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Effect of *Bothrops leucurus* venom in chick biventer cervicis preparations

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Abstract

Bothrops leucurus is a poorly studied pitviper found in northeastern Brazil. We examined the action of *B. leucurus* venom (5–100 μg/ml) on contractile responses in chick biventer cervicis preparations. Muscle damage was assessed by quantifying the release of creatine kinase (CK) and by histological analysis. *B. leucurus* venom dose-dependently inhibited the contractile responses of indirectly stimulated preparations, the maximum inhibition with 100 μg of venom/ml being 74.0 ± 6.6% (mean ± SEM) after 120 min. The venom also reduced contractures to exogenous acetylcholine (55 and 110 μM) and K^+ (13.4 mM) (85–100% reduction with 100 μg of venom/ml) and increased the release of CK (348 ± 139 U/ml in controls vs 1260 ± 263 U/ml with 20 μg of venom/ml after 120 min, $p < 0.05$). The accompanying morphological changes included multivacuolated, swollen, amorphous fibers and agglutinated myofibrils. These results indicate that *B. leucurus* venom can adversely affect neuromuscular transmission and produce muscle damage in avian preparations.

Keywords: *Bothrops leucurus*; Myotoxin; Neuromuscular junction; Neurotransmission; Phospholipase A_2; Venom

1. Introduction

The venoms of Central and South American pitvipers of the genus *Bothrops* produce extensive local tissue damage, including edema, hemorrhage and necrosis, as well as systemic effects such as coagulopathies, renal failure and hemorrhage (Rosenfeld, 1971; Gutiérrez and Lomonte, 1989; Watt, 1989; Fan and Cardoso, 1995; Ministério da Saúde, 1998). These effects result from the concerted action of various enzymes (especially phospholipases A_2 and proteases) and toxins, such as myotoxins (Gutiérrez and Lomonte, 1995), present in these venoms.

Although there is no convincing evidence of neurotoxicity following envenomation of humans or experimental animals by *Bothrops* venoms, studies using isolated preparations have shown that the venoms of *Bothrops jararacussu* (Rodrigues-Simioni et al., 1983), *Bothrops insularis* (Cogo et al., 1993), *Bothrops neuwiedi* (Borja-Oliveira et al., 2002), *Bothrops pirajai* (Costa et al., 1999) and other *Bothrops* species (Zamunér et al., 1997) adversely affect

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neuromuscular transmission in avian and mammalian nerve–muscle preparations. *B. jararacussu* venom also inhibits neurotransmission in frog nerve–muscle preparations (Rodrigues-Simioni et al., 1983). This inhibition involves primarily a postsynaptic action mediated by PLA_2 myotoxins (Homsi-Brandeburgo et al., 1988; Heluany et al., 1992; Cogo et al., 1998; Soares et al., 2000).

Bothrops leucurus, popularly known as the white-tailed jararaca or lancehead because of the white tail color when juvenile, ranges from the state of Ceará in northeastern Brazil to the state of Espírito Santo in the southeast and occurs in dry or humid habitats at altitudes up to approximately 500 m (Campbell and Lamar, 1989; Porto and Teixeira, 1995; Lira-da-Silva et al., 1996). Since little is known of the toxinology of this species, we have examined the neuromuscular action of *B. leucurus* venom in chick biventer cervicis preparations and also assessed the histological damage caused by this venom.

2. Materials and methods

2.1. Reagents

Acetylcholine, bovine casein, bovine serum albumin, 4-nitro-3-(octanoyloxy)-benzoic acid, Tris base and *d*-tubocurarine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Ninety-six-well plates were from Corning Incorporated (Corning, NY, USA). Other reagents were of analytical grade from local suppliers.

2.2. Venom

Venom collected by manual milking of adult *B. leucurus* of both sexes in the Department of Zoology, Institute of Biology, Federal University of Bahia, was desiccated and stored at 4 °C until used. Desiccated *Bothrops jararaca* venom was obtained from the Instituto Butantan, São Paulo, Brazil, and stored at 4 °C.

