even more accurate structure-based inhibitors for PLA_2-like proteins.

1. Introduction

Snakebite envenomings are an important global health issue. According to recent estimates, at least 421,000 ophidian accidents occur each year, of which 20,000 cases result in death, although these number may be underestimated by poor reporting in the areas with the highest frequencies of accidents, such as regions in Asia, Africa and Latin America [1]. Specifically, in the Latin America region, the high estimates expect approximately 130,000 accidents, in which 1.8% of cases result in death *per* year, and *Bothrops* genus snakes (American lance heads from Viperidae family) are the most important responsible genus [2]. In addition to the high mortality, the local myotoxicity of bothropic snake bites may cause permanent sequelae and disability, with member amputation in cases where the *anti*-venom is not quickly administered [3]. Most of these accidents happen in rural areas, and these consequences may incapacitate rural workers [4]. This scenario led the World Health Organization to classify snakebite envenomings as a neglected tropical disease [5].

Two groups of toxins, metalloproteinases and phospholipase A_2 (PLA_2) proteins, may cause the local myonecrosis which is the main effect caused by *Bothrops* snake bite. For some snakes of the Viperidae family, PLA_2 protein is the most abundant toxin in their venoms [6,7], and its rapid damage is one of the major difficulties in treating snake venom accidents because *anti*-venom therapy is not able to reverse its effects [4]. PLA_2s are small (approximate molecular weight of 14 kDa) and stable proteins, which frequently have 7 disulfide bridges. These

Abbreviations: Phospholipase A_2, PLA_2; Membrane-Docking Site, MDoS; Membrane-Disruption Site, MDiS; Bothropstoxin I, BthTX-I; Phrenic nerve-diaphragm, PND; Resting potential, RP; Polyethylene glycol, PEG; PEG4000, PEG4k; Protein Data Bank identification code, PDB id; Root-mean square deviation, RMSD; *p*-bromophenacyl bromide, BPB.

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enzymes are able to hydrolyze the *sn*-2 ester bond of phospholipids in the lipid bilayer of micelles, vesicles and membranes, releasing lysophospholipids and fatty acids through a catalytic mechanism that is dependent on calcium ions [8,9]. A subtype of PLA_2s, the PLA_2-like proteins, also named the PLA_2-homologues due to their similar tertiary structures and common evolutionary ancestor, do not possess catalytic activity, but still present high myotoxicity and a wide range of pharmacological activities [9,10]. The proteins from PLA_2-like group have different residues at 49 positions (Lys, Ser and Arg) and may present similar myotoxic mechanisms of action, although the most studied subgroup is the Lys49-PLA_2 proteins [10].

Since the identification of PLA_2-like toxins in the 1980s, these proteins have been investigated using many different approaches to elucidate their toxic mechanism [10]. The large charge distribution on the toxins' surface and their various pharmacological and toxic effects enhance the complexity and hinder our comprehension of their mechanisms of action. Functional studies highlighted the importance of their dimeric oligomerization [11,12], as well as the C-terminal region, which has a high distribution of basic and hydrophobic residues, for myotoxicity [13,14]. Based on the different dimeric orientations observed in available crystallographic structures, PLA_2-like proteins from the *Bothrops* genus were classified as being in: i) an **inactive state**, whose toxins are native with void hydrophobic channels and ii) an **active state**, whose toxins were complexed to either hydrophobic molecules or inhibitors. Recently, based on structural and functional studies, a myotoxic mechanism has been proposed that involves specific regions in the C-terminal region, the MDoS (Membrane-Docking Site) and the MDiS (Membrane-Disruption Site) [15]. The entrance of a hydrophobic molecule in the toxin hydrophobic channel would induce an allosteric modification of the protein, with dimer reorientation [16] from the **inactive** to **active state,** enabling the MDoS to dock with the cell membrane and the MDiS to perturb cell membrane integrity [15]. After the toxin penetrates the lipid bilayer and disturbs the phospholipids and membrane integrity, ion homeostasis is disrupted [9]. Consequently, the calcium influx increases membrane damage by inducing hypercontraction of myofilaments, mitochondrial damage, calcium-dependent proteases and cytosolic PLA_2s activity, leading to muscle necrosis [9,17]. All of these indirect effects of PLA_2-like activity enhance cytotoxicity [17].

