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The C-terminal region of a Lys49 myotoxin mediates Ca^{2+} influx in C2C12 myotubes

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ABSTRACT

Myotoxins are abundant components of snake venoms, being a significant public health problem worldwide. Among them, Lys49 phospholipase A_2 homologue myotoxins cause extensive necrosis in skeletal muscle tissue. Their mechanisms of action are still poorly understood, but there is evidence that the C-terminal region is involved in membrane damage leading to myotoxicity. To investigate the effect of the C-terminal peptide 115–129 of *Agkistrodon contortrix laticinctus* myotoxin on the plasma membrane of myoblasts and myotubes, the entry of Ca^{2+} was monitored by fluorescence imaging, and the ensuing cytotoxicity was determined. The myotoxin synthetic peptide was found to act selectively on myotubes, which were rapidly overloaded with Ca^{2+} with ensuing necrosis. The profile of intracellular Ca^{2+} increase induced by the C-terminal peptide, but not by its scrambled version control, reproduces the second, prominent wave of the biphasic response documented in previous studies using whole Lys49 myotoxins. These observations provide relevant insights into the mechanism of action of this family of toxins, with implications for the understanding of their structure–function relationships.

1. Introduction

Necrosis of skeletal muscle is a frequent manifestation of snakebite envenomings, which may lead to permanent tissue loss and disability (Warrell, 2004; Gutiérrez et al., 2006). Such effect is caused by venom components that directly target muscle fibers, most notably toxins with phospholipase A_2 (PLA_2) structure (Gutiérrez and Ownby, 2003; Lomonte et al., 2003a). PLA_2s are common and abundant venom components which, in addition to myotoxicity, may also display a potent presynaptic neurotoxic action (Montecucco et al., 2008). Venom PLA_2s found in snakes of the family Viperidae are classified within the structural group IIA, with subunits of 121–122 amino acid residues characterized by a specific pattern of disulfide bonds (Schaloske and Dennis, 2006). Among them, two subgroups can be discerned. One consists of enzymatically active PLA_2s with a characteristic Asp49, which is a key residue for their catalytic mechanism. The other subgroup includes proteins with a conserved PLA_2 fold, but presenting the replacement of Asp49 with Lys49 (or other amino acids), which lack PLA_2 activity. The latter toxins have been termed, therefore, PLA_2 homologues (Lomonte et al., 2003a, 2009). Despite their radical difference in catalytic ability, both Asp49 and Lys49 proteins are myotoxic, implying two distinct mechanisms of action.

Asp49 PLA_2 myotoxins depend on their enzymatic activity to damage skeletal muscle fibers (Gutiérrez and Ownby, 2003). In contrast, the catalytically inactive Lys49 PLA_2

Abbreviations: p-Acl, C-terminal peptide 115–129 of *Agkistrodon contortrix laticinctus* myotoxin; p-Scr, scrambled version peptide; PLA_2, phospholipase A_2; $[Ca^{2+}]_c$, cytosolic calcium ion concentration; EGTA, ethylene glycol tetraacetic acid.

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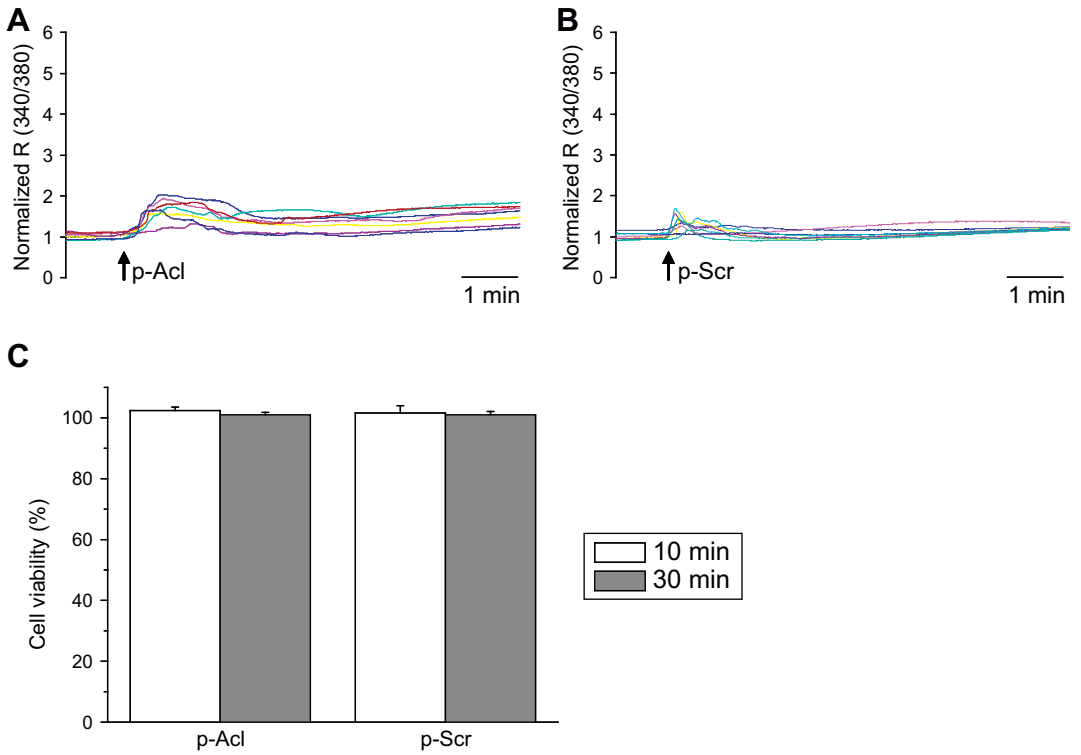

