Brazilian Journal of Medical and Biological Research (2003) 36: 617-624
ISSN 0100-879X

The pharmacological effect of *Bothrops neuwiedii pauloensis* (jararaca-pintada) snake venom on avian neuromuscular transmission

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Abstract

The neuromuscular effects of *Bothrops neuwiedii pauloensis* (jararaca-pintada) venom were studied on isolated chick biventer cervicis nerve-muscle preparations. Venom concentrations of 5-50 µg/ml produced an initial inhibition and a secondary increase of indirectly evoked twitches followed by a progressive concentration-dependent and irreversible neuromuscular blockade. At venom concentrations of 1-20 µg/ml, the responses to 13.4 mM KCl were inhibited whereas those to 110 µM acetylcholine alone and cumulative concentrations of 1 µM to 10 mM were unaffected. At venom concentrations higher than 50 µg/ml, there was pronounced muscle contracture with inhibition of the responses to acetylcholine, KCl and direct stimulation. At 20-24ºC, the venom (50 µg/ml) produced only partial neuromuscular blockade (30.7 ± 8.0%, N = 3) after 120 min and the initial inhibition and the secondary increase of the twitch responses caused by the venom were prolonged and pronounced and the response to KCl was unchanged. These results indicate that *B.n. pauloensis* venom is neurotoxic, acting primarily at presynaptic sites, and that enzyme activity may be involved in this pharmacological action.

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Research supported by FAPESP, CNPq, CAPES and FAEP-UNICAMP.

Received July 3, 2002
Accepted November 5, 2002

Key words
- Chick biventer cervicis
- Myotoxicity
- Neurotoxicity
- Phospholipase A_2
- Presynaptic action

Introduction

Snakes of the genus *Bothrops* are the most important cause of snakebites in Brazil. The main complications in lethal cases are acute renal failure, shock, acute respiratory failure, and sepsis (1,2). The mechanism of respiratory failure is not clearly understood since *Bothrops* venoms do not produce apparent signs of neurotoxicity after snakebites. In some cases, respiratory failure was associated with pulmonary edema (1). Nevertheless, Zamuner et al. (3) reported that *Bothrops neuwiedii* venom caused head drop, loss of balance and respiratory failure in chicks *in vivo* after injection of a 0.55 mg/kg. The same report described inhibition of the twitch-tension response to *B. neuwiedii* venom in chick biventer cervicis preparations, but found no decrease in the responses

to exogenous acetylcholine or KCl. Subsequently, Borja-Oliveira et al. (4) observed intraspecific variation in the neuromuscular activity of 16 lots of *B. neuwiedii* venoms in chick nerve-muscle preparations. At low concentrations (5-20 µg/ml), most of the venoms reduced the twitch-tension without completely abolishing the contracture to exogenous acetylcholine, thus suggesting a presynaptic action. The neuromuscular blockade with these venoms varied from 2 to 100% and the electrophoretic profile of the venoms which had the highest neuromuscular potency also had an additional electrophoretic band compared to the other venoms. *B.n. pauloensis* venom also markedly increased the frequency of miniature endplate potentials (mepps) and the incidence of giant mepps in the mouse phrenic nerve-diaphragm (Durigon AM, Borja-Oliveira CR, Dal Belo CA, Oshima-Franco Y, Cogo JC, Lapa AJ, Souccar C and Rodrigues-Simioni L, unpublished data). In the present study, we examined the neurotoxic action of one of the most neurotoxic samples of *B.n. pauloensis* venom screened in chick biventer cervicis preparations.

Table 1. Time to 50% blockade by *Bothrops neuwiedii pauloensis* venom of twitches elicited by field stimulation in chick biventer cervicis preparations.

Concentration (µg/ml)	Time to 50% blockade (min)	N
1	>120	3
5	64.5 ± 4.3	4
10	46.5 ± 3.5	4
50	30.7 ± 3.0	3
100	24.0 ± 4.2	3

The venom concentration of 20 µg/ml (N = 7) was not included in the Table because its effect was not significantly different from those obtained with the other concentrations. Data are reported as the mean ± SEM of the number of experiments (N) shown. All of the data reported in the Table were significantly different from each other ($P<0.05$, (Student *t*-test).

Material and Methods

Venom and reagents

Bothrops neuwiedii pauloensis venom, collected in São Paulo, was provided by the Instituto Butantan (São Paulo, SP, Brazil). *d*-Tubocurarine chloride was purchased from Abbott Laboratórios do Brasil Ltda. (São Paulo, SP, Brazil) and acetylcholine chloride from Sigma (St. Louis, MO, USA).

