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RESEARCH ARTICLE

Neuromuscular activity of *Bothrops fonsecai* snake venom in vertebrate preparations

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ABSTRACT

The neuromuscular activity of venom from *Bothrops fonsecai*, a lancehead endemic to southeastern Brazil, was investigated. Chick biventer cervicis (CBC) and mouse phrenic nerve-diaphragm (PND) preparations were used for myographic recordings and mouse diaphragm muscle was used for membrane resting potential (RP) and miniature end-plate potential (MEPP) recordings. Creatine kinase release and muscle damage were also assessed. In CBC, venom (40, 80 and 160µg/ml) produced concentration- and time-dependent neuromuscular blockade (50% blockade in 85±9 min and 73±8 min with 80 and 160µg/ml, respectively) and attenuated the contractures to 110µM ACh (78–100% inhibition) and 40mM KCl (45–90% inhibition). The venom-induced decrease in twitch-tension in curarized, directly-stimulated preparations was similar to that in indirectly stimulated preparations. Venom (100 and 200µg/ml) also caused blockade in PND preparations (50% blockade in 94±13 min and 49±8 min with 100 and 200µg/ml, respectively) but did not alter the RP or MEPP amplitude. In CBC, venom caused creatine kinase release and myonecrosis. The venom-induced decrease in twitch-tension and in the contractures to ACh and K^+ were abolished by preincubating venom with commercial antivenom. These findings indicate that *Bothrops fonsecai* venom interferes with neuromuscular transmission essentially through postsynaptic muscle damage that affects responses to ACh and KCl. These actions are effectively prevented by commercial antivenom.

KEYWORDS: *Bothrops fonsecai*, myotoxicity, neuromuscular blockade, neurotransmission, post-synaptic

INTRODUCTION

Bothrops fonsecai is an uncommon terrestrial species of lancehead endemic to mountainous regions such as the Serra da Mantiqueira in the states of Minas Gerais, Rio de Janeiro and São Paulo in southeastern Brazil, where it is found in open areas and along the edges of forests dominated by *Araucaria angustifolia* pines at elevations of 1000–1800 m (Martins et al, 2002; Campbell and Lamar, 2004; Tashima et al, 2008). Taxonomically, *Bothrops fonsecai* is closely related to *Bothrops alternatus*, *Bothrops cotiara* and *Bothrops itapetiningae*, that together form the *alternatus* species group (Salomão et al, 1997; Salomão et al, 1999; Martins et al, 2001; Wüster et al, 2002), all of which are stout-bodied species (Martins et al, 2001). The diet of *B. fonsecai* is exclusively mammalian, as is that of *B. alternatus* and *B. cotiara*, whereas *B. itapetiningae* feeds predominantly on mammals (~43%) but also includes lizards (23%), anurans

(~21%), centipedes (9.5%) and birds (2.4%) (Martins et al, 2002).

The limited geographic distribution of *B. fonsecai* and the difficulty in obtaining venom have resulted in few detailed studies of this species´ venom, with most investigations dealing with biochemical aspects. Rosenfeld et al (1959) reported that the coagulant activity of *B. fonsecai* venom was ~5 times greater than that of *B. alternatus*, *B. cotiara* and *B. itapetiningae* and ~60% greater than that of *B. jararaca* whereas its fibrinolytic activity was only 20% of the latter species. Subsequently, Nahas et al (1979) showed that this venom contained thrombin-like activity but was unable to produce prothrombin activator when preincubated with serum, factor V, and phospholipid. More recently, Queiroz et al (2008) reported that *B. fonsecai* venom contains PLA_2, proteolytic and hyaluronidase activities that are neutralized to varying degrees by commercial equine bothropic antivenom produced by the Butantan Institute (São Paulo, SP, Brazil), *i.e.*, virtually no neutralization of PLA_2 activity, ~15–20% neutralization of proteolytic activity and ~70% neutralization of hyaluronidase activity, despite the high overall immunoreactivity of the venom with this antivenom in ELISA. These authors also showed that the SDS-PAGE profile of *B. fonsecai* venom was similar to that of various other *Bothrops* species, with prominent protein bands at ~14 kDa (corresponding to PLA_2), 20–26 kDa (probably corresponding to class P1 snake venom metalloproteinases - SVMPs) and 50–60 kDa (probably corresponding to class P3 SMVPs and other proteins). In immunoblotting, commercial bothropic antivenom reacted with these major protein bands. In addition to these studies, proteomic analyses have shown that *B. fonsecai* venom contains a variety of peptides (Tashima et al, 2012) and the major protein/toxin classes found in other *Bothrops* venoms, *i.e.*, PLA_2, SVMPs, serine proteases, C-type lectins, L-amino acid oxidase and disintegrins (Tashima et al, 2008). Approximately 30% and 43% of *B. fonsecai* venom proteins are PLA_2 and SVMPs, respectively, whereas in *B. cotiara*, no PLA_2 were detected and SVMPs accounted for 73% of proteins (Tashima et al, 2008).

