

RESEARCH ARTICLE

The neuromuscular activity of *Micrurus pyrrochryptus* venom and its neutralization by commercial and specific coral snake antivenoms

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ABSTRACT

The neuromuscular activity of *Micrurus pyrrochryptus* venom was studied in chick biventer cervicis (BC) and mouse phrenic nerve-diaphragm (PND) preparations. The venom (0.5-50µg/ml) caused irreversible, time- and concentration-dependent blockade, with BC being more sensitive than PND (50% blockade with 10µg/ml in 22±3min and 62±4min, respectively; mean±SEM, n=6; p<0.05). In BC preparations, venom (0.5µg/ml) progressively abolished ACh-induced contractures, whereas contractures to exogenous KCl and muscle twitches in curarized preparations were unaffected. The venom neither altered creatine kinase release (venom: 25.8±1.75IU/l vs control: 24.3±2.2IU/l, n=6, after 120min), nor it caused significant muscle damage (50µg of venom/ml vs control: 3.5±0.8% vs 1.1±0.7% for PND; 4.3±1.5% vs 1.2±0.5% for BC, n=5). The venom had low PLA₂ activity. Neurotoxicity was effectively neutralized by commercial *Micrurus* antivenom and specific antivenom. These findings indicate that *M. pyrrochryptus* venom acts postsynaptically on nicotinic receptors, with no significant myotoxicity.

KEYWORDS: Coral snake venom, neuromuscular blockade, neurotoxin, nicotinic receptor, postsynaptic

INTRODUCTION

Coral snakes constitute a large taxonomic group of more than 120 species and subspecies divided into three genera, *Leptomicrurus*, *Micruroides* and *Micrurus*, with a distribution ranging from the United States to Argentina (Scrocchi, 1990; Roze, 1996; da Silva and Sites, 1999; da Silva and Sites, 2001).

Micrurus venoms are highly-neurotoxic, with clinical manifestations of palpebral ptosis, ophthalmoplegia and respiratory paralysis (in severe cases), indicating neuromuscular blockade (Vital Brazil and Vieira, 1996; da Silva and Bucaretychi, 2003; Warrell, 2004; Bucaretychi et al, 2006; Manock et al, 2008). Experimental studies have shown that the neuromuscular blockade is caused by pre- and postsynaptic neurotoxins from these venoms (Vital Brazil

et al, 1976; Vital Brazil et al, 1977; Vital Brazil, 1980; Goularte et al, 1995; Vital Brazil et al, 1995; Serafim et al, 2002; Abreu et al, 2008). However, only a few of these toxins have actually been purified and studied *in vivo* and *in vitro* (Alapé-Girón et al, 1996b; Francis et al, 1997; Dal Belo et al, 2005), primarily because of the difficulty in maintaining these snakes in captivity (Serapicos and Merusse, 2002; Oliveira et al, 2005) and their low venom yields (de Roodt et al, 1998).

Although bites by *Micrurus* spp. in South America are relatively rare, severe cases of respiratory paralysis can be life-threatening if adequate therapeutic interventions are not implemented. Antivenom administration is the only specific treatment for coral snake bites, although ancillary measures, such as, mechanical ventilation and the administration of cholinesterase inhibitors (the latter

for postsynaptically-active venoms), can also be useful (Coelho et al, 1992; Vital Brazil and Vieira, 1996; Bucarechi et al, 2006). Experimentally, the neutralizing capacity of antivenoms against neurotoxins is frequently studied in neuromuscular preparations *in vitro*, following pre-incubation of venom with antivenom (Barfaraz and Harvey, 1994; Alapé-Girón et al, 1996a; Alapé-Girón et al, 1997; Hodgson and Wickramaratna, 2002; Abreu et al, 2008).

M. pyrrhocryptus, which occurs in central Argentina, Bolivia, Paraguay and Brazil (in the states of Mato Grosso and Mato Grosso do Sul), was originally a subspecies of *Micrurus frontalis*, but was elevated to species status based on morphological features that distinguished it from *M. frontalis* (da Silva and Sites, 1999; Ministerio de Salud, 2007). Little is known about the composition of *M. pyrrhocryptus* venom (Hoge and Lancini, 1959; de Roodt 2002; Dokmetjian et al, 2009) and its neutralization by antivenom.

In this work, we studied the effects of *M. pyrrhocryptus* venom on neuromuscular transmission in avian and mammalian neuromuscular preparations, and examined the neutralization of neurotoxicity by commercial antivenom and specific antiserum.

MATERIAL AND METHODS

Animals

Adult male Swiss white mice (28-35gm) were supplied by the Multidisciplinary Center for Biological investigation (Cemib) at Unicamp. HY Line chicks (4-10 days old) were obtained from Globo Aves (Jaguariúna, SP, Brazil). The animals were housed at 24°C with free access to food and water. The experiments were done in accordance with the guidelines of the Brazilian College for Animal Experimentation (Cobea) and were approved by the institutional Ethics Committee on Animal Use (CEUA/Unicamp, Protocol No. 1550-1).

