

Effect of calcineurin inhibitors on myotoxic activity of crotoxin and *Bothrops asper* phospholipase A₂ myotoxins in vivo and in vitro

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Abstract

Previous studies have shown that calcineurin activity plays a critical role in the myotoxic activity induced by crotoxin (CTX), a group II phospholipase A₂ (PLA₂) with neurotoxic and myotoxic actions. In order to address whether calcineurin is also important for the activity of non-neurotoxic group II PLA₂ myotoxins we have compared the effects of calcineurin inhibition on the myotoxic capacity of CTX and the non-neurotoxic PLA₂s, myotoxin II (Mt II) and myotoxin III (Mt III) from *Bothrops asper* venom. Rats were treated with cyclosporin A (CsA) or FK506, calcineurin inhibitors, and received an intramuscular injection of either CTX, Mt II or Mt III into the tibialis anterior. Animals were killed 24 h after injection of toxins. Tibialis anterior was removed and stored in liquid nitrogen. Myofibers in culture were also treated with CsA or FK506 and exposed to CTX, Mt II and Mt III. It was observed that, in contrast to CTX, CsA and FK506 do not attenuate myotoxic effects induced by both Mt II and Mt III in vivo and in vitro. The results of the present study suggest that calcineurin is not essential for the myotoxic activity of Mt II and Mt III, indicating that distinct intracellular pathways might be involved in myonecrosis induced by neurotoxic CTX and non-neurotoxic *Bothrops* sp. PLA₂ myotoxins. Alternatively, calcineurin dependent fast fiber type shift might render the muscle resistant to the action of CTX, without affecting its susceptibility to *Bothrops* sp. myotoxins.

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1. Introduction

Secretory phospholipases A₂ (sPLA₂s) are enzymes that catalyze the hydrolysis of the *sn*-2 acyl bond of glycerophospholipids, in a calcium-dependent fashion, generating free fatty acids and lysophospholipids (Six and Dennis, 2000). These enzymes are widely distributed in pancreatic secretions, inflammatory exudates, and in snake and arthropod venoms (Balsinde et al., 1999) and in some invertebrates (Nevalainen et al., 2004a,b; Foradori et al., 2005). A variety of actions are attributed to venom sPLA₂s, which include neurotoxic,

myotoxic, edema-inducing, platelet-aggregating, cardiotoxic and anticoagulant effects (Gutierrez and Lomonte, 1995; Arni and Ward, 1996; Lomonte et al., 2003b). Venom sPLA₂s belong to groups I (terrestrial elapids and sea snake venoms), II (viperid snake venoms) and III (bee and lizard venoms) (Six and Dennis, 2000; Valentin and Lambeau, 2000). Group II sPLA₂s include, from the pharmacological standpoint, neurotoxic and non-neurotoxic types (Mebs and Ownby, 1990).

A well-characterized example of neurotoxic sPLA₂ myotoxin is crotoxin (CTX), a venom component of the South American rattlesnake *Crotalus durissus terrificus* (Hendon and Fraenkel-Conrat, 1971; Mebs and Ownby, 1990; Salvini et al., 2001). Among the non-neurotoxic sPLA₂ myotoxins, two different types are currently described: the “classical” Asp-49 PLA₂s, which catalyze the hydrolysis of the ester

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bond in the *sn*-2 position of glycerophospholipids in a Ca^{2+} dependent manner; and the “variant” Lys-49 PLA₂s which have been described as devoid of enzymatic activity due to replacement of Asp-49 by Lys, together with other modifications in the calcium-binding loop (Lomonte et al., 2003b). Although unable to hydrolyze the ester bond in the *sn*-2 position of glycerophospholipids, a host of studies have shown evidence that Lys-49 PLA₂s are able to destabilize cell membranes by catalytically independent mechanisms (Rufini et al., 1992; Falconi et al., 2000; Ward et al., 2002; Lomonte et al., 2003b).

Myotoxins II (Mt II) and III (Mt III) are well-characterized Lys-49 and Asp-49 myotoxic PLA₂s, respectively, present in *Bothrops asper* venom (Kaiser et al., 1990; Francis et al., 1991; Gutierrez and Lomonte, 1995). Similar myotoxic PLA₂s have been described in many *Bothrops* sp. venoms (Gutierrez and Lomonte, 1995). Although it is well accepted that most of the skeletal muscle fiber deleterious effects of Mt II and Mt III are a direct result of cell membrane damage (delta-lesions, hypercontraction of myofilaments, mitochondrial swelling, flocculent densities and rupture of mitochondrial membranes, disruption of intracellular membranes and pycnosis of nuclei) (Gutierrez and Ownby, 2003), it is reasonable to consider that these proteins might also trigger specific intracellular biochemical pathways that lead to cell modifications in structure and function.

In fact it has been recently shown that calcineurin, a Ca^{2+} -calmodulin-dependent phosphatase, is important for myotoxicity driven by a neurotoxic sPLA₂, CTX (Miyabara et al., 2004a), involving nitric oxide production (Miyabara et al., 2004b). This phosphatase has been involved in muscle differentiation (Abbott et al., 1998), fiber type determination (Chin et al., 1998) and hypertrophy (Musaro et al., 1999). Calcineurin binds to and activates nuclear factor of activated T cells (NFAT) (Rao et al., 1997; Crabtree, 1999), which targets the nucleus, dimerizes with other nuclear factors and modulates transcription of target genes (Rao et al., 1997).

In order to get further insight on the molecular mechanisms involved in sPLA₂ action in skeletal muscle we decided to investigate whether other PLA₂s are also influenced by calcineurin pharmacological inhibition. We have used Mt II and Mt III (isolated from the venom of *B. asper*) as representatives of Lys-49 and Asp-49 PLA₂ subtypes, respectively, which differently from CTX are non-neurotoxic proteins in vivo (Lomonte et al., 2003a). The results show that, in contrast with CTX, the calcineurin inhibitors CsA and FK506 do not attenuate myotoxic effects induced by both *B. asper* myotoxins.

2. Materials and methods

The protocols used in this study are in agreement with ethical principles in animal research followed by the Brazilian College of Animals Experimentation (COBEA) and were approved by the Institute of Biomedical Sciences/University of Sao Paulo—Ethical Committee for animals research (CEEAA).