Isolated chick biventer cervicis nerve-muscle preparation

The biventer cervicis muscle was removed from chicks as described by Ginsborg and Warriner (5) and mounted under a tension of 0.5 g in a 5 ml organ bath containing aerated (95% O_2, 5% CO_2) Krebs solution, pH 7.5, 37ºC, of the following composition: 118.6 mM NaCl, 4.69 mM KCl, 1.88 mM $CaCl_2$, 1.17 mM KH_2PO_4, 1.17 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 11.65 mM glucose. Indirect (0.1 Hz, 0.2 ms, 6-7 V) and direct (0.1 Hz, 0.2 ms, 50 V) stimulation with a Grass S4 stimulator (Quincy, MA, USA) was used to stimulate the muscle and contractions and contractures were recorded via a force displacement-transducer (BG 25 GM Kulite, Leonia, NJ, USA) coupled to a Gould RS 3400 recorder (Cleveland, OH, USA). The preparations were allowed to stabilize for at least 15 min before the addition of venom (1, 5, 10, 20, 50 or 100 µg/ml). Contractures to exogenously applied submaximal concentrations of 110 µM acetylcholine (for 60 s or cumulative concentrations of 1 µM to 10 mM) and 13.4 mM KCl (for 120-160 s) were obtained in the absence of nerve stimulation prior to the addition of venom and at the end of the experiment, in order to test for the presence of neurotoxic and myotoxic activities (6). Some experiments were done at 20-24ºC or after the addition of 11.7 µM *d*-tubocurarine.

Statistical analysis

Each experiment was repeated at least three times. The results are reported as the mean ± SEM. The Student *t*-test was used for statistical analysis of the data, with values of $P<0.05$ indicating statistical significance.

Results

Effect of *B.n. pauloensis* venom on the isolated chick biventer cervicis preparation

At 37ºC, *B.n. pauloensis* venom (1-100 µg/ml) produced a concentration-dependent

