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Paralyzing and myotoxic effects of a recombinant bothropstoxin-I (BthTX-I) on mouse neuromuscular preparations

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Abstract

As a first step to investigate the structure–function relationship of bothropstoxin-I (BthTX-I), a myotoxin from *Bothrops jararacussu* snake venom, our group previously cloned a recombinant toxin (rBthTX-I) in *Escherichia coli*. The aim of this work was to characterize the biological activities of this rBthTX-I (1.0 μM) in both phrenic-diaphragm and extensor digitorum longus preparations in vitro, by means of myographic and morphologic techniques. Native BthTX-I (1.0 μM) was used as a standard. The influence of heparin (27.5 μg/ml) upon the biological activities of both toxins was also investigated. rBthTX-I had similar effects to the native toxin inducing blockage of both directly and indirectly evoked contractions in phrenic-diaphragm preparations, and muscle damage characterized by edema, round fibers, and cell areas devoid of myofibrils. Interestingly the paralyzing activity of rBthTX-I was slightly more potent than the native toxin. Heparin prevented paralyzing and myotoxic effects of both the native and recombinant toxins. This work shows that rBthTX-I was expressed in a fully active form, and presents a biological profile similar to the native toxin.

Keywords: Myotoxin; Phospholipase A_2; Muscle damage; Ultrastructure

Introduction

Myotoxic phospholipases A_2 (PLA_2s) have been identified in many snake venoms (Gutierrez and Ownby, 2003). These proteins are usually classified into two categories: the Asp49 PLA_2s, catalytically active, and the Lys49 PLA_2s, devoid of significant catalytic activity. Nevertheless, Lys49 PLA_2s induce severe myonecrosis by a non-hydrolytic mechanism (Gutierrez and Ownby, 2003). Current evidence indicates that Lys49 PLA_2s bind to the plasma membrane of muscle cells and alter their permeability, although a specific receptor has not been identified yet, and their action mechanism is still not well understood (Lomonte et al., 2003).

Bothropstoxin-I (BthTX-I) is Lys49 PLA_2 isolated from venom of the South American snake *Bothrops jararacussu* (Homsi-Brandeburgo et al., 1988). In addition to a potent myotoxic action (Homsi-Brandeburgo

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et al., 1988), it also promotes the blockage of both directly and indirectly evoked contractions in isolated neuromuscular preparations (Oliveira et al., 2003). However, the relationship between the myotoxic and paralyzing activities of BthTX-I has not been fully understood, and further studies are necessary to evaluate whether these actions are mediated by the same structural site.

Molecular biology techniques such as cloning and site-directed mutagenesis have been used as important tools for studying Lys49 PLA_2s structure–function relationship (Chioato et al., 2002; Ward et al., 2002). As a first step to investigate the structural basis underlying the functional properties of BthTX-I, our group (Spencer et al., 2000) developed an expression system for production of a soluble and immunoreactive recombinant BthTX-I (rBthTX-I) in *E. coli* (BL 21). The aim of this work was to characterize, by means of myographic and morphologic techniques, the biological properties of this rBthTX-I on mouse neuromuscular preparations, using native BthTX-I as standard. Since heparin has been shown to inhibit some biological activities of Lys49 PLA_2s (Lomonte et al., 1994, 2003), we also evaluated the interaction of both recombinant and native toxins with heparin.

Material and methods

Materials

Native BthTX-I was isolated from *B. jararacussu* venom (Spencer et al., 1998) and recombinant BthTX-I was expressed in *E. coli* BL 21 according to procedures described by Spencer et al. (2000). Heparin (Liquemine) was purchased from Roche laboratories. All other reagents were of analytical grade. BthTX-I, rBthTX-I and heparin were dissolved in physiological saline solution (PSS, 0.9% NaCl).

