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Research Article

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

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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 phospolipase 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

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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 PLA2 s, 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 PLA2 s [–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 thio-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,

ATCC). C2C12 cells were maintained at sub-confluent levels in growth medium consisting of Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% foetal bovine serum (EuroClone). To induce differentiation (5–6 days), cells were grown to 80% confluence and then the medium was replaced with DMEM supplemented with 2% horse serum (Gibco) and changed every 24–48 h.

Calcium measurements. Cells, plated on cover slips (24 mm diameter), were loaded with fura-2 by incubation with 3 μM fura-2/AM at 37 °C for about 30 min in modified Krebs–Ringer Buffer (see below) containing 0.04% pluronic (Molecular Probes, Inc., Eugene, OR). To prevent fura-2 leakage and sequestration, 250 μM sulfinpyrazone was present throughout the loading procedure and $[Ca^{2+}]c$ measurements. The cover slips were washed with a modified Krebs–-Ringer Buffer (mKRB, 140mM NaCl, 2.8mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 10mM HEPES, 11mM glucose pH 7.4), mounted on a thermostated chamber (Medical System Corp., New York, USA) at 37 °C, placed on the stage of an inverted microscope (Zeiss, Axiovert 100 TV) equipped for single cell fluorescence measurements and imaging analysis (TILL Photonics, Martinsried, Germany). Where indicated, a Ca^{2+}-free EGTA (200 μM)-containing medium was used. The sample was alternatively illuminated (t = 200 ms) by monochromatic light (at 340 and 380 nm wave lengths), every second for 10–20 min after toxin exposure, through a 40x oil immersion objective (NA = 1.30; Zeiss). The emitted fluorescence was passed through a dichroic beamsplitter (455DRPL), filtered at 505–530 nm (Omega Optical and Chroma Technologies, Brattleboro, VT, USA) and captured by a cooled CCD camera (Imago, TILL Photonics). For presentation, the ratios (F340/F380) of different cells were off-line normalized to the resting value measured within the first minute of the experiment.

Cytotoxicity assay. Myoblasts and differentiated myotubes were grown in 96-well plates and then exposed to toxins and lipid mixtures for ten or thirty minutes; their viability was then measured with the MTS (3-(4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl-2-(4-sulfonphenyl)-2H-tetrazolium, inner salt) assay. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used and instructions from the manufacturer were followed.

Results

Characterization of the snake myotoxins. In this study we have compared the effects of Asp49 and Lys49 myotoxins from two *Bothrops* species on both $[Ca^{2+}]c$ and viability of muscle cells in culture. The myotoxins were purified following previously described procedures and their SDS-PAGE profiles are depicted in Figure 1. In addition to the major bands at ~14 kDa, weak bands with electrophoretic mobility corresponding to oligomers of the two Lys49 myotoxins could be observed. This was noted in previous studies and may correspond to a natural tendency of these toxins to oligomerize [33]. Purified Mt-I (Asp49) preserved a high PLA2 activity (282.0 ± 24.6 μmol/min/mg), whereas no significant phospholipid hydrolysis was catalysed by the two Lys49 toxins Mt-II and BthTX-I (0.028 ± 0.003 and 0.62 ± 0.42 μmol/min/mg, respectively), which is a further indication of their purity.

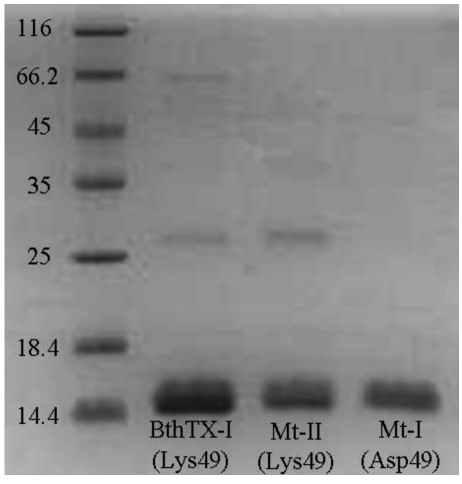

Figure 1. Biochemical characterization of the snake myotoxins. SDS-PAGE of *Bothrops* myotoxins showing their purity and the presence of oligomers of the Lys49 toxins.

Effect of the *Bothrops* myotoxins on cytosolic $[Ca^{2+}]$ of C2C12 myoblasts. Earlier work has shown that the myogenic cell line C2C12 represents a good model for the study of myotoxicity induced by class II PLA2 myotoxins [21, 22, 33, 34] and it was reported that undifferentiated C2C12 myoblasts are less sensitive than myotubes to various snake myotoxins, as measured by lactic dehydrogenase release [21]. This finding opened the possibility that few or no acceptors for myotoxins are present on the myoblast cell surface. However, Figure 2 shows that the three *Bothrops* myotoxins studied here did interact with myoblasts, in as much as they caused a transient $[Ca^{2+}]c$ rise within a few seconds after addition (Fig. 2A and C). However, all cells returned to their basal $[Ca^{2+}]c$ within 1–2 minutes and the toxins interactions did not have any effect on cell viability at the concentration used (50 μg/ml) (not shown); myoblasts were damaged only when much higher concentrations of myotoxin were used,