In contrast to these biochemical studies, very little is known of the biological activities of *B. fonsecai* venom. The lethality of this venom in mice (LD_{50} 56.8mg/kg, i.p.) is similar to that of *B. cotiara* (53.8mg/kg) and slightly greater than that of *B. alternatus* (63.7mg/kg) and *B. itapetiningae* (74.4mg/kg) (Queiroz et al, 2008). Although the high content of PLA_2 and SVMPs indicated by proteomic analysis suggest that this venom probably causes myonecrosis and hemorrhage mediated by PLA_2 (Gutiérrez and Ownby, 2003) and SVMPs (Escalante et al, 2011), respectively (as in other *Bothrops* venoms), there has been no detailed investigation of these activities in this venom. The presence of PLA_2 also suggests that *B. fonsecai* could adversely affect neurotransmission in vertebrate neuromuscular preparations *in vitro*, particularly since several *Bothrops* snake venoms have been shown to block neurotransmission by a combination of pre- and postsynaptic mechanisms, the latter involving muscle damage (myotoxicity) (Cogo et al, 1998; Rodrigues-Simioni et al, 2004; Zamunér et al, 2004; Durigon et al, 2005; Cogo et al, 2006; Cavalcante et al, 2011; Zamunér et al, 2011; Moraes et al, 2012). Various studies have shown that venom PLA_2 are the principal components involved in this neuromuscular damage (Gallaci and Cavalcante, 2010; Galbiatti et al, 2012; Floriano et al, 2013). Based on these considerations, in this work we investigated the neuromuscular activity of *B. fonsecai* venom in chick biventer cervicis and mouse phrenic nerve-diaphragm muscle preparations.

MATERIALS AND METHODS

Reagents and venom

Acetylcholine was obtained from Sigma Chemical Co (St Louis, MO, USA). Commercial kits for the measurement of creatine kinase (CK) activity were from Laborlab (Capão Bonito, SP, Brazil). The salts for physiological solutions were from Synth (São Paulo, SP, Brazil) and Merck (Rio de Janeiro, RJ, Brazil). *Bothrops fonsecai* venom was obtained from five adult specimens of both sexes captured in the Serra da Mantiqueira mountain range in the municipality of Campos do Jordão, São Paulo state. The snakes were maintained in the Serpentarium of the Centro de Estudos da Natureza at the Universidade do Vale do Paraíba (UNIVAP; license SMA 15380/2012). The venom was milked manually, lyophilized and stored at 4°C until used.

Animals

Male HY-LINE W36 chicks (4–8 days old) were supplied by Granja Globo Aves Agrícola Ltda. (Campinas, SP, Brazil) and male Swiss white mice (25–30gm) were obtained from Anilab S/A (Paulínia, SP, Brazil). The animals were housed at 25°C on a 12hrs light/dark cycle and had free access to food and water. All procedures were done in accordance with the general guidelines of the Brazilian Society of Laboratory Animal Science (SBCAL) and were approved by the Committee for Ethics in Animal Use (CEUA/UNIVAP, Protocol No A102/CEP/2007).

Chick biventer cervicis (CBC) preparations

Chicks were killed with an overdose of a mixture of xylazine plus ketamine (Syntec, Cotia, SP, Brazil) administered i.p. and biventer cervicis (CBC) muscle preparations were mounted as described by Ginsborg and Warriner (1960), under a resting tension of 1gm in a 4ml organ bath containing warmed (37°C), aerated (95%,v/v, O_2 - 5%,v/v, CO_2) Krebs solution (pH 7.5) of the following composition (mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65. Field (*i.e.*, nerve) stimulation was done using bipolar electrodes coupled to a Grass S4 stimulator (supramaximal stimuli at 0.1Hz and 0.2ms). The preparations were allowed to stabilize for 15–20min prior to testing with venom. Venom (in 200ml) was added to the organ bath (final concentrations: 40, 80 and 160µg/ml) and muscle contractions were recorded for up to 120min via an isometric transducer (BG-25 GM Kulite) connected to a Gemini 7070 recorder (Ugo Basile). The venom concentrations used here were chosen based essentially on the concentration range (10–200µg/ml) over which other *Bothrops* venoms have been shown to be active in CBC and PND (see below) preparations (Rodrigues-Simioni et al, 2004; Zamunér et al, 2004; Abreu et al, 2007; Moraes et al, 2012). The incubation time of 120min, which is sufficient for complete blockade by various *Bothrops* venoms, was likewise chosen based on previous studies. Muscle responses to exogenous acetylcholine (ACh, 110µM) and potassium

chloride (KCl, 40mM) were obtained in the absence of field stimulation before and after venom addition. In some experiments, the preparations were stimulated directly (20V) after complete neuromuscular blockade induced by incubation with d-tubocurarine (dTc, 10µg/ml); a venom concentration of 160µg/ml was used in these experiments. In control experiments, 200µl of Krebs solution alone was added to the organ bath and the muscle contractions then recorded for up to 120min. The anesthetic used here (xylazine/ketamine) did not significantly affect the twitch responses to electrical stimulation or the contractures to exogenous ACh and K^+ when compared to preparations from chicks anesthetized with isoflurane.

In experiments with 40 and 160µg of venom/ml, aliquots (200µl) of the bathing solution were obtained before and 15, 30, 60, 90 and 120min after venom addition for the quantification of muscle creatine kinase (CK) release using commercial kits and a Bio2000 Analyzer. CK activity in the bathing solution was expressed as U/l, in which one unit corresponded to the amount of enzyme that produced 1µmol of NADH/min under the assay conditions.

In some experiments, the ability of commercial bothropic antivenom to neutralize the neuromuscular activity of the venom was assessed by preincubating venom with antivenom at a venom:antivenom ratio of 5:1 at 37°C for 30min prior to testing the residual activity in CBC preparations. The antivenom used was raised in horses immunized with a mixture of *Bothrops* venoms (*B. alternatus*, *B. jararaca*, *B. jararacussu*, *B. moojeni* and *B. neuwiedi*) (Cardoso et al, 2009) and the venom:antivenom ratio was based on the manufacturer´s stated neutralizing capacity (1 ml neutralizes 5mg of *B. jararaca* venom; Butantan Institute, São Paulo, SP, Brazil). The venom concentration (200µg/ml) and venom:antivenom ratio used in these experiments were chosen to allow direct comparison with similar work using other *Bothrops* venoms (Zamunér et al, 2004).