Animals

Adult male mice weighing 25–30 g were maintained under a 12 h light–dark cycle (lights on at 07:00) in a temperature controlled environment ($22 \pm 2\,^{\circ}C$) for at least 10 days prior to the experiments. Food and water were freely available. Animal procedures were in accordance with the guidelines for animal care prepared by the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

Myographic study

Mice were killed by exsanguination after ether anesthesia. The phrenic-diaphragm (PD) preparation was removed and mounted vertically in a conventional isolated organ-bath chamber containing 20 ml of physiological solution with the following composition (mmol/L): NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $NaHCO_3$, 15; Na_2HPO_4, 1; glucose, 11. This solution was gassed with O_2 (95%) + CO_2 (5%) and kept at $35 \pm 1\,^{\circ}C$. The preparation was attached to an isometric force transducer (Gould, GM2/GM3) for recording twitch tension. The transducer output was displayed on a polygraph recorder (Beckman, R-511A). Resting tension was 2 g. Indirect contractions were evoked by supramaximal strength pulses (0.5 Hz; 0.5 ms), delivered by an electronic stimulator (Narco Bio-System) and applied on the phrenic nerve by suction electrode. Direct contractions were evoked by supramaximal pulses (0.2 Hz, 5 ms) through a bipolar electrode positioned on opposite sides of the muscle. Experiments of direct contractions were performed in the presence of pancuronium bromide (2×10^{-6} M). The preparations were allowed to stabilize for 45 min before the addition of BthTX-I (1 μM), rBthTX-I or a mixture of each toxin (1 μM) plus heparin (27.5 μg/ml). These mixtures were pre-incubated at 37 °C for 15 min.

Morphological study

The myotoxic activity of both BthTX-I and rBthTX-I were assayed on the extensor digitorum longus (EDL) muscle, since it can be isolated without injury. EDL muscles were isolated and submitted to the same incubation conditions as used for the myographic study. Subsequently, EDL muscles were removed from the bath and frozen in liquid nitrogen. Transverse sections (8 μm thick) were cut at $-20\,^{\circ}C$ in a cryostat and stained with haematoxylin and eosin (HE) for examination by light microscopy (Mc Manus and Mowry, 1960). Muscle samples were also fixed in Karnovsky's fixative (2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4) for 4 h, and washed in 1% osmium tetroxide. The tissue was dehydrated in ascending concentrations of acetone and embedded in Epon resin. Sections (1.5 μm) were stained with uranyl acetate and lead citrate, and examined by electron microscope.

Morphological damage was quantified in HE stained preparations, using an Analysis Imaging System (Leica, Qwin). The number of fibers with lesions was expressed as a percentage of the total number of cells (muscle damage index), in three non-overlapping, non-adjacent areas of each muscle, observed at the same magnification.

Statistical analysis

Results are expressed as mean ± SE. Data were analyzed by Student's *t*-test (for comparison of two

samples) and analysis of variance complemented by the Tukey-Kramer test (for comparison of more than 2 samples). Values of $P<0.05$ were considered significant.

Results

BthTX-I and rBthTX-I induced a time-dependent blockage of directly and indirectly evoked contractions (Fig. 1A and B). However, the paralyzing effect of rBthTX-I was slightly more potent than the native toxin. The mean time required to reduce muscle contraction amplitude by 50% ($t_{1/2}$) was significantly less ($P<0.05$) for rBthTX-I than BthTX-I, both for direct (38.1 ± 3.6, $n=5$ vs. 56.2 ± 7.4, $n=4$) and indirect (12.6 ± 0.5, $n=3$ vs. 22.0 ± 2.9, $n=4$) contractions. The effects of both toxins were not reversed when preparations were washed for at least 30 min with toxin-free physiological solution (data not shown). Heparin mixed with BthTX-I or rBthTX-I did not induce the contraction blockages (Figs. 2A and B), indicating that it was able to neutralize the paralyzing effect of both native and recombinant toxins. Heparin on its own did not significantly affect muscle contraction.