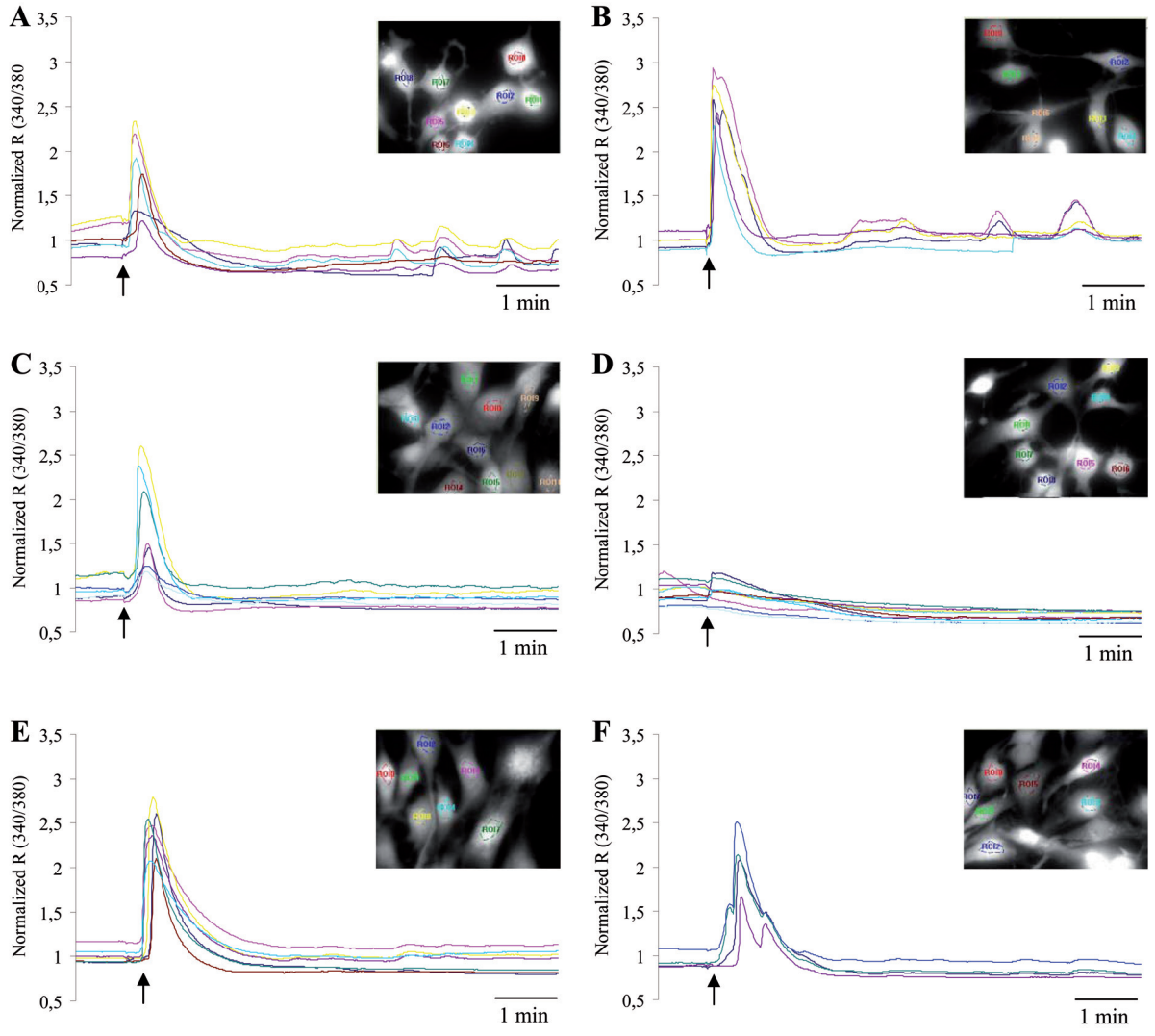

Figure 2. Effect of snake myotoxins on $[Ca^{2+}]_c$ in C2C12 myoblasts. Cells were loaded with fura-2 as described in the experimental procedures. Various effectors (myotoxins or PLA2) were added (arrows) and the effect on $[Ca^{2+}]_c$ was followed as a change in the fura-2 fluorescence ratio (340/380 nm) in different cells identified in the corresponding insets. Each trace represents the 340/380 ratio change with time of a single cell; in this and the following experiments each panel is representative of experiments carried out under the same conditions in at least four different cell preparations. For presentation, the ratios were normalised to the resting value. BthTX-I (Lys49) (*A*), Mt-II (Lys49) (*B*), Mt-I (Asp49) (*C*) and pPLA2 (*D*) were added at 50 μg/ml final concentration in Ca^{2+}-containing buffer, whereas in panels E and F, experiments with BthTX-I (Lys49) and Mt-II (Lys49) (50 μg/ml each) were performed in Ca^{2+}-free EGTA-containing medium.

within the range of 200–250 μg/ml [22]. The bovine pancreatic PLA2, which is not myotoxic, had no effect on $[Ca^{2+}]$c (Fig. 2D).

The transient $[Ca^{2+}]$c increase induced by the myotoxins was due primarily to Ca^{2+} mobilization from intracellular stores, as its amplitude and duration were found to be hardly different in cells bathed in a Ca^{2+}-free, EGTA-containing medium (Fig. 2E and F). Small, but reproducible, differences among the toxins tested were noticed in terms of shape and amplitude of the evoked $[Ca^{2+}]$c transients, but overall the effects were very similar. This clearly demonstrates that myotoxin acceptors are present on the myoblast and that this binding is not sufficient to trigger later events of cytotoxicity, thus discarding the hypothesis of myoblast being insensitive because of a lack of toxin acceptors.