2.1. In vivo experiments

2.1.1. Animals

Sixty male Wistar rats weighing 195 ± 6 g were used. The rats were at random divided into twelve groups ($n=5$ for each group) and housed in standard plastic cages in an animal room with controlled environmental conditions. Rats received standard food (Purina® chow) and had ad libitum access to food and water.

2.1.2. Toxins isolation

CTX was purified from *C. durissus terrificus* crude venom by gel filtration chromatography (Landucci et al., 1994), and analyzed by a tricine-SDS-polyacrylamide gel electrophoresis (Schagger and von Jagow, 1987).

Mt II and Mt III were isolated by ion-exchange chromatography on CM-Sephadex C-25 from *B. asper* venom of adult specimens, as previously described by Lomonte and Gutierrez (1989). A final purification step involving reverse-phase HPLC on a C8 column was introduced. Homogeneity was demonstrated by analytical reverse phase HPLC.

2.1.3. Treated groups

Three groups received CsA (Sandimmun®, Novartis, Switzerland; diluted 1:5 with physiologic saline) that was intraperitoneally administered, twice a day, at a dose of 10 mg/kg body weight (b.w.), during 5 days. On the 6th day, each animal received one intramuscular injection of CTX (0.17 mg/kg b.w.) or Mt II (0.1 mg/kg b.w.) or Mt III (0.1 mg/kg b.w.) into the middle belly of the left tibialis anterior (TA) muscle, which was evaluated 24 h after the toxin injection.

Other three groups received FK506 (Prograf®, Fujisawa, Ireland) that was intraperitoneally administered, twice a day, at a dose of 1 mg/kg body weight (b.w.), during 5 days. On the 6th day, each animal received one intramuscular injection of CTX (0.17 mg/kg b.w.) or Mt II (0.1 mg/kg b.w.) or Mt III (0.1 mg/kg b.w.) into the middle belly of the left tibialis anterior (TA) muscle, which was evaluated 24 h after the toxin injection. The doses of toxins used in the present study were chosen aiming significant necrosis in the muscle injected without decrease in total body weight.

2.1.4. Control groups

Animals received an intramuscular injection of saline (NaCl 0.9%) into the left TA muscle. Other groups were injected only with CTX (0.17 mg/kg b.w.) or Mt II (0.1 mg/kg b.w.) or Mt III (0.1 mg/kg b.w.) into the same muscle. All groups were killed 24 h after saline or toxin injections. Finally, other two groups were treated with CsA alone or FK506 alone at the same period and doses of the treated groups, but did not receive injection of toxins.

2.1.5. Histology

Animals were weighed, killed by an overdose of ketamine and xylazine and the TA muscles were removed, immediately frozen in molten isopentane and stored in liquid nitrogen. Frozen muscles were cut through mid-portion (end-plate region)

generating 10 μm cross-sections using a cryostat (Leica CM3050, Germany).

Unfixed histological sections were stained with aqueous toluidine-blue-borax solution (both 1% w/v) to reveal the general morphology (Morini et al., 1998; Salvini et al., 1999). The regions of muscle injuries, in which active macrophages are present, were localized by the histochemical detection of lysosomal acid phosphatase activity (ac-phosphatase, Gomori lead method) (Bancroft, 1996).

2.1.6. Muscle injury

The presence of muscle fiber injury was evaluated by light microscopy (Nikon Eclipse E600). Muscle damage was identified by the presence of myonecrotic fibers, such as hypercontracted fibers, clear areas among the muscle fibers, which indicated tissue disruption, intracellular vacuoles and cell infiltration. Presence of ac-phosphatase activity was also considered as a marker of necrosis and phagocytosis. It is well known that normal muscle fibers do not have positive ac-phosphatase reaction, which indicates high concentration of lysosomes, and is considered an evident proof of tissue necrosis and phagocytosis (Carpenter and Karpati, 1984).

2.2. In vitro experiments

2.2.1. Primary culture of myofibers

Skeletal muscles from hind limb of neonatal rats (0–4 days age) were removed and immersed in 5 ml Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Cultilab) containing 0.2% collagenase (type I; Gibco) for 3 h at 37 °C (Bischoff, 1986; Liu et al., 1997; Shefer and Yablonka-Reuveni, 2005). After dissociation, myofibers were maintained in DMEM 10% FBS, penicillin (100 U/ml) and streptomycin (0.1 mg/ml) in a humidified atmosphere with 5% CO₂, at 37 °C. Myofibers were standardized in 24-well plates, in which each well was coated with 30 μl phosphate buffer saline (PBS) containing laminin (1 $\mu\text{g}/\mu\text{l}$; Sigma).

2.2.2. Treatments

After establishment of myofibers, they were incubated with DMEM 10% FBS containing CsA (10^{-6} M) or FK 506 (10^{-6} M) for 24 h. Then, the myofibers were exposed to CTX (133 ng/ μl ; $n=3$ wells) or Mt II (133 ng/ μl ; $n=3$ wells) or Mt III (133 ng/ μl , $n=3$ wells) for 3 h.

2.2.3. Myofiber membrane damage

Membrane damage induced by CTX, Mt II and Mt III was assessed by cytochemistry using a fixable membrane wound

marker (FITC-dx 10,000 M.W., Sigma) as previously described (Clarke et al., 1994; Clarke and Feedback, 1996).

2.3. Quantitative analysis

The amount of TA necrosed myofibers in tissue sections was expressed as necrosed myofibers/ mm^2 (N.M./ mm^2). The data was obtained by analyzing digital images acquired by Metamorph® software (Universal Imaging Corporation, Down-towtown, PA, USA).

In cell culture experiments, myofiber alterations were classified in two ways: (a) the number of damaged myofibers (i.e. fibers FITC-dx positive) in primary cultures was expressed as mean percentage of 50 counted fibers per well (total of 150 fibers per group). The data was obtained by analyzing digital images acquired by a confocal microscope (Nikon Eclipse TE 300, Japan). The number of intact myofibers (intact) in culture was obtained by using a light and confocal microscope. (b) The percentage of dead myofibers (dead) in culture was obtained by the following equation: $\text{dead} = [T - (I + \text{FITC-dx})]/T$. T means the total number of myofibers before the incubation of toxins; I means intact (FITC-dx negative) myofibers after incubation with toxins. By using these two patterns of myofiber alteration, it was possible to detect fibers that had alterations in plasma membrane permeability but have not disappeared (FITC-dx positive) and cells that were in a more advanced stage of degeneration that were not stained with FITC-dx and had disappeared by microscopic analysis.