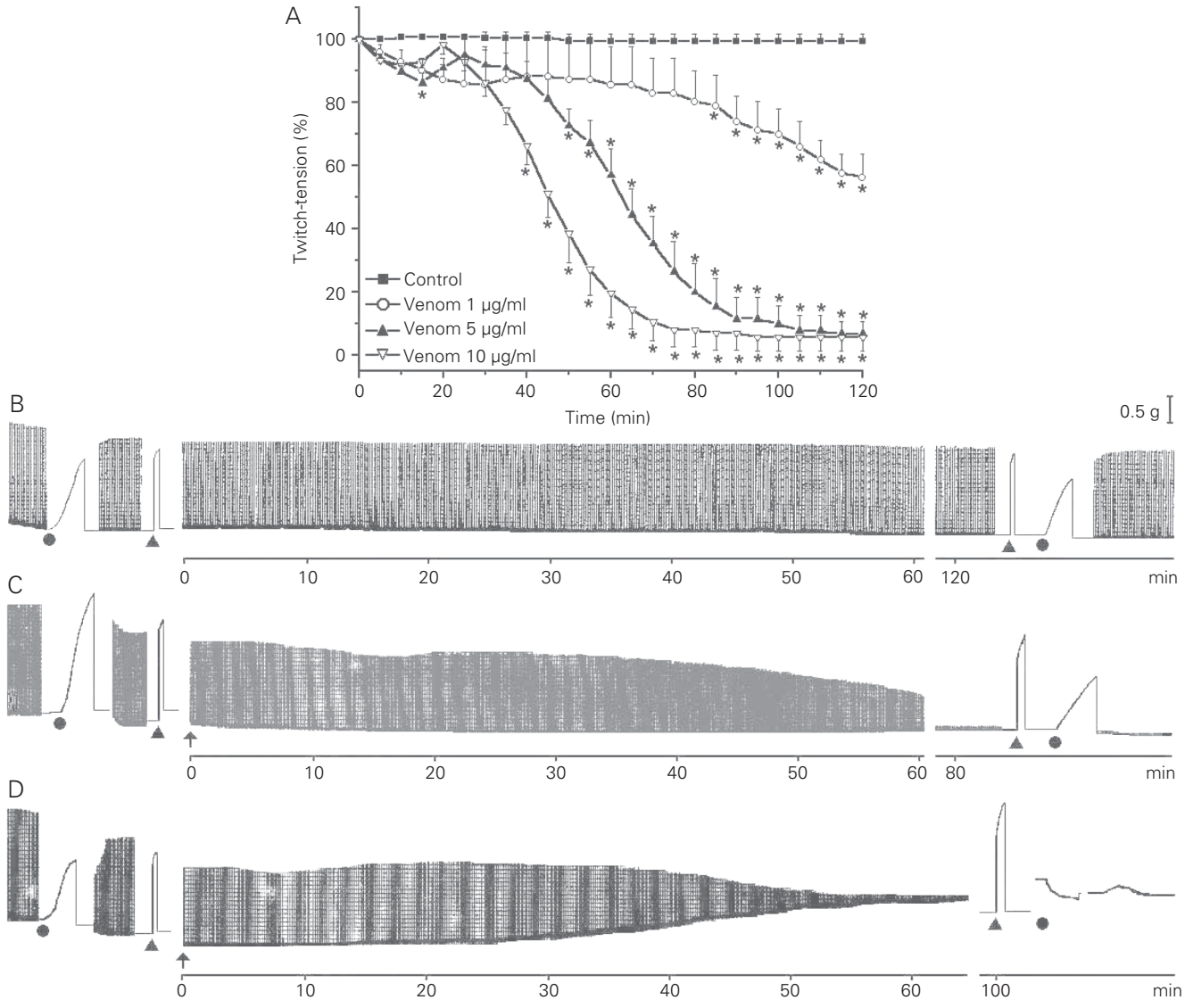


Figure 1. Effect of low concentrations of *Bothrops neuwiedii pauloensis* venom (1, 5 and 10 µg/ml) on chick biventer cervicis nerve-muscle preparations at 37ºC. *A*, Concentration-time response curves for the neuromuscular blocking activity of the venom compared to Krebs solution alone (control). Each point represents the mean ± SEM of 3-6 experiments. *B*, Twitch-tension responses by a control (Krebs solution alone) preparation. *C* and *D*, Venom-treated preparations (5 and 10 µg/ml, respectively). *B.n. pauloensis* venom was added (arrow) after allowing the preparations to stabilize for 15 min. Note the contracture induced by 10 µg of venom/ml. The responses to exogenous 110 µM acetylcholine (triangles) and 13.4 mM KCl (circles) were obtained before and after the addition of venom. These results are representative of 3-6 experiments per venom concentration. *P<0.05 compared to the control preparations (Student *t*-test).

neuromuscular blockade in indirectly stimulated chick biventer cervicis preparations (Table 1, Figures 1 and 2). In most experiments, the venom (5-50 µg/ml) caused an initial inhibition and a secondary increase of indirectly evoked twitches followed by a progressive neuromuscular blockade (Figures 1 and 2).

At concentrations of 5-20 µg/ml, the venom irreversibly blocked twitches evoked by field stimulation within 120 min, without inhibiting acetylcholine-induced contractures, at 37ºC (Figures 1 and 3). At 20 µg/ml, the venom produced complete blockade without affecting the responses to cumulative concentrations of acetylcholine (1, 3, 10 and 30 µM, and 0.1, 0.3, 1, 3 and 10 mM, respectively) obtained before and after the venom addition (data not shown). The contractions induced by KCl were completely blocked at venom concentrations >10 µg/ml (Figures 1D and 3). At the same concentrations (>10