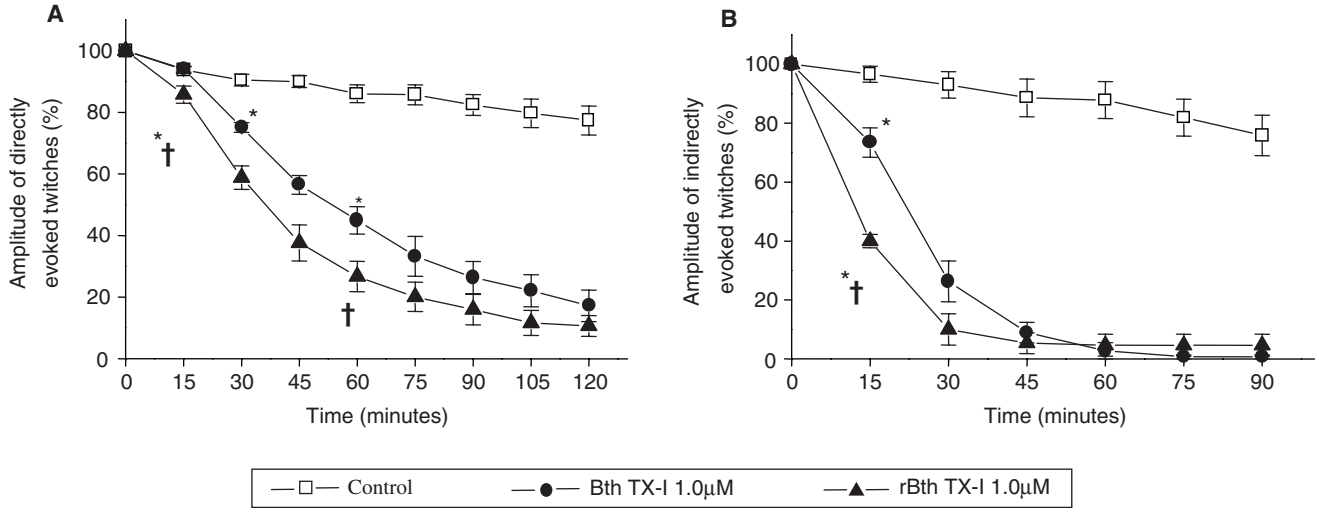

Fig. 1. Effects of native bothropstoxin-I (BthTX-I) and recombinant bothropstoxin-I (rBthTX-I) on (A) directly and (B) indirectly evoked twitches in mouse phrenic-diaphragm preparations. Results are expressed as mean ± S.E.M of at least three different preparations. *indicates the point at which differences to controls become significant. †indicates significant differences between recombinant and native toxin, (A) from t_{15} to t_{60} and (B) at t_{15} ($P<0.05$).

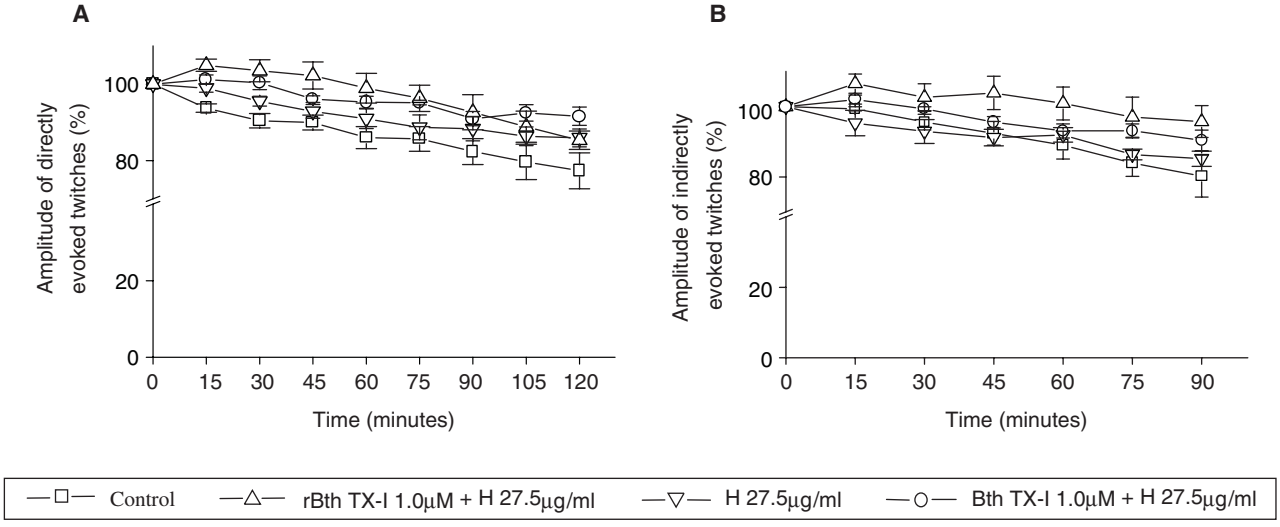

Fig. 2. Effects of native bothropstoxin-I (BthTX-I) or recombinant bothropstoxin-I (rBthTX-I) pre-incubated with heparin (H) on directly (A) and indirectly (B) evoked twitches in mouse phrenic-diaphragm preparations. Results are expressed as mean ± S.E.M. of at least four different preparations.

