Calcium imaging of muscle cells treated with snake myotoxins reveals toxin synergism and presence of acceptors


Abstract. Snake myotoxins have a great impact on human health worldwide. Most of them adopt a phospholipase A2 fold and occur in two forms which often co-exist in the same venom: the Asp49 toxins hydrolyse phospholipids, whilst Lys49 toxins are enzymatically inactive. To gain insights into their mechanism of action, muscle cells were exposed to Bothrops myotoxins, and cytosolic Ca$^{2+}$ and cytotoxicity were measured. In both myoblasts and myotubes, the myotoxins induced a rapid and transient rise in cytosolic [Ca$^{2+}$], derived from intracellular stores, followed, only in myotubes, by a large Ca$^{2+}$ influx and extensive cell death. Myoblast viability was unaffected. Notably, in myotubes Asp49 and Lys49 myotoxins acted synergistically to increase the plasma membrane Ca$^{2+}$ permeability, inducing cell death. Therefore, these myotoxins may bind to acceptor(s) coupled to intracellular Ca$^{2+}$ mobilization in both myoblasts and myotubes. However, in myotubes only, the toxins alter plasma membrane permeability, leading to death.

Keywords. Snake myotoxins, myoblasts, myotubes, PLA2, calcium imaging.

Introduction

Many species of poisonous snakes produce venoms containing toxins that rapidly cause extensive muscle damage, leading to a major pathology of the affected limb which, in some cases, may be followed by systemic myotoxicity, i.e. rhabdomyolysis [1–4]. The venom composition is complex and varies greatly not only with the snake genus, but also with species and even within the same animal species living in different ecological areas [5–7]. In any case major components of these venoms, both in terms of protein mass and of role in pathogenesis of envenomation [8], are toxins which target muscles and are therefore termed myotoxins [1–3, 9]. Over a hundred different myotoxins have been characterized so far [1, 10, 11]. The vast majority of these toxins are proteins of 14 kDa which adopt the typical fold of the phospholipase A2 (PLA2) enzymes with seven intra-chain disulfide bridges that confer high stability to them [10, 12]. The catalytic site of snake venom PLA2 is characterized by the presence of four key residues: His48, Asp49, Tyr52 and Asp99. His48 hydrogen binds the water molecule used for hydrolysis and the Asp49 plays the essential role of coordination...
and positioning the Ca\(^{2+}\) ion which binds and polarizes both the phosphate and the sn-2 carbonyl groups of the phospholipid molecule during hydrolysis [12]. There is evidence that the PLA2 activity of this Asp49 group of myotoxins is implicated in the intoxication of muscle cells, but this is not the only determinant, as other activities are involved as well [9].

A distinct group of PLA2 homologue myotoxins exhibits a replacement of the key Asp49 residue with other residues, with consequent loss of Ca\(^{2+}\)-binding and enzymatic activity. The most abundant of these catalytically-inert myotoxins have a lysine in position 49 and are therefore termed Lys49 myotoxins [3, 13]. The major toxicity-determining site in these PLA2 homologues is segment 115–129 of the C-terminal region, which includes a variable combination of positively-charged and hydrophobic/aromatic residues, with the ability to alter the bilayer membrane integrity [3, 10].

Frequently, the Asp49 and the Lys49 myotoxins are present within the same venom, though in variable proportions. This co-presence must have a relevant adaptive role, which is as yet unexplained [3, 13].

Even less is known on the cell binding step of myotoxic PLA2s, which is clearly essential for the display of their toxicity [14]. Receptors for myotoxins isolated from the venom of *Oxyuranus scutellatus* have been identified [15, 16] and it has been suggested that the receptor of the Lys49 myotoxin is the VEGF (vascular endothelial growth factor) receptor itself [17, 18]. The presence of specific plasma membrane receptors is also supported by the selectivity of some myotoxins for type I muscle fibers [19, 20]. The finding that various snake myotoxins are more toxic to differentiated myotubes than to undifferentiated myoblasts [21, 22] may indeed suggest the presence of specific toxin receptors in the differentiated, but not in the undifferentiated, cells. In addition, there is evidence that negatively-charged lipids in the plasma membrane may play a role in the binding of some myotoxic PLA2s [23–26].

Previous studies have documented that, in general, snake venom myotoxins damage muscle cells by acting on the sarcolemma, causing a major alteration of its permeability with consequent loss of membrane potential and cytosolic components and massive entry of external calcium [3, 9]. Calcium overload alone is sufficient to trigger a series of intracellular degeneration events, leading rapidly to necrotic cell death [11, 27].

The Asp49 myotoxins produce lysophospholipids (LysoPL) and fatty acids (FA), and LysoPL+FA were recently shown to permeabilize the plasma membrane of neurons to external calcium, leading to a prolonged increase of the cytosolic [Ca\(^{2+}\)]. [Ca\(^{2+}\)]c [28]. It is therefore possible that Asp49 myotoxins act similarly on muscle cells. On the other hand, the Lys49 myotoxins are catalytically-inactive PLA2 homologues which, nonetheless, are very effective inducers of a membrane leakage with calcium entry [9, 22]. However, no quantitative measurements and visualization of cytosolic calcium concentration ([Ca\(^{2+}\)]c) on muscle cells have been reported so far. Therefore, we have performed an extensive analysis of the activities of both Asp49 and Lys49 myotoxins on muscles cells in culture, using calcium imaging and sensitive cytotoxicity assays. Unexpected results of great relevance for the understanding of the molecular mechanism of action of these toxins were obtained, and their implications for the pathogenesis of muscle damage induced by snake venoms are discussed.

**Materials and methods**

**Myotoxins.** Bothrops asper and *B. jararacussu* crude venoms were pools obtained from snakes collected in Costa Rica and Brazil, respectively. Myotoxic PLA2 were purified by cation-exchange chromatography on carboxymethyl-Sephadex C-25 (Pharmacia, Sweden) as previously described: Mt-I (Bothrops asper myotoxin, Asp49) [29], Mt-II (Bothrops asper myotoxin II, Lys49) [30] and BthTX-I (Bothrops jararacussu myotoxin I, Bothropstoxin-I, Lys49) [31]. Purity of the toxins was assessed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE) with Coomassie Blue staining and by reverse-phase high performance liquid chromatography on a C4 column (250×4.6 mm; Vydac), eluted at 1.0 ml/min with a gradient from 0 to 60% acetonitrile in 0.1% trifluoroacetic acid. Bovine pancreatic PLA2 (pPLA2) was obtained from Sigma (P-8913, USA). PLA2 activity was measured with an sPLA2 assay kit (Cayman Chemicals, Ann Arbor, MI, USA), using the 1,2-dithio of diheptanoyl phosphatidylcholine analog, which serves as a substrate for most PLA2, with the exception of cytosolic PLA2. Absorbance was measured every minute after adding the substrate to obtain fifteen time points. Upon hydrolysis of the tho-ester bond at the sn-2 position by PLA2, free thiols were detected using DTNB (5,5-dithio-bis-(2-nitrobenzoic acid)).

**Lipid mixture preparations.** 1-myristoyl-lysophosphatidylcholine (mLysoPC, Sigma) and oleic acid (OA, Sigma) mixture (mLysoPC+OA) was prepared as previously described [32].

**Cell culture.** The cell model used as toxin target was the murine skeletal muscle C2C12 line, obtained from the American Type Culture Collection (CRL-1772,