2.4. Statistical analysis

Multiple comparisons of mean values were performed with analysis of variance (ANOVA) and a post-hoc Tukey's test to compare mean values when appropriate. Significant level accepted was below 5% ($p < 0.05$).

3. Results

3.1. In vivo experiments

Mt II, Mt III and CTX induced significant rise in TA/body weight ratio as compared to their respective controls (41%, 47% and 47%, $p < 0.05$, respectively; Table 1). As expected, CsA alone and FK506 alone did not promote significant alterations in TA/body weight ratio (Table 1). The TA muscle weights from animals treated with CsA or FK506 and injected with Mt II, Mt III and CTX were also increased in comparison to their controls [70% (MtII+CsA), 58% (MtIII+CsA) and 35% (CTX+CsA); 65% (MtII+FK506), 35% (MtIII+FK506) and 59% (CTX

Table 1
TA/body weight ratio in controls and injured groups expressed as percentage

Controls		Injured		CsA+Injured		FK506+Injured	
Saline	0.17±0.02	CTX	0.25±0.01**	CTX+CsA	0.23±0.02*	CTX+FK506	0.27±0.03**
CsA	0.18±0.01	Mt II	0.24±0.04*	Mt II+CsA	0.29±0.01**	Mt II+FK506	0.28±0.02**
FK506	0.18±0.02	Mt III	0.25±0.03**	Mt III+CsA	0.27±0.03**	Mt III+FK506	0.23±0.02*

Results are mean±standard deviation; $n=5$ for each group; * $p < 0.01$ vs. saline; ** $p < 0.001$ vs. saline.

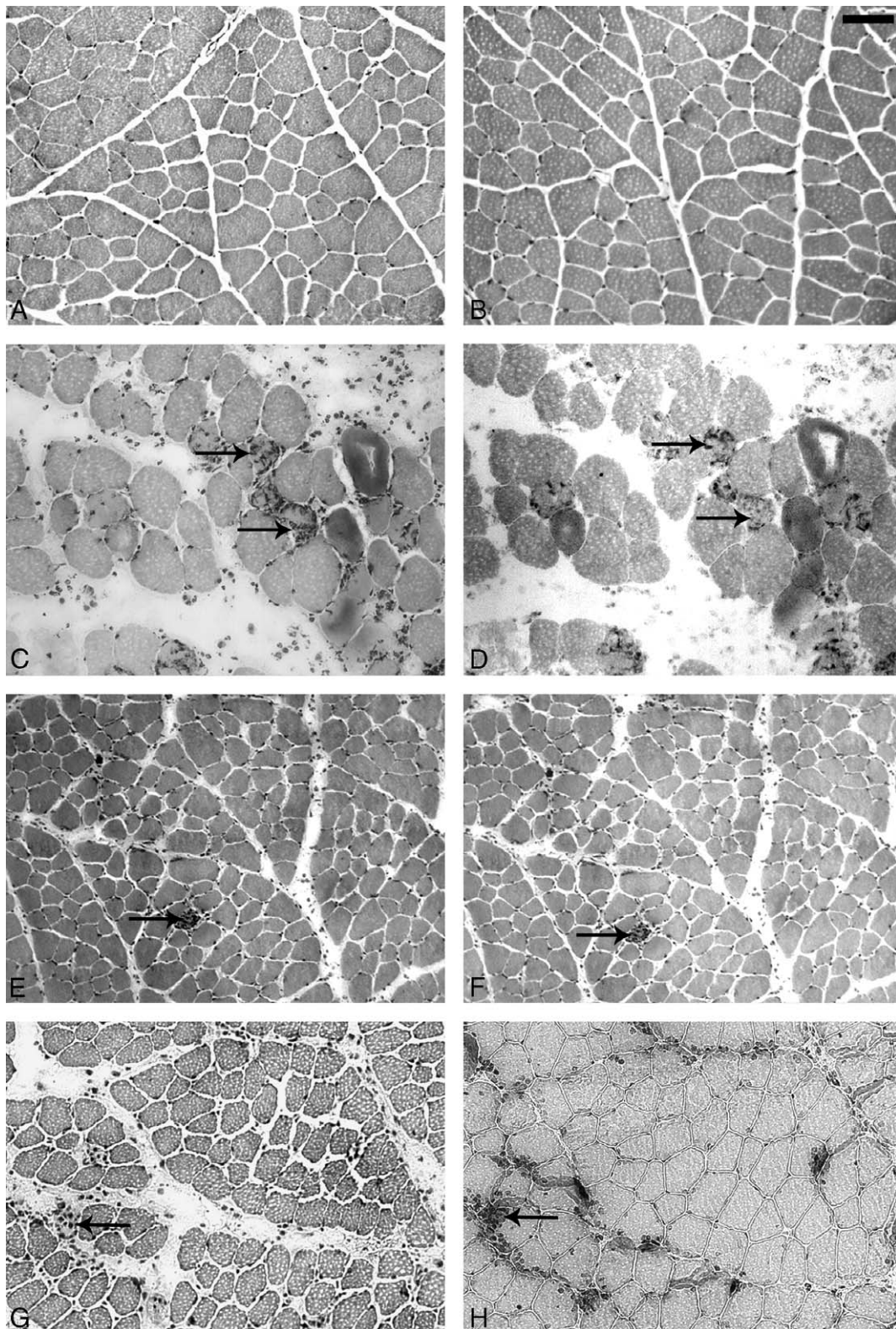


Fig. 1. Cross-sections of the middle belly of TA muscles. In animals injected with saline or treated with CsA during 6 days (A and B, respectively) the TA muscle had normal morphology. (C and D) TA injected with CTX and analyzed after 24 h. (E and F) TA treated with CsA and injected with CTX. (G and H) TA treated with FK506 and injected with CTX. Sections were stained with either toluidine blue (A, B, C, E, G) or acid-phosphatase (D, F, H). Note the presence of clear areas among the muscle fibers, which result from the muscle fiber disruption and edema (C, D) and several necrotic fibers with cell infiltration (arrows; C, E, G) are positive to ac-phosphatase reaction, which indicate presence of necrosis and macrophages in process of phagocytosis (arrows; D, F, H). TA muscles treated with CsA or FK506 and injected with CTX had minimal cell infiltration (E, G) and positive ac-phosphatase reaction (F, H). Bar: 50 μ m.