Figs. 3 and 4, respectively, show light and ultrastructural microscopy of (A) control and (B) heparin exposed EDL muscle. In both situations, most fibers have a normal polygonal appearance with acidophilic sarcoplasm and peripheral nuclei. They are clearly delimited by a thin layer of connective tissue, the

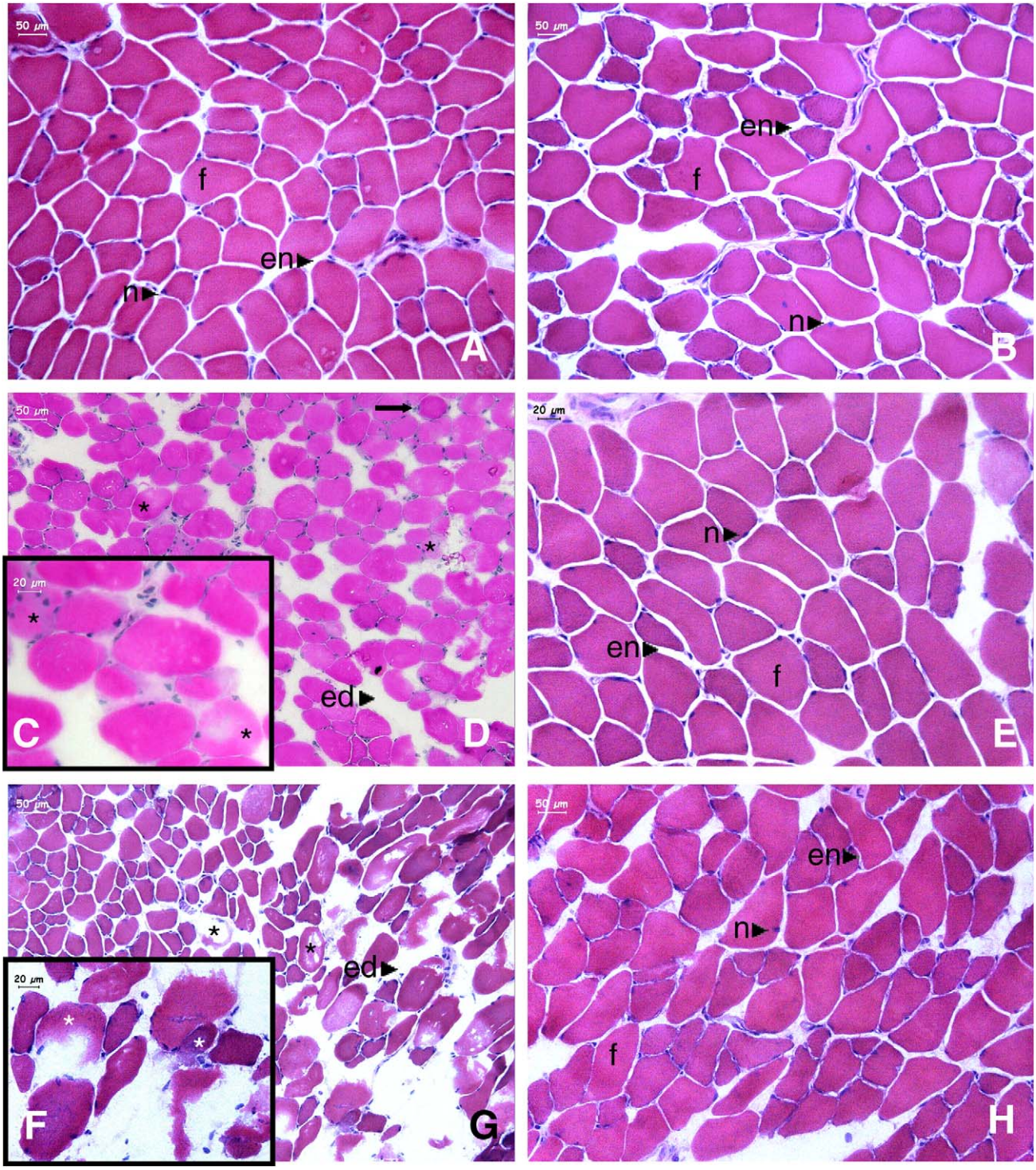

Fig. 3. Light micrographs of mouse EDL muscles submitted to haematoxilin and eosin (HE) stain. (A) Control muscle and (B) heparin exposed muscle with normal fiber appearance. Note the polygonal aspect of fibers (f) and endomysium (en). C, D, F and G: muscle exposed to native bothropstoxin-I (BthTX-I) or recombinant bothropstoxin-I (rBthTX-I) showing edema (ed), round fibers (arrow) with loss of myofibrils (*). E and H: muscle exposed to heparin pre-incubated BthTX-I and rBthTX-I, most fibers presenting normal aspect.

