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A STUDY OF THE MYOTOXICITY OF BOTHROPSTOXIN-I USING MANGANESE IN MOUSE PHRENIC NERVE-DIAPHRAGM AND *EXTENSOR DIGITORUM LONGUS* PREPARATIONS

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ABSTRACT

Bothropstoxin-I (BthTX-I) from *Bothrops jararacussu* snake venom has a predominantly postsynaptic action that is responsible for this toxin´s myotoxicity. However, BthTX-I also has a presynaptic action that is counteracted by Mn^{2+}, a reversible neuromuscular blocker that acts predominantly presynaptically. In this work, we used two nerve-muscle preparations (mouse phrenic nerve-diaphragm - PND and *extensor digitorum longus* - EDL) to investigate the ability of Mn^{2+} to protect against the myotoxicity of BthTX-I. The preparations were incubated with Tyrode solution (control), BthTX-I, or Mn^{2+} alone. BthTX-I (1.4 μM) produced irreversible blockade in both preparations, whereas the blockade by Mn^{2+} (0.9 mM) was total and reversible in PND but just partially reversible in EDL. Pretreating the preparations with Mn^{2+} resulted in 100% and 80% protection against BthTX-I-induced blockade, respectively. However, when Mn^{2+} (0.9 or 1.8 mM) and BthTX-I (1.4 μM) were co-incubated for 30 min before testing, the blockade was faster and sustained. Washing the preparations resulted in complete, sustained recovery in those exposed to 1.8 mM Mn^{2+} but not to 0.9 mM Mn^{2+}. Morphological analysis showed that the extent of fiber damage by BthTX-I (1.4 μM) was 82% (PND) and 68.5% (EDL), and that Mn^{2+} (0.9 mM) afforded 40% protection in both preparations and reduced the increase in muscle fiber cross-sectional area by 20% and 15%, respectively, compared to BthTX-I alone. Mn^{2+} (0.9 mM) significantly attenuated the release of creatine kinase by BthTX-I. The low creatine kinase activity resulted from a protective action of Mn^{2+} on the sarcolemma and from direct inactivation of the released enzyme. These results show that Mn^{2+} prevents membrane disruption by BthTX-I and can protect against the myotoxicity and neurotoxicity caused by this toxin.

Key words: *Bothrops jararacussu*, bothropstoxin-I, manganese, nerve-muscle preparations, snake venom

INTRODUCTION

Procedures for minimizing the effects of snake venoms and their toxins include the use of antivenoms, which can also be useful for studying venom variability, cross-reactivity and cross-neutralization. Other approaches include the use of molecules with specific characteristics, such as heparin, which has binding sites for growth factors implicated in muscle differentiation and regeneration [10]. Heparin can partially neutralize the effects of bothropstoxin-I (BthTX-I), a predominantly postsynaptic-acting, homodimeric, Lys-49 phospholipase A_2 myotoxin from *Bothrops jararacussu* snake venom, in skeletal muscle [21]. Other molecules, such as manganese ions (Mn^{2+}), can partially prevent the paw edema and gastrocnemius myonecrosis caused by BthTX-I, and reverts the blockade of neurotransmission in phrenic nerve-hemidiaphragm preparations [31]. Manganese can also counteract the effects of a calcium-dependent Asp49 PLA_2 myotoxin from *Naja nigricollis* snake venom [8] on electrical and contractile activities of rat cardiac muscle.

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BthTX-I causes a range of pharmacological effects, including irreversible paralysis of directly- and indirectly-evoked contractions in nerve-muscle preparations [11,28], myonecrosis and mouse paw edema [1,12,14,22]. Despite its very low catalytic activity [29], BthTX-I induces extensive myonecrosis by an unknown mechanism [7,9,12,25]. Lys-49 PLA_2s produce no detectable phospholipid hydrolysis [29] and the dimeric form of the protein is essential for the Ca^{2+}-independent for membrane damaging activity [4].

Oshima-Franco *et al.* [22] recently showed that at concentrations as low as 0.35 μM BthTX-I elicited giant miniature end-plate potentials and a transitory increase in the quantal content of end-plate potentials, with no change in the membrane resting potential. These findings suggested a presynaptic action for this toxin.

In this work, we investigated whether Mn^{2+}, which protects against the neuromuscular blockade caused by BthTX-I, can also protect against the myotoxicity caused by this toxin. This protection was assessed using two neuromuscular preparations (mouse phrenic nerve-diaphragm and *extensor digitorum longus*) that differ in their contractile, electrical and metabolic activities.

MATERIAL AND METHODS

Reagents and preparation of BthTX-I

Manganese chloride was from Sigma-Aldrich Co. (St. Louis, MO, USA). Reagents for histological procedures were from Leica (Vienna, Austria) and Merck (Rio de Janeiro, Brazil). Bothropstoxin-I was purified from *B. jararacussu* venom as described by Homsi-Brandeburgo *et al.* [12].

Animals

Male Swiss white mice (26-32 g) supplied by the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) were housed at 25 ± 3ºC on a 12 h light/dark cycle with access to food and water *ad libitum*. This project (protocol number 510-1) was approved by the institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP) and was done within the guidelines of the Brazilian College for Animal Experimentation (COBEA).