2.3. Animals

HY-Line W36 chicks 4–8 days old were obtained from Granja Ito S/A (Campinas, SP, Brazil) and housed at 24–28 °C with access to water and food ad libitum. The experiments reported here were done within the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

Table 1
Protein content and PLA_2 and proteolytic activities of *B. leucurus* venom

Venom	Protein (% dry wt)	PLA_2 (A_{425} nm/mg)	Protease (U/mg)
B. leucurus	92.7 ± 6.0	2.84 ± 0.87*	94.4 ± 14.2
B. jararaca	79.9 ± 1.3	0.30 ± 0.03	60.5 ± 13.9

The values are the mean ± SEM of three determinations done in duplicate. *$p < 0.05$ compared to *B. jararaca*.

2.4. Protein content

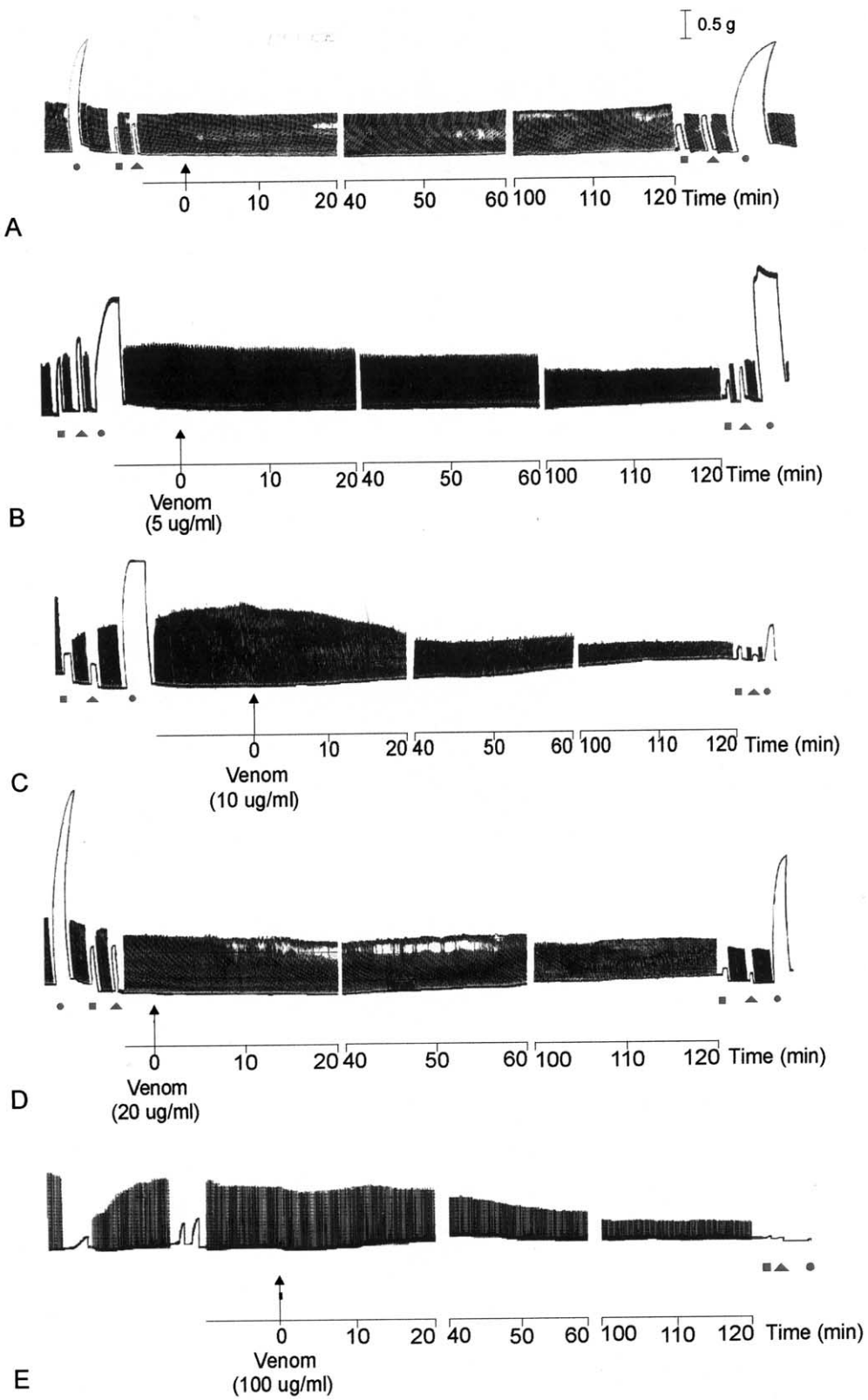
The protein content of the venom was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.5. Enzymatic activities

