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RESEARCH REPORT

Biological characterization of *Bothrops marajoensis* snake venom

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ABSTRACT

This study describes the effects of *Bothrops marajoensis* venom (Marajó lancehead) on isolated neuromuscular preparations of chick biventer cervicis (CBC) and mouse phrenic nerve-diaphragm (PND). At low concentrations (1µg/ml for CBC and 5µg/ml for PND), the venom exhibited a neuromuscular blocking without any damaging effect on the muscle integrity. At higher concentration (20µg/ml for PND), together with the neuromuscular blockade, there was a moderate myonecrosis. The results show differences between mammalian and avian preparations in response to venom concentration; the avian preparation was more sensitive to venom neurotoxic effect than the mammalian preparation. The possible presynaptic mechanism underlying the neuromuscular blocking effect was reinforced by the observed increase in MEPPs at the same time (at 15min) when the facilitation of twitch tension occurred. These results indicate that the *B. marajoensis* venom produced neuromuscular blockade, which appeared to be presynaptic at low concentrations with a postsynaptic component at high concentrations, leading to muscle oedema. These observations demand the fractionation of the crude venom and characterization of its active components for a better understanding of its biological dynamics.

KEYWORDS: Marajó lancehead, neuromuscular junction, neurotoxicity, myotoxicity, presynaptic effects

INTRODUCTION

The Neotropical pitvipers of the genus *Bothrops* (Viperidae family) are one of the most frequent causes of snakebite accidents in Latin America (Brasil, 2001). This genus comprises more than 30 species, although the relationship among members of this group remains poorly understood (Hoge and Romano, 1973; Campbell and Lamar, 1989).

Bothrops marajoensis (Marajó lancehead) is found in savanna Marajo island (State of Pará, Brazil) and possibly in coastal lowlands of the Amazon Delta (Hoge and Romano, 1973; Campbell and Lamar, 1989). This species is part of the *Bothrops atrox* complex, which comprises a number of populations of medium to large-sized pitvipers distributed throughout the tropical parts of Central to South America (Wüster et al, 1998). A toxinological characterization of the venoms from this snake complex has a particular importance for the clinical diagnosis and the production of effective antivenom, as well as for a better understanding of the relationships among *Bothrops* species.

Envenoming by *Bothrops* snakes are characterized by pronounced local effects including hemorrhage, edema, pain and myonecrosis, as well as systemic effects, such as coagulopathies and renal failure (Rosenfeld, 1984). Although *Bothrops* venoms in general do not produce apparent signs of neurotoxicity after snakebites, *in vitro* studies indicate that several of these venoms produce neuromuscular blockade in amphibian, avian and mammalian neuromuscular preparations (Rodrigues-Simioni et al, 1983;

Cogo et al, 1993; Costa et al, 1999; Lôbo de Araújo et al, 2002; Borja-Oliveira et al, 2003; Prianti et al, 2003).

Considering the rich Brazilian biodiversity and the fact that snake bite has been considered as a neglected disease (Williams et al, 2010), this work was aimed at contributing to the knowledge of snake venom by the characterization of the biological properties of *B. marajoensis* venom.

MATERIAL AND METHODS

Animals

Male HY-LINE W36 chicks (4-8 days old) were supplied by Granja Ito S/A (Campinas, SP, Brazil) and male Swiss white mice (26-32g) were supplied by the Multidisciplinary Center for Biological Research of the University of Campinas (Cemib/Unicamp). Animals were housed at 25ºC under a 12hr light/dark cycle and had free access to food and water. All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (Cobea), protocol number 959-1.

Venom, drugs and reagents

B. marajoensis crude venom was generously donated by Professor Sérgio Marangoni (Unicamp, Campinas, Brazil). D-tubocurarine (Abbott, Brazil); halothane (Cristália, Brazil); historesin JB-4 (LKB-Bromma, Sweden) and acetylcholine iodide (Sigma, St Louis, MO, USA), as well as Tyrode's solution reagents: 137mM NaCl, 2.7mM KCl, 1.8mM $CaCl_2$, 0.49mM $MgCl_2$, 0.42mM NaH_2PO_4, 11.9mM $NaHCO_3$ and 11.1.mM glucose. Ingredients of the Krebs solution (118.7mM NaCl, 4.7mM KCl, 1.88mM $CaCl_2$, 1.17mM KH_2PO_4, 1.17mM $MgSO_4$, 25mM $NaHCO_3$ and 11.65mM glucose), were purchased from laboratory product distributors.