Neuromuscular activity
Phrenic nerve-diaphragm preparation (PND)

The phrenic nerve and diaphragm [2] were excised from anesthetized mice (chloral hydrate, 300 mg/kg, i.p.) sacrificed by exsanguination. The diaphragm was removed and a hemidiaphragm with its nerve branch was mounted under a resting tension of 5 g in a 5 mL organ bath containing aerated (95% O_2 – 5% CO_2) Tyrode solution (pH 7.4, 37ºC) of the following composition (in mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1. The phrenic nerve was stimulated supramaximally (0.1 Hz, 0.2 ms) using a Grass S48 stimulator and bipolar electrodes applied to the nerve. Isometric muscle tension was recorded by a force displacement transducer (Load Cell BG-10 GM) coupled via a Gould universal amplifier to a Gould model RS 3400 physiograph. The preparations were allowed to stabilize for at least 15 min before the addition of Mn^{2+} (0.9 mM for 120 min), BthTX-I (1.4 μM for 120 min) or Mn^{2+} (0.9 mM for 60 min) followed by BthTX-I (1.4 μM for 60 min). The concentrations used were chosen based on previous work [21]. Control experiments were done using Tyrode solution alone. In some experiments, BthTX-I and 0.9 mM or 1.8 mM Mn^{2+} were preincubated for 30 min at room temperature prior to testing.

Extensor digitorum longus preparation (EDL)

Mice were anesthetized with chloral hydrate (300 mg/kg, i.p.) and the EDL was carefully removed by sectioning the tendon without damaging the muscle structure in order to avoid the artifactual release of creatine kinase. The preparation was mounted vertically under a resting tension of 0.5 g in a 3.5 mL organ bath containing aerated Tyrode solution (see above). A bipolar platinum ring electrode was placed around the muscle for indirect stimulation with a Grass S48 stimulator (0.1 Hz, 0.2 ms). Isometric muscle tension was recorded by a force displacement transducer (Load Cell BG - 10 GM) coupled to a Gould RS 3400 physiograph. The preparations were washed three times at 15 min intervals to allow the tissue to stabilize before the addition of Mn^{2+} (0.9 mM), BthTX-I (1.4 μM) or Mn^{2+} (0.9 mM) followed by BthTX-I (1.4 μM). Control experiments were done using Tyrode solution alone.

Myotoxicity
Creatine kinase (CK) activity

Since phrenic nerve-hemidiaphragm preparations have a spontaneous release of CK resulting in elevated basal levels, this activity was measured only in EDL preparations. For the quantification of CK activity, samples (100 μL) of the EDL bathing solution were withdrawn from the organ bath at 0, 15, 30, 60, 90 and 120 min after each of the treatments (n=12-20 measurements per group and time interval) and the volume withdrawn was replaced with an equal volume of Tyrode solution. The samples collected were stored for 2 h at 4ºC until CK activity (expressed in units/L) was measured using a commercial kit (CK NAC EC 2.7.3.2, Randox® 335, England, UK).

To determine whether the decrease in CK activity was attributable to the ability of Mn^{2+} to prevent sarcolemmal rupture and further enzyme release, or whether Mn^{2+} inactivated the enzyme after its release from the muscle, aliquots (100 µL) were taken from the incubation bath before (t_0 min) and 60 min after (t_{60} min) toxin addition to the bath and assayed. The EDL preparations were subsequently removed from the bathing solution and 0.9 mM Mn^{2+} was added to the incubation bath for 30 min. Samples (100 µL) were collected at the end of the incubation (t_{90} min) and CK was assayed as above described.

Histopathological analysis

After a 120 min incubation with Tyrode solution, Mn^{2+}, BthTX-I or Mn^{2+} followed by BthTX-I, the PND and EDL muscles were rapidly removed from the bath and fixed in Bouin's solution for 24-48 h, after which the tissues were washed three times with a solution of water and ammonia followed by dehydration and embedding in Historesin (Leica). Sections 2 µm thick were cut using a Leica RM 2035 microtome (Leica Instruments Gmbh, Nubloch/Heidelberg) and stained with 0.5% toluidine blue for examination by light microscopy. Morphological damage was quantified by counting the number of fibers with lesions (edema, intense myonecrosis characterized by atrophy of the muscle fibers with a hyaline aspect, sarcolemmal disruption and lysis of the myofibrils) and this value was expressed as a percentage of the total number of cells counted in six non-overlapping, non-adjacent areas of each muscle. This procedure was used in all control and treated preparations (n = 5-11 preparations/treatment).

Cross-sections (2 µm thick) of historesin-embedded diaphragms or EDL were stained with 0.5% toluidine blue and examined with a Nikon Eclipse E800 microscope. The images were captured with a digital imaging system (CoolSnap-Pro Color, Media Cybernetics, USA). For each group (control and treated preparations), the areas of 125 fibers in five non-overlapping, non-adjacent regions of each sample was measured using Image Pro Plus Software.

Statistical analysis

The results were expressed as the mean ± S.E.M. of the number of mice or experiments. Statistical comparisons between groups were done using Student's *t*-test, with $p<0.05$ indicating significance.