Phrenic nerve-diaphragm muscle (PND) preparation

Phrenic nerve-diaphragm preparations were obtained from mice killed with an overdose of a mixture of xylazine plus ketamine (Syntec) administered i.p. The preparations were mounted under a tension of 2gm in 5ml tissue baths containing Tyrode solution (pH 7.4, 37°C) of the following composition (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, as described by Bülbring (1946). The preparations were stimulated indirectly (supramaximal voltage, 0.1Hz, 0.2ms) with stimuli delivered from a Grass S4 stimulator. The resulting muscle tension was recorded using a force displacement transducer (BG 25 GM Kulite) coupled to a Gould RS 3400 recorder. The preparations were allowed to stabilize for at least 15min before the addition of venom (100 and 200µg/ml).

Resting membrane potential (RP) and miniature end-plate potentials (MEPPs)

The resting membrane potential (recorded at or distant from the end-plate region) and miniature end-plate potentials (MEPPs) were recorded from mouse diaphragm muscle using conventional microelectrode techniques, as described by Dal Belo et al (2005). The dissected muscle was mounted in a Lucite chamber containing Tyrode solution (pH 7.4) at 30°C and gassed with 5%,v/v, CO_2-95%,v/v, O_2. The RP and MEPPs of several fibers in each muscle were recorded using glass microelectrodes filled with 3M KCl (resistance 10–20W) placed inside the muscle fiber. The recordings were taken at 0 (basal) and 15, 30, 60, 90 and 120min after addition of venom (100 and 200µg/ml).

Histological analysis

At the end of the 120min incubation with venom (40, 80 and 160µg/ml) the CBCs were fixed in Bouin solution containing 4% formol followed by dehydration in a graded ethanol series (70%, 80%, 95% and three times in 100%, 30min each) and embedding in paraplast. After polymerization at 60°C, the tissues were mounted in blocks and sections 5 mm thick were cut with a microtome (Leica model RM 2145, Leica Microsystems, Heerbrugg, Switzerland). The sections were stained with hematoxylin and eosin and examined by light microscopy using an Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) prior to photographing. The extent of muscle damage was calculated by expressing the number of damaged fibers as a percentage of the total number of fibers (125) counted per muscle (one microscopic field analyzed in each of 10 sections per muscle; four muscles per incubation with saline or venom).

Statistical analysis

The contractile responses to electrical stimulation were expressed as a percentage of the basal values of each preparation (taken as 100% prior to the addition of venom or control Krebs or Tyrode solution). The post-venom responses to exogenous ACh and KCl were also expressed as a percentage of the responses obtained before the addition of venom or control solution. Myonecrosis was expressed as the percentage of damaged fibers relative to the total number of fibers counted and creatine kinase release was expressed in absolute values (U/L). All data (absolute values and percentages) were expressed as the mean ±SEM of the number of experiments indicated. Statistical comparisons were based on the absolute values or percentages and were done using Student's unpaired *t*-test or by analysis of variance (ANOVA) followed by the Bonferroni test. A value of $p<0.05$ indicated significance.

RESULTS

Effects of *B. fonsecai* venom in CBC neuromuscular preparation

In CBC preparations, venom (40, 80 and 160µg/ml) produced blockade of indirectly evoked twitches (Figure 1A, B). The time required for the venom to cause a 50% reduction in the contractile responses, *i.e.*, 50% blockade or t_{50}, at concentrations of 80 and 160µg/ml was 84.9±9.3min and 73.3±7.6min, respectively (mean±SEM, n=6 each); the lowest concentration (40µg/ml) produced <50% blockade after 120min compared to a maximum blockade of 85.6±14% with the highest venom concentration. None of the venom concentrations tested had any significant effect on the baseline tension of the preparation, *i.e.*, there was no muscle contracture (Figure 1A, traces 2 and 3). However, incubation with all venom concentrations attenuated the contractures to exogenous ACh and KCl (Figure 1A). The inhibition of