Phospholipase A_2 (PLA_2) activity was measured using the assay of Holzer and Mackessy (1996) modified for 96-well plates (Beghini et al., 2000). The standard assay contained 200 μl of buffer (10 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 100 mM NaCl), 20 μl of water, 20 μl of venom (stock solution, 10 mg/ml) and 20 μl of substrate (4-nitro-3-(octanoyloxy)-benzoic acid, 3 mM in acetonitrile) in a final volume of 260 μl. After the addition of substrate, the mixture was incubated for 30 min at 37 °C and the absorbance read at 5 min intervals. The changes in absorbance were determined with a multiwell plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA), and the enzymatic activity was expressed as the increase in absorbance after 30 min (A_{425} nm) per mg of venom.

Proteolytic activity was quantified by the method of Kunitz (1947) using bovine casein as substrate. To 1.9 ml of 1% casein in 100 mM Tris–HCl, pH 7.5, was added 0.1 ml of venom solution and the mixture incubated for 20 min at 37 °C. The reaction was terminated by adding 2 ml of 5% trichloroacetic acid followed by vigorous mixing and standing on ice for 30 min. After centrifugation (2000*g*, 15 min, 25 °C), the absorbance of the supernatant was read at 280 nm in a Uvikon 810 spectrophotometer (Kontron Instruments, Milan, Italy). One unit of enzyme activity corresponded to an increase of 1.0 absorbance unit per min and was expressed as units per mg of venom.

Fig. 1. Muscle responses in indirectly stimulated chick biventer cervicis preparations. (A) Control (Krebs solution only). (B), (C), (D) and (E), *B. leucurus* venom at 5, 10, 20 and 100 μg/ml, respectively. Note the neuromuscular blockade, muscle contracture and alterations in the responses to acetylcholine (55 and 110 μM, squares and broad triangles) and K^+ (13.4 mM, circles) in venom-treated preparations after 120 min. The preparations were mounted and stimulated as described in Section 2. These recordings are representative of 3 (E), 7 (A, C) and 8 (B, D) experiments each.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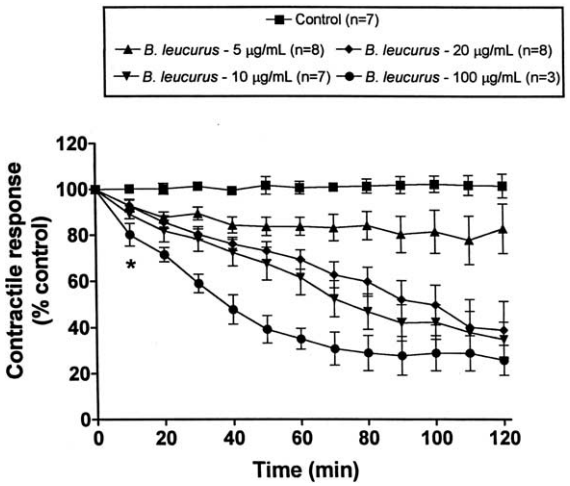

Fig. 2. Mean muscle responses in indirectly stimulated control and *B. leucurus* venom-treated chick biventer cervicis preparations. The preparations were mounted and stimulated as described in Section 2. The points represent the mean ± SEM of the number of experiments indicated. All points from 10 min (*) onwards (except for the last three intervals for 5 μg/ml) were significantly different ($p < 0.05$) from the controls.

Table 2
Inhibition by *B. leucurus* venom of muscle contractures produced by acetylcholine (ACh) and K^+

Drug	Venom (μg/ml)	*n*	Post-venom contractures (%)
ACh (55 μM)	0 (C)	7	114.3 ± 25.3
	5	6	57.1 ± 14.3*
	10	5	55.6 ± 11.1
	20	3	25.0 ± 3.8**
	100	3	6.4 ± 6.4*
ACh (110 μM)	0 (C)	7	109.1 ± 27.3
	5	6	62.3 ± 18.8
	10	5	58.3 ± 16.7
	20	3	33.3 ± 10.0*
	100	3	15.4 ± 15.4*
K^+ (13.4 mM)	0 (C)	7	98.6 ± 14.3
	5	6	105.0 ± 25.0
	10	5	44.4 ± 22.2
	20	3	31.6 ± 15.8*
	100	3	0.0 ± 0.0**

The preparations were mounted and the contractures recorded as described in Section 2. The values (mean ± SEM) represent the percentage of response *remaining* 120 min after venom addition compared to the pre-venom value (100%). (C) = control. *$p < 0.05$ and **$p < 0.01$, compared to the pre-venom response.