Effect of the *Bothrops* Lys49 myotoxins on $[Ca^{2+}]$c of C2C12 myotubes. C2C12 myoblasts can be induced to differentiate into multinucleated myotubes, which have many of the biochemical and physiological features of mature muscle fibres [35]. The pattern of $[Ca^{2+}]$c changes induced by the snake myotoxins in differentiated C2C12 myotubes had never been studied before and it was very different from that obtained in undifferentiated cells. Figure 3 shows that the two Lys49 myotoxins, Mt-II and BthTX-I, caused a rapid

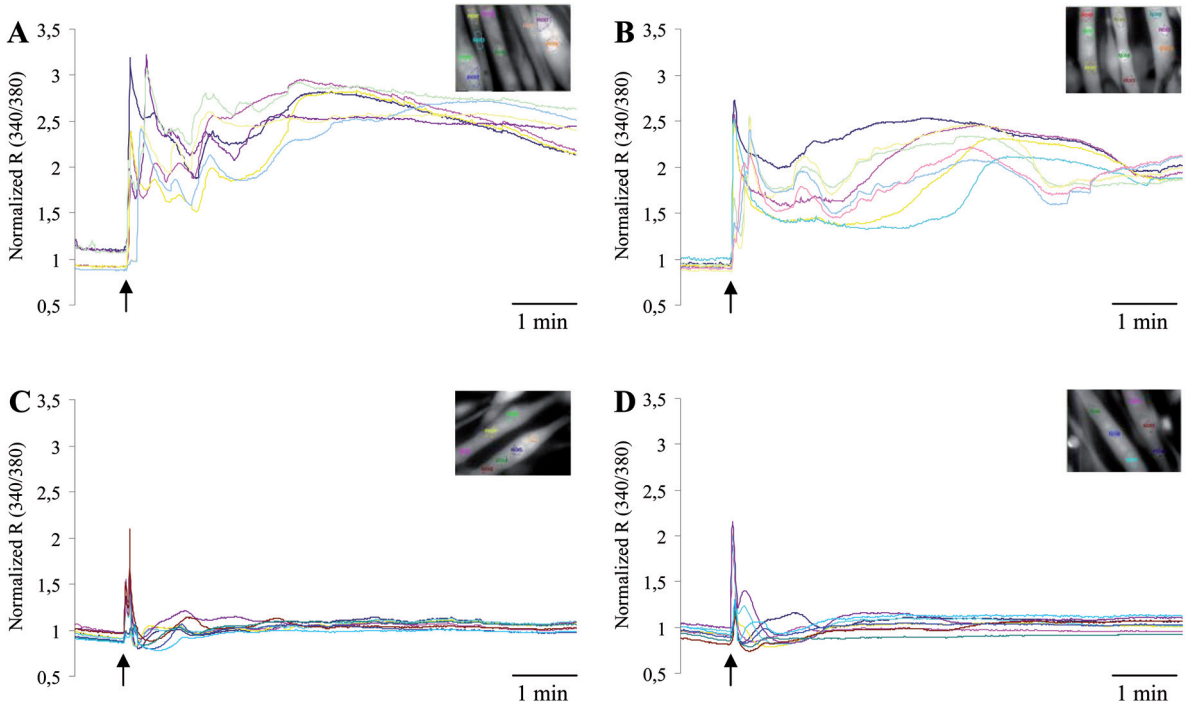

Figure 3. Effect of *Bothrops* Lys49 myotoxins on Ca^{2+} homeostasis in C2C12 myotubes loaded with fura-2. Experiments were carried out as in Figure 2 in Ca^{2+}-containing medium (*A* and *B*) or Ca^{2+}-free EGTA-containing medium (*C* and *D*) after the addition (arrows) of BthTX-I (50 μg/ml) (*A* and *C*), and Mt-II (50 μg/ml) (*B* and *D*). Owing to the elongated shape of myotubes (see insets), in this and in the following figures, the individual traces refer to different cells or to different regions of the same cell.

increase in $[Ca^{2+}]c$, similar to that recorded in myoblasts, followed, however, by a second prolonged $[Ca^{2+}]c$ rise, often characterized by slow waves (Fig. 3A and B). This second, large $[Ca^{2+}]c$ increase was abolished by incubating the cells in Ca^{2+}-free, EGTA-containing medium (Fig. 3C and D); noteworthy, in the latter condition, the first $[Ca^{2+}]c$ peak was also smaller than that observed in Ca^{2+} medium, unlike the situation of myoblasts, where removal of extracellular Ca^{2+} resulted in a marginal reduction of the myotoxin induced $[Ca^{2+}]c$ increase. In addition, when the toxins were applied to myotubes incubated in Ca^{2+}-free, EGTA-containing medium, the duration of the $[Ca^{2+}]c$ rise was very short, i.e. the $[Ca^{2+}]c$ was back to resting levels in 2–5 seconds, again at variance with the situation observed in myoblasts (compare Fig. 2E,F and Fig. 3C,D). Figure 3 also shows that there is some heterogeneity in the kinetics of the $[Ca^{2+}]c$ increases induced by the myotoxins in different cells, though after 10 min similar levels of high $[Ca^{2+}]c$ were attained in all cells. Taken together, these findings document the massive and rapid entry of Ca^{2+} from the extracellular medium induced in myotubes and show the validity of the imaging method in the study of the cellular pathogenesis induced by the Lys49 myotoxins.