+FK506) respectively; $p < 0.05$, Table 1] and no significant differences were found when compared to that from animals receiving only Mt II, Mt III and CTX.

TA muscles from control groups injected with saline or treated only with CsA had normal morphology, with no signals of lesion (Fig. 1A, B). Also, the muscles from animals treated only with FK506 had no signals of injury (data not shown). CTX was able to induce edema, tissue disruption and myonecrosis in TA muscles injected with CTX as observed by the presence of clear spaces among muscle fibers with inflammatory cells, hypercontracted fibers (Fig. 1C) and several necrotic fibers positive to ac-phosphatase reaction (Fig. 1D). On the other hand, TA muscles pre-treated with CsA or FK506 and injured with CTX showed minimal cell infiltration (Fig. 1E, F,

G, H), in comparison with muscles from rats only injected with CTX (Fig. 1C, D). In addition, pre-treatment with CsA and FK506 decreased significantly the number of necrosed fibers of muscle injured by CTX, as assessed by ac-phosphatase reactions (~80% and 59% as compared to controls, $p < 0.05$; Fig. 4).

Mt II and Mt III, in contrast to CTX, neither damaged nor altered the wet weight of the muscles neighboring the injected TA muscle, such as soleus and extensor digitorum longus (data not shown), confirming the restricted nature of the myotoxicity induced by these myotoxins (Gutierrez and Ownby, 2003). Figs. 2 and 3 show the results of experiments using Mt II and Mt III, respectively. The TA muscles injected with Mt II or Mt III showed empty spaces among the muscle fibers, cell infiltration

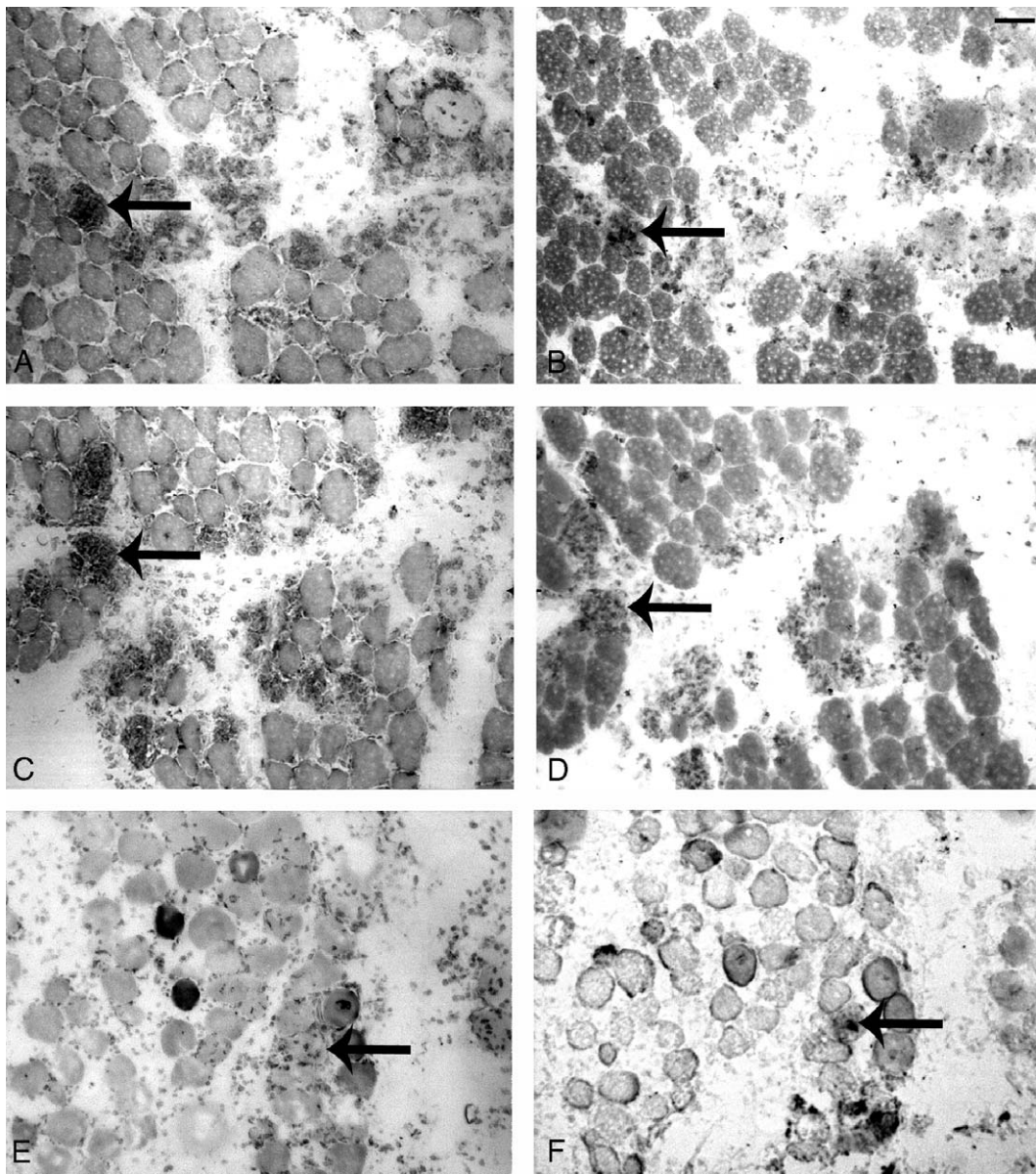


Fig. 2. Serial cross-sections of TA muscles. (A, B) Muscles only injected with Mt II. (C, D) Muscles treated with CsA and injured with Mt II. (E, F) Muscles treated with FK506 and injured with Mt II. (A, C, E) Toluidine blue staining and (B, D, F) ac-phosphatase reaction. The muscles only injected with Mt II (A, B) show similar signals of injury to the group treated with CsA or FK506 and injured with Mt II (C, D, E, F): clear spaces among the muscle fibers (A, B, C, D, E, F), intense cellular infiltration and necrosed myofibers (arrow; A, C, E) with positive ac-phosphatase reaction (arrow; B, D, F). Bar: 50 μm .