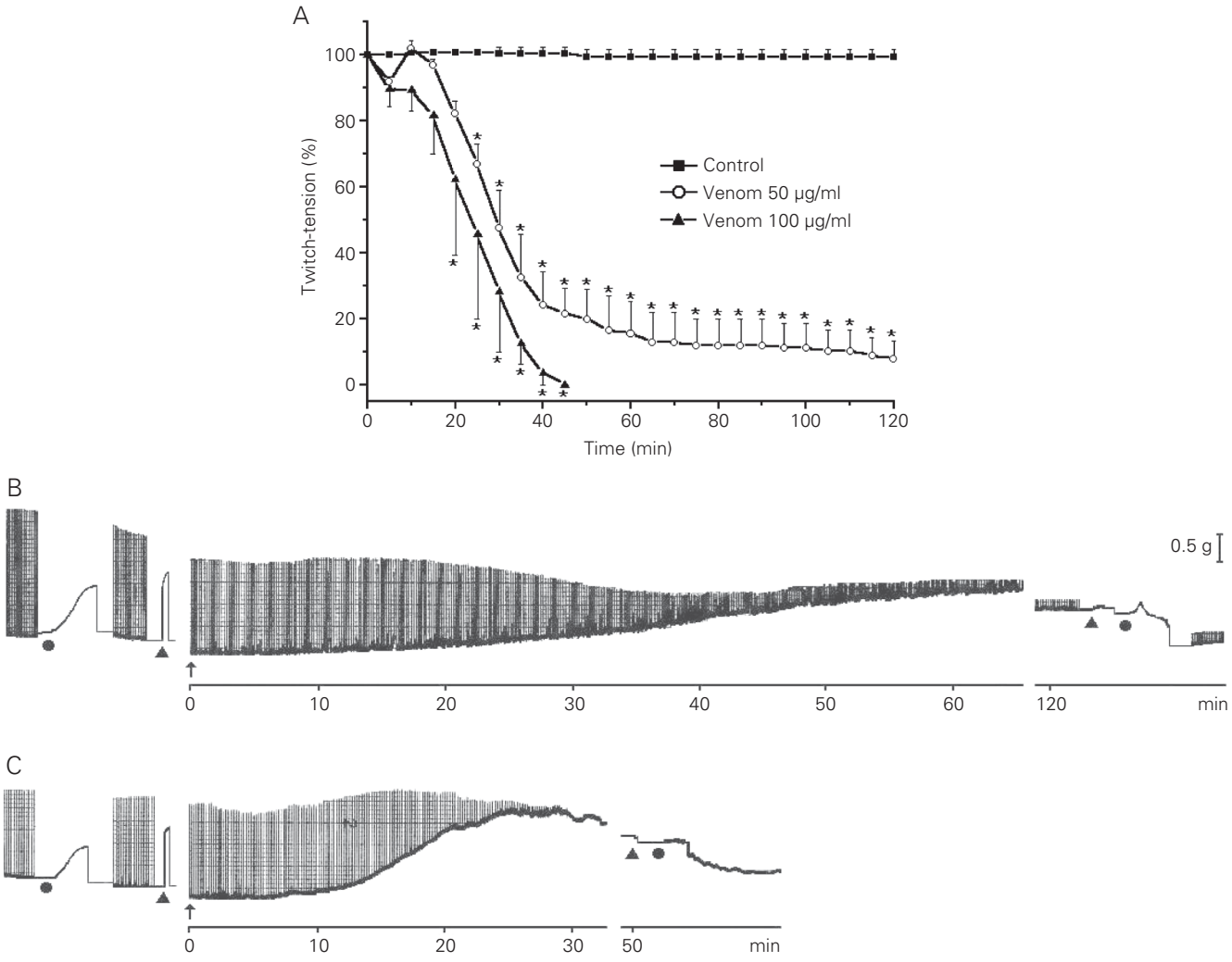


Figure 2. Effect of high concentrations (50-100 µg/ml) of *Bothrops neuwiedii pauloensis* venom on chick biventer cervicis preparations at 37ºC. *A*, Concentration-time response curves for the neuromuscular blocking activity of the venom compared to Krebs solution alone (control). Each point represents the mean ± SEM of 3-6 experiments. *B* and *C*, Venom-treated preparations (50 and 100 µg/ml, respectively). *B.n. pauloensis* venom was added (arrow) after allowing the preparations to stabilize for 15 min. Note that the contractions induced by the venom were concentration dependent. The responses to exogenous 110 µM acetylcholine (triangles) and 13.4 mM KCl (circles) were obtained before and after the addition of venom. These results are representative of 3-4 experiments per venom concentration. *P<0.05 compared to the control preparations (Student *t*-test).

µg/ml), the venom also caused muscle contractures, the extent of which was concentration dependent (Figures 1 and 2). Higher venom concentrations (50-100 µg/ml) abolished the response to acetylcholine (Figures 2 and 3).

At 20-24ºC, the venom (50 µg/ml) produced only partial blockade (30.7 ± 8.0%, N = 3) after 120 min (Figures 3 and 4). Under these conditions, the initial inhibition and the secondary increase of the twitch responses produced by the venom were prolonged and pronounced and the response to KCl was unchanged, whereas the contractions to acetylcholine were partially inhibited (30 ± 14% inhibition, N = 3, $P<0.05$; Figures 3 and 4).

The twitches elicited by direct muscle stimulation in curarized (11.7 µM *d*-tubocurarine) preparations at 37ºC were not significantly depressed by 90-min incubation with low venom concentrations (<20 µg/ml) (Figure 5A). At 50 µg/ml, the venom caused partial blockade (60 ± 7% inhibition, N = 3, $P<0.05$) of directly evoked contractions (Figure 5B).

Discussion

In Brazil, the snakes of the genera *Crotalus* and *Micrurus* are the only ones that produce signs of peripheral neurotoxicity after snakebites, such as palpebral ptosis and respiratory paralysis. Nevertheless, *Bothrops* snakebites may also produce respiratory failure associated with pulmonary edema in some cases (1).