RESULTS

Neuromuscular activity

The neuromuscular activity of BthTX-I in PND and EDL preparations was assessed using a single concentration of toxin (1.4 µM) which produced irreversible neuromuscular blockade. The twitch-tension responses of preparations incubated with Tyrode solution, Mn^{2+} (0.9 mM) or BthTX-I (1.4 µM) for 120 min are shown in Figure 1 (Panels A,B for PND and C,D for EDL preparations). The time required for 1.4 µM BthTX-I to cause 50% neuromuscular blockade in PND and EDL preparations was practically the same (32.8 ± 3.3 min, n=6, and 32.9 ± 3.2 min, n=11, respectively), but the time for 100% blockade was slightly greater for EDL than for PND preparations at the end of the 120 min incubation (Fig. 1A,C). The twitch-tension blockade was irreversible, even after washing (data not shown).

Mn^{2+} alone (0.9 mM) produced complete, reversible blockade of the twitch-tension response in PND preparations, whereas in EDL preparations the blockade was partial and sustained (Fig. 1A,C). Pretreatment with 0.9 mM Mn^{2+} for 60 min prevented the typical blockade caused by BthTX-I in both preparations (Fig. 1B,D), with 100% protection in PND and 80% protection in EDL, as seen after washing the preparations (data not shown). The different sensitivities of PND and EDL preparations to Mn^{2+} alone contrasted with their similar sensitivites to BthTX-I.

When BthTX-I and Mn^{2+} 0.9 mM were preincubated for 30 min before testing in PND preparations, total blockade occurred within 1 - 2 min and was initially reversed by washing but was then followed by a gradual decrease of the twitch-tension and neuromuscular blockade typical of BthTX-I (Fig. 2A). In contrast, with Mn^{2+} 1.8 mM, after washing, a complete and maintained protection of BthTX-I blockade was observed, indicating that the effect of Mn^{2+} depended on the concentration of this ion (Fig. 2B).

Myotoxic activity

Creatine kinase activity

BthTX-I (1.4 µM) caused a progressive release of CK from EDL muscle that reached 1,261 ± 113 U/L (n=20) after 120 min (Fig. 3). Pre-incubation with Mn^{2+} (0.9 mM, 60 min) markedly attenuated the CK release (95± 18 U/L, n=17; corresponding to ≥ 80% reduction; $p<0.05$). In the protocols used to evaluate the influence of Mn^{2+} on already released CK (U/L), in which 0.9 mM Mn^{2+} was added to organ baths used to incubate EDL with BthTX-I for 30 min, the

