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NEUROMUSCULAR ACTIVITY OF *Bothrops neuwiedi pauloensis* SNAKE VENOM IN MOUSE NERVE-MUSCLE PREPARATIONS

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ABSTRACT: The pharmacological effects of *Bothrops neuwiedi pauloensis* venom on mouse phrenic nerve-diaphragm (PND) preparations were studied. Venom (20 μg/ml) irreversibly inhibited indirectly evoked twitches in PND preparations (60 ± 10% inhibition, mean ± SEM; $p<0.05$; $n=6$). At 50 μg/ml, the venom blocked indirectly and directly (curarized preparations) evoked twitches in mouse hemidiaphragms. In the absence of Ca^{2+}, venom (50 μg/ml), produced partial blockade only after an 80 min incubation, which reached 40.3 ± 7.8% ($p<0.05$; $n=3$) after 120 min. Venom (20 μg/ml) increased (25 ± 2%, $p<0.05$) the frequency of giant miniature end-plate potentials in 9 of 10 end-plates after 30 min and the number of miniature end-plate potentials which was maximum (562 ± 3%, $p<0.05$) after 120 min. During the same period, the resting membrane potential decreased from – 81 ± 1.4 mV to – 41.3 ± 3.6 mV 24 fibers; $p<0.01$; $n=4$) in the end-plate region and from – 77.4 ± 1.4 to –44.6 ± 3.9 mV (24 fibers; $p<0.01$; $n=4$) in regions distant from the end-plate. These results indicate that *B. n. pauloensis* venom acts primarily at presynaptic sites. They also suggest that enzymatic activity may be involved in this pharmacological action.

KEY WORDS: giant MEPPs, myotoxicity, neurotoxicity, presynaptic action.

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INTRODUCTION

According to the Brazilian Ministry of Health, from 1990 to 1993, bites by *Bothrops* spp. accounted for 90.5% of the accidents involving snakes in which the genus was known or suspected and for about 70% venomous snakebites in Brazil (14). The main complications in lethal cases are acute renal failure, shock, acute respiratory failure, and sepsis (2, 5, 6, 15). In some cases, respiratory failure has been associated with pulmonary edema (15). Although *Bothrops* venoms do not produce signs of neurotoxicity after snakebite, in recent years venoms of several *Bothrops* species, including *B. jararacussu* (17), *B. insularis* (8, 9), *B. neuwiedi* (3, 4, 21, 22), and *B. pirajai* (10) have been found to affect neuromuscular transmission in avian and mammalian isolated nerve-muscle preparations and in chick *in vivo*.

B. neuwiedi venom caused head-drop, loss of balance and respiratory failure in chicks *in vivo*, and inhibited twitch-tension response in biventer cervicis preparations, without decreasing responses to acetylcholine or KCl, all of which suggested a presynaptic action (21). More recently, Borja-Oliveira *et al*. (3) investigated the neurotoxicity of several lots of *B. neuwiedi* venoms in biventer cervicis and observed that not all lots exhibited neurotoxicity *in vitro*. *B. n. pauloensis* venom neurotoxicity in these preparations is temperature-dependent (4). In this work, the initial observations on neuromuscular action of *B. n. pauloensis* venom in chick preparations were extended using myographic and electrophysiological techniques in mouse nerve-muscle preparations.

MATERIALS AND METHODS

Venom and reagents

B. n. pauloensis venom, collected from snakes caught in São Paulo State, was provided by the Instituto Butantan (São Paulo, SP, Brazil). D-tubocurarine chloride was from Abbot Laboratórios do Brasil Ltda and neostigmine methylsulfate from Roche (Basel, Switzerland).

Mouse phrenic nerve-diaphragm preparation

Adult male Swiss white mice (28-35 g) were supplied by the University Animal House. Phrenic nerve and diaphragm were obtained from mice anesthetized with chloral hydrate (300 mg/kg, IP) and sacrificed by exsanguination. Nerve-muscle

preparation was mounted as described by Bülbring (7). Hemidiaphragms and phrenic nerves were mounted in 5 ml 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. The preparations were mounted on an electrode that enabled indirect (supramaximal voltage, 0.1 Hz, 0.2 ms) and direct (50 V, 0.1 Hz, 2 ms) muscle stimulation with impulses 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 preparation was allowed to stabilize for at least 15 min before venom addition (10, 20, 50, or 100 μg/ml). In some cases, neostigmine (7.4 μM) was used to reverse venom effects. Before direct stimulation, the preparations were curarized (d-tubocurarine, 7.3 μM). In some experiments, the Ca^{2+} (1.8 mM) in the Tyrode solution was replaced with Sr^{2+} (4 mM).