Zamuner et al. (3) reported the neurotoxic action of *B. neuwiedii* venom in chicks *in vivo* and *in vitro*. In isolated chick nerve-muscle preparations, *B. neuwiedii* venom (10-50 µg/ml) completely blocked neuromuscular transmission without depressing the response to acetylcholine. These results suggested a presynaptic site of action for this venom. However, not all *B. neuwiedii* venoms exhibit significant neurotoxicity on isolated neuromuscular preparations (4).

The present report describes the neuromuscular activity of the venom of one of the most toxic subspecies of *B. neuwiedii*, *B.n. pauloensis*, screened by Borja-Oliveira et al. (4). This venom produced concentration-dependent neuromuscular blockade in chick biventer cervicis preparations. Complete blockade at a low concentration (5 µg/ml) was not accompanied by inhibition of the responses to KCl and acetylcholine. These observations indicate that at low concentrations the venom had no inhibitory effect on postsynaptic acetylcholine receptors and its action was not dependent on myotoxicity.

At high concentrations (>10 µg/ml), the venom has a myotoxic effect, including the inhibition of KCl-induced contractures, pronounced muscle contracture and the partial inhibition of contractions in response to direct muscle stimulation in curarized preparations. Recently, Soares et al. (7) reported the effect of *B. neuwiedii* venom and a Lys49 myotoxic phospholipase A_2 homologue from

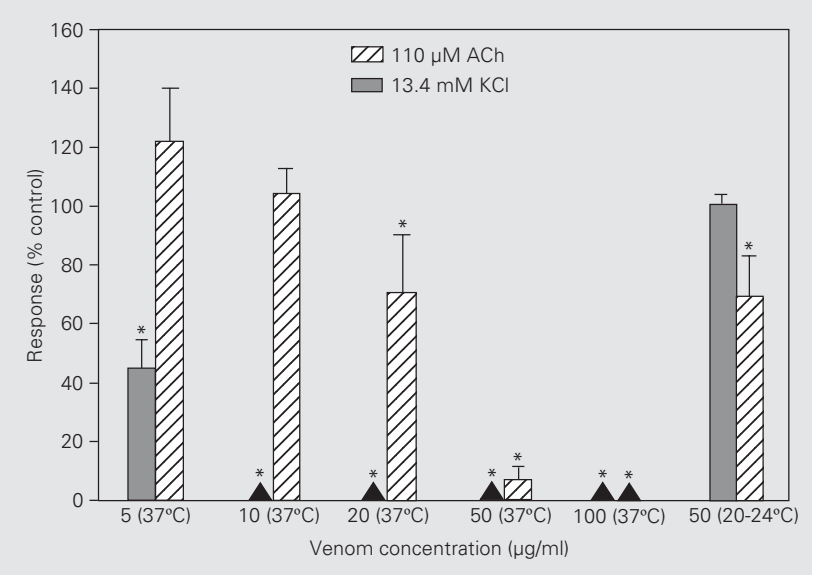


Figure 3. Effect of *Bothrops neuwiedii pauloensis* venom on muscle contractions induced by 13.4 mM KCl and 110 µM acetylcholine (ACh) in the chick biventer cervicis preparations after 120-min incubation compared to the responses observed in Krebs controls (100%). Data are reported as means ± SEM for 3-6 experiments per concentration. The preparations were incubated with venom at the concentrations and temperatures indicated on the histogram. The triangles on the X-axis indicate that the venom totally blocked the response to the agonist. *$P<0.05$ compared to the corresponding control (Krebs solution alone) (Student *t*-test).