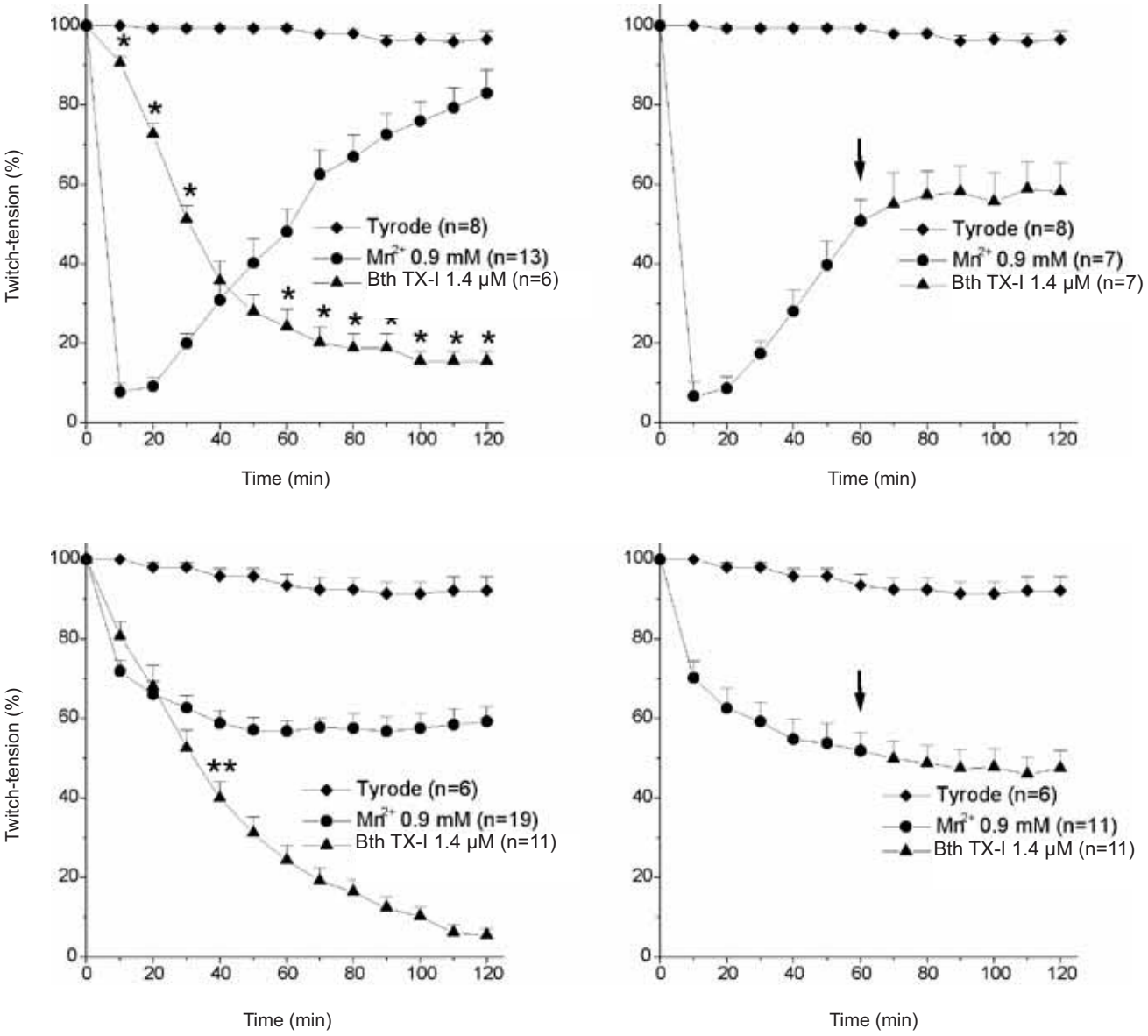

Figure 1. Twitch-tension responses of indirectly stimulated (0.1 Hz, 0.2 ms) PND (**A** and **B**) and EDL (**C** and **D**) preparations. The preparations were incubated for 120 min with Mn^{2+} (0.9 mM), BthTX-I (1.4 µM), or Tyrode solution. BthTX-I alone caused irreversible neuromuscular blockade (**A** and **C**). Mn^{2+} caused blockade with spontaneous reversal in PND (**A**) but not in EDL (**C**) preparations. Mn^{2+} attenuated the neuromuscular blockade caused by BthTX-I (**B** and **D**). Arrows indicate the addition of BthTX-I. Each point is the mean ± S.E.M. of the number of experiments indicated in parentheses. ***p*<0.05. ****All points from this time interval onwards were significantly different (*p*<0.05) from 0.9 mM Mn^{2+}.

enzymatic activity decreased markedly to 30 ± 7.0 (t_0 min, n=6), 950 ± 78 (t_{60} min, n=6) and 193 ± 28 (t_{90} min, n=6).

Histopathological analysis

Figures 4 and 5 show cross-sections of PND and EDL muscle fibers after 120 min of indirect electrical stimulation, respectively. The fibers were well-preserved after incubation with Tyrode solution alone (Figs. 4A and 5A) and also with 0.9 mM Mn^{2+} alone in PND preparations (p>0.05, n=6), but not in EDL preparations, where slight changes in the polygonal profile were observed (8.2 ± 1.1% n=6, different from the characteristic lesions seen in myonecrosis) (Figs. 4B and 5B).