Effect of the *Bothrops* Asp49 myotoxin on cytosolic $[Ca^{2+}]$ of C2C12 myotubes. The effect of the Mt-I (Asp49) toxin, which has PLA2 activity, on $[Ca^{2+}]c$ of myotubes was somewhat different from that observed with the Lys49 myotoxins in the same cell preparation. Addition of the toxin caused a first, small $[Ca^{2+}]c$ peak (Fig. 4A), followed by a return of $[Ca^{2+}]c$ close to basal levels within one minute; this first Ca^{2+} peak was observed also in a medium in which Ca^{2+} was replaced by Sr^{2+} (Fig. 4B), which does not support the hydrolytic activity of PLA2 enzymes or in Ca^{2+} free medium supplemented with EGTA (not shown). This latter finding indicates that Mt-I (Asp49) toxin is capable of binding to myotubes and promoting an early $[Ca^{2+}]c$ increment in the absence of extracellular Ca^{2+} and catalytic activity. In Ca^{2+}-containing medium, after a variable period of time from addition of the toxin (2–3 minutes), the $[Ca^{2+}]c$ started to increase again, but slowly and with an irregular pattern. This novel finding clearly documents the different modes of cytosolic Ca^{2+} alteration by the Asp49 and the Lys49 myotoxins. This slow and irregular $[Ca^{2+}]c$ increase was kinetically very different from that elicited by the Lys49 myotoxins and somehow reminiscent of that observed in neurons treated with PLA2 neurotoxins or with their phospholipid hydrolysis products: lysophospholipids and fatty acids [28]. We thus tested the

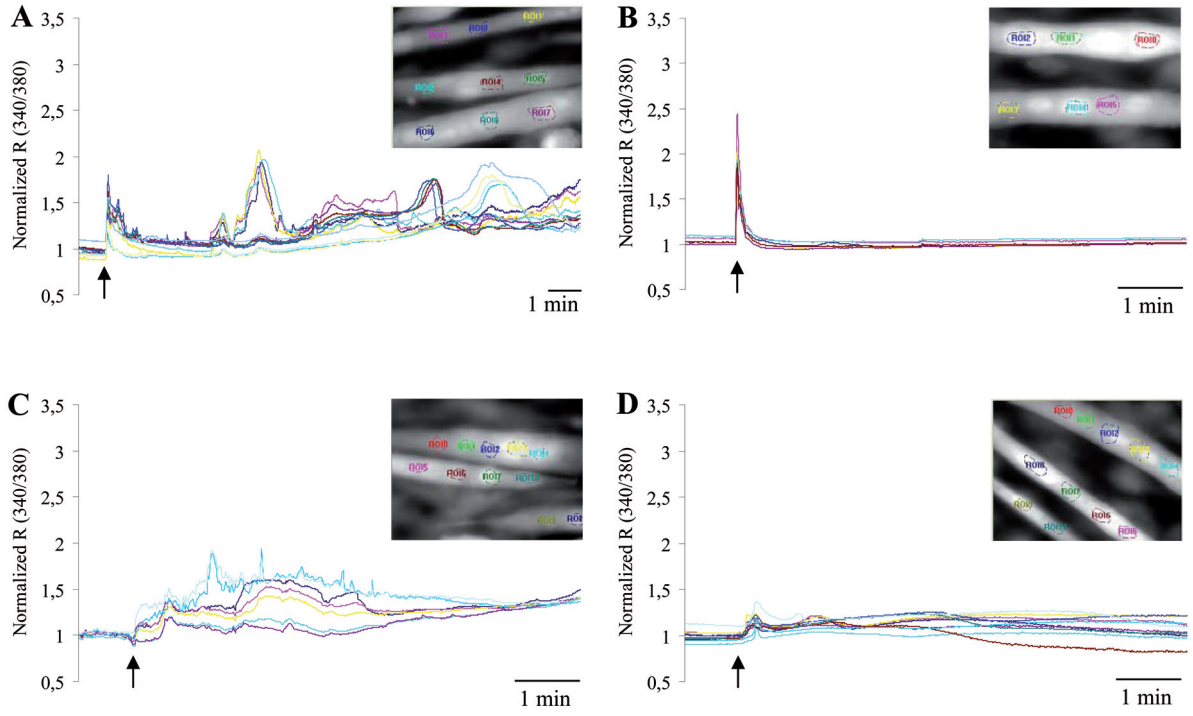

Figure 4. Effect of a *Bothrops* Asp49 myotoxin and the products of PLA2 activity on $[Ca^{2+}]_c$ in C2C12 myotubes. Experiments were carried out as in Figure 2, in the presence of external Ca^{2+} (*A* and *C*) or in a medium where Ca^{2+} was replaced by Sr^{2+} (*B*) or Ca^{2+}-free EGTA-containing medium (*D*). The arrows indicate the addition of Mt-I (50 μg/ml) (*A* and *B*) or mLysoPC+OA (100 μM) (C and D).

effect of an equimolar mixture of 1-myristoyl-lysophosphatidylcholine (mLysoPC) and oleic acid (OA) on the $[Ca^{2+}]$c of C2C12 myotubes (Fig. 4C); indeed, the mLysoPC+OA mixture induced a rise in $[Ca^{2+}]$c, dependent on external calcium (Fig. 4D) and with a kinetic profile very similar to the prolonged and irregular $[Ca^{2+}]$c caused by the Mt-I (Asp49) toxin. Taken together, these data suggest that there are two phases in the rise of $[Ca^{2+}]$c caused by the *Bothrops* Lys49 myotoxins: the first one being transient and dependent on intracellular calcium stores and the second one, more sustained, and clearly dependent on extracellular Ca^{2+}. In the case of the Asp49 myotoxin, the first $[Ca^{2+}]$c rise is similar (albeit smaller) to that induced by the two Lys49 toxins, while the second one appears to be slower in onset, more irregular and kinetically similar to that induced by mLysoPC+OA. This may be taken as an indication that these myotoxins bind to similar acceptor sites, but that they then follow different biochemical and biophysical pathways to increase membrane permeability.