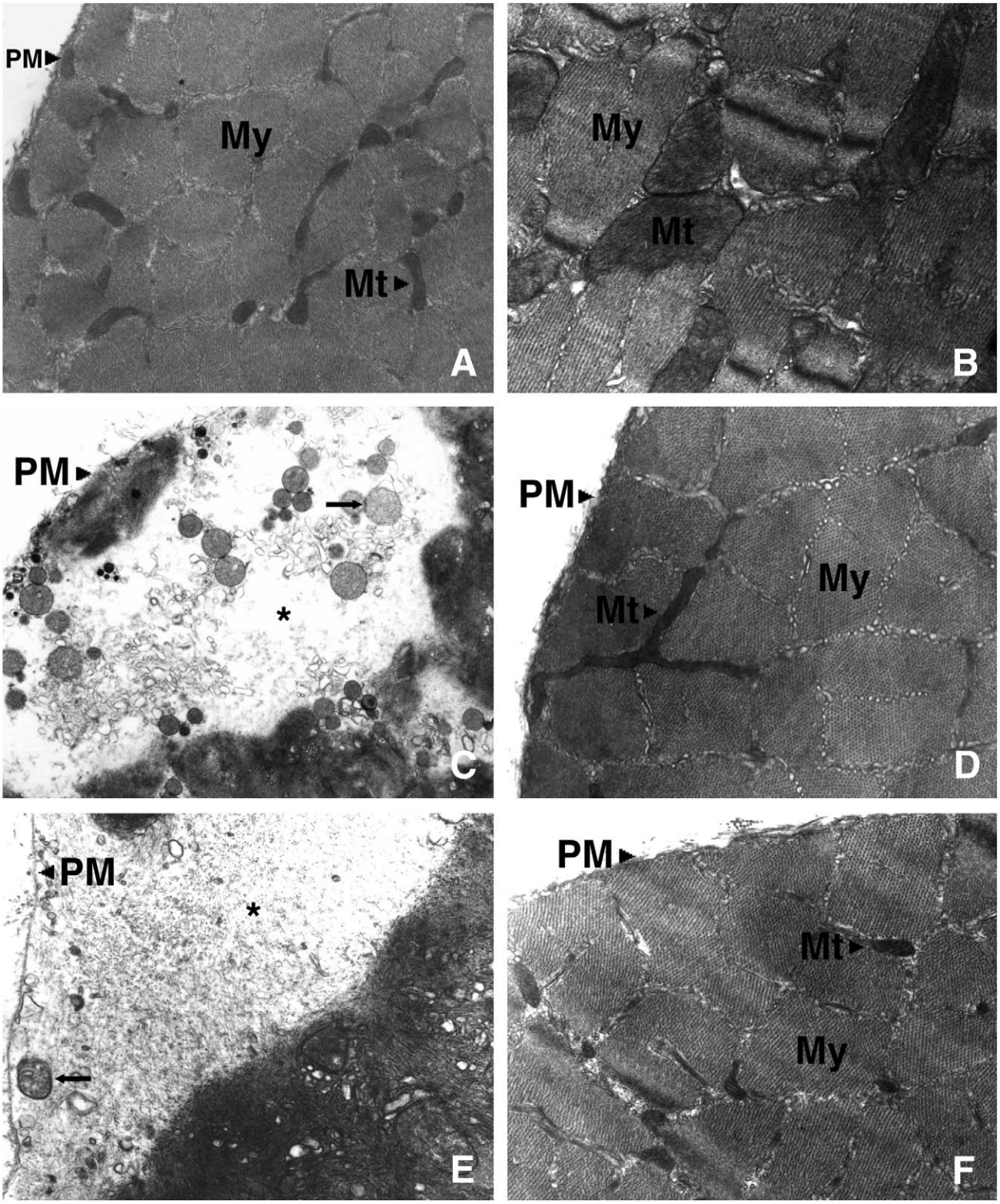

Fig. 4. Electron micrographs of mouse EDL muscle. (A) Control muscle fibers and (B) heparin exposed muscle fibers showing normal morphology with plasma membrane (PM), myofibrils (My) and mitochondria (Mt), A: 15 900 × and B: 27 600 × . C and E: Muscle exposed to BthTX-I and rBthTX-I. Note fiber disorganization or loss of myofibrils (*) and mitochondrial swelling with reduced or ruptured cristae (arrows), C: 17 000 × and E: 7750 × . D and F muscle exposed to heparin pre-incubated native bothropstoxin-I (BthTX-I) and recombinant bothropstoxin-I (rBthTX-I) presenting morphological aspect similar to controls, D and F: 20 400 × .

endomysium (Fig. 3A and B). Ultra-structural analysis revealed organized myofibrils and normal mitochondria (Fig. 4A and B). Few fibers from the control ($2.6 \pm 0.5\%$, $n = 5$) and heparin-treated muscles ($1.2 \pm 0.7\%$, $n = 5$) were injured.

After 120 min of BthTX-I or rBthTX-I exposure, EDL muscles had fibers with different degrees of damage. The most common features are round fibers and edema in endomysium connective tissue, characterized by larger spaces between fibers. Many cells had cytoplasm areas devoid of myofibrils, some with central nuclei (Fig. 3D and G). The muscle damage indices of preparations submitted to BthTX-I or rBthTX-I were 30.3 ± 7.8 ($n = 5$) and 29.3 ± 7.8 ($n = 5$), respectively. Ultra-structural analysis revealed hypercontraction or the loss of myofilaments, leaving sarcoplasmic spaces apparently devoid of myofibrils. Mitochondria showed swelling with reduced or ruptured cristae (Fig. 4C and E).

On the other hand, preparations exposed to heparin pre-incubated BthTX-I and rBthTX-I showed low muscle damage indices ($2.6 \pm 0.5\%$, $n = 5$ and 4.6 ± 1.7, $n = 5$, respectively). Most fibers presented normal aspects (Figs. 3E and H, 4D and F).

Discussion

In order to investigate the structure–function relationship of BthTX-I, a recombinant toxin (rBthTX-I) was first cloned in *E. coli* BL 21 (Spencer et al., 2000). This rBthTX-I behaved similarly to the native toxin when assayed by enzyme-linked immunosorbent assay, analytical ion exchange, and reverse phase chromatography (Spencer et al., 2000). This work characterized the biological activities of the recombinant toxin in mouse neuromuscular preparations, using a native toxin isolated from *B. jararacussu* snake venom as a standard.