2.6. Chick biventer cervicis preparation

The preparation was isolated and mounted as described by Ginsborg and Warriner (1960). The muscle was suspended under a resting tension of 0.5 g in 4 ml of aerated (95% O_2–5% CO_2) Krebs solution (composition, in mM: NaCl 118.6, KCl 4.69, $CaCl_2$ 1.88, KH_2PO_4 1.17, $NaHCO_3$ 14.99 and glucose 11.65, pH 7.4) at 37 °C, and was stimulated either directly (20–30 V, 0.1 Hz, 0.2 ms), in the presence of *d*-tubocurarine (*d*-Tc, 4 μg/ml), or indirectly (5–6 V, 0.1 Hz, 0.2 ms). When *d*-Tc was used, the preparations were initially incubated under indirect stimulation until full blockade by *d*-Tc was achieved, after which the voltage was increased to the level indicated above for direct stimulation in order to obtain adequate muscle responses. After the addition of venom (0.2 ml; final concentration, 5–100 μg/ml), the muscle contractions were recorded for up to 120 min via an isometric transducer (BG-25 GM Kulite, Semiconductor Products Inc.) coupled to a 4-channel Gould 3400 recorder (Gould Instrument Systems, Valley View, OH, USA) or a 2-channel Ugo Basile Gemini 7070 recorder (Ugo Basile, Varese, Italy). The responses to acetylcholine (55 and 110 μM) and K^+ (13.4 mM) were tested before and after venom addition. In control experiments, 0.2 ml of Krebs solution alone was added to the organ bath.

2.7. Creatine kinase release

Aliquots (20 μl) of bath solution were removed before and 10, 20, 30, 40, 80 and 120 min after the addition of venom to the preparations. An equal volume of Krebs solution replaced the volume withdrawn for creatine kinase (CK) measurements. CK levels were quantified using a commercial kit (NAC Laborlab S/A, São Paulo, SP, Brazil) and were expressed in U/l.

2.8. Morphological analysis

After incubation with venom (5, 10 and 20 μg/ml) for 120 min, the preparations were removed and fixed in Bouin solution followed by washing with ammonium hydroxide for 30 min. The muscles were then dehydrated in a graded series of ethanol (70, 80, 95 and 3 × 100%, 30 min each) before embedding in historesin. After resin polymerization at 60 °C, the tissues were mounted in blocks and sections 2 μm thick were cut with a microtome (Leica model RM 2145, Leica Microsystems Ltd, Heerbrugg, Switzerland). The sections were stained with toluidine blue and examined by light microscopy using an Olympus microscope (Olympus Optical Co. Ltd, Tokyo, Japan) prior to photographing.

2.9. Statistical analysis

Where appropriate, the results were expressed as the mean ± SEM of the number of experiments indicated. The significance of the differences between groups was assessed using Student's *t*-test. A value of $p < 0.05$ indicated significance.