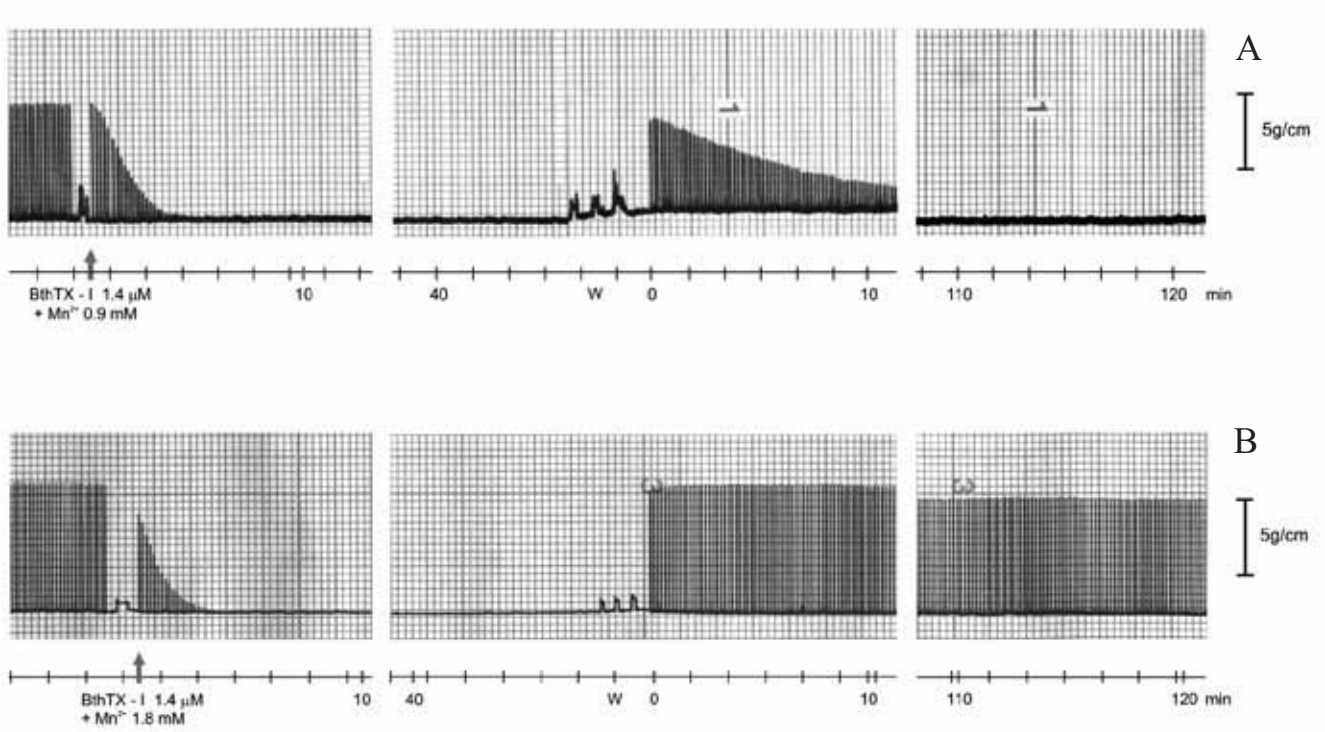

Figure 2. Neuromuscular activity of a mixture of Mn^{2+} (0.9 or 1.8 mM) + BthTX-I (1.4 µM) preincubated for 30 min at room temperature (37º C) prior to testing. Note that BthTX-I produced neuromuscular blockade with either of the Mn^{2+} concentrations. However, washing (**W**) the preparation treated with the Mn^{2+} (0.9 mM) + BthTX-I (1.4 µM) mixture resulted in an initial recovery followed by the blockade typical of BthTX-I (**A**). In contrast, washing the preparation treated with the Mn^{2+} (1.8 mM) + BthTX-I (1.4 µM) mixture resulted in a recovery that was sustained for 120 min (**B**). These results show that the protective effect of Mn^{2+} is concentration-dependent.

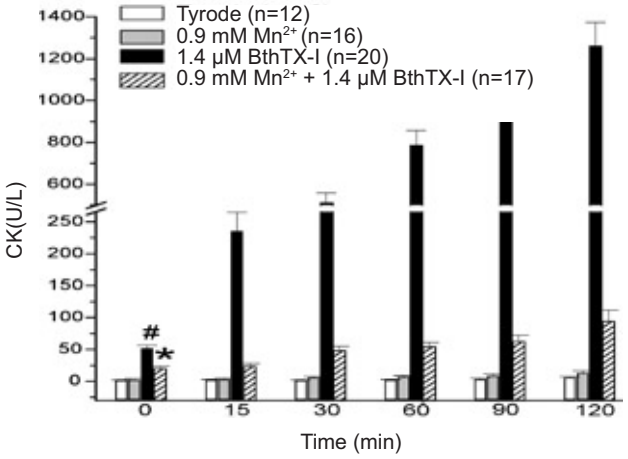

Figure 3. CK release from mouse EDL muscle preparations during a 120 min incubation with Tyrode solution alone, Mn^{2+} (0.9 mM), BthTX-I (1.4 µM), and Mn^{2+} (0.9 mM, 60 min) followed by BthTX-I (1.4 µM, 60 min). Co-incubation with Mn^{2+} prevented the release of CK by BthTX-I. Each column is the mean ± S.E.M. of the number of experiments indicated in parentheses. #All points from this time interval onwards were significantly different ($p<0.05$) from the controls and 0.9 mM Mn^{2+} alone. *All points from this time interval onwards were significantly different ($p<0.05$) from 1.4 µM BthTX-I alone.

Incubation of PND or EDL preparations with BthTX-I resulted in myonecrosis, with PND being significantly ($p<0.05$) more susceptible to damage than EDL (Figs. 4C and 5C). Panel C of Figures 4 and 5 shows myonecrotic fibers with lysed (clear, pulverulent sarcoplasmic areas) and/or densely-clumped tortuous myofibrils, with consequent alterations in the myofiber arrangement. These panels also show that most small, dark myofibers had increased in size and had lost their polygonal shape to become round.

Quantification of the percentage of damaged fibers and their cross-sectional areas corroborated the histopathological analysis. The percentage of damaged fibers in PND preparations treated with BthTX-I alone (82 ± 3.1%, n=11) was significantly higher than in EDL preparations (68.5 ± 2.9%, n=6, $p<0.05$). Pretreating PND and EDL preparations with Mn^{2+} followed by BthTX-I (Figs. 4D and 5D) reduced significantly the percentage of damaged fibers to 49.2 ± 4.5% (n=10) and 41.3 ± 2.9% (n=6), respectively ($p<0.05$).

Figure 6 shows that the cross-sectional areas of control muscle fibers (preparations incubated with