Chick biventer cervicis preparation (CBC)

Male chicks were killed by halothane inhalation and the biventer cervicis muscles were removed (Ginsborg and Warriner, 1960) and mounted under a tension of 1*g* in a 5ml organ bath containing Krebs solution (pH 7.5, 37ºC) aerated with 95% (v/v) O_2 and 5% (v/v) CO_2. The preparations were allowed to stabilize for at least 20min before the addition of a single concentration of the venom. A bipolar platinum ring electrode was placed around the muscle and coupled to a Grass S48 stimulator (0.1Hz, 0.2ms, 4-8V). Isometric muscle contractions and contractures were recorded via a force displacement transducer (Load Cell BG-10 GM) coupled to a physiograph (Gould, Model RS 3400). Muscle responses to exogenous acetylcholine (ACh, 110µM) and potassium chloride (KCl, 20mM) were obtained in the absence of field stimulation prior to venom addition (1, 5, 10 and 20µg/ml) and by the end of the experiment.

Mouse phrenic nerve-diaphragm preparation (PND)

Mice were killed by exsanguinations after halothane anesthesia, and the phrenic-diaphragm preparations were removed and mounted under a tension of 5*g* in an organ-bath chamber containing 5ml of Tyrode's solution (pH 7.4, 37 ºC), aerated with 95% (v/v) O_2 and 5% (v/v) CO_2 (as described in Bülbring, 1946, for rats). The preparation was stabilized for at least 20min before the venom addition (1, 5, 10, 15 and 20µg/ml). Indirect contractions were evoked by supramaximal strength pulses (0.1Hz; 0.2ms), delivered from a Grass S48 stimulator and applied to the phrenic nerve by a bipolar electrode. Direct contractions were evoked by supramaximal pulses (0.1Hz, 2ms). Experiments of direct contractions were performed in the presence of d-tubocurarine (5µg/ml) previously to venom addition (5 and 20µg/ml). Isometric muscle twitch tension was recorded by a force displacement transducer (Load Cell BG-10 GM) coupled to a physiograph (Gould, Model RS 3400).

Miniature end-plate potentials (MEPPs) were recorded in mouse hemidiaphragm muscle, using conventional microelectrode techniques. The dissected muscle was mounted in a lucite chamber containing aerated (95%, v/v, O_2 and 5%, v/v, CO_2) Tyrode solution (pH 7.4; 27-30°C) with or without *B. marajoensis* (15µg/ml). Intracellular microelectrodes filled with 3M KCl (resistance 10-25MΩ) were used. Microelectrode placement was considered adequate when the rise time of MEPPs was less than 1ms. MEPPs were recorded on a oscilloscope (Tektronix, Beaverton, OR, USA) and digitized using an analog-to-digital converter (Lynx, SP, Brazil; CAD 12/36, resolution 12bits) coupled to a microcomputer (Microtec, SP, Brazil) loaded with a software (AqDados 5, Lynx) for measurement and analysis.

Morphological and morphometrical analyses

The diaphragm muscles were incubated with venom (5 and 20µg/ml) for 120min and muscle fragments were immediately fixed for 24hr in Bouin's fixative, washed with a solution of ammonium hydroxide, dehydrated in increasing ethanol concentrations (70, 95 and 100%, v/v) and embedded in historesin JB-4 (LKB-Bromma, Sweden). 5µm thick sections were cut using a Leica RM 2035 microtome (Leica, Vienna, Austria) and stained with hematoxylin-eosin (HE) for examination by light microscopy. Control preparations were incubated with physiological solution alone. The extent of muscle damage was assessed by counting the number of normal and damaged fibers in four non-overlapping areas of histological slides per preparation for each venom dose (n=4). Photomicrographs were obtained using a Zenalumar Zeiss light microscope (Carl Zeiss, Jena, Germany).