Synergism of the Asp49 and Lys49 myotoxins. The evolutionary advantage of presenting catalytically-active Asp49 PLA2 toxins together with catalytically-inactive Lys49 variants within the same venom is not evident [3]. One possibility is that the two toxins cooperate somehow in membrane perturbation and permeabilization. When myotubes were treated at the same time with low doses (12.5 μg/ml) of Mt-I (Asp49) and Mt-II (Lys49), doses that by themselves do not induce the second and sustained phase of Ca^{2+} increase (Fig. 5A and B), a marked rise in the second large $[Ca^{2+}]$c increase, with no effect on the first transient peak, was observed (Fig. 5C). The amplitude of the rise of $[Ca^{2+}]$c induced by the low doses of the two toxins together was not additive, but rather appeared to be synergistic. Notably, no synergism was observed on the transient $[Ca^{2+}]$c increase in myoblasts, where the mixture of 25 μg/ml of each of the two *B. asper* myotoxins did not cause cell death or altered $[Ca^{2+}]$c (only the transient $[Ca^{2+}]$c peak similar to that shown in Fig. 2 was observed) (not shown). This synergistic activity was in keeping with the data of Figures 3 and 4, which suggested two different modes of inducing the large rise in the plasma membrane permeability to Ca^{2+} by Mt-I (Asp49) and Mt-II (Lys49) toxins. If the cell action of Mt-I (Asp49) is indeed mediated by the production of lysophospholipids and fatty acids, then the simple addition of their mixture to the cell should synergize with the Lys49 myotoxin. Figure 5D shows that this is indeed the case, indicating that the presence within the membrane of mLysoPC and OA made it more sensitive to the action

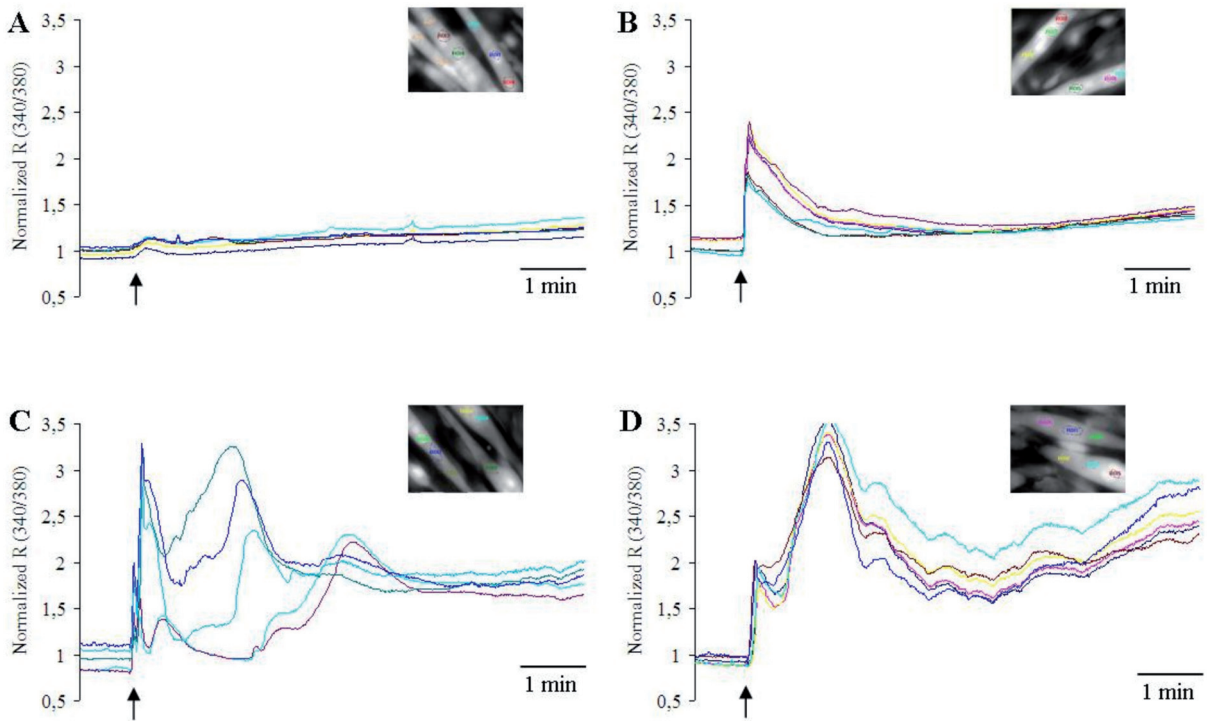

Figure 5. Synergism of Lys49 and Asp49 *Bothrops asper* myotoxins on $[Ca^{2+}]_c$ in C2C12 myotubes loaded with fura-2. Experiments were carried out as in Figure 3, in the presence of external Ca^{2+}. Where indicated by arrows, Mt-I (12.5 μg/ml) (*A*), or Mt-II (12.5 μg/ml) (*B*), or Mt-I (12.5 μg/ml) + Mt-II (12.5 μg/ml) (*C*), or Mt-II (12.5 μg/ml) + mLysoPC+OA (100 μM) (*D*) were added.

of Mt-II (Lys49). Among the components of the lipid mixture, when applied alone, only mLysoPC was effective on cell death, while FA was essentially inactive (Fig. 6E).