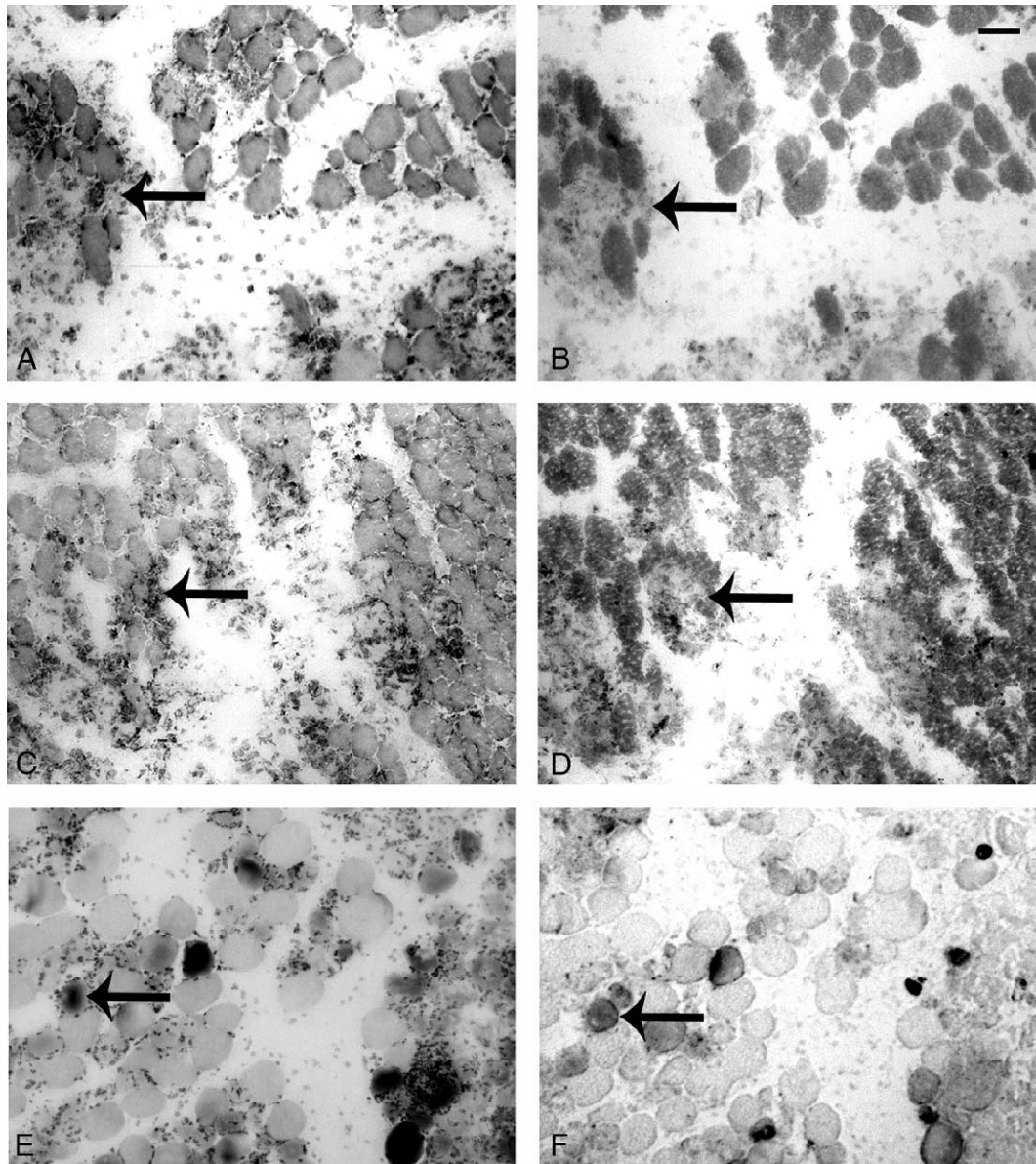


Fig. 3. Serial cross-sections of TA muscles. (A, B) Muscles only injected with Mt III. (C, D) Muscles treated with CsA and injured with Mt III. (E, F) Muscles treated with FK506 and injured with Mt III. (A, C, E) Toluidine blue staining and (B, D, F) ac-phosphatase reaction. The muscles only injected with Mt III (A, B) show similar signals of injury to the group treated with CsA or FK506 and injured with Mt III (C, D, E, F): clear spaces among the muscle fibers (A, B, C, D, E, F), intense cellular infiltration and necrosed myofibers (arrow; A, C, E) with positive ac-phosphatase reaction (arrow; B, D, F). Bar: 50 μ m.

and necrosed myofibers with positive ac-phosphatase reaction (Figs. 2A, B and 3A, B, respectively). Interestingly, the muscles pre-treated with CsA or FK506 and injured with Mt II or Mt III also had similar signs of lesion as compared to the groups injected only with Mt II and Mt III, i.e. presence of edema and myonecrosis (Figs. 2C, D, E, F and 3C, D, E, F, respectively). The amount of necrosis, expressed as necrosed fibers per mm^2 (N.M./ mm^2), from muscles of rats pre-treated with CsA or FK506 and injected with Mt II or Mt III were also not different from those groups only injured with Mt II and Mt III (Fig. 4).

3.2. *In vitro* experiments

As expected, the isolated myofibers treated only with CsA or FK506 did not have membrane injury as assessed by FITC-dx

labeling (data not shown). CTX, on the other hand, was able to induce myofiber membrane damage and myofiber death (27% and 62% respectively, relative to total number of cells before treatment, Fig. 5). Pre-treatment with CsA and FK506 decreased significantly the amount of dead and FITC-dx positive myofibers exposed to CTX as compared to the group exposed only with CTX (43% and 29% respectively, $p < 0.05$; Fig. 5).

Similarly to CTX, Mt II induced myofiber membrane damage and myofiber death (Mt II: 29% and 68%, respectively; Fig. 6). Interestingly, pre-treatment with CsA or FK506 and incubation with Mt II had similar amount of dead and FITC-dx positive myofibers as compared to groups only exposed to Mt II (Fig. 6).

Mt III induced higher amount of myofiber membrane damage than myofiber death (80% and 20% respectively, Fig.