Electrophysiology

Membrane resting potentials and 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% O_2, 5% CO_2) Tyrode solution (pH 7.4) at 30°C. The resting potential and MEPPs were recorded using glass microelectrodes filled with 3 M KCl (resistance 10-20 MΩ) inserted into the muscle fiber and the recordings were displayed on a Tektronix oscilloscope. Measurements were obtained 15, 30, 60, 90, and 120 min after the addition of venom (20 μg/ml) and were recorded on a Gould RS 3400. The resting potential was measured at or distant from the end-plate regions.

Statistical analysis

Each experiment was repeated at least three times. The results were expressed as mean ± S.E.M. Student's t-test was used for statistical analysis of data. Values of $p < 0.05$ were considered significant.

RESULTS

Venom effect on indirectly and directly stimulated mouse diaphragm

B. n. pauloensis venom (20-100 μg/ml) produced concentration-dependent neuromuscular blockade in indirectly stimulated mouse nerve-muscle preparation (Table 1 and Figure 1). At 20 μg/ml, the venom produced 60 ± 10% blockade (p<0.05; n = 6) after 120 min (Figure 1A, B); a concentration of 50 μg/ml or 100 μg/ml caused complete twitch-tension blockade (Figure 1A, C, D). In the absence of Ca^{2+}, venom (50 μg/ml), produced partial blockade only after an 80 min incubation, which reached 40.3 ± 7.8% (p<0.05; n = 3) after 120 min. In Ca^{2+}-free solution, the results differed significantly (p<0.05) from that obtained in experiments with Ca^{2+} after 35 min incubation with venom (Figure 2). There were no venom-induced contractures. The twitch inhibition was not reverted by neostigmine (not shown) or by several washes with Tyrode solution (Figure 1B-D). No significant neuromuscular blockade was observed at concentrations < 20 μg/ml (not shown).

Venom concentration of 50 μg/ml inhibited contractions induced by direct muscle stimulation in curarized preparations (80 ± 8% inhibition after 120 min; p<0.05; n = 6) (Figure 3B). No significant neuromuscular blockade was observed at concentrations < 50 μg/ml (Figure 3A).

Venom effect (20 μg/ml) on the resting membrane potential and MEPPs

Venom caused a decrease in the resting membrane potential from – 81 ± 1.4 mV to – 41.3 ± 3.6 mV (24 fibers; p<0.01; n = 4) in the end-plate region and from – 77.4 ± 1.4 to – 44.6 ± 3.9 mV (24 fibers; p<0.01; n = 4) in regions distant from the end-plate (Figure 4).

Changes in MEPPs amplitude and frequency were observed in preparations incubated with *B. n. pauloensis* venom when compared to preparations incubated with Tyrode solution (control). There was also an increase (25 ± 2%, p<0.05) in the number of giant miniature end-plate potentials observed in 9 of 10 end-plates after a 30 min incubation (Figure 5). The intracellular recordings showed an increase in MEPPs frequency, which was maximum (562 ± 3%, p<0.05) after 120 min incubation with venom (20 μg/ml). There was also a visible increase in the frequency of 0.6-0.7 mV potentials after 30 min and 0.4-0.5 mV after 120 min, and the generation of

MEPPs with large amplitude (>1.2 mV) during 120 min incubation, which was not observed in control experiments (Figure 6).

Table 1: Time to 50% blockade by *B. n. pauloensis* venom of twitches elicited by indirect stimulation in phrenic nerve-diaphragm preparations.

Concentration (μg/ml)	Time to 50% blockade (min)	n
10	-	4
20	100.7 ± 7.0	6
50	40.0 ± 12	4
100	28.3 ± 6.4	3

All data in the table were significantly (p<0.05) different from each other. Values are mean ± SEM of the number of experiments (n) shown.