Myotoxin-induced cytotoxicity in C2C12 myotubes and myoblasts. A prolonged increase of $[Ca^{2+}]c$ is known to be cytotoxic in most cell models and also *in vivo* [11, 36, 37]. We have thus investigated the cytotoxic effects of the myotoxins, using the highly sensitive assay based on the reduction of MTS (see the experimental procedures). Figure 6A, B and C shows the dose/response profiles of the cytotoxicity of the three myotoxins on myotubes, assessed after 10 and 30 minutes. Cytotoxicity was found to be clearly time- and dose-dependent, a finding which complements the above described sustained $[Ca^{2+}]c$ in the presence of myotoxins. The two Lys49 myotoxins, isolated from different *Bothrops* species, appeared to have a similar cytotoxic potency, while the Asp49 myotoxin was slightly less potent. At doses > 25 μg/ml cytotoxicity was evident as early as after 10 minutes of incubation with either group of myotoxins. Most importantly, the cytotoxic effect was dramatic when low doses (12.5 μg/ml) of the two toxins, Mt-I (Asp49) and Mt-II (Lys49), are added together: in the latter case only one third of cells was viable after 10 minutes and all cells were dead after 30 minutes (Fig. 6D), whilst when added alone the two toxins caused only minor effects. Figure 7A shows that the synergism on cytotoxicity was present at a concentration of toxins (5 μg/ml) at which either one, applied alone, had no effect on cell viability (Fig. 6B and C). Furthermore, the two toxins added together displayed a clear synergistic effect also in their capacity to induce the prolonged $[Ca^{2+}]c$ rise (Fig. 7B), but not on the initial peak. As predicted, in the absence of external Ca^{2+}, Mt-I (Asp49) was completely non toxic (Fig. 6C) and did not synergize with Mt-II (Lys49) (not shown), consistent with the notion that this toxin requires Ca^{2+} and PLA2 activity for its biological action. In contrast, the two Lys49 myotoxins were still toxic in Ca^{2+}-free medium (Fig. 6A and B), in full agreement with previous findings [3, 10, 23, 38]. Cell death under these conditions was, however, slightly lower after 30 min of incubation. It should be noted that no cytotoxicity was detected in myoblasts even at high concentration of the three myotoxins (50 μg/ml) after 30 min of exposure (not shown).

Discussion

Here, we have described the effects on muscle cell $[Ca^{2+}]c$ and viability of highly purified preparations of three different snake myotoxins: two Lys49 PLA2