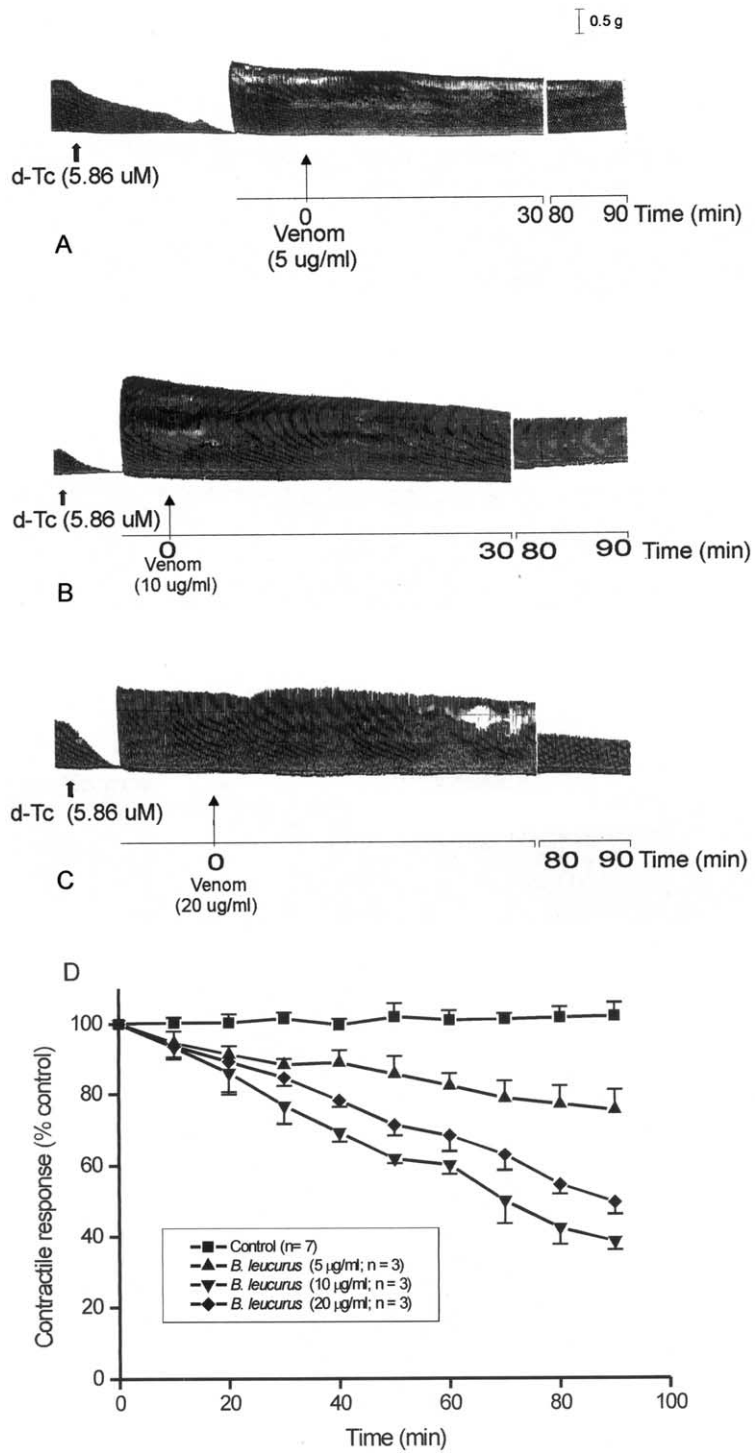

Fig. 3. Muscle responses in directly stimulated chick biventer cervicis preparations. (A), (B) and (C), *B. leucurus* venom at 5, 10 and 20 μg/ml, respectively. (D) Mean values (± SEM, $n = 3$) for the inhibition observed at the above concentrations. To allow for direct stimulation, the preparations were first incubated with 5.85 μM *d*-tubocurarine (*d*-Tc) under indirect stimulation (5–6 V), as described in Section 2. Following blockade by *d*-Tc, the voltage was increased to 20–30 V, which accounts for the increase in the contractile responses seen in (A) to (C). All points from 20 min onwards were significantly different ($p < 0.05$) from the controls.

3. Results

3.1. Protein content and enzymatic activities

Table 1 shows the protein content and the PLA_2 and proteolytic activities of *B. leucurus* venom compared with those of *B. jararaca* (positive control). Although the mean values for all three parameters were greater in *B. leucurus* venom, only PLA_2 activity was significantly different from *B. jararaca* venom.

3.2. Chick biventer cervicis preparations

In control preparations, there were no significant changes in the amplitude of the muscle contractions in response to indirect stimulation over a 120 min period, and the contractures to acetylcholine and K^+ were also unaltered ($n = 7$; Fig. 1(A)). *B. leucurus* venom (5–100 μg/ml) produced progressive, irreversible (by washing) inhibition of the muscle contractions in indirectly stimulated preparations, with maximum inhibition of 16.9 ± 10.8, 64.8 ± 7.6, 60.7 ± 12.4 and 74.0 ± 6.6% for 5, 10, 20 and 100 μg/ml, respectively, after 120 min; there was no significant difference between the levels of inhibition produced by the last three venom concentrations (Figs. 1(B)–(E) and 2).