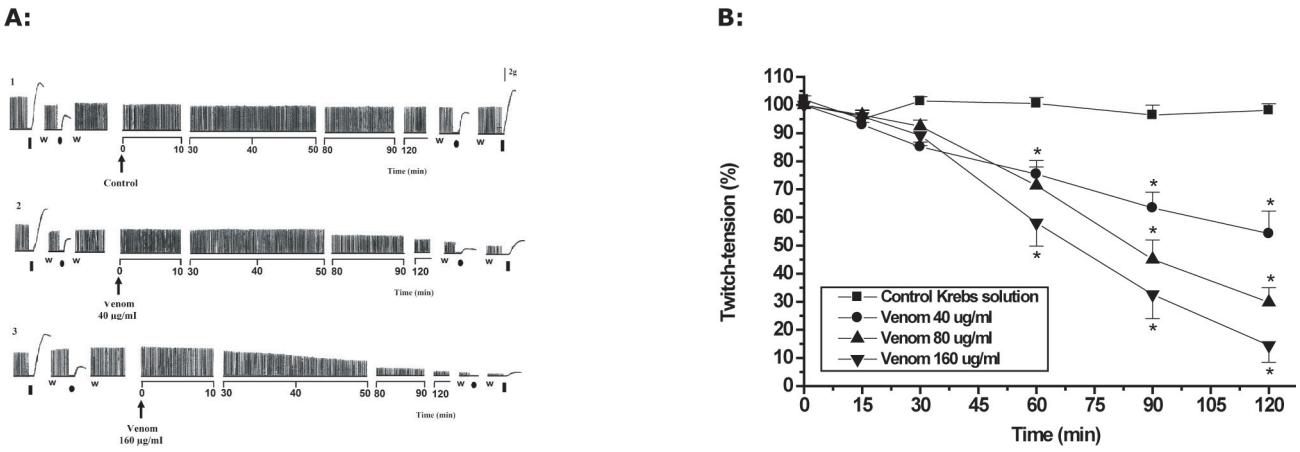

Figure 1. Representative recordings (A) and mean responses (B) for the neuromuscular blockade caused by *B. fonsecai* venom in CBC preparations. The preparations were mounted and stimulated as described in the Methods. In A, recording 1 represents a control (Krebs solution only) preparation and recordings 2 and 3 represent the responses to *B. fonsecai* venom (40µg/ml and 160µg/ml, respectively). Note the progressive decrease in the contractile responses in the presence of venom, especially with the highest concentration, and the marked attenuation of contractures to exogenous ACh (110µM; black squares) and KCl (40mM; black circles) after 120min. w – wash. In B, the points are the mean±SEM (n=6). *p<0.05 compared to control preparations.

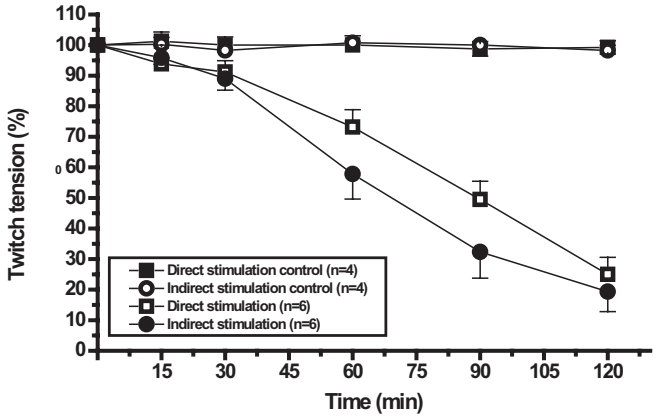

Figure 2. Neuromuscular blockade caused by *B. fonsecai* venom (160µg/ml) in directly and indirectly stimulated CBC preparations. There was no difference in the time required for 50% blockade or in the extent of blockade after 120min in each case. The points are the mean±SEM (n=6). *p<0.05 compared to control preparations.

contractures to ACh (110mM) was 78.5±9.2%, 88.7±6.8% and 100±0% for 40, 80 and 160 µg/ml, respectively (n=4 each), and that of KCl (40mM)-induced contractures was 45.2±6.7%, 67.5±2.6% and 89.7±1.5% for 40, 80 and 160 µg/ml, respectively (n=4 each), compared to pre-venom values.

In directly stimulated CBC, venom (160µg/ml; n=4) produced a progressive reduction in contractile force that was not significantly different from that seen in indirectly stimulated preparations (Figure 2); the t_{50} in these preparations was 87.5±8.1min for direct stimulation compared to 73.3±7.6min for indirect stimulation (n=6 each).

Effects of *B. fonsecai* venom in PND neuromuscular preparation

In PND preparations, venom (100 and 200µg/ml) caused blockade with t_{50} of 94±13 min and 49±8min, respectively, and t_{90} (time to 90% blockade) of 161±8min and 90±15min (n=4 each), respectively (Figure 3), indicating that this preparation was less sensitive to venom than CBC preparations (see previous section). There were no significant changes in the RP or in the amplitude of MEPPs in diaphragm muscle incubated with venom (100 and 200µg/ml; n=3 each) for up to 120min (data not shown), nor was there any increase in baseline tension, *i.e.*, no muscle contracture.

Muscle damage assessed by CK release and histological analysis

Incubation with venom resulted in a progressive increase in CK release during the experiment (Figure 4A) that was suggestive of muscle damage. This damage was confirmed by histological analysis, which showed extensive myonecrosis that involved delta lesions, as well as the presence of edematous and vacuolated cells (Figure 4B, C).