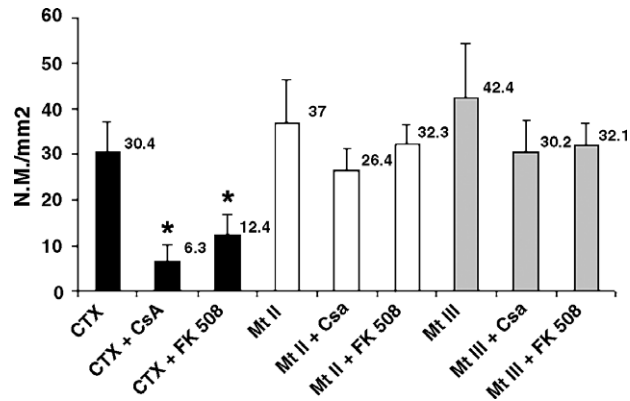


Fig. 4. Necrosed myofibers/mm² in the injected (CTX, Mt II and Mt III) and treated with CsA or FK506 and injected groups (CTX+CsA, Mt II+CsA, Mt III+CsA, CTX+FK506, Mt II+FK506, Mt III+FK506). Note the drastic reduction of myotoxic activity of CTX in the presence of CsA and FK506; differently, CsA and FK506 have no effect on the myotoxic activity of Mt II and Mt III.

7). CsA and FK506 pre-treatment did not alter the proportion of damaged and dead myofibers as compared to the group only exposed to Mt III (Fig. 7).

4. Discussion

The present study shows that pre-treatment with CsA or FK506 leads to a significant minimization of injury in TA muscles and myofibers exposed to CTX, a neurotoxic

heterodimeric PLA₂. On the other hand, pre-treatment with these calcineurin inhibitors does not have any protective effect upon skeletal muscles and myofibers exposed to the non-neurotoxic *B. asper* PLA₂ myotoxins (Mt II and Mt III). We have used CsA and FK506, in order to exclude the possibility that the effects described herein are due to other effects of these drugs not related to calcineurin inhibition. FK506 has distinct molecular structure as compared to CsA, however it binds to the same surface of calcineurin (Huai et

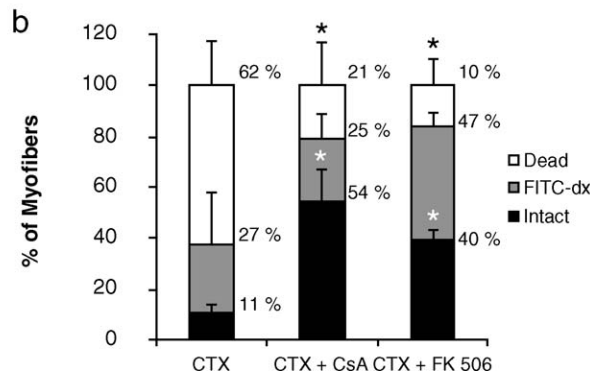
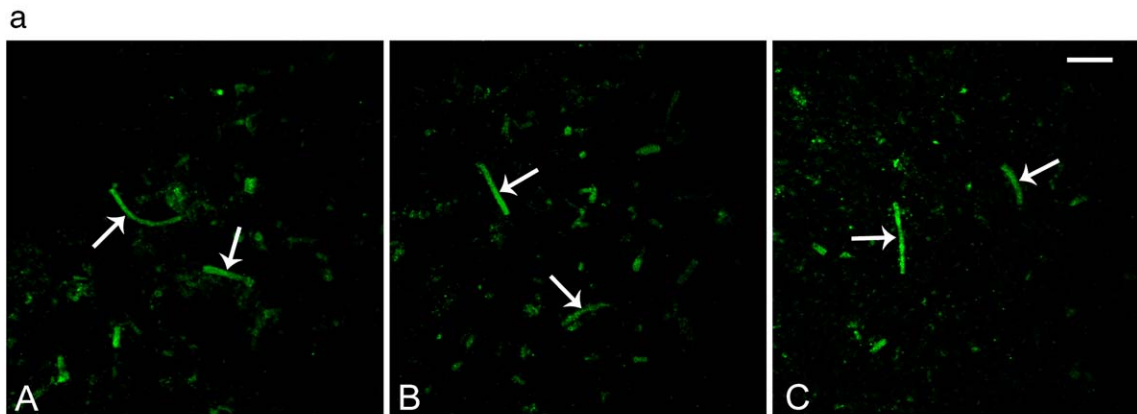


Fig. 5. (a) Confocal images of primary myofibers in culture stained with FITC-dx exposed to CTX (A), treated with CsA or FK506 and exposed to CTX (B and C, respectively). (b) Comparison of percent of dead, FITC-dx positive and intact myofibers in culture in the groups treated with CTX (CTX), treated with CsA or FK506 and exposed to CTX (CTX+CSA and CTX+FK506; respectively). Data are expressed as mean±S.D.; n=3–4 wells; *p<0.05 vs. matched CTX.