In addition to the neuromuscular inhibition, venom concentrations of 10–100 μg/ml produced gradual muscle contracture which occurred from 5 min after venom addition onwards (Fig. 1(C)–(E)). This contracture corresponded to an increase of 28–55% above the resting tension. However, this phenomenon varied with the venom concentration, occurring in only 14.3% of the experiments with 10 μg of venom/ml, in 43.3% with 20 μg/ml and in 100% with 100 μg/ml, and showed no consistent dose-dependence in its magnitude. Venom (5–100 μg/ml) also decreased the acetylcholine-induced contractures, particularly after 120 min at higher concentrations (20 and 100 μg/ml), and this response was not significantly affected by increasing the acetylcholine concentration from 55 to 110 μM (Table 2). A reduction in the responses to K^+ was seen only at higher venom concentrations after 120 min (Table 2).

B. leucurus venom also inhibited directly stimulated preparations to a similar extent to that seen with indirect stimulation after 90 min (Fig. 3).

3.3. Creatine kinase release

B. leucurus venom caused a time-dependent increase in CK release from chick biventer cervicis preparations (Fig. 4). However, at no time point was *B. leucurus* venom more potent than *B. jararaca* venom in releasing CK.

3.4. Morphological analysis

Preparations incubated with Krebs solution alone (controls) showed a normal muscle morphology with regular

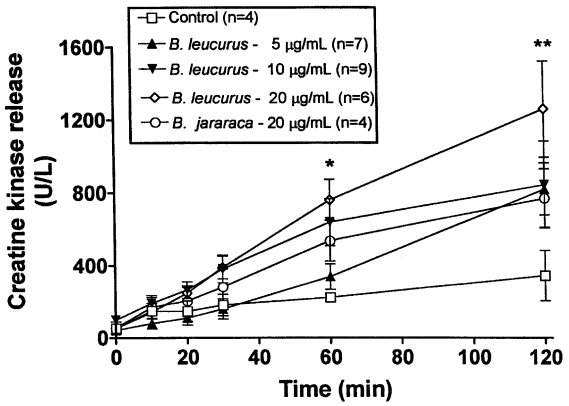

Fig. 4. CK release from chick biventer cervicis preparations incubated with *B. leucurus* venom. The preparations were mounted and the CK levels determined as described in Section 2. Control preparations were incubated with Krebs solution alone. The CK release by *B. jararaca* venom is shown for comparison. The points represent the mean ± SEM of 4–9 experiments. $*p < 0.05$ and $**p < 0.01$ for *B. leucurus* venom concentrations at 60 and 120 min, respectively, compared to the controls.

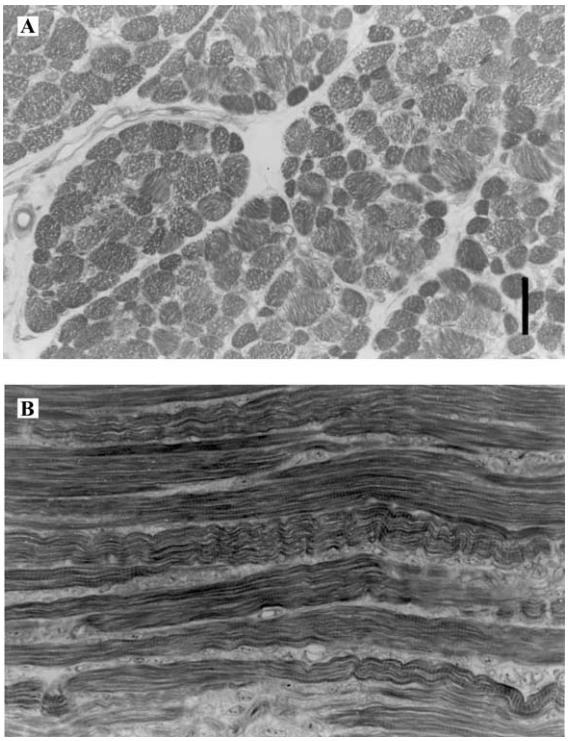

Fig. 5. Cross (A) and longitudinal (B) sections of chick biventer cervicis muscle preparations incubated with Krebs solution alone (control). Note the regular fiber fascicles in (A) and the moderate undulations and cross-striations of the myofibrils in (B). Toluidine blue staining. Bar = 25 μm for both panels.