Neutralization of the neuromuscular action of venom by commercial antivenom

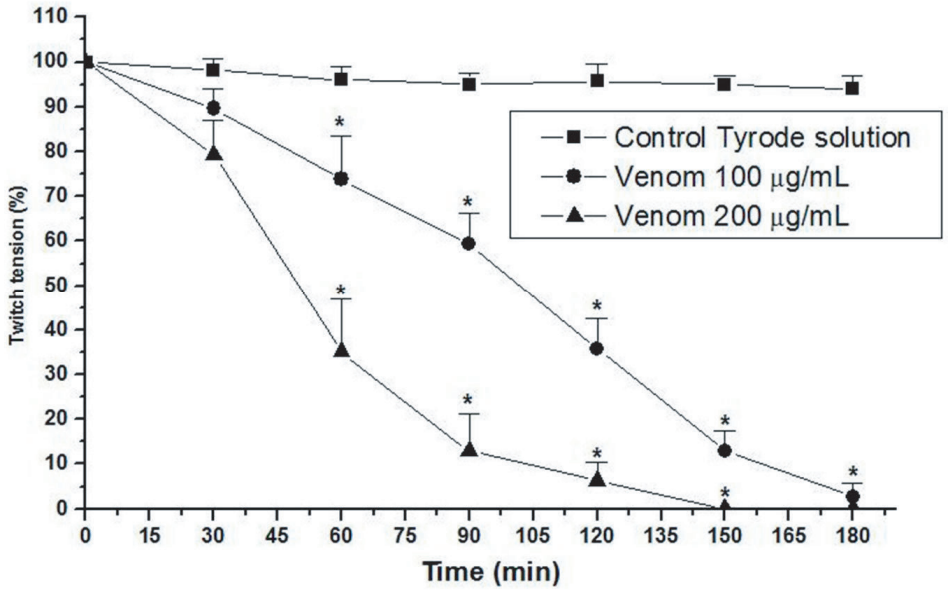

Figure 3. Twitch-tension responses of mouse PND preparations incubated with *B. fonsecai* venom (100 and 200µg/ml). The points are the mean±SEM (n=4). *p<0.05 compared to control preparations.

Preincubation of venom with commercial bothropic antivenom at a venom:antivenom ratio of 5:1 resulted in complete neutralization of the venom-induced neuromuscular blockade in CBC preparations (Figure 5). The inhibition of contractile responses to ACh and K^+ seen with venom alone was also completely prevented by antivenom (% contractures at the end of the incubation relative to basal: ACh 108±13, K^+ 104±12).

DISCUSSION

The results of this study show that, in common with other *Bothrops* species (Cogo et al, 1993; Prianti et al, 2003; Zamunér et al, 2004; Rodrigues-Simioni et al, 2004; Abreu et al, 2007; Cavalcante et al, 2011; Rodrigues-Simioni et al, 2011), *B. fonsecai* venom adversely affects neuromuscular responses in vertebrate nerve-muscle preparations. The mechanism responsible for this neuromuscular blockade appears to involve essentially extensive damage to the skeletal muscle contractile machinery as shown by (1) the marked attenuation of the responses to exogenous ACh and KCl, (2) the very similar responses to venom in directly and indirectly stimulated preparations and (3) the extensive myonecrosis confirmed by CK release and histological analysis.

The potency of *B. fonsecai* venom in producing neuromuscular blockade in CBC was similar to that of several other *Bothrops* venoms (80–200µg/ml) (Zamunér et al, 2004), but considerably less than that of *Bothrops* venoms with a known presynaptic action (blockade at <20µg/ml) (Rodrigues-Simioni et al, 2004, 2011; Cavalcante et al, 2011), *i.e.*, *B. fonsecai* venom caused only slight/moderate blockade (<33%) at a concentration of 40µg/ml. This low potency agrees with the suggestion that this venom interferes with neuromuscular responses primarily by acting postsynaptically.

CBC preparations were more sensitive to *B. fonsecai* venom than PND preparations. Greater sensitivity of avian compared to mammalian preparations has also been seen with other *Bothrops* venoms *e.g.*, *B. alcatraz* (Moraes et al, 2012), *B. insularis* (Cogo et al, 1993), *B. marajoensis* (Cavalcante et al, 2011) and *Bothriopsis* (*Bothrops*) *bilineata smaragdina* (Rodrigues-Simioni et al, 2011), whereas in some cases the PND is more sensitive, *e.g.*, *B. neuwiedi goyazensis* (= *B. neuwiedi*) (Abreu et al, 2007), or both preparations may show similar sensitivity, e.g., *B. neuwiedi pauloensis* (= *B. pauloensis*). Table 1 summarizes the sensitivity of CBC and PND to several *Bothrops* venoms based on the times required for 50% and 90% neuromuscular blockade (t_{50} and t_{90}, respectively). Direct comparison between many of these studies is complicated by the fact that the same concentrations have not been systematically used for all venoms in each preparation, nor have the same lots of venom been used in different studies of the same species. Despite these limitations, the patterns of PND sensitivity noted above (less than, similar to or greater than CBC) can be seen. In addition, the CBC is particularly sensitive to blockade by venoms with marked presynaptic activity that contain presynaptically active PLA_2, *e.g.*, *B. insularis* (Cogo et al, 1993; Cogo et al, 1998; Cogo et al, 2006), *B. pauloensis* (=*B. neuwiedi pauloensis*) (Borja-Oliveira et al, 2003, Borja-Oliveira et al, 2006; Rodrigues-Simioni et al, 2004), *B. marajoensis* (Cavalcante et al, 2011; Galbiatti et al, 2012) and *B. b. smaragdina* (Rodrigues-Simioni et al, 2011; Floriano et al, 2013).

The lower sensitivity of PND to *Bothrops* venoms compared to CBC could reflect anatomical differences in the muscle fibers and form of innervation (monofocal in PND and multifocal in CBC) of the two preparations, with CBC being more sensitive to exogenous agents such as ACh and K^+ (see Cavalcante et al, 2011 and discussion therein). Another possibility is that the lower sensitivity of PND may reflect a mammalian adaptation to provide resistance to *Bothrops* venoms since mammals are an important part of the diet