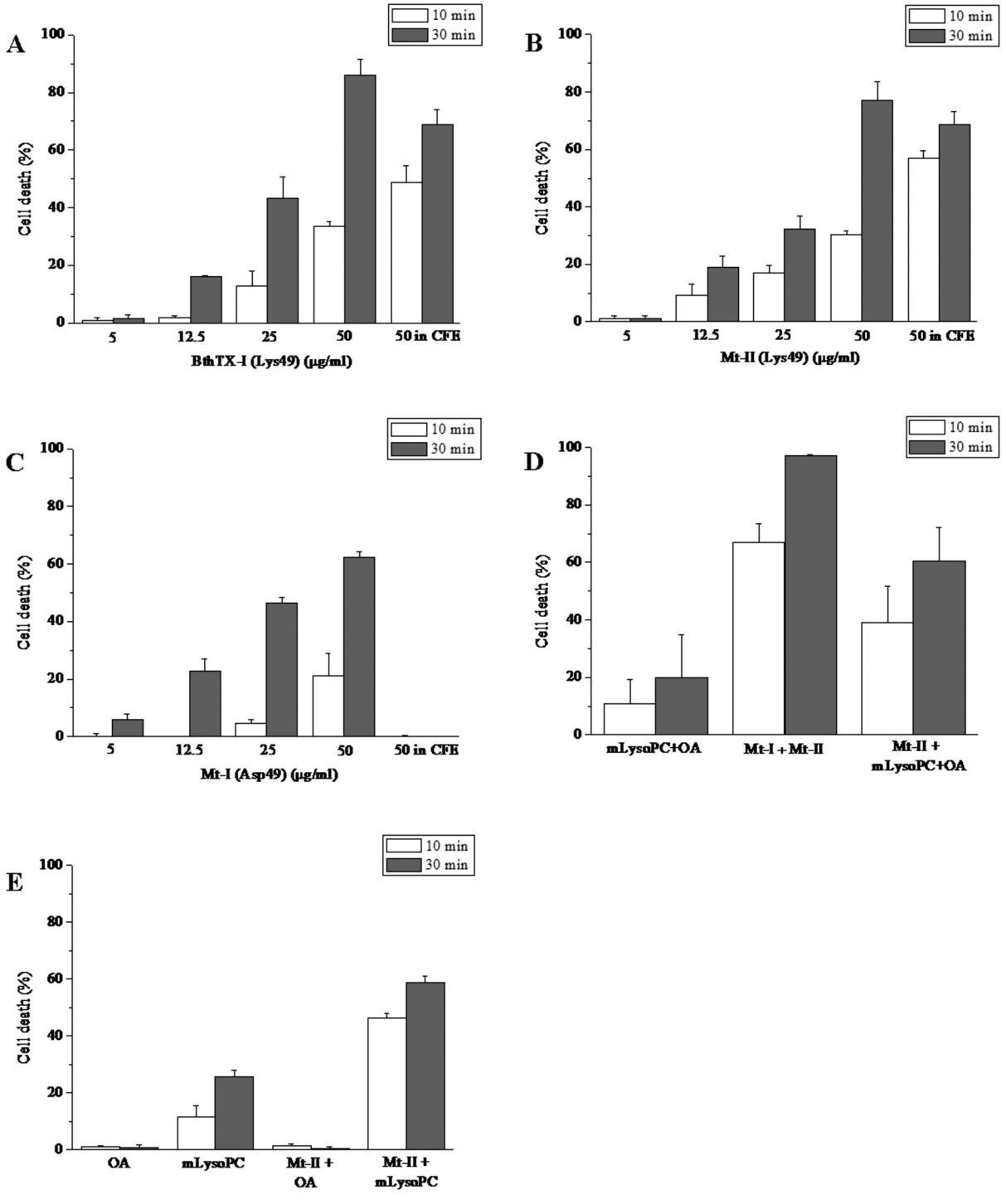

Figure 6. Viability of C2C12 myotubes upon incubation for different time periods with *Bothrops* myotoxins alone or in combination with either the mLysoPC+OA lipid mixture or the single lipid components. BthTX-I (Lys49) (*A*), Mt-II (Lys49) (*B*), Mt-I (Asp49) (*C*) were incubated at the indicated doses for 10 (empty bars) or 30 (grey bars) minutes in a Ca^{2+}-containing or Ca^{2+}-free EGTA medium (CFE). Panel D shows the effect of a mixture of: mLysoPC (100 μM) and OA (100 μM); Mt-I (12.5 μg/ml) and Mt-II (12.5 μg/ml); Mt-II (12.5 μg/ml) and mLysoPC+OA (100 μM each). Panel E shows the effect of OA (100 μM) or mLysoPC (100 μM) alone or in combination with Mt-II (12.5 μg/ml). Bars represent mean values ± SEM estimated in three or more experiments run in duplicates.

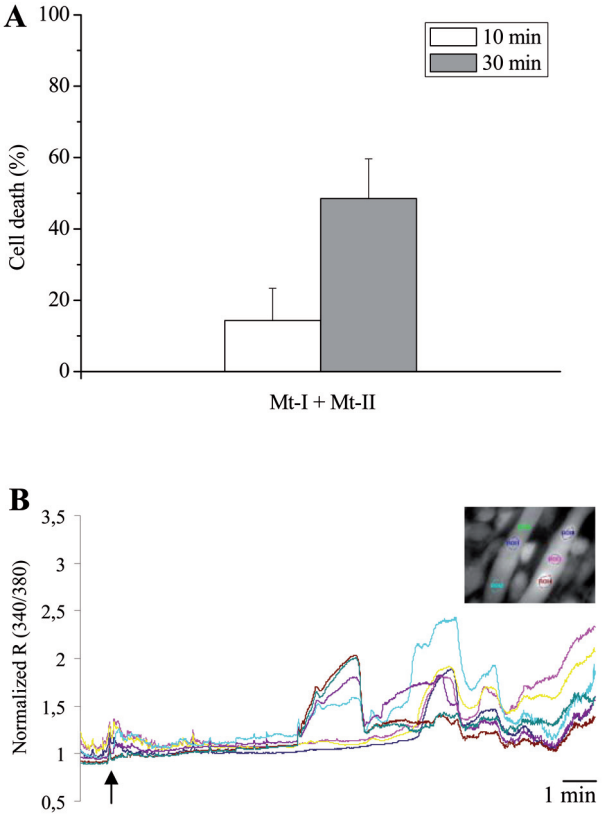

Figure 7. Synergic effects of low doses of Lys49 and Asp49 *Bothrops asper* myotoxins in C2C12 myotubes. Panel A. Viability of C2C12 myotubes after 10 or 30 minutes of incubation with the two myotoxins. Panel B. Changes in $[Ca^{2+}]_c$ after addition (arrow) of Mt-I (Asp49) (5 μg/ml) + Mt-II (Lys49) (5 μg/ml) in Ca^{2+}-containing medium.

homologue toxins which are enzymatically inactive (Mt-II and BthTX-I) and one active enzyme which has the conserved and essential Asp residue in position 49 (Mt-I). A first important observation is that the three snake PLA2 myotoxins are capable of binding to both undifferentiated and differentiated skeletal muscle cell lines in culture, causing a rapid and transient rise in $[Ca^{2+}]$c, which originates from intracellular stores. This is indirect but compelling evidence that not only myotubes, but also myoblasts, are capable of binding these myotoxins. The chemical nature of these acceptors was not identified and is beyond the purpose of the present work. Recently, the VEGF receptor was found to bind a catalytically inactive Lys49 myotoxin from *Agkistrodon piscivorus* [17], but not the active PLA2 Asp49 myotoxins of the same and related venoms [18]. However, the VEGF receptor does not appear to be involved in the present findings because the addition of VEGF to C2C12 myoblasts and myotubes does not induce a transient $[Ca^{2+}]$c peak and the VEGF-R2 inhibitor VI did not prevent the cell toxicity and Ca^{2+} response profile induced by the myotoxins (not shown). The important result is that the *Bothrops* myotoxins used here interact with cell membrane acceptor(s) coupled to intracellular calcium store mobilization, and this is a relevant indication for future attempts of identification of the acceptor molecule(s).

