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 block, 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 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 25°C 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); histoestin 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, 1.88mM CaCl_2, 0.49mM MgCl_2, 11.9mM NaHCO_3, and 11.1mM 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 War-riner, 1960) and mounted under a tension of 1g in a 5ml organ bath containing Krebs solution (pH 7.5, 37°C) 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). Miniature end-plate potentials (MEPPs) were recorded in mouse hemidiaphragm muscle, using conventional micro-electrode 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. Micro-electrode 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 histoestin 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
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/ml) 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 36±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 MEPPs 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 acido-philic 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).

**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

**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 (*).
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. pauloensisvenoms (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 elapid venom.

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
PLA₂; phospholipases A₂ (PLA₂)

REFERENCES

Borja-Oliveira CR, Soares AM, Zamunér SR et al. 2002. Intraspecific variation in the neurotoxic and myotoxic activities of Bothrops neuwiedi venom snakes. J. Venom Anim. Toxins incl Trop Dis, 8, 88-101.
Borja-Oliveira CR, Durigon AM, Vallin AC et al. 2003. The pharmacological effect of Bothrops neuwiedi (jararaca pintada) snake venom on avian neuromuscular transmission. Braz J Med Biol Res, 36, 617-24.
Borja-Oliveira CR, Kassab BH, Soares AM et al. 2007. Purification and n-terminal sequencing of two presynaptic neurotoxic PLA₂, neuwidetoxin-I and neuwidetoxin-II, from Bothrops neuwiedi pauloensis (jararaca pintada) venom. J Venom Anim Toxins incl Trop Dis, 13, 103-21.
Brasil. 2001. Ministério da Saúde. Fundação Nacional de Saúde. Manual de diagnóstico e tratamento de acidentes por animais peçonhentos. Brasília: MS/FUNASA.
Bülbring E. 1946. Observations on the isolated phrenic nerve diaphragm preparation of the rat. Br J Pharmacol, 120, 3-26.
Campbell JA and Lamar WW. The venomous reptiles of Latin America. Comstock Publishing Associates, New York, USA.
Cogo JC, Prado-Franceschi J, Cruz-Höfling MA, Corrado AP and Rodrigues-Simioni L. 1993. Effect of Bothrops insularis venom on the mouse and chick nerve-muscle preparation. Toxicon, 31, 1237-47.
Costa PD, Toyama MH, Marangoni S, Rodrigues-Simioni L and Cruz-Höfling MA. 1999. Effects of Bothrops pirajai venom on the mouse extensor digitorum longus (EDL) muscle preparation. Toxicon, 37, 1143-53.
Dal-Belo CA, Leite GB, Toyama MH et al. 2005. Pharmacological and structural characterization of a novel phospholipase A₂ from Micrurus dumerili carinicauda venom. Toxicon, 46, 736-50.
Ginsborg BL and Warriner J. 1960. The isolated chick biventer cervicis nerve muscle preparation. Br J Pharmacol Chemother, 15, 410-11.
Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S and Harris JB. 1994. Screening of snake venoms for neurotoxic and myotoxic effects using simple in vitro preparations from rodents and chicks. Toxicon, 32, 257-65.
Hodgson WC and Wickramaratna JC. 2002. In vitro neuromuscular activity of snake venoms. Clin Ex Phar Phy, 29, 807-14.
Hoge AR and Romano AS. 1973. Sinopse das serpentes peçonhentas do Brasil. Serpentes, Elapidae e Viperidae. Mem Inst Butantan, 36, 109-207.
Lewis RL and Gutmann L. 2004. Snake venoms and the neuromuscular junction. Semin Neurol, 24, 175-79.
Lôbo-Araújo A, Donato JL, Leite GB et al. 2002. Neuromuscular action of Bothrops lanceolatus (fer do lance) venom and a postsynaptic effect involving muscle edema. Toxicon, 31, 1237-47.
Rodrigues-Simioni L. 1993. Effect of Bothrops jararacussu venom on the mouse and chick nerve-muscle preparation. Toxicon, 31, 1237-47.
Rodrigues-Simioni L. 2004. Comparison of the neurotoxic and myotoxic effects of Brazilian snake venoms. J. Venom Anim. Toxins incl Trop Dis, 10, 525-39.
Rodrigues-Simioni L, Cogo JC, Prado-Franceschi J, Cruz-Höfling MA, Corrado AP and Lopes-Martins RA et al. 2003. Effect of Bothrops jararaca venom in chick biventer cervicis preparations. Toxicon, 41, 595-603.
Rodrigues-Simioni L, Borgese N and Ceccarelli B. 1983. The effects of Bothrops jararacussu venom and its components on frog nerve-muscle preparation. Neuroscience, 10, 475-89.
Rodrigues-Simioni L, Floriano RS, Rostelato-Ferreira S et al. 2011. Presynaptic action of Bothriopsis bilineata smargadina (forest viper) venom in vitro. Toxicon, 58, 140-45.
Rodrigues-Simioni L, Zamunér SR, Cogo JC et al. 2004. Pharmacological evidence for a presynaptic action of venoms from Bothrops lanceolatus (jararaca ilhoa) and Bothrops neuwiedi (jararaca pintada). Toxicon, 43, 633-638.
Rosenfeld G. Symptomatology, pathology and treatment of snake bites in South America. Venomous Animals and their Venoms. Academic Press, New York, USA.
Vital Brazil O and Fontana MD. 1984. Ações pré-juncionais e pós-juncionais da peçõna da cobra coral Micrurus corallinus na junção neuromuscular. Mem Inst Butantan, 47/48, 13-26.
Wüster W, Golay P and Warrell DA. 1998. Synopsis of recent developments in venomous snake systematics, Toxicon, 36, 299-307.
Zamunér SR, da Cruz-Höfling MA, Corrado AP, Hyslop S and Rodrigues-Simioni L. 2004. Comparison of the neurotoxic and myotoxic effects of Brazilian Bothrops venoms and their neurologic action by commercial antivenom. Toxicon, 44, 259-71.
Williams D, Gutierrez JM, Harrison R et al. 2010. Global Snake Bite Initiative Working Group; International Society on Toxinology. The Global Snake Bite Initiative: an antidote for snake bite. Lancet, 375, 89-91.