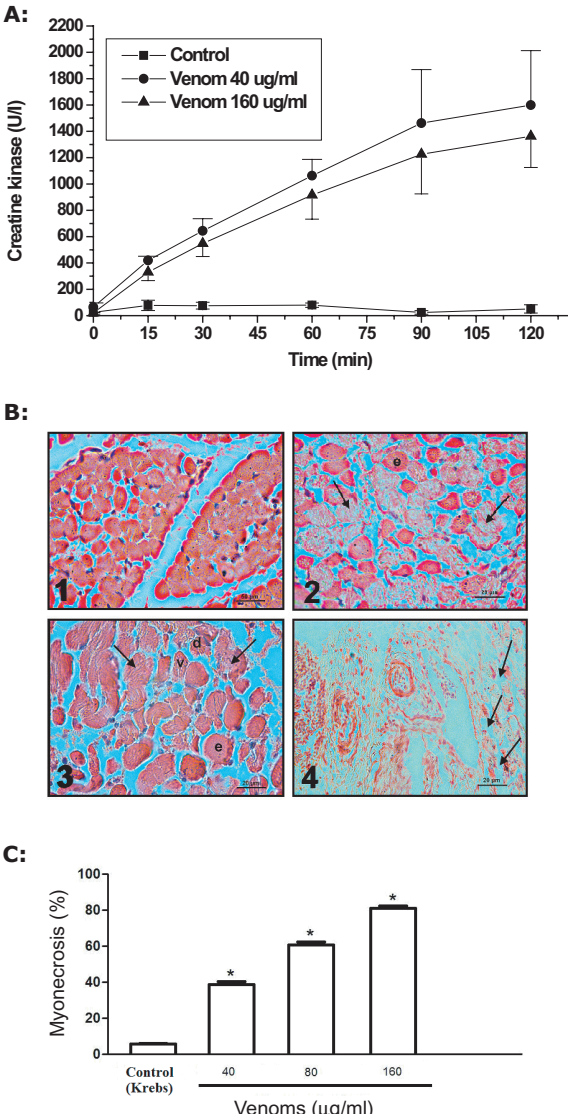

Figure 4. Muscle damage caused by *B. fonsecai* venom in CBC preparations. A. Creatine kinase (CK) release during incubation with venom (40 and 160µg/ml). CK release in response to an intermediate venom concentration (80µg/ml) was not examined since the responses to 40 and 160µg/ml were very similar. The points are the mean±SEM (n=6). *p<0.05 compared to control preparations. B. Morphological alterations in chick BC muscle incubated with Krebs solution alone (panel 1) or *B. fonsecai* venom (panels 2, 3 and 4 for 40, 80 and 160µg/ml, respectively). Panel 1 shows normal fibers with a polygonal shape and peripherally-located nuclei and panels 2–4 show extensive cell damage and myonecrosis. Arrows – myonecrosis, d – delta lesions, e – edematous cells, v – vacuolated cells. Transversal sections, HE staining. Scale bars in micrometers. C. Quantitative analysis of venom-induced myonecrosis. The extent of myonecrosis was calculated by expressing the number of myonecrotic fibers as a percentage of the total number of fibers counted in the muscles of each group. The extent of myonecrosis increased with the venom concentration. The points are the mean±SEM (n=6). *p<0.05 compared to the control group (Krebs alone).

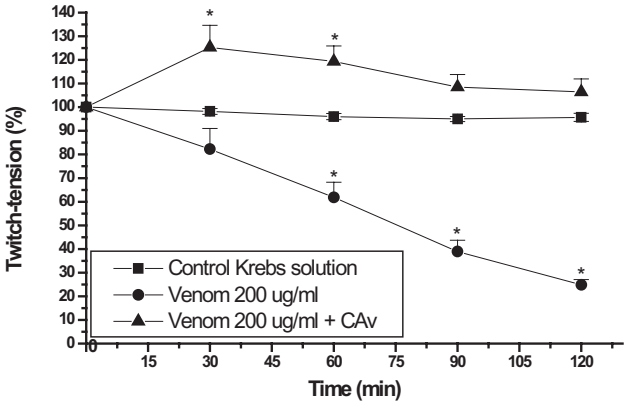

Figure 5. Neutralization by commercial antivenom (CAv) of the neuromuscular effects of B. fonsecai venom (200µg/ml) in CBC preparations. Venom was preincubated with antivenom at a venom:antivenom ratio of 5:1 for 30min at 37ºC prior to testing. The points are the mean±SEM (n=4). *p<0.05 compared to control preparations.

of many species in this genus (Martins et al, 2002). Table 1 summarizes the dietary composition for various *Bothrops* species and shows that while for several of these species mammals are indeed an important component, only in *B. fonsecai* (and the related *B. alternatus* and *B. cotiara*; not shown in the Table) is the diet exclusively mammalian (Martins et al, 2002). However, closer analysis suggests that the relationship between prey type and sensitivity to *Bothrops* venoms is not strictly direct or particularly strong, as shown by various exceptions: (1) for *B. insularis*, a semi-arboreal species that feeds almost exclusively on birds (there are no mammals on the Island of Queimada Grande off the coast of São Paulo State where this species is endemic) (Table 1), the CBC is still more sensitive than the PND to this species´ venom, *i.e.*, the logic applied to the sensitivity of mammalian prey does not apply here; a similar situation occurs with the island species *B. alcatraz* found on the Island of Alcatraz (also off the coast of São Paulo State) and that feeds primarily on centipedes (67% of diet) and anurans (33% of diet) (Martins et al, 2002) yet has a venom that produces blockade in CBC and PND, with the latter being less sensitive (Moraes et al, 2012); indeed, at a concentration of 10µg/ml, this venom produces blockade in CBC with t_{50} and t_{90} (41±4min and 68±8min, respectively) similar to those of mainland *B. pauloensis* [=*B. neuwiedi* in Rodrigues-Simioni et al (2004)] shown in Table 1; (2) the sensitivity of CBC and PND to the venom of *B. pauloensis*, a mainland terrestrial species that feeds on mammals (35% of diet) but few birds (3% of diet), is similar to that of *B. insularis* that feeds primarily on birds (Table 1. Comparison of the times required for B. fonsecai and other Bothrops venoms to produce neuromuscular blockade in CBC and PND preparations 1; Rodrigues-Simioni et al, 2004), and (3) for *B. neuwiedi* (includes *B. n. goyazensis* and other subspecies; Silva, 2004; Silva and Rodrigues, 2008), which has an almost totally mammalian diet, the PND is more sensitive than CBC (Abreu et al, 2007) , *i.e.*, the opposite of that seen with various other *Bothrops* venoms. Overall, these observations indicate that there is scope for detailed investigation into the relationship between prey type and sensitivity to *Bothrops* venoms.