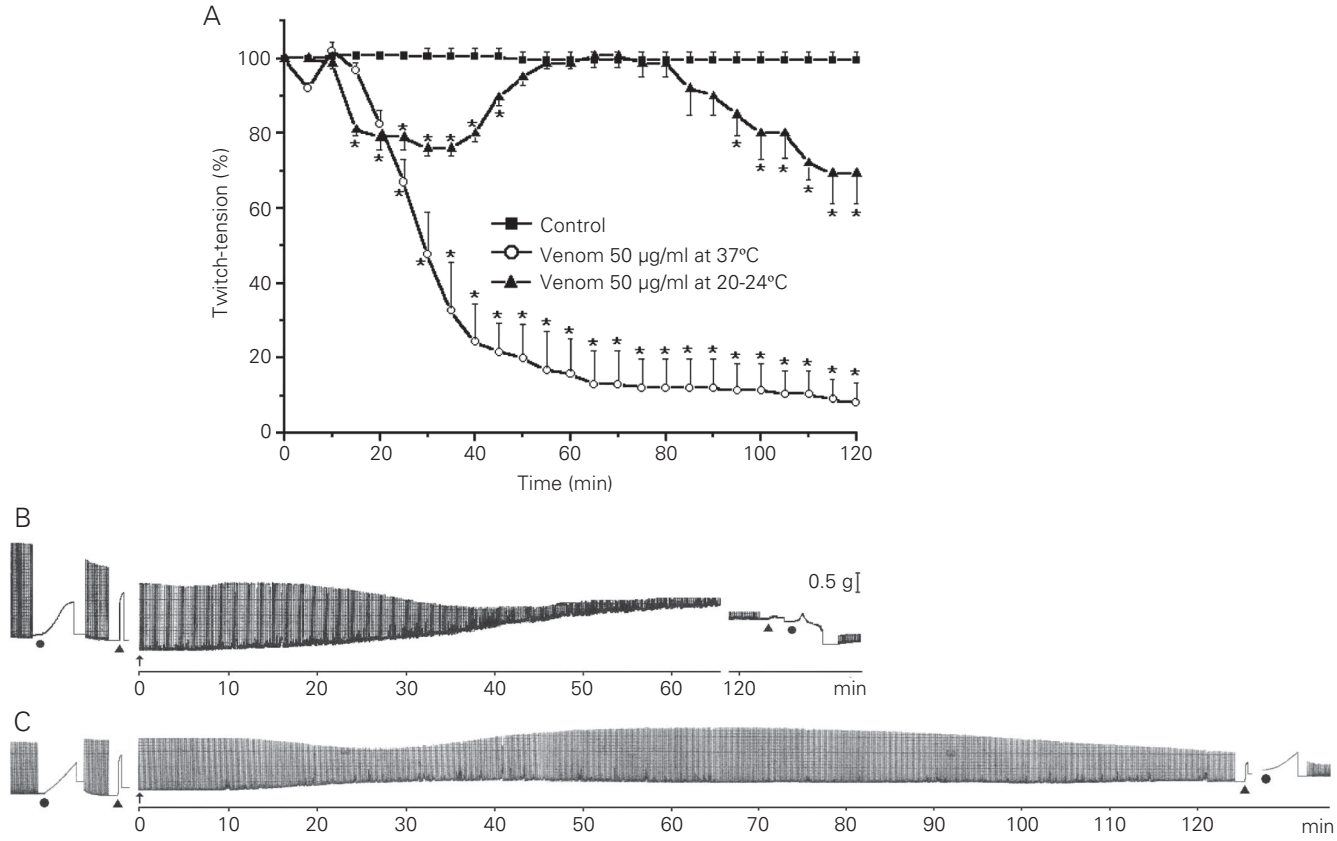
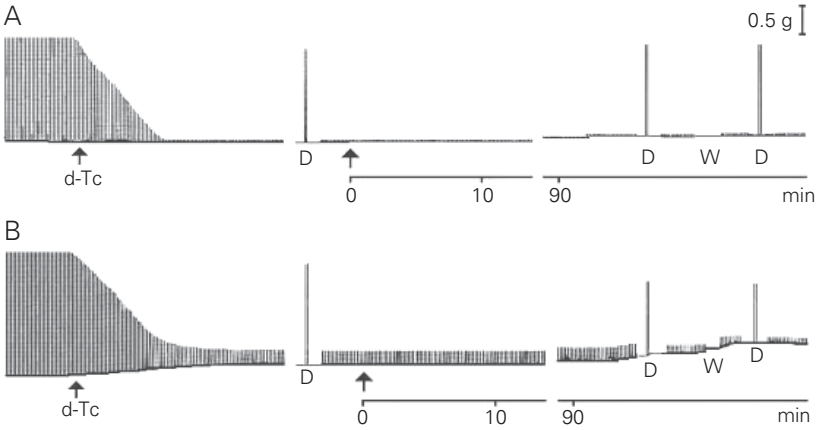


Figure 4. Effect of *Bothrops neuwiedii pauloensis* venom (50 µg/ml) on chick biventer cervicis preparations at 20-24ºC compared to 37ºC. *A*, Concentration-time response curves for the neuromuscular blocking activity of the venom compared to Krebs solution alone (control) are shown. Each point represents the mean ± SEM of 3-6 experiments. *B*, Venom-treated preparations (50 µg/ml) at 37ºC. *C*, A low incubation temperature (20-24ºC) prevented the neuromuscular action of the venom (50 µg/ml). *B.n. pauloensis* venom was added (arrow) after allowing the preparations to stabilize for 15 min. The responses to exogenous 110 µM acetylcholine (triangles) and 13.4 mM KCl (circles) were obtained before and after the addition of venom. These results are representative of 3-4 experiments. *P<0.05 compared to the control preparations (Student *t*-test).