Statistical analysis

Results were expressed as mean ±SEM. Data were analyzed by using the Student's t-test (for comparison of two samples) and analysis of variance complemented by the Tukey-Kramer test (for comparison of more than two samples). P-values <0.05 were considered significant.

RESULTS

Myographic Studies

Chick biventer cervicis preparation (CBC)

In control preparation, there was no detectable change in the amplitude of muscle contractions in response to indirect stimulation over a period of 120min. However, *B. marajoensis* venom (1, 5 and 20µg/ml) induced a concentration-dependent and irreversible neuromuscular blockade of indirectly evoked twitches in CBC (Figure 1A).

At very low venom concentrations (1 and 5µg/ml) the twitch-tension was reduced by, 79.8±8% and 83.2±6%, respectively, and the contractures caused by the exogenous

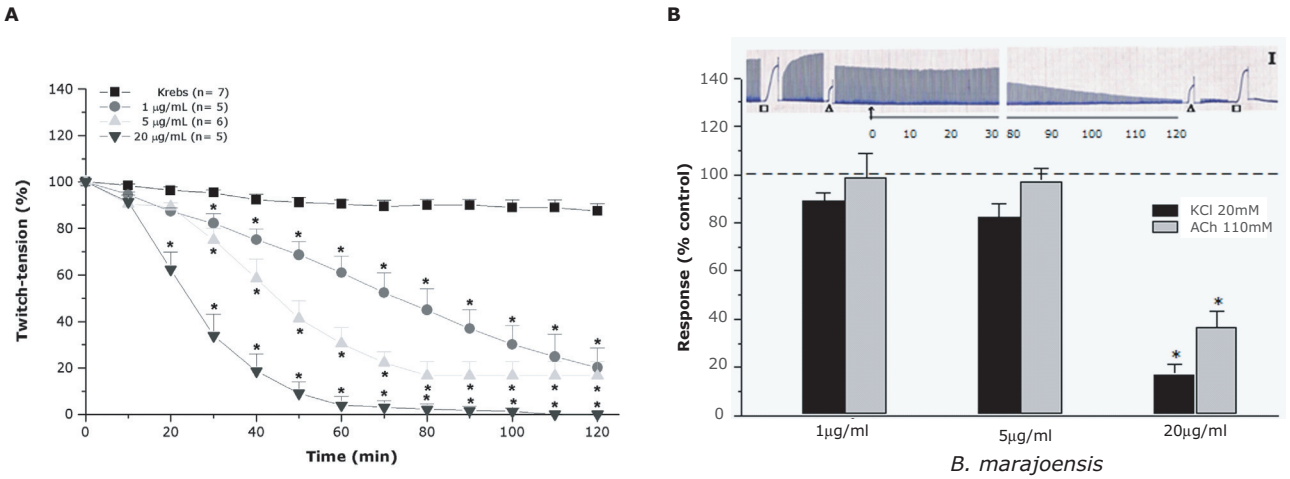

Figure 1. A. Effects of *Bothrops marajoensis* venom on CBC preparations to field contractions. The ordinate represents the % amplitude of twitches relative to the initial amplitude. The abscissa indicates the time (min) after the addition of each dose of venom to the organ bath. The points are the mean ±SEM of 5-7 experiments; * and + indicate, respectively, the point at which differences between each venom dose (1, 5 and 20µg/ml) relative to the control become significant ($p<0.05$). **B.** Acetylcholine (ACh) and potassium (KCl) effects: The points represent mean ±SEM of 5 experiments when compared with the control ($p<0.05$). Insert: Myographic register of the preparation incubated with venom at 5µg/ml; time zero indicates the moment of the venom addition: Closed box shows the time of KCl addition and closed triangle that of ACh addition.