The myonecrosis observed here most likely reflected the action of myotoxic PLA_2 in this venom since these toxins are abundant in *Bothrops* venoms and are important contributors to this damage (Gutiérrez and Ownby, 2003)

Table 1. Comparison of the times required for *B. fonsecai* and other *Bothrops* venoms to produce neuromuscular blockade in CBC and PND preparations

Species	Venom conc. (µg/ml)	CBC		PND		Diet (% composition)[1]			Reference
		t_{50}	t_{90}	t_{50}	t_{90}	Mammals	Birds	Others[2]	
B. fonsecai	80	85±9	NC	-----	-----	100	0	0	This work
	100	-----	-----	94±13	161±8				
	160	73±8	122±7	-----	-----				
	200	-----	-----	49±8	102±15				
B. insularis	10	30±2	43±4	-----	-----	0	83	17[3]	Rodrigues-Simioni et al (2004) (CBC)
	80	~10	-----	44±1	-----				Cogo et al (1993) (CBC and PND)
B. jararacussu	10	-----	-----	46±8	~90	70	0	30[4]	Souza et al (2002) (PND)
	20	-----	-----	18±5	~38				Zamunér et al (2004) (CBC)
	200	48±7	NC	-----	-----				
B. marajoensis	20	25±3	~50	36±4	~60	Primary prey[5]	Few birds	Primarily anurans and lizards	Cavalcante et al (2011)
B. neuwiedi pauloensis	10	47±4	~70	-----	-----	35	3	62[7]	Borja-Oliveira et al (2003) (CBC)
(= *B. pauloensis*)[6]		42±4	63±4						Rodrigues-Simioni et al (2004) (CBC)
	20	-----	-----	101±7	NC				Durigon et al (2005) (PND)
	50	31±3	-----	40±12	~110				
	100	24±4	-----	28±6	~52				
B. neuwiedi goyazensis	50	102±9	NC	60±11	NC	93	0	7 (lizards)	Abreu et al (2007)
(= *B. neuwiedi*)[6]	100	~118	NC	~20	~55				
	200	76±10	NC	-----	-----				
Bothriopsis bilineata	1	~43	~60	NC	NC	36	0	64[9]	Rodrigues-Simioni et al (2011)
smaragdina[8]	10	~16	~27	~100	NC				
	30	~14	~25	~70	~95				

The results are expressed as the time (min, mean ±SEM) required for the venoms to produce 50% (t_{50}) and 90% (t_{90}) neuromuscular blockade. For clarity, the values have been rounded to the nearest whole integer. Values estimated from graphs in the original publications are preceded by ~ and have no SEM. CBC – chick biventer cervicis preparation, PND – phrenic nerve-diaphragm preparation. NC – not calculated because t_{90} was not reached at this concentration within the experimental time-scale used (generally 120min). Only species/venoms for which neuromuscular activity has been assessed in both preparations are shown. [1]Dietary information from Martins et al (2002). [2]Includes varying proportions of centipedes, anurans, lizards and snakes. [3]Anurans – 8%, centipedes – 6% and snakes – 3%. [4]Anurans – 16%, lizards – 10%, snakes – 2% and centipedes – 2%. [5]Estimated abundance based on *Bothrops atrox* (Martins et al, 2002), to which *B. marajoensis* is taxonomically closely related (Salomão et al, 1997; Salomão et al, 1999). The dietary percentages for *B. atrox* are: mammals 45.5%, birds 2.6% and others 51.5% (primarily anurans – 33.5% and lizards – 14.2%). [6]Based on the nomenclature of Silva (2004), and Silva and Rodrigues (2008). [7]Lizards – 23%, anurans – 20%, centipedes – 11% and snakes 8%. [8]Based on data for *Bothrops* (=*Bothriopsis*) *bilineatus*. [9]50% anurans and 14% lizards+snakes.

and to neuromuscular blockade (Gallaci and Cavalcante, 2010). Muscle damage induced by myotoxic PLA_2 involves a series of intracellular responses triggered by membrane permeabilization (via membrane phospholipid degradation in the case of catalytically active PLA_2 or membrane perturbation in the case of catalytically inactive PLA_2) and the subsequent entry of extracellular calcium that can lead to muscle contracture, activation of intracellular proteolytic pathways and mitochondrial damage (Montecucco et al, 2008). As shown here, in neither of the preparations used did *B. fonsecai* venom cause muscle contracture (an increase in baseline tension generally associated with extensive uncontrolled entry of extracellular Ca^{2+}) nor were there significant changes in the membrane resting potential in mouse diaphragm muscle. Nevertheless, the attenuated responses to exogenous K^+, the release of CK and the histological findings indicated the occurrence of muscle damage. Although no myotoxic PLA_2 have yet been characterized from *B. fonsecai* venom, this venom does contain PLA_2 activity (Queiroz et al, 2008) and SDS-PAGE and 2D electrophoretic analyses have demonstrated the presence of PLA_2 in this venom (Queiroz et al, 2008; Tashima et al, 2008). These PLA_2 could be responsible for the neuromuscular actions of the venom. In relation to this, it is worth noting that the venom of the taxonomically related *B. alternatus* contains a basic PLA_2 that is active at vertebrate neuromuscular junctions (Ponce-Soto et al, 2007; Ponce-Soto et al, 2009).