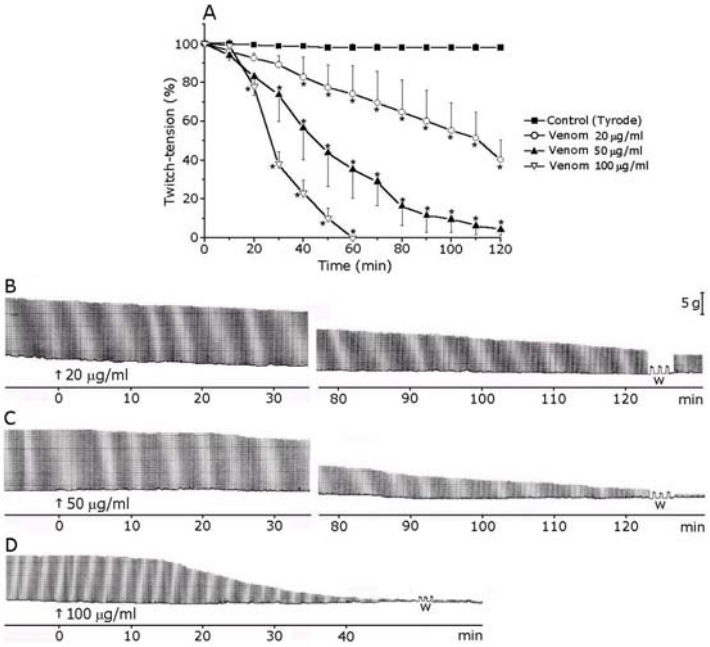

Figure 1. *B. n. pauloensis* venom effect on indirectly stimulated mouse phrenic nerve-diaphragm preparations. A shows average responses to venom compared to Tyrode control (*p<0.05). Each point represents mean ± S.E.M. of 3-6 experiments. B-D shows responses to venom (added at arrow) in indirectly stimulated preparations. Recordings are representative of 3-6 experiments.

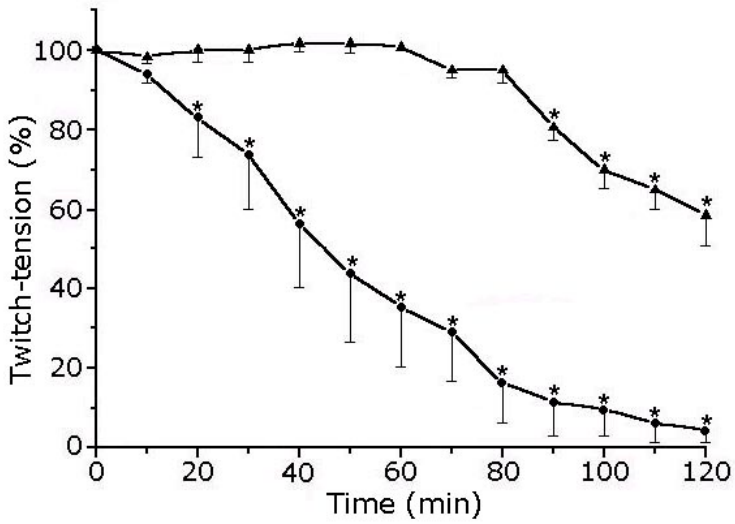

Figure 2. The effect of replacing 1.8 mM Ca^{2+} for 4.0 mM Sr^{2+} in the blocking activity of *B. n. pauloensis* venom (50 μg/ml) as measured by the development of twitch-tension in indirectly stimulated mouse diaphragm. Preparations incubated with normal Tyrode solution plus venom (●) and Ca^{2+}-free Tyrode solution plus 4.0 mM Sr^{2+} and venom (▲). Each point represents mean ± S.E.M. of 3-6 experiments. All points indicated by an asterisk differed significantly from control preparations (p< 0.05).

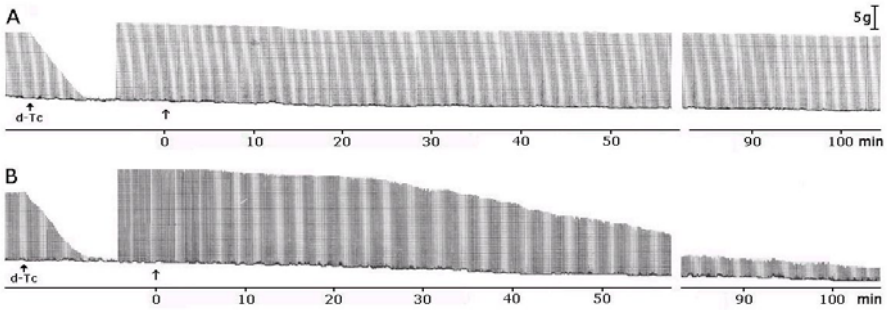

Figure 3. *B. n. pauloensis* venom effect on directly stimulated mouse phrenic nerve-diaphragm preparations. A and B show preparations treated with *d*-tubocurarine (7.3 μM) before venom addition (20 μg/ml and 50 μg/ml, respectively, added at arrow) and directly stimulated diaphragm. Recordings are representative of 3-6 experiments.