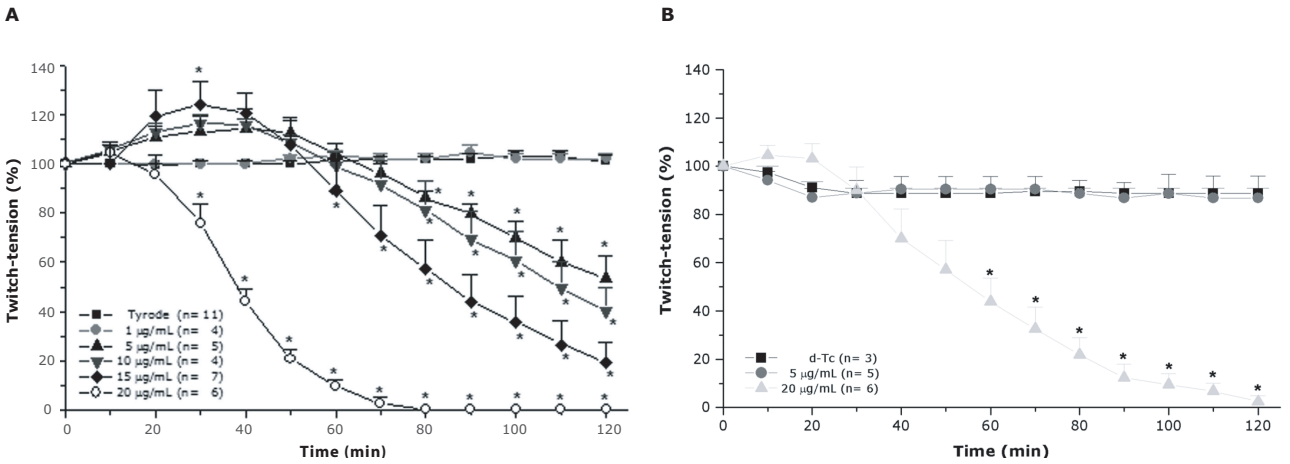

Figure 2. Effects of *Bothrops marajoensis* venom on indirectly (**A**) and directly (**B**) evoked twitches in PND. The ordinate represents the % amplitude of twitches relative to the initial amplitude. The abscissa indicates the time (minutes) after the addition of each venom dose (5 and 20µg/ml) to the organ bath. The points are the mean ±SEM of 3-6 experiments; * indicates the point at which differences between each venom dose relative to the control become significant ($p<0.05$).

addition of ACh and KCl (99.7±10, 97.3±5 and 89.0±4, 82.1±6, respectively) were unchanged. These finds indicated a lack of effect on nicotinic receptor function and muscle fiber integrity (5µg/ml, Figure 1B).

At higher concentration (20µg/m) 50% blockade of twitches was observed after 25±3 min (n=5). This neuromuscular blockade was accompanied by a significant reduction of KCl (17.1±4%, n=5) and ACh (36.7±6%, n=5)-induced contractures (Figure 1B).

Mouse phrenic nerve-diaphragm preparation (PND)

Control preparations did not display significant changes in the amplitude of muscle contractions in response to indirect stimulation over a period of 120min. B. marajoensis venom (1, 5, 10, 15 and 20µg/ml) induced a concentration and time-dependent blockade of indirectly evoked twitches (Figure 2A). At 5, 10 and 15µg/ml, the venom caused a progressive increasing of indirectly evoked twitches (13±6%, 16.3±4% and 24±10% at 30min, respectively) followed by a partial neuromuscular blockade (53.4±9%, 60±10% and 79.5±8%, respectively) (n=5-7) after 120min incubation.

In contrast, the venom significantly blocked both indirectly and directly evoked twitches at 20µg/ml (Figure 2A and B); no significant difference was observed for the time to reach 50% blockade of twitches in both patterns of stimulation used (36.4±2min and 56±8min, respectively (n=6). Differently from 20µg/ml venom, 5µg/ml did not induce any effect on curarized preparations directly stimulated observed during 120min (Figure 2B). The effects of the venom on both directly and indirectly stimulated muscle contractions could not be reversed by washing the preparations with Tyrode solution at the end of the experiment (data not shown).