Metalloproteinases, which are abundant in *Bothrops* venoms (Escalante et al, 2011) and present in *B. fonsecai* venom (Tashima et al, 2008), could also theoretically contribute to the muscle damage observed here, perhaps by cleaving surface proteins important for the postsynaptic interaction of PLA_2 with skeletal muscle membrane and/or the binding of ACh to postsynaptic nicotinic receptors, as suggested for the venoms of *B. alcatraz* (Moraes et al, 2012) and *B. leucurus* (Prianti et al, 2003). In this regard, a proteolytic fraction from *Bothrops lanceolatus* venom has been shown to produce contracture and irreversible neuromuscular blockade in CBC, with a decrease in the responses to exogenous ACh but no effect on the contracture to K^+; the fraction produced membrane depolarization but had no presynaptic effect on miniature endplate potential (MEPP) frequency or amplitude, *i.e.*, the effect was solely postsynaptic (Lôbo de Araújo et al, 2002).

Preincubation of venom with commercial antivenom at the venom:antivenom ratio recommended by the manufacturer completely prevented the venom-induced decrease in the contractile responses to electrical stimulation and restored the contractures to exogenous ACh and K^+. This finding indicated that although *B. fonsecai* venom was not included in the venom pool used in the immunization protocols during antiserum production, there was apparently sufficient cross-reactivity among these venoms to ensure good neutralization of this activity. This cross-reactivity may be explained not only by the presence of shared antigenically-related proteins among these venoms but also by the fact that the venom pool used in immunization contained venom from *B. alternatus*, a species taxonomically closely related to *B. fonsecai* (Salomão et al, 1977; Salomão et al, 1999; Wüster et al, 2002). The neutralization of the neuromuscular actions of *B. fonsecai* venom seen here agreed with the high ELISA cross-reactivity of this venom (and those of *B. cotiara* and *B. itapetiningae*) with commercial antivenom that was similar to those of *B. jararaca*, *B. jararacussu* and *B. moojeni*, whereas *B. alternatus* and *B. neuwiedi* showed lower cross-reactivity (Queiroz et al, 2008). In a study of the neuromuscular actions of several *Bothrops* venons (200µg/ml) in CBC and their neutralization by commercial antivenom (at a venom:antivenom ratio of 5:1), Zamunér et al (2004) noted that only the venom of *B. moojeni* was completely neutralized in a manner similar to that seen here for *B. fonsecai*; the other venoms (*B. erythromelas*, *B. jararaca*, *B. jararacussu* and *B. neuwiedi*) showed variable neutralization, even though the latter three were part of the immunization pool. Similarly, Queiroz et al (2008) noted that despite the high ELISA cross-reactivity of *B. fonsecai* venom with antivenom there was considerable variation in the neutralizing capacity towards selected enzymatic activities, e.g., virtually no neutralization of PLA_2 activity, ~15–20% neutralization of proteolytic activity and ~70% neutralization of hyaluronidase activity. The lack of neutralization of PLA_2 activity is interesting in view of the suggestion made above that PLA_2 may be involved in the venom-induced neuromuscular alterations and the finding that antivenom completely neutralized the neuromuscular effects of the venom. This discrepancy may be explained by the fact that whereas PLA_2 activity assays depend on catalytically active enzymes, many of the PLA_2 that exert marked neuromuscular effects are basic PLA_2 with very low or no enzymatic activity (catalytically inactive) (Gallacci and Cavalcante, 2010).

The finding that *B. fonsecai* venom contains the major protein/toxin classes present in other *Bothrops* venoms (Queiroz et al, 2008; Tashima et al, 2008; Tashima et al, 2012) suggests that bites by this species will probably result in local (edema, pain, inflammation, hemorrhage and necrosis) and systemic (coagulopathy, internal bleeding, hypotension, circulatory shock and renal failure) manifestations similar to the general pattern observed for other venoms of this genus (Warrell, 2004; França and Málaque, 2009). However, there are no detailed literature reports of envenoming by *Bothrops* spp. in which *B. fonsecai* has been conclusively identified as the offending species; this situation partly reflects the remote, sparsely populated (by humans) regions where this species occurs. Whilst bites by this species probably have occurred, the general lack of identification of the offending snake to the species level at the attending clinics or hospitals and the probably similar manifestations to those of other *Bothrops* spp. means that most bites by this species have generally gone unnoticed and been (wrongly) attributed to more common *Bothrops* species, *e.g.*, *B. alternatus*, *B. jararaca* and/or *B. jararacussu* that are responsible for most cases of *Bothrops* envenoming in southeastern Brazil. This lack of information makes it difficult to precisely assess the relevance of the present findings to envenoming by this species.

CONCLUSIONS

The results of this study show that *B. fonsecai* venom can adversely affect muscle contraction in vertebrate neuromuscular preparations, essentially by interference with nicotinic receptor functioning and direct damage to the muscle. This profile of activity is similar to that reported for *B. alcatraz*

(Moraes et al, 2012) and *B. leucurus* (Prianti et al, 2003) venoms.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CBC; chick biventer cervicis preparation
CK; creatine kinase
MEPP; miniature end-plate potential
PND; phrenic nerve-diaphragm preparation
RP; resting membrane potential

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