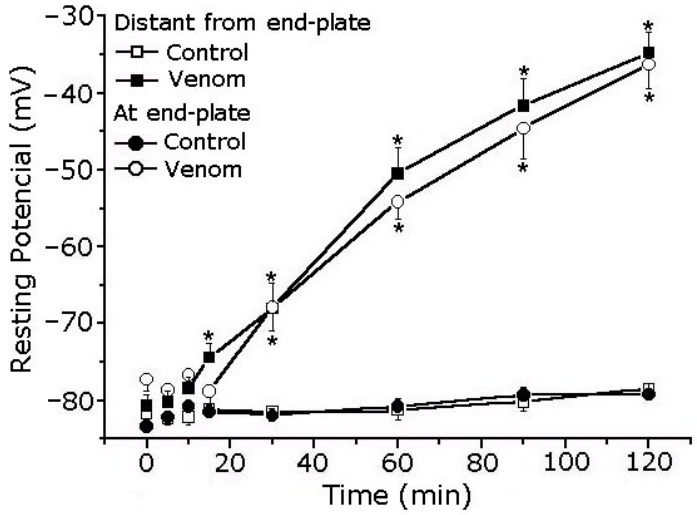

Figure 4. Resting potential (RP) of mouse diaphragm preparations obtained at or distant from the end-plate regions. There was no significant difference in effects between these two regions. Preparations incubated with Tyrode solution showed no change in RP. The depolarizing effect of *B. n. pauloensis* venom (20 μg/ml) was gradual. The points are mean ± S.E.M. of four experiments. All points indicated by an asterisk differed significantly from the control preparations ($p < 0.05$).

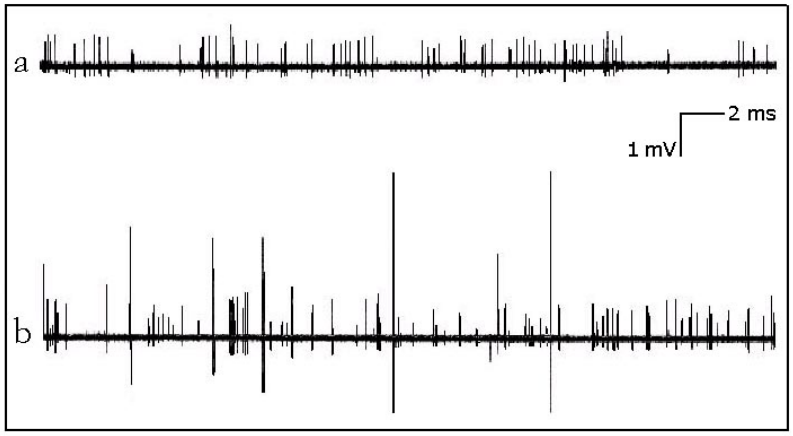

Figure 5. *B. n. pauloensis* venom (20 μg/ml) effect on MEPPs amplitude in mouse diaphragm. In (a), preparations exposed to Tyrode solution and, in (b), venom effect. Note in (a) that most MEPPs have amplitudes ≤0.9 mV and; in (b), the large potentials (>1.8 mV) appear 30 min after venom addition. These phenomena are indicative of presynaptic events. Recordings are representative of four experiments.

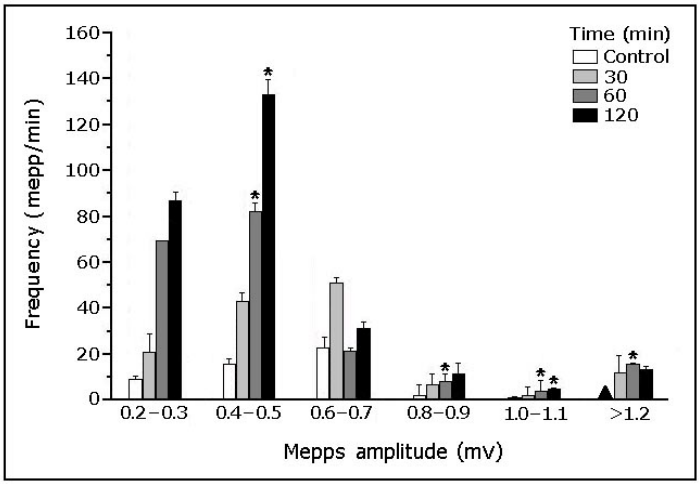

Figure 6. *B. n. pauloensis* venom (20 μg/ml) effect on MEPPs frequency and amplitude in mouse diaphragm after 30, 60, and 120 min incubation compared to Tyrode control (white columns). The columns indicated by an asterisk are significantly different from control responses. Symbol (▲) on the X-axis indicates non-existent reading of bioelectric potentials. Note that 60 min after venom addition to the bath, an evident increase in MEPPs frequency is observed ($p<0.05$). Each point represents mean ± S.E.M. of four experiments.