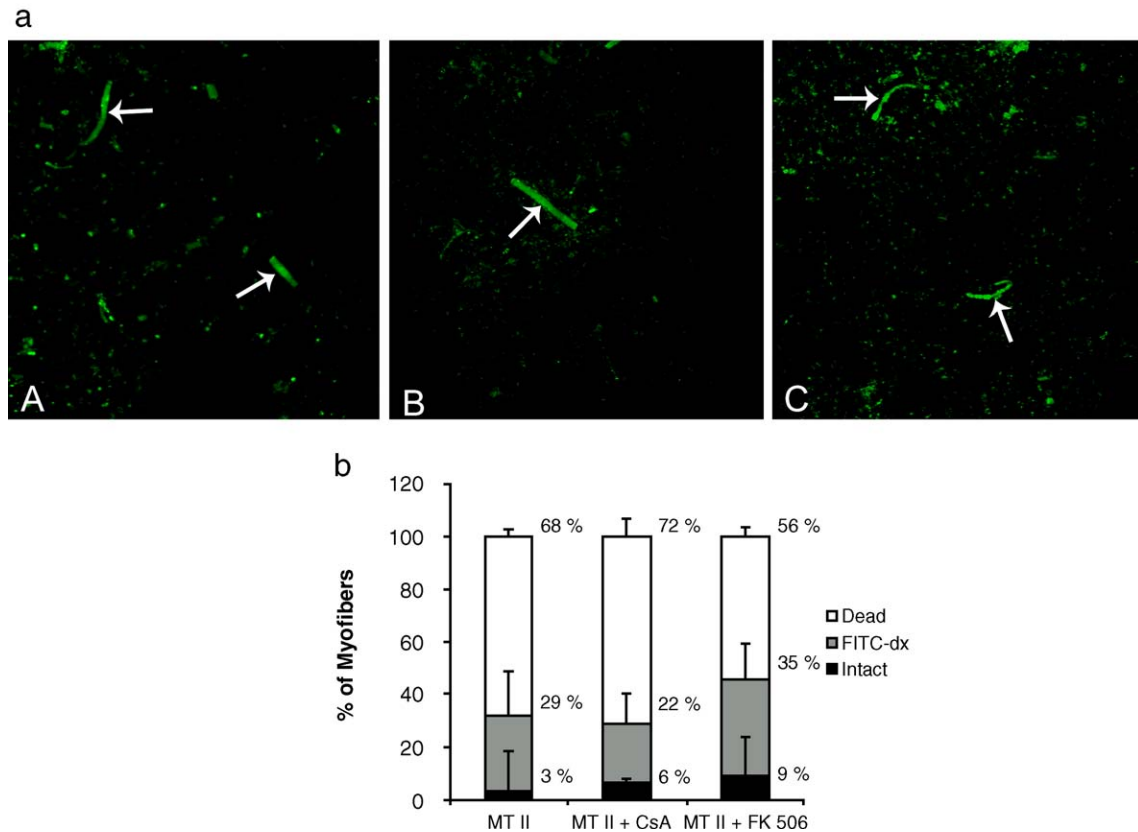


Fig. 6. (a) Confocal images of primary myofibers in culture stained with FITC-dx exposed to Mt II (A), treated with CsA or FK506 and exposed to Mt II (B and C, respectively). (b) Comparison of percent of dead, FITC-dx positive and intact myofibers in culture in the groups treated with Mt II (Mt II), treated with CsA or FK506 and exposed to Mt II (Mt II+CSA and Mt II+FK506; respectively). Data are expressed as mean \pm S.D.; $n=3-4$ wells.

al., 2002) and efficiently inhibits calcineurin with 100-fold lower concentration as compared to CsA (Liu et al., 1991). The doses of CsA and FK506 utilized in the present work in vivo (20 and 2 mg/kg/day b.w., respectively) and in vitro (10^{-6} M) have been previously studied and are able to significantly reduce calcineurin activity ($\sim 60\%$) (Lai et al., 1998; Dunn et al., 1999; Friday et al., 2000; Dunn et al., 2001; Serrano et al., 2001; Miyabara et al., 2005). Calcineurin inhibition by CsA (Fig. 1B) drives no histological signals of skeletal muscle injury such as necrosed fibers and cellular infiltration. Similar results were obtained when rats were injected with FK506 (data not shown). Accordingly, TA/body weight ratio is also not altered in CsA and FK506 treated animals (Table 1).

As expected, at 24 h after injection of CTX, Mt II and Mt III the TA/body weight ratio was increased as compared to control, indicating that these myotoxins induced an inflammatory process and consequently edema (Table 1). In addition, both CsA and FK506 were not able to significantly reduce TA/body weight ratio induced by Mt II, Mt III and CTX in TA muscles (Table 1), indicating that the dose of CsA and FK506 administered were not high enough to elicit an anti-inflammatory effect in a severely injured muscle.

Our results show that the three myotoxins tested in this work promoted efficient injury in TA muscle and membrane damage in isolated myofibers in culture. Although the histological signs

resulting from intramuscular injection of these myotoxins result in similar damage, these PLA₂s have different characteristics in their toxicological profile. It is well known that CTX is classified as a basic neurotoxic PLA₂ myotoxin because it acts on the neuromuscular junction, primarily at a presynaptic level, inhibiting the release of the neurotransmitter acetylcholine (ACh) (Harris, 1991); consequently its lethal dose 50% (LD₅₀) value is extremely low. On the other hand, Mt II and Mt III are non-neurotoxic PLA₂ myotoxins that display high LD₅₀ values (Gutierrez et al., 1986; Gutierrez and Lomonte, 1995). CTX and *Bothrops* sp. PLA₂ myotoxins also have differences regarding their ability to induce local and systemic muscle damage (Gutierrez and Ownby, 2003). CTX is able to induce muscle damage not only in the injected muscle (local myotoxicity), but also in muscles remotely located (systemic myotoxicity) (Salvini et al., 2001). In contrast, Mt II and Mt III only induce local myotoxicity. These distinct effects might be due to the specificity of these myotoxins for differentiated muscle cells, i.e. on their affinity for highly specific targets in muscle cell plasma membrane (Gutierrez and Ownby, 2003) and also for targeting other cell types (Lomonte et al., 1994, 1999).

The data showing that both myonecrosis and cytotoxicity induced by neurotoxic PLA₂ myotoxin (CTX) were inhibited by CsA treatment, combined with no myoprotective effect of CsA in damaging induced by non-neurotoxic PLA₂ myotoxins (Mt II