Figure 5. Effect of *Bothrops neuwiedii pauloensis* venom on the twitches elicited by direct muscle stimulation. *A* and *B*, Venom-treated chick biventer cervicis preparations at 20 and 50 µg/ml, respectively (arrow, time zero). Direct stimulation (D) was applied after treatment with *d*-tubocurarine (7.3 µM d-Tc) and after several washes (W) with Krebs solution. These results are representative of 3 and 5 experiments for *A* and *B*, respectively.



this venom, namely BnSP-7, on chick biventer cervicis preparations. The phospholipase A_2 BnSP-7 inhibited the twitch-tension and KCl-induced contractures only at high concentrations. In addition, both BnSP-7 and the crude venom released creatine kinase from the mouse gastrocnemius muscle and induced a dose-dependent edema.

Since the blockade of the responses to indirect stimulation and to KCl as well as the incidence of contractures were temperature dependent, enzyme activity may be involved in the neuromuscular action of *B.n. pauloensis* venom. *B. neuwiedii* venom contains phospholipases (7-11) and the venom used in the present study had significant phospholipase A_2 activity (Borja-Oliveira CR, Kassab BH, Durigon AM, Soares AM, Toyama MH, Novello JC, Giglio JR, Marangoni S and Rodrigues-Simioni L, unpublished results). The data available do not permit us to identify the putative enzyme.

The neuromuscular action produced by *B.n. pauloensis* venom is consistent with the available data about phospholipase A_2 neurotoxins. Crotoxin, the main neurotoxin of *Crotalus durissus terrificus* venom, causes a triphasic change (depression, facilitation and final blockade) of acetylcholine release by the nerve terminals (12-15), similar to that observed with other snake ß-neurotoxins, such as ß-bungarotoxin (16-18), notexin (19), taipoxin (20,21), and textilotoxin (22,23). In mouse hemi-diaphragm nerve-muscle preparations, by reducing the external Ca^{2+} concentration, ß-bungarotoxin classically produces an initial transient inhibition of twitches (phase 1) followed by a prolonged facilitatory phase (phase 2) and finally a blocking phase (phase 3). The facilitatory effect of these toxins on mammalian nerve-muscle preparations is independent of phospholipase A_2 activity. Indeed, the increase in the number of twitches produced by *B.n. pauloensis* venom was present at 20-24ºC, although the neuromuscular blocking action (phase 3) was inhibited. The third phase (complete and irreversible inhibition of neurotransmission) produced by ß-neurotoxins depends on temperature and on the presence of Ca^{2+} in the medium and is probably due to phospholipase A_2-mediated destruction of membrane phospholipids in motor nerve terminals. The enzymatic activity of ß-neurotoxins is not significantly correlated with their toxicity (24), but is obligatory for the full expression of neurotoxic activity since its inhibition prevents lethality (25).

Acknowledgments

The authors thank Gildo Bernardo Leite for technical assistance, and Instituto Butantan for providing the venom sample.

References

1. Ribeiro LA, Albuquerque MJ, Pires de Campos VAF, Katz G, Takaokam NY, Lebrão ML & Jorge MT (1998). Óbitos por serpentes peçonhentas no Estado de São Paulo: avaliação de 43 casos, 1988/98. *Revista da Associação Médica Brasileira*, 44: 312-318.
2. Bucaretchi F, Herrera SRF, Hyslop S, Baracat ECE & Vieira RJ (2001). Snakebites by *Bothrops* spp in children in Campinas, São Paulo, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, 43: 329-333.
3. Zamuner SR, Prado-Franceschi J & Rodrigues-Simioni L (1996). The screening of *Bothrops* venoms for neurotoxic activity using the chick biventer cervicis preparation. *Toxicon*, 34: 314-315.
4. Borja-Oliveira CR, Soares AM, Zamuner SR, Hyslop S, Giglio JR, Prado-Franceschi J & Rodrigues-Simioni L (2002). Intraspecific variation in the neurotoxic and myotoxic activities of *Bothrops neuwiedii* snake venoms. *Journal of Venomous Animals and Toxins*, 8: 88-101.
5. Ginsborg BL & Warriner J (1960). The isolated chick biventer cervicis nerve-muscle preparation. *British Journal of Pharmacology*, 15: 410-411.
6. Harvey AL, Barfaraz A, Thompson E, Faiz A, Preston S & Harris JB (1994). Screening of snake venoms for neurotoxic and myotoxic effects using simple *in vitro* preparations from rodents and chicks. *Toxicon*, 32: 257-265.
7. Soares AM, Guerra-Sá R, Borja-Oliveira CR, Rodrigues VM, Rodrigues-Simioni L, Rodrigues V, Fontes MRM, Lomonte B, Gutiérrez JM & Giglio JR (2000). Structural and functional characterization of BnSP-7, a Lys49 myotoxic phospholipase A_2 homologue from *Bothrops neuwiedii* venom. *Archives of Biochemistry and Biophysics*, 378: 201-209.