DISCUSSION

Although *Bothrops* venoms produce no clinical signs of neurotoxicity after snakebite, the venoms of several species can cause neuromuscular blockade *in vitro* and produce signs of peripheral muscular weakness in chicks. *B. n. pauloensis* venom produced neuromuscular blockade which was not reversed by neostigmine or washing the preparations. The MEPPs frequency of mouse isolated hemidiaphragm preparations was increased by venom and giant MEPPs were observed. Effects on muscle membrane were also observed, including the inhibition of muscle contractions in response to direct stimulation (seen at high venom concentrations - 50 μg/ml) and extrajunctional depolarizations in mouse preparations. These results suggest that *B. n. pauloensis* venom neuromuscular blocking action was caused mainly by neurotoxic presynaptic and myotoxic components.

B. neuwiedi venom neuromuscular activity has been the subject of previous reports. Soares *et al*. (21) described the effect of the venom and an isolated component, a Lys49 myotoxic phospholipase A_2 homolog, on chick biventer cervicis preparations, in which inhibition of twitch-tension and KCl-induced contractures was observed.

High concentrations of venom and of its myotoxic component were necessary to cause only partial neuromuscular blockade. Indeed, as reported by Harvey *et al*. (11), low venom concentrations frequently reveal the presence of neurotoxins, while high concentrations are required to demonstrate the presence of myotoxic components. Borja-Oliveira et al. (3) reported intraspecific variation in *B. neuwiedi* venom neuromuscular activity in chick biventer cervicis preparations based on 17 venom lots from various regions of southeastern Brazil. The samples which had the highest neuromuscular potency also had an additional electrophoretic band in relation to the other venoms and, at low concentrations, most of the venoms reduced the twitch-tension without abolishing the contracture to exogenous acetylcholine, suggesting a presynaptic action. *B. n. pauloensis* venom shows individual variation in its composition and there may be occasional complete lack of some toxins (16,21). Several myotoxic variants have been identified in *B. neuwiedi* venoms from different geographic regions (16).

B. neuwiedi venom is not the first of the *Bothrops* genus to show presynaptic activity. *B. insularis* venom also has a presynaptic action (8) which probably involves the Ca^{2+}- and temperature-dependent presynaptic phospholipase A_2 present in this venom (9). The observation that *B. n. pauloensis* venom neuromuscular effect was Ca^{2+}-dependent indicated that enzymatic activity was necessary for neuromuscular action. Several divalent ions, including Sr^{2+}, can bind to the same site, allowing neuromuscular transmission, but cannot substitute for Ca^{2+} in catalysis. For this reason, they are useful phospholipase A_2 activity inhibitors (20).

Similar to *B. insularis* venom (8), *B. n. pauloensis* venom caused the early appearance of giant MEPPs that may be considered as the initial manifestation of a presynaptic action. This early agrees with the action of this venom on membrane resting potential seen at the same time (30 min). Crotoxin, the major neurotoxin from *Crotalus durrissus terrificus* venom, also causes an initial fall in MEPPs frequency in frog neuromuscular junction, followed by a secondary rise which was characterized by the appearance of large spontaneous potentials, i.e., giant MEPPs (12, 18). At frog neuromuscular junction, giant MEPPs are described as spontaneous potentials with amplitude of more than twice the modal MEPPs average and a slower rising phase (1). A recent comparative study about the pharmacological activities of *B. insularis*, *B. neuwiedi,* and *C. d. terrificus* in chick neuromuscular preparations (19) indicated that bothropic venoms may contain components which act presynaptically

at the skeletal neuromuscular junction in a manner similar to *C. d. terrificus venom* and its main toxin, crotoxin.

The neuromuscular blocking action of some bothropic venoms reported in recent years appears to involve Ca^{2+}-independent (*B. jararacussu*) and Ca^{2+}-dependent (*B. insularis* and *B. n. pauloensis*) pathways. The first pathway is intriguing because high Ca^{2+} inhibits the neuromuscular blockade seen with bothropstoxin-I from *B. jararacussu* venom (13), the second pathway, which requires Ca^{2+} for its toxic action, may involve PLA_2 activity of some as yet unidentified venom constituents. Thus, the classic concept of neurotoxicity involving mainly elapid and some crotalid venoms may need reexamination in view of the presynaptic actions of some bothropic venoms.

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