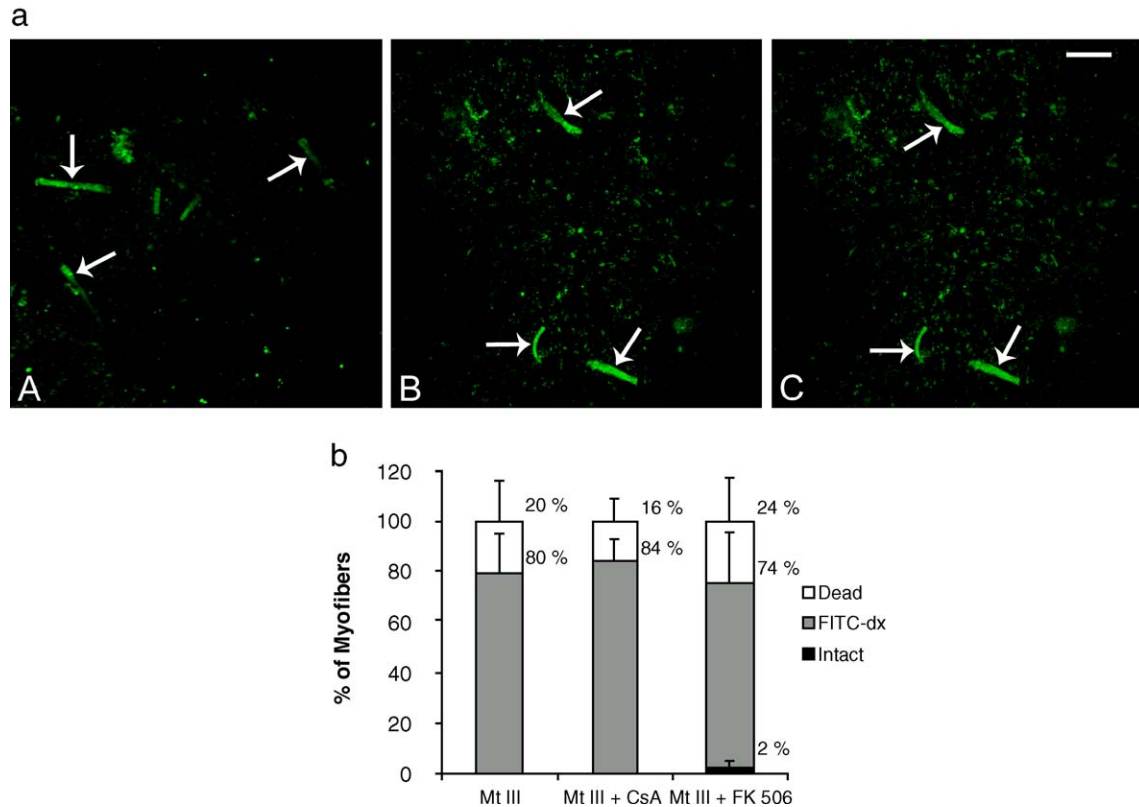


Fig. 7. (a) Confocal images of primary myofibers in culture stained with FITC-dx exposed to Mt III (A), treated with CsA or FK506 and exposed to Mt III (B and C, respectively). (b) Comparison of percent of dead, FITC-dx positive and intact myofibers in culture in the groups treated with Mt III (Mt III), treated with CsA or FK506 and exposed to Mt III (Mt III+CSA and Mt III+FK506; respectively). Data are expressed as mean \pm S.D.; $n=3-4$ wells.

and Mt III), raise the possibility that different intracellular pathways mediate myonecrotic effects of CTX and *B. asper* PLA₂ myotoxins. As previously demonstrated (Miyabara et al., 2004a), pretreatment with CsA allows a myoprotective effect in muscles injected with CTX, indicating that calcineurin is important for myotoxicity driven by CTX, involving nitric oxide production (Miyabara et al., 2004b).

Although the molecular basis underlying the differential dependence of these myotoxic PLA₂s on calcineurin is still unknown, several possibilities could be envisioned to explain this difference: (a) CTX, and related neurotoxic PLA₂s, may bind to receptors in skeletal muscle plasma membrane, such as the M-type PLA₂ receptor (Lambeau and Lazdunski, 1999), thus promoting intracellular signaling pathways leading to calcineurin activation, whereas the action of *B. asper* myotoxins is likely to depend on a direct damage to the integrity of sarcolemma. (b) The heterodimeric nature of CTX might play a role in the calcineurin dependent myotoxic effect; accordingly, subunit A might be somehow involved in calcineurin activation. (c) Since calcineurin is involved in muscle fiber type determination (Chin et al., 1998), our results may reflect the different specificity of these PLA₂s toward different fiber types. CTX and other neurotoxic PLA₂ myotoxins have preference for type I fibers (Harris et al., 1975; Salvini et al., 2001), whereas types I and II fibers present a similar susceptibility to a viperid Lys-49 myotoxic PLA₂ (Morini et al., 1998). From this perspective, the effect of calcineurin

would not depend on activation of intracellular degenerative mechanisms, but instead would be related with fiber type determination. (d) Another possibility might include the propagation of a signal in a CsA-sensitive manner, starting from CTX binding to myofibers leading to cell death. Both Mt II and Mt III would not trigger such process, therefore the degree of damage would not be affected by CsA. In fact certain cytokines, such as interleukin-1 β , can be secreted by muscle cells to act as an endocrine/paracrine signal (Curfs et al., 1997) to induce cell damage and are dependent on calcineurin (Ho et al., 1996). (e) Finally, it should be taken in consideration that CTX is able to exert myotoxicity systemically, while Mt II and Mt III promote myolesion restricted to the injected muscle, which could contribute to the differences in sensitivity to CsA.

A primary goal of this study was to assess whether the protective effect of calcineurin inhibition against CTX injection in skeletal muscle depends on the in vivo context. We have chosen to isolate and culture skeletal muscle myofibers because, in contrast to permanent lines such as C2C12 cells (Lomonte et al., 1999), CTX promotes severe plasma membrane damage as detected by FITC-dx labeling, then leading to disappearance of cell structure (Fig. 5a). The results clearly show that addition of CsA or FK506 to the cultures markedly decreases the proportion of dead cells as a result of CTX treatment. These results show that the in vivo context is not necessary for the protective effects of calcineurin inhibition on skeletal fiber

damage caused by CTX. As expected from the *in vivo* findings, Mt II and Mt III damaging effects were not altered by calcineurin inhibition, reinforcing the idea that the calcineurin pathway is specifically activated by CTX and not by these non-neurotoxic PLA₂s.

The data of the present work, combined with two previous publications (Miyabara et al., 2004a,b) highlight that although the gross effect of CTX and *Bothrops* sp. myotoxic PLA₂s in muscle cells is similar, including early cell membrane destruction (Gopalakrishnakone et al., 1984; Gutierrez et al., 1984) that leads to similar histological signals of lesion, specific intracellular pathways might also be activated, leading to different outcomes at the molecular level. These differential effects might explain differences in potency, site of action (muscle vs. nervous system) and extent of myotoxicity (local vs. systemic). Accordingly, elucidation of such intracellular pathways activated by PLA₂s might provide useful information on muscle degeneration mechanisms, which, in turn, may be helpful for the development of future therapeutic approaches to be employed in neuromuscular diseases.

In conclusion, the results presented herein show that calcineurin is an essential pathway mediating CTX myotoxicity *in vivo* and *in vitro*, which might be specific since calcineurin inhibitors do not attenuate muscle damage induced by other myotoxins such as *B. asper* Mt II and Mt III.

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