One of the main themes in current toxinology is the development of alternatives to the conventional serum therapy that does not efficiently neutralize snake venom injuries. Currently, the majority of studies with this objective focus on compounds isolated from plants used in folk medicine in developing countries [18,19]. Functional and structural studies of the toxins complexed to these compounds led to the proposal of three different possibilities for the mechanism by which the PLA_2-like proteins are inhibited [20–22]. On the other hand, only a few studies were performed to evaluate the inhibitory effects of divalent cations against snake venoms and, more particularly, PLA_2-like toxins [23–25], despite their role in physiological functions and their use in the treatment of diseases [26,27]. Zinc ions are relatively harmless to humans and have been used in different treatments. It is an effective antioxidant and anti-inflammatory agent, and it may have beneficial effects on myocardial pathologies and atherosclerosis [28]. Zinc has been administered to prevent specific types of cancer [27,29,30] and blindness in patients with age-related macular degeneration, to complement the treatment of diarrhea, to assist the immune system, and to treat the common cold, Wilson's disease, and sickle cell disease [26].

Here, the complex between bothropstoxin I (BthTX-I), a PLA_2-like toxin from *Bothrops jararacussu* venom, and zinc ions was studied using functional, calorimetric and structural methods to obtain new insights into the mechanism by which the PLA_2-like proteins are inhibited. As result of this study, it has been shown that zinc ions are able to inhibit the myotoxic effect through two different ways. Furthermore, by comparing this structure with the crystallographic structures of other PLA_2-like proteins, more details of the current myotoxic mechanism [15] were revealed, particularly those related to the conformational changes of the MDiS region and the hydrophobic channel.

2. Materials and methods

2.1. Animals

Male Swiss mice (25–30 g) obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/Unicamp) were housed 10/cage at 23 °C on a 12 h light/dark cycle, with the lights on at 6 a.m. The animals had free access to food and water. For *in vitro* protocols, the animals were euthanized with isoflurane immediately prior the experiments according to the guidelines of the Brazilian College for Animal Experimentation (COBEA); this study was approved by the institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 3312-1).

2.2. Protein purification

BthTX-I was isolated from *Bothrops jararacussu* snake venom by ion-exchange chromatography on CM-Sepharose, as previously described [31].

2.3. Twitch-tension experiments

Mouse phrenic nerve-diaphragm preparations (PND) were mounted under a resting tension of 1 g in a 5 mL organ bath containing aerated (95% O_2 and 5% CO_2) Tyrode's solution (composition, in mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1, pH 7.0) at 37 °C and allowed to stabilize for 10 min prior to use, as described elsewhere [32,33]. Supramaximal stimuli (0.1 Hz and 0.2 ms) were delivered to the nerve from a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA), and the muscle twitches were recorded using a TRI201AD force displacement transducer coupled to a Quad Bridge Amp and LabChart 6.0 software (all from AD Instruments Pty Ltd., Bella Vista, Australia). The preparations were incubated with BthTX-I (20 μg/mL), $ZnCl_2$ (0.4 mM) or BthTX-I (20 μg/mL) + $ZnCl_2$ (0.4 mM) for 120 min or until complete neuromuscular blockade, and the changes in twitch-tension were recorded.

2.4. Intracellular recordings

The effects of BthTX-I (20 μg/mL), $ZnCl_2$ (0.4 mM) or BthTX-I (20 μg/mL) + $ZnCl_2$ (0.4 mM) on membrane resting potential (RP) were recorded using a mouse hemidiaphragm muscle mounted in a Lucite chamber containing Tyrode's solution (composition shown above), as previously described [32,34]. The RP was measured using an amplifier (AM 502 Tektronix) and digitized by an A/D converter CAD12/36 12 bits (Lynx, São Paulo, SP, Brazil) coupled to a microcomputer (Microtec, São Paulo, SP, Brazil) loaded with AqDados 5 software (Lynx, São Paulo, SP, Brazil). The RP were monitors in different regions of the muscle considering basal values as t_0 (control); the effects of BthTX-I (20 μg/mL), $ZnCl_2$ (0.4 mM) or BthTX-I (20 μg/mL) + $ZnCl_2$ (0.4 mM) were analyzed in different intervals (t_{15}, t_{30}, t_{60}, t_{90} and t_{120}).

2.5. Crystallographic studies of BthTX-I in the presence of zinc ions

Cocrystallization experiments were performed with lyophilized samples of BthTX-I and $ZnCl_2$. The protein was dissolved in ultrapure water, and the concentration was measured by determining the absorbance at 280 nm with a NanoDrop 2000c (Thermo Scientific™) using the theoretical protein molecular weight and extinction molar coefficient. Crystals were obtained by the hanging drop vapor diffusion method at 18 °C [35]. Hexagonal shape crystals were obtained from drops of 0.8 μL of protein (10.1 mg/mL), 0.09 μL of $ZnCl_2$ (1 M), 0.32 μL of $MnSO_4$ (12.5 mM) and 0.48 μL of reservoir solution composed of 0.1 M TRIS-HCl, pH 8.5,