The rBthTX-I presented a qualitatively similar biological profile to the native toxin inducing both paralyzing and myotoxic effects. Interestingly, the paralyzing activity of rBthTX-I was slightly more potent than the native toxin. The reason for this was unclear and further structural characterization of rBthTX-I is needed to help resolve this question. Spectroscopy could reveal important structural characteristics of the recombinant toxin, such as intrinsic tryptophan fluorescence emission (ITFE) for dimer forming ability, and ultra-violet circular dichroism for secondary structure.

This work also showed that heparin neutralizes the myotoxic and paralyzing activities of both native and recombinant toxins. Since it has previously been shown that heparin binds to the C-terminus of several Lys49 PLA_2s (Lomonte et al., 1994, 2003), we suggest that the C-terminal region is involved in both the myotoxic and paralyzing activities of BthTX-I.

Based on current evidence, it is conceivable that both the myotoxic and paralyzing activities of BthTX-I result from its ability to alter cell membrane integrity. A calcium influx following membrane destabilization could be responsible for a variety of cell degenerative mechanisms such as myofilament hypercontraction, mithocondrial alterations, and the activation of calcium–dependent proteases and phospholipases, which amplify the muscle-damaging process (Gutierrez and Ownby, 2003). Muscle cell depolarization, a consequence of membrane leakiness, could lead to the muscle paralysis. This hypothesis however, should be tested more directly. The development of a recombinant BthTX-I is an essential step in enabling future site-directed mutagenesis studies to help clarify the structural basis underlying BthTX-I activities.

In summary, this work shows that the rBthTX-I cloned in *E. coli* (BL 21) was expressed in a fully active form, and presents a similar biological profile to the native toxin purified from the *B. jararacussu* venom. This rBthTX-I could be an important tool for performing structure–function relationship studies.

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