8. Vidal JC, Badano BN, Stoppani AO & Boveris A (1966). Inhibition of electron transport chain by purified phospholipase A from *Bothrops neuwiedii* venom. *Memórias do Instituto Butantan*, 33: 913-920.
9. Daniele JJ, Bianco ID & Fidelio GD (1995). Kinetic and pharmacologic characterization of phospholipases A_2 from *Bothrops neuwiedii* venom. *Archives of Biochemistry and Biophysics*, 318: 65-70.
10. Daniele JJ, Bianco ID, Delgado C, Carrilo DB & Fidelio GD (1997). A new phospholipase A_2 isoform isolated from *Bothrops neuwiedii* (yarará chica) venom with novel kinetic and chromatographic properties. *Toxicon*, 35: 1205-1215.
11. Geoghegan P, Angulo Y, Cangelosi A, Diaz M & Lomonte B (1999). Characterization of a basic phospholipase A_2-homologue myotoxin isolated from the venom of the snake *Bothrops neuwiedii* (yarará chica) from Argentina. *Toxicon*, 37: 1735-1746.
12. Brazil OV & Excell BJ (1971). Action of crotoxin and crotactin from the venom of *Crotalus durissus terrificus* (South American rattlesnake) on the frog neuromuscular junction. *Journal of Physiology*, 212: 34P-35P.
13. Hawgood BJ & Smith JW (1977). The mode of action at the mouse neuromuscular junction of the phospholipase A-crotapotin complex isolated from venom of the South American rattlesnake. *British Journal of Pharmacology*, 61: 597-606.
14. Chang CC & Lee JD (1977). Crotoxin, the neurotoxin of South American rattlesnake venom, is a presynaptic toxin acting like beta-bungarotoxin. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 296: 159-168.
15. Hawgood BJ & Santana de Sa S (1979). Changes in spontaneous and evoked release of transmitter induced by the crotoxin complex and its component phospholipase A_2 at the frog neuromuscular junction. *Neuroscience*, 4: 293-303.
16. Chang CC, Chen TF & Lee CY (1973). Studies of the presynaptic effect of ß-bungarotoxin on neuromuscular transmission. *Journal of Pharmacology and Experimental Therapeutics*, 184: 339-345.
17. Strong PN, Goerke J, Oberg SG & Kelly RB (1976). ß-bungarotoxin, a pre-synaptic toxin with enzymatic activity. *Proceedings of the National Academy of Sciences, USA*, 73: 178-182.
18. Abe T, Alema S & Miledi R (1977). Isolation and characterization of presynaptically acting neurotoxins from the venom of *Bungarus* snakes. *European Journal of Biochemistry*, 80: 1-12.
19. Harris JB, Karlsson E & Thesleff S (1973). Effects of an isolated toxin from Australian tiger snake (*Notechis scutatus scutatus*) venom at the mammalian neuromuscular junction. *British Journal of Pharmacology*, 47: 141-146.
20. Cull-Candy SG, Fohlman J, Gustavsson D, Lullmann-Rauch R & Thesleff S (1976). The effects of taipoxin and notexin on the function and fine structure of the murine neuromuscular junction. *Neuroscience*, 1: 175-180.
21. Kamenskaya MA & Thesleff S (1974). The neuromuscular blocking action of an isolated toxin from the elapid (*Oxyuranus scutellactus*). *Acta Physiologica Scandinavica*, 90: 716-724.
22. Su MJ, Coulter AR, Sutherland SK & Chang CC (1983). The presynaptic neuromuscular blocking effect and phospholipase A_2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis. Toxicon*, 21: 143-151.
23. Barnett D, Howden MEH & Spence I (1979). Pre- and postsynaptic neurotoxins in the venom of the common brown snake (*Pseudonaja t. textilis*). *Proceedings of the Australian Physiological and Pharmacological Society*, 10: 240P (Abstract).
24. Lambeau G, Barhanin J, Schweitz H, Qar J & Lazdunski M (1989). Identification and properties of very high affinity brain membrane-binding sites for a neurotoxic phospholipase from the taipan venom. *Journal of Biological Chemistry*, 264: 11503-11510.
25. Jeng TW & Fraenkel-Conrat H (1978). Chemical modification of histidine and lysine residues of crotoxin. *FEBS Letters*, 87: 291-296.