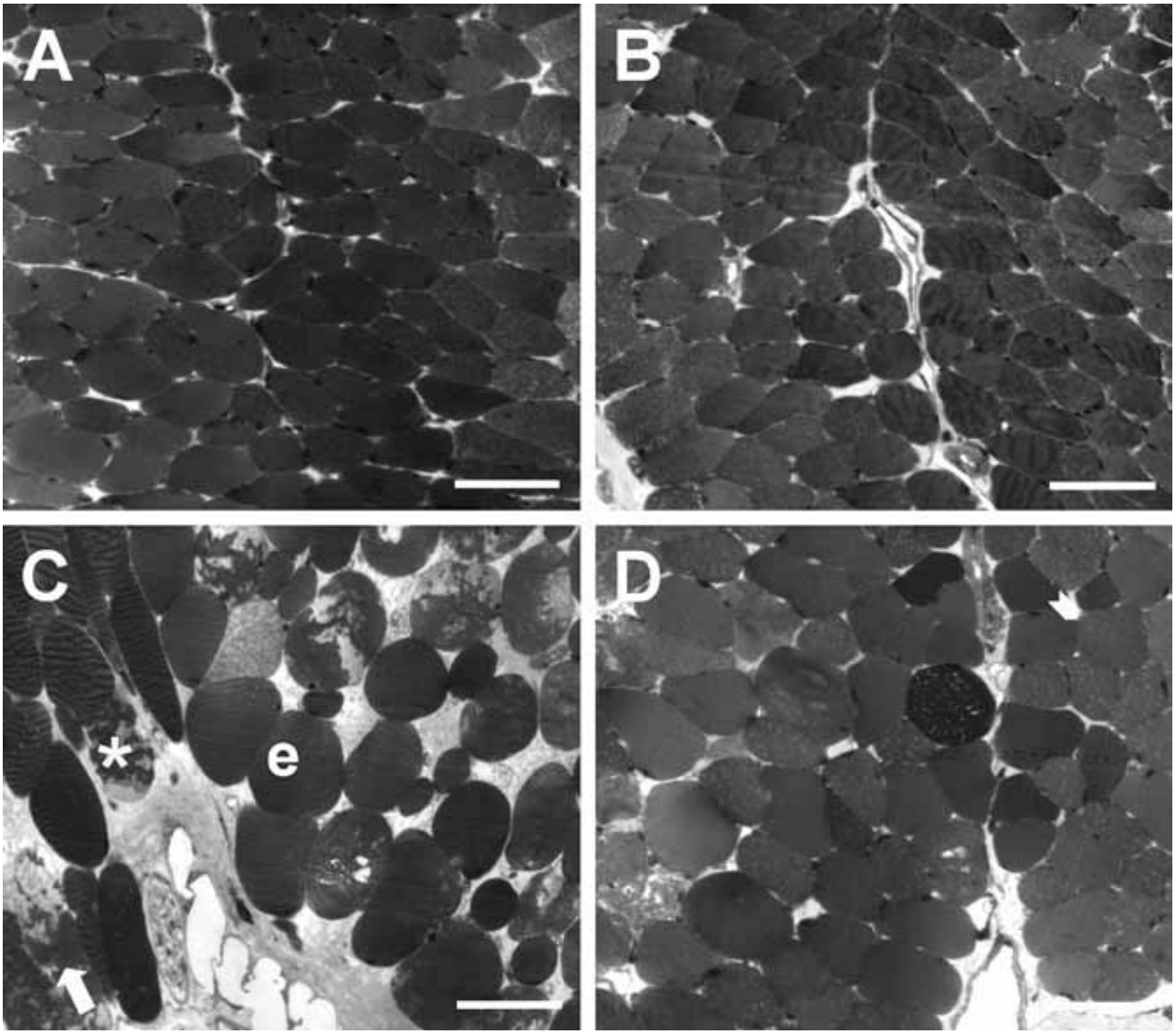

Figure 4. Cross-sections (2 μm thick) of PND preparations after a 120 min incubation. In muscles incubated with Tyrode solution (**A,** control) and 0.9 mM Mn^{2+} (**B**), the fibers had a normal appearance. Note the polygonal aspect of the fibers and the peripheral nuclei. (**C**) Muscle incubated with 1.4 μM BthTX-I showing edema (**e**), intense myonecrosis characterized by atrophy of the muscle fibers with a hyaline aspect (*****), sarcolemmal disruption, and lysis of the myofibrils (**arrow**). (**D**) Muscle incubated with 0.9 mM Mn^{2+} (60 min) followed by 1.4 μM BthTX-I (60 min) showing some fibers with a normal aspect (**arrowhead**). Bar = 50 μm.

Tyrode solution) were 1,887 ± 93 μm^2 (n=5) for PND and 940 ± 37 μm^2 (n=5) for EDL. Incubation with Mn^{2+} significantly increased (by 54%, $p<0.05$) the cross-sectional area of diaphragm fibers but had little effect on the cross-sectional area of EDL fibers (5.7% increase); the latter were not significantly different from the controls. BthTX-I markedly increased the cross-sectional area of PND (206%) and EDL (167%) muscle fibers to 3,885 ± 141 μm^2 (n=5) and 1,574 ± 58 μm^2 (n=5), respectively ($p<0.05$ compared to the corresponding controls). Pretreatment with Mn^{2+} significantly reduced ($p<0.05$) the increase in fiber cross-sectional area caused by BthTX-I to 3,107 ± 109 μm^2 (n=5) and 1,346 ± 48 μm^2 (n=5) for PND (20% reduction) and EDL (15% reduction) preparations, respectively, when compared with BthTX-I alone.