The effect on $[Ca^{2+}]$c of the myotoxins is clearly different in myoblasts and myotubes; the toxins induced only a transient early increase in $[Ca^{2+}]$c in myoblasts, while provoking a biphasic $[Ca^{2+}]$c increase in myotubes, i. e. a transient early reversible increment followed by a second and sustained elevation. This second increase clearly depends on Ca^{2+} influx from the extracellular fluid after plasma membrane damage, damage that can occur also in the absence of external Ca^{2+} [3, 9]. The biophysical and structural nature of this large membrane permabilization is beyond the scope of the present work. What is clearly shown here is that muscle videoimaging is a very sensitive and appropriate method to study the action of myotoxins and that this method provides an accurate description of the kinetics of the process.

Despite the fact that the end result of the action of both Lys49 and Asp49 PLA2 myotoxins is similar, i. e. plasma membrane perturbation leading to a prominent Ca^{2+} influx and irreversible cell damage, the mechanisms involved are different. Lys49 PLA2 homologues are capable of altering membrane integrity by a non-catalytic mechanism which is likely dependent on the membrane-perturbing effect of a stretch of hydrophobic and cationic residues located at the C-terminal region of these proteins [3, 39, 40]. In contrast, our present observations indicate that the sustained increment in $[Ca^{2+}]$c induced by Asp49 PLA2 Mt-I myotoxin depends on its enzymatic activity and is mechanistically related to the action of the products of phospholipid hydrolysis within the membrane. The present findings agree with previous observations that inhibition of the catalytic activity of *Bothrops* spp. myotoxic Asp49 PLA2 s abrogates *in vivo* myotoxicity [41, 42]. Such mechanistic divergence in membrane damage explains the different kinetics observed in the $[Ca^{2+}]$c increments caused by Lys49 and Asp49 myotoxic PLA2 s in myotubes.

Of major interest is the present finding that the two classes of snake PLA2 myotoxins, i. e. the PLA2 active Asp49 and the PLA2 inactive Lys49, act synergistically in myotubes. This is an important result because it provides a good explanation for the presence of the two types of evolutionarily close toxins in the same venom: the synergic effect allows the same pathological effect to be achieved with a lower amount of venom protein. This is clearly an economical advantage for the snake and therefore it is an evolutionary benefit. Indeed, the concomitant presence of Asp49 and Lys49 PLA2

variants in the same venom has been described in various viperid species [42 – 45]. The molecular basis of this synergistic action is not clear. In fact, there is evidence that the Asp49 myotoxins act via phospholipid hydrolysis. On the other hand, it has been proposed that, after binding, the C-terminal portion of the Lys49, myotoxin inserts into the lipid bilayer and disrupts it in such a way as to cause a large increase of plasma membrane permeability, so high as to permit the leakage of lactic dehydrogenase and creatine kinase [3, 39, 40, 46]. The finding that a mixture of lysophosphatidylcholine and fatty acid added to the muscle cells greatly increases the activity of a Lys49 myotoxin suggests that the action of a Asp49 myotoxin renders the plasma membrane lipid bilayer more susceptible to the action of the Lys49 toxin. Among the two lipids, the lysophospholipid is active whilst the fatty acid is not. However, as previously found in neurons, their equimolar mixture is much more effective [32]. These findings provide new understanding concerning the actions of these myotoxins.

The present results tend to exclude the possibility that the inactive Lys49 toxins act by binding to a membrane channel, thus increasing its permeability to Ca^{2+}, as it is difficult to envisage how mLysoPC and OA could synergize with this mode of action. Moreover, cell depolarization with gramicidin, which should activate voltage-operated Ca^{2+} channels, did not alter the activity of the myotoxins (not shown). Alternatively, and more likely, it is possible that lysophospholipids and fatty acids favour the disruption of the lipid bilayer organization by the Lys49 myotoxins by changing the membrane curvature or by other more specific effects. In any case, the present data clearly show that the membrane lesion caused by the Lys49 toxins is achieved with a smaller amount of protein in the presence of the Asp49 toxin. This synergism is of adaptive value to the snake, providing a rationale for the emergence of Lys49 PLA2 myotoxins from their catalytically-active Asp49 PLA2 precursors during evolution.

In conclusion, we have shown here that the action of myotoxins from snake venoms on muscle cells begins with the activation of membrane acceptors coupled to intracellular Ca^{2+} stores, which is rapidly followed by a toxin dependent alteration of membrane permeability to ions (and other molecules). Most important, we here demonstrate the existence of a potent synergism between catalytically inactive and active toxins (both commonly present in the same venom), a feature that may lead to the development of novel therapeutic strategies in addition to the current use of specific antivenoms. In particular, drugs that could inhibit the PLA2 activity may turn out to be effective also on the catalytically inactive toxins by abolishing the synergism between the two classes of poisonous proteins.

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