Fig. 1. Effects of peptides on $[Ca^{2+}]_c$ and viability of C2C12 myoblasts. In panels A and B cells were loaded with fura-2 as described in Section 2. The effect on $[Ca^{2+}]_c$ was followed as a change in the fura-2 fluorescence ratio (340/380 nm) in different cells after addition (arrows) of p-Acl (panel A) or p-Scr (panel B) at 250 μg/ml. Each trace represents the 340/380 ratio change with time of a single cell. For presentation, the ratios were normalized to the resting value. Panel C represents the cell viability, measured with the MTS assay, after treatment with peptides for 10 (empty bars) or 30 min (grey bars).

homologues utilize as major determinant of toxicity a site encompassing residues 115–129 in the C-terminal region, which includes a variable combination of cationic and hydrophobic/aromatic residues (Lomonte et al., 2003a; Chioato and Ward, 2003). There is evidence that both types of myotoxins alter the plasma membrane permeability and trigger a series of ensuing degenerative events, but the molecular mechanisms involved are still poorly characterized, and the identity of target acceptor moieties remains unknown (Montecucco et al., 2008).

Previous studies have shown that Lys49 PLA_2 homologues preferentially affect differentiated skeletal muscle myotubes, in comparison to their immature myoblast precursors (Angulo and Lomonte, 2005; Cintra-Francischinelli et al., 2009). Upon exposure to Lys49 myotoxins, a rapid increase in intracellular Ca^{2+} concentration occurs in C2C12 myotubes (Villalobos et al., 2007). This rise is characterized by a consistent biphasic pattern: an initial calcium mobilization from intracellular stores, followed by a massive influx from the extracellular milieu (Cintra-Francischinelli et al., 2009). Ultimately, such alterations in membrane permeability and intracellular Ca^{2+} levels bring the muscle cells to the point of irreversible damage and necrotic death.

Short synthetic peptides representing the C-terminal region of Lys49 myotoxins have cytolytic and muscle-damaging activities similar to their parent proteins, although they display a lower potency (Lomonte et al., 1994, 2003b; Núñez et al., 2001; Angulo and Lomonte, 2005; Gebrim et al., 2009). Their precise mode of action is not known and, therefore, it is of interest to characterize in detail the effects of such kind of small molecules on muscle cell Ca^{2+} homeostasis. In this study we used the relevant cellular model of C2C12 myoblast and myotube cultures, and advanced Ca^{2+} imaging which provides time and space resolved information on live cells. The synthetic C-terminal peptide 115–129 of *Agkistrodon contortrix laticinctus* (ACL) myotoxin (Selistre de Araujo et al., 1996; sequence numbering according to Renetseder et al., 1985) was prepared and studied in the present work. By comparing the results obtained here with those previously recorded upon exposure to Lys49 myotoxins, relevant insights into the mechanism of action of these proteins were obtained, with implications for the understanding of their structure–function relationships.

2. Materials and methods

2.1. Synthetic peptides

A 13-mer peptide (KKYKAYFKFKCKK; p-Acl), corresponding to the sequence 115–129 of ACL myotoxin, a Lys49 PLA_2 homologue from the venom of the snake *A. contortrix laticinctus*, and a scrambled version (FKFKYKKACKKYK; p-Scr) were synthesized at the Protein Chemistry Laboratory of the University of Padova. They were purified with a C18 reverse phase NovaPak column, and were tested at a final concentration of 250 μg/ml (146 μM), selected on the basis of