DISCUSSION

Mn^{2+} inhibits Ca^{2+} influx into nerve endings thereby preventing acetylcholine (ACh) release and

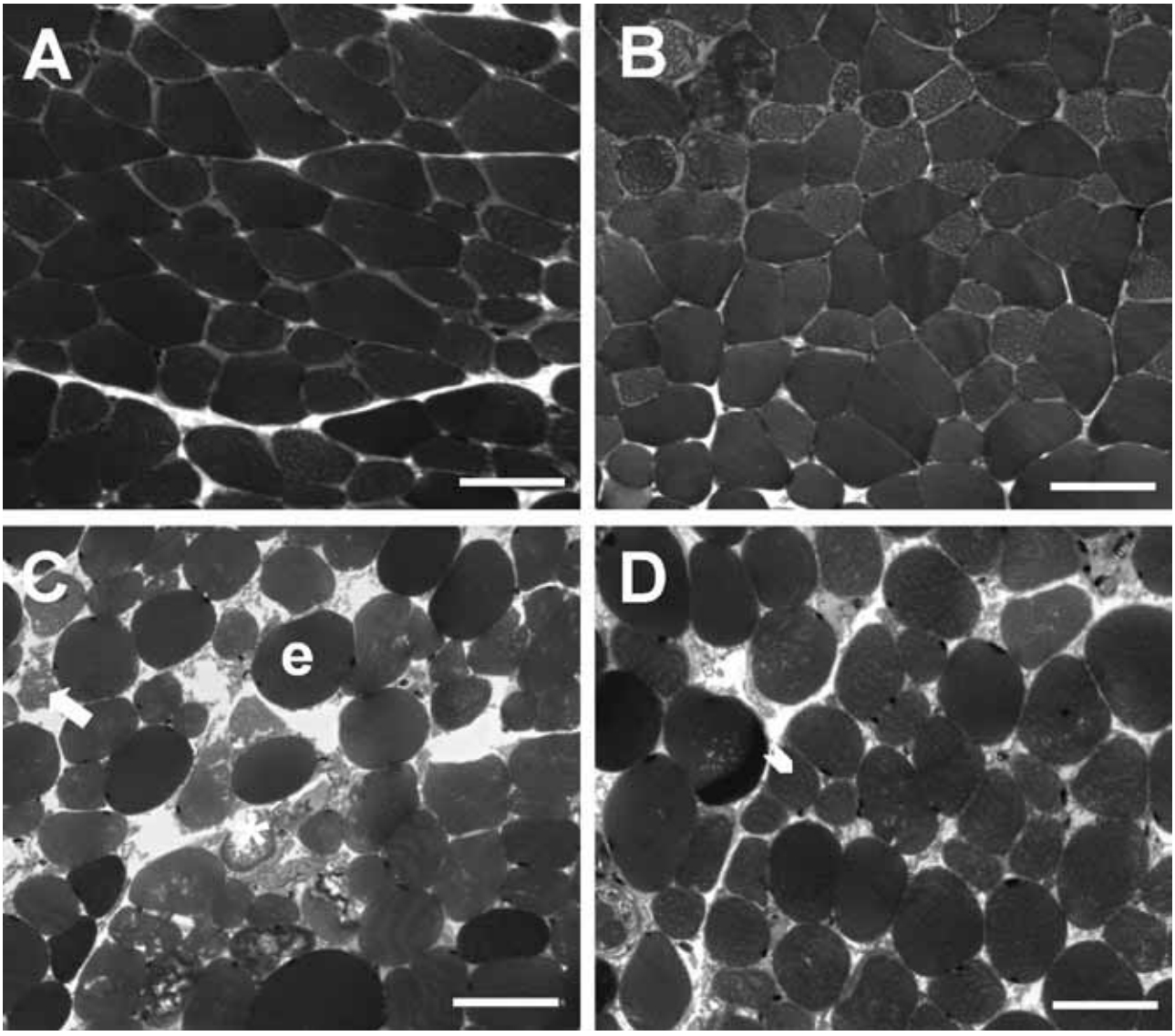

Figure 5. Cross-sections (2 μm thick) of EDL preparations after a 120 min incubation. In muscle incubated with Tyrode solution alone (**A,** control) and 0.9 mM Mn^{2+} (**B**), the fibers had a normal appearance. Note the polygonal aspect of the fibers and the peripheral nuclei. (**C**) Muscle incubated with BthTX-I 1.4 μM showing edema (**e**), intense myonecrosis characterized by atrophy of the muscle fibers that have a hyaline aspect (*****), sarcolemmal disruption, and lysis of the myofibrils (**arrow**). (**D**) Muscle incubated with 0.9 mM Mn^{2+} (60 min) followed by 1.4 μM BthTX-I (60 min) showing some fibers with a normal aspect (**arrowhead**). Bar = 50 μm.

causing total but reversible neuromuscular blockade in hemidiaphragm preparations [16]. The spontaneous reversal of the blockade caused by Mn^{2+} in mouse PND preparations has been studied [22,31] based on findings that Mn^{2+} is taken up and stored in nerve terminals where it can mimic Ca^{2+}-functioning [27]. The spontaneous restoration of diaphragm twitch-tension responses after blockade induced by Mn^{2+} is attributable to the availability of free extracellular Ca^{2+} that is essential for reestablishing neuromuscular transmission [22,31].