Intracellular recordings of miniature endplate potentials (MEPPs) of mouse diaphragm preparations after *B. marajoensis* venom addition (15µg/ml) revealed a significant increase in MEEPs frequency from 18.2±3.6 at time zero to 27.4±9.7 after 15min (n=9; $p<0.05$); this effect was progressively decreasing until complete lack of MEPPs in all the experiments done (data not shown).

Morphological Studies

Transversal sections of control hemidiaphragm muscles showed normal polygonal fiber morphology with an acidophilic sarcoplasm and peripheral nuclei (Figure 3A). The same pattern was observed in preparations exposed to 5µg/ml of venom (Figure 3B), and there were no significant muscle fibers damage (5.5±1.9%, n=4) when compared with controls (0.4±0.1%, n=4). In contrast, preparations exposed to 20µg/ml of venom showed a range of structural changes, including endomysial edema, presence edematous fibers and loss of myofibrils (Figure 3C). There were significant muscle damage indices (26.8±3.6%; n=4) relative to control (0.4±0.1%; n=4) ($p<0.05$).

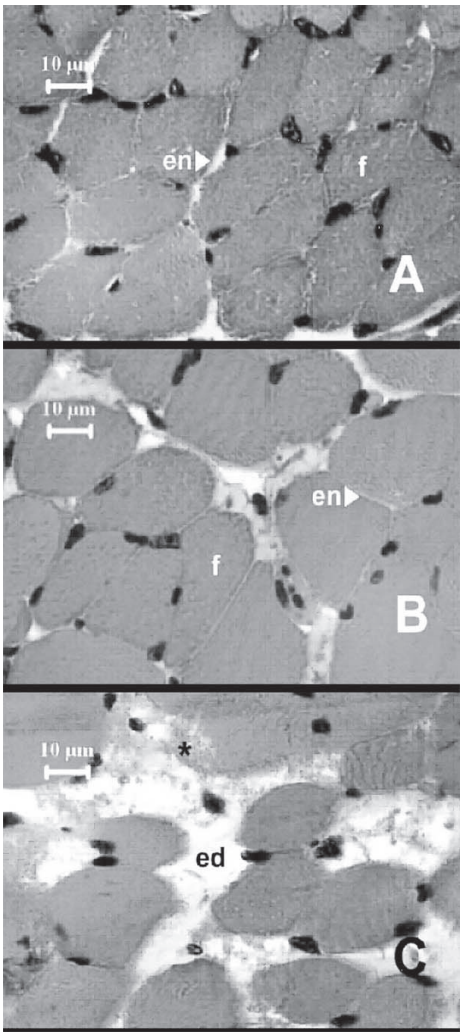

Figure 3. Light micrographs of mouse diaphragm muscles. **A.** Control muscle with normal fiber **(f)** morphology. Note the polygonal aspect and the intact endomysium **(en)**. **B.** Muscle exposed to 5 µg/ml of *B. marajoensis* venom showing well preserved morphology. Observe a slight edema **(ed)**. **C.** Muscle exposed to 20µg/ml of venom showing edema **(ed)**, round fibers **(rf)**, hypercontracted fibers **(arrow)** and heavily stained fibers **(*)**.

DISCUSSION

Studies have shown that *Bothrops* venoms can cause neuromuscular blockade in amphibian, avian and mammalian preparations *in vitro* (Rodrigues-Simioni et al, 1983; Cogo et al, 1993; Costa et al, 1999; Borja-Oliveira et al, 2002; Borja-Oliveira et al, 2003; Prianti et al, 2003; Zamunér et al, 2004), as demonstrated in the present study to *B. marajoensis* venom. Different sensitivities between avian (biventer cervicis) and mammalian (phrenic nerve-diaphragm) preparations regarding to the effects of animal venoms and toxins have already been described for *Bothrops* venoms, including *B. marajoensis*, and were attributed to differences in muscle fibers and in the kind of innervation (Harvey et al, 1994; Hodgson and Wickramaratna, 2002). Nevertheless, the studies conducted simultaneously on mammalian and avian preparations are extremely useful to understand the mechanism of action of animal venoms and toxins (Harvey et al, 1994).