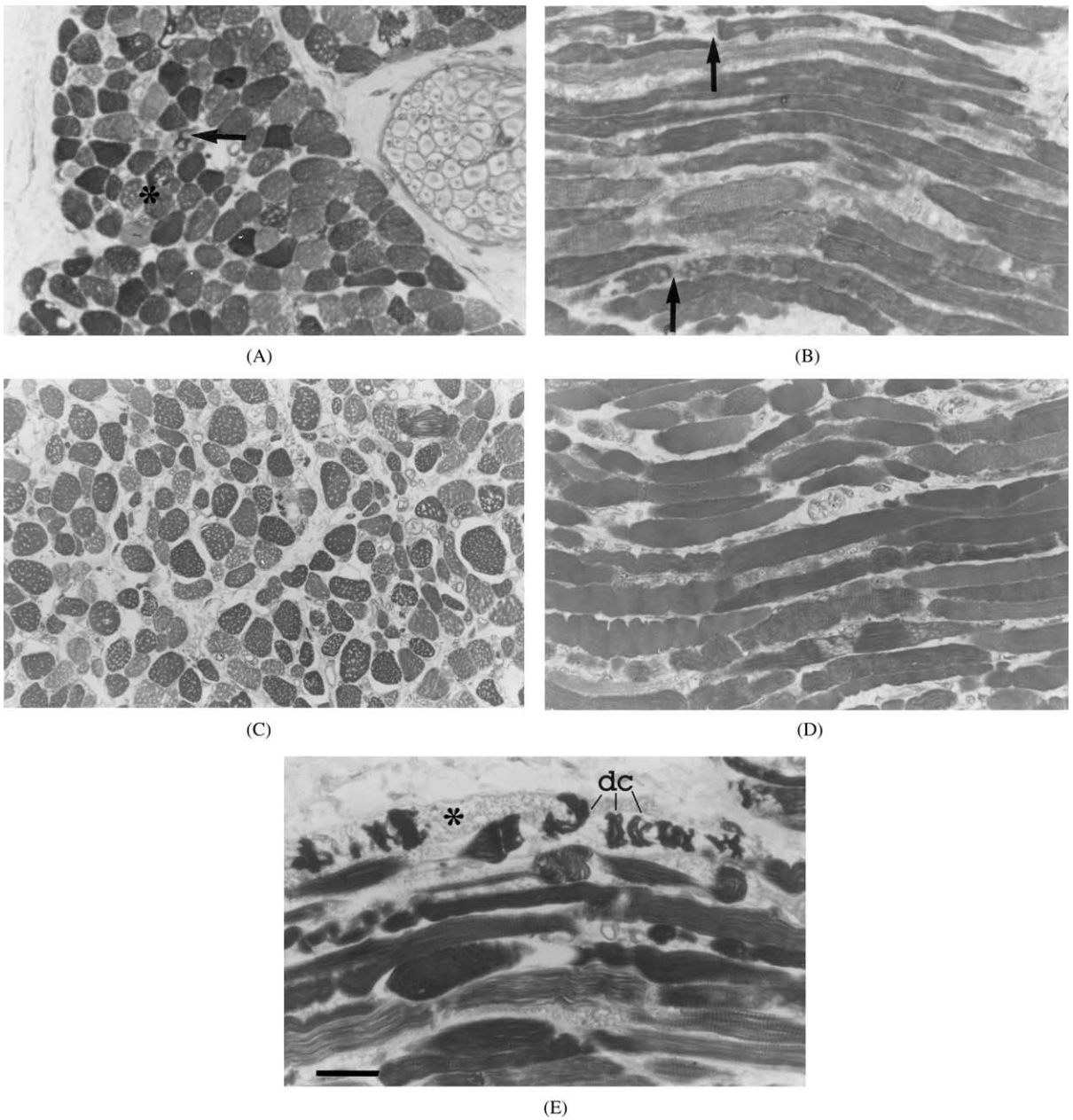

Fig. 6. Light micrographs of chick biventer cervicis muscle preparations incubated with *B. leucurus* venom. (A) and (B), 5 μg/ml, (C) and (D), 10 μg/ml, (E), 20 μg/ml. (A) and (C) are cross-sections and (B), (D) and (E) are longitudinal sections. Note in (A) the disorganization of the fascicles and the appearance of dark vacuolated cells (black asterisk) and of cells with contorted, dark ribbons caused by the agglutination of myofibrils (arrow). (B) shows fibers with a dark contracted amorphous appearance (arrows). (C) shows a marked increase in the number of dark vacuolated cells and in muscle fascicle disorganization. The longitudinal section in (D) shows fibers without striations and/or with hypercontracted or lysed regions. (E) shows densely clumped myofibrils (dc) interposed with flocculent areas (*) resulting from degraded myofibrils. Toluidine blue staining. Bar = 25 μm for all panels.