In contrast to PND, the blockade caused by Mn^{2+} in EDL was partial but sustained until the end of the incubation period; this divergence in reactivity probably reflects differences in the biochemical composition of the muscle fiber types involved [5,6,13,17,26,30,32]. However, when both preparations were preincubated for 60 min with Mn^{2+}, there was no blockade by BthTX-1**.** In experiments using preincubated mixtures, Mn^{2+} showed concentration-dependent protection after washing of the preparations,

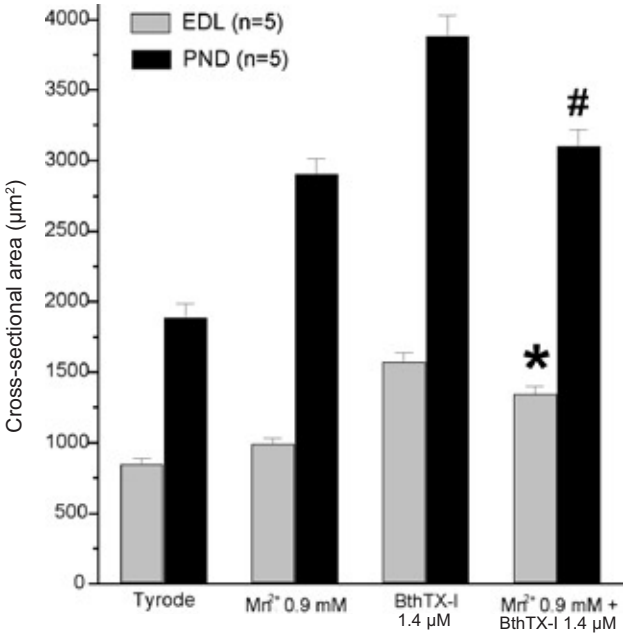

Figure 6. Cross-sectional areas of fibers from PND and EDL muscles. The areas were calculated as described in Methods. Each column is the mean ± S.E.M. of five preparations. (#) and (*), $p<0.05$ compared to 1.4 µM BthTX-I alone in PND and EDL preparations, respectively.

indicating that the protection was not attributable to the formation of acid-base complexes, in contrast to the inhibition seen with suramin or heparin [3,21].

Since BthTX-I acts presynaptically at low concentrations [22], the protective effect of Mn^{2+} against the neurotoxicity in both preparations may have been partially mediated by a presynaptic action of this ion. However, BthTX-I is predominantly postsynaptic in action and Mn^{2+} prevented membrane depolarization in preparations pretreated with this ion, thus raising the possibility that, under certain conditions, Mn^{2+} may also act postsynaptically [22]. The histological findings showing the ability of Mn^{2+} to partially protect against tissue damage support the latter hypothesis.

The finding that Mn^{2+} completely abolished the neurotoxicity of BthTX-I but only partially prevented the myotoxicity in both preparations suggests either that the concentration of Mn^{2+} was insufficient to neutralize all of the myotoxicity, or that part of the myotoxicity was mediated by a mechanism insensitive to this ion. The mechanisms involved in BthTX-I-induced myotoxicity are unclear, although it is known that Lys-49 PLA_2s cause no detectable phospholipid hydrolysis and that the sarcolemmal damage is calcium-independent [4,29]. It may be speculated that BthTX-I acts on Ca^{2+} targets, such as calcium channels located in the muscle fiber membrane, in a manner similar to Mn^{2+}. BthTX-I may cause myonecrosis by altering the sarcolemmal permeability, with consequent edema (as shown by the significant increase in fiber cross-sectional area) and cell membrane disruption. Pretreatment with Mn^{2+} significantly reduced the fiber cross-sectional area, indicating that this cation probably minimized sarcolemmal rupture by preventing the permeabilization of this organelle and reducing the intracellular edema.

Venom-induced necrosis involves the action of specific factors and/or a combination of secondary, non-specific reactions in the affected tissues [23,24]. In general, the quantification of CK release combined with morphological analysis is recommended for a more consistent assessment of myotoxicity [9,15,18-20]. As a quantitative biochemical marker of skeletal muscle damage, CK release provided an additional parameter for confirming the histological results. Indeed, there was a correlation between the CK levels and the pathological alterations observed with BthTX-I. This correlation disappeared following treatment with Mn^{2+}. Mn^{2+} significantly prevented the release of CK but only partially protected against the myonecrosis caused by BthTX-I. These divergent findings suggested that the low CK activity measured in the bath did not correspond to the true amount of enzyme released. A direct inactivation of the enzyme by this ion could explain the drastic change in the bath content of CK. Indeed, our results for experiments in which 0.9 mM Mn^{2+} was added to bathing solution in which EDL had been incubated with BthTX-I for 30 min before being removed (and that showed high CK activity) strongly indicated that Mn^{2+} was able to directly inactivate the catalytic site of CK. These findings suggest that Mn^{2+} decreased the release of CK by muscle and, at the same time, directly inhibited the activity of enzyme already released into the bath.

In conclusion, Mn^{2+} antagonizes the neurotoxicity and myotoxicity of BthTX-I, but is more effective against the former activity. This difference may reflect the fact that the main target for BthTX-I is the muscle sarcolemma, whereas the main target for Mn^{2+} is the presynaptic axolemma.

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