Mouse phrenic-diaphragm preparation is focally-innervated and mediates electrically-evoked twitches. In contrast, chick biventer cervicis contains fibers that have either focal or multiple innervation, therefore being able to respond to either electrical stimulation or exogenous nicotinic agonists, respectively (Hodgson and Wickramaratna, 2002). This characteristic enables discrimination between pre- and post-junctional effects of animal venoms and toxins (Harvey et al, 1994; Hodgson and Wickramaratna, 2002).

Most *Bothrops* venoms produce neuromuscular blockade at concentrations ranging from 50-200µg/ml, and it is associated with extensive muscle damage (Zamunér et al, 2004). However, some *Bothrops* venoms, as *B. insularis* and *B. pauloensis* (Cogo et al, 1993; Rodrigues-Simioni et al, 2004) and *Bothriopsis bilineata* (Rodrigues-Simioni et al, 2011), induce blockade at much lower concentrations (≤5µg/ml) without causing apparent muscle damage; *B. marajoensis* venom appears to belong to the latter group, since neuromuscular blockade was observed at concentrations of 1-20µg/ml.

In CBC preparations, low concentrations of *B. marajoensis* venom (1µg/ml) blocked neuromuscular transmission without depressing the responses to exogenous ACh and KCl; *i.e.*, there was no blockade of postsynaptic acetylcholine receptors or interference with the muscle contractile mechanisms. In PND preparations, 5µg/ml of venom depressed the contractions to indirect stimulation without showing any effect on the responses to direct stimulation, inducing very mild diaphragm muscle edema.

Such neuromuscular blockade characteristics have been attributed to presynaptic-acting venoms and/or toxins (Harvey et al, 1994; Lewis and Gutmann, 2004) as those of *Crotalus durissus terrificus* (Rodrigues-Simioni et al, 2004), *Micrurus* species (Vital Brazil and Fontana, 1984; Dal-Belo et al, 2005) and other *Bothrops*, *B. insularis* (Cogo et al, 1993), *B. pauloensis* (Rodrigues-Simioni et al, 2004; Borja-Oliveira et al, 2007) and *B. bilineata* (Rodrigues-Simioni et al, 2011), which did not show any detectable effect on the nicotinic

receptor and, in some cases, showed only a mild muscle alteration, corroborating with the increase of MEPPs frequency on PND after *B. marajoensis* venom addition.

The neuromuscular blockade observed at high concentrations (≥10µg/ml) of *B. marajoensis* venom probably is a combination of the presynaptic action mentioned above and a postsynaptic effect involving muscle edema, corroborating the progressive attenuation of the contracture responses to exogenous KCl and ACh seen at concentrations ≥10µg/ml in CBC preparations. These results agree to the observed blockade of directly stimulated PND preparations, as well as the histological findings. Similar data have also been shown for *B. insularis* and *B. pauloensis* venoms (Cogo et al, 1993; Rodrigues-Simioni et al, 2004). Indeed, the neuromuscular blockade induced by *B.* marajoensis venom always preceded the morphological changes. Moreover, the effectiveness of this venom in causing neuromuscular blockade is similar to elapidics venoms.

CONCLUSIONS

In conclusion, *B. marajoensis* venom produced neuromuscular blockade in avian and mammalian nerve-muscle preparations *in vitro*; this blockade appeared to be presynaptic at low concentrations (≤5µg/ml) with a postsynaptic component at high concentrations (≥10µg/ml). In addition, high venom concentrations caused only muscle fibers edema. Further studies are needed to identify the biologically-active venom components responsible for these actions.

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COMPETING INTEREST STATEMENT

None declared.

LIST OF ABBREVIATIONS

CBC; chick biventer cervicis
PND; mouse phrenic nerve-diaphragm
NMJ; neuromuscular junction
ACh; acetylcholine
PLA_2; phospholipases A_2 (PLA_2)

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