muscle fiber fascicles, visible striated myofibrils and a uniform intensity of cell staining (Fig. 5). Muscles incubated with *B. leucurus* venom (5, 10 and 20 μg/ml) showed darker compacted cells, vacuolization, membrane lesions, fiber hypercontracture and degradation of the myofibrils, which gave the cells an amorphous appearance and resulted in dark contorted masses (Fig. 6). There were no major qualitative differences between the morphological changes observed

with 10 and 20 μg of venom/ml and, in both cases, unaffected fibers outnumbered affected ones (this was more pronounced with 10 μg of venom/ml). The morphological effects of higher concentrations (100 μg/ml) were not tested because of the limited amount of venom available.

4. Discussion

Little is known about the toxinology of *B. leucurus*. In a study of the biological activities of South American snake venoms, Sanchez et al. (1992) showed that the lethality (i.c.v., i.v. and i.p.), as well as the coagulant, edematogenic, hemorrhagic, and necrosis-inducing activities of *B. leucurus* venom were similar to those of several other *Bothrops* species, including *B. jararaca*. As shown here, *B. leucurus* venom produced dose- and time-dependent neuromuscular inhibition in chick nerve–muscle preparations, as also reported for the venoms of *B. insularis* (Cogo et al., 1993), *B. neuwiedi* (Zamunér et al., 1997) and *B. jararacussu* (Rodrigues-Simioni et al., 1983) in this preparation.

This neuromuscular action apparently involved interference with neurotransmission, as shown by the reduced response to acetylcholine after incubation with the venom (Harvey et al., 1994). Such inhibition of neurotransmission could involve damage to the nicotinic receptors by proteases present in this venom (Table 1), as well as damage to muscle fibers. The action on muscle is based on the observation that the venom inhibited contractile responses to direct stimulation, depressed the responses to K^+, and caused muscle damage as seen histologically. That muscle damage was the main cause of the diminished contractile responses is also supported by the fact that to date no true antagonists or blockers of post-synaptic nicotinic receptors (similar to those in elapid venoms) have been identified in *Bothrops* venoms.

The release of CK, a commonly used indicator for cardiac and skeletal muscle damage and venom-induced myonecrosis, by *B. leucurus* venom may be related to the proteolytic and PLA_2 activity of the venom and agrees with the ability of this venom to cause hemorrhage and necrosis (Sanchez et al., 1992). This finding also agrees with other studies showing that *Bothrops* venoms can release CK in vitro and in vivo (Gutiérrez and Lomonte, 1989; Moura-da-Silva et al., 1991; Oshima-Franco et al., 2000). The cellular damage was confirmed by histological examination which showed that *B. leucurus* venom produced morphological alterations at all concentrations examined (5–20 μg/ml). This observation agreed with the ability of *Bothrops* venoms to cause myonecrosis (Gutiérrez and Lomonte, 1989, 1995; Costa et al., 1999).

Bothrops myotoxins show variable amounts of PLA_2 activity (Gutiérrez and Lomonte, 1995) and, together with hemorrhagic metalloproteases (Gutiérrez and Rucavado, 2000), are the principal cause of local damage by these venoms. Although the PLA_2 activity of *B. leucurus* venom was greater than that of *B. jararaca*, the relative contribution of this enzyme to the pharmacological actions of *B. leucurus* venom remains to be established. In other *Bothrops* venoms, PLA_2 contributes to local edema and inflammation (Selistre et al., 1990; Daniele et al., 1995; Castro et al., 2000), myotoxicity (Gutiérrez and Lomonte, 1995), and lethality (Nisenbom et al., 1986; Cogo et al., 1998).

In conclusion, *B. leucurus* venom showed neuromuscular and myotoxic actions in avian nerve–muscle preparations. A similar action in mammals could perhaps contribute to the local and systemic effects seen after envenomation by this species.

Acknowledgements

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