Venom, antivenoms and venom neutralization

M. pyrrhocryptus venom, obtained from snakes captured in Santiago del Estero, Argentina, and specific antivenom were gifts from Dr Alejandro U Vogt (The Centro Zootoxicológico de Misiones, Argentina). The specific antivenom (SAV, batch 115) was raised in horses by the Instituto Nacional de Producción de Biológicos of the Administración Nacional de Laboratorios e Institutos de Salud (A.N.L.I.S.) "Dr Carlos G. Malbrán", Ministerio de Salud (Buenos Aires, Argentina). The venom of *M. pyrrhocryptus* is the main immunogen of this antivenom. Commercial *Micrurus* antivenom (Batch No. 03.2184), produced by immunizing horses with a pool of *M. frontalis* and *M. corallinus* venoms, was obtained from the Instituto Butantan (São Paulo, SP, Brazil). Both antivenoms consisted of F(ab')₂ equine immunoglobulin fragments.

The neutralizing capacity of the antivenoms was studied by pre-incubating venom (10µg/ml) with each antivenom for 30min at 37°C, at a venom:antivenom ratio of 1.5mg of venom:1.0ml of antivenom, before adding to the organ bath.

Chick biventer cervicis preparation

Biventer cervicis muscles obtained from chicks previously anesthetized with halothane were mounted as previously described (Ginsborg and Warriner, 1960). The preparations were suspended under a resting tension of 1gm in 5ml of Krebs solution of the following composition: 136mM NaCl, 5mM KCl, 2.5mM CaCl₂, 23.8mM NaHCO₃, 1.2mM MgSO₄, 1.2mM KH₂PO₄ and 11mM glucose), maintained at 37°C or 22°C (the latter used to verify the involvement of venom PLA₂ activity in neuromuscular blockade) and aerated with a mixture of 95%, v/v, O₂ + 5%, v/v, CO₂. The preparations were stimulated indirectly with supramaximal pulses (6V, 0.2ms and 0.1Hz) delivered by a Grass S4 electronic stimulator (Grass Instrument Co, Quincy, MA, USA) and were allowed to stabilize for at least 15min before the addition of drugs, venom or venom:antivenom mixtures. In some experiments, the muscle contractures to exogenous carbachol (carbamylcholine - CCh, 8µM), acetylcholine (ACh, 110µM) and KCl (20mM) were obtained before and after incubation of the tissues with venom. In order to determine the kinetics of ACh contracture inhibition, preparations under indirect stimulation and incubated with a single venom dose (0.5µg/ml) were assayed in different time points (5, 10, 15 and 30min) (n=5-10 preparations per time interval).

Mouse phrenic-nerve diaphragm muscle preparation

Whole diaphragms along with the phrenic nerves were removed from mice anesthetized with isoflurane and sacrificed by exsanguination. The left diaphragm was mounted essentially as described for rats (Bülbring, 1946). The preparation was suspended under a constant tension of 5gm in a 5ml organ bath containing aerated (95%, v/v, O₂ + 5%, v/v, CO₂) Tyrode solution (pH 7.4, 37°C) of the following composition: 137mM NaCl, 2.7mM KCl, 1.8mM CaCl₂, 0.49mM MgCl₂, 0.42mM NaHPO₄, 11.9mM NaHCO₃, and 11.1mM glucose. In some experiments, CaCl₂ was replaced by 4mM SrCl₂ to assess the influence of venom PLA₂ activity on the venom-induced neuromuscular blockade. Supramaximal pulses (0.1Hz, 0.2ms, 3-6V) and tetanic stimuli (50Hz, 0.2ms) delivered by a Grass S4 stimulator were applied by electrodes placed on the motor nerve. Isometric muscle tension was recorded using a Load Cell BG 50gm force-displacement transducer (Kulite Semiconductor Products Inc., Leonia, NJ, USA) coupled to a physiograph (Gould RS 3400, Cleveland, OH, USA). The preparations were allowed to stabilize for at least 20min before the addition of drugs or venom.

Reversal of neuromuscular blockade by neostigmine, 3,4-diaminopyridine and washing

The reversibility of the venom-induced blockade was assessed by incubating the preparations with neostigmine (10µg/ml) or 3,4-diaminopyridine (10µg/ml), or by extensive washing, after 50% neuromuscular blockade had been achieved.

PLA₂ activity

Venom PLA₂ activity was assayed in 10mM Tris-HCl, pH 8.0, containing 10mM CaCl₂, essentially as described elsewhere (Abreu et al, 2008). The assays were done in triplicate, and the activity expressed as the increase in absorbance at 425nm measured in a multiwell plate reader

(SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA). Venom from the South American rattlesnake, *Crotalus durissus terrificus*, was used as a positive control in this assay. *C. d. terrificus* venom was a gift from JC Cogo (Universidade do Vale do Paraíba, São José dos Campos, SP, Brazil).

Creatine kinase (CK) activity

Samples of organ bath solution (100 μ l) were collected before and after a 120min incubation with venom (5 μ g/ml in BC preparations; CK release by PND preparations was not examined); the initial 100 μ l aliquot was replaced by fresh solution. The samples were stored at 4°C, and CK activity was assayed within 4hrs after the experiment, using a commercial kit (Sigma Chemical Co, St Louis, MO, USA). CK activity was also assayed in control experiments without venom. Enzyme activity was expressed in international units per liter (IU/l), with one unit of activity corresponding to the phosphorylation of 1nmol of creatine/min at 25°C.

Light microscopy

At the end of the experiments, when complete blockade had been achieved at venom concentrations of 5, 10 and 50 μ g/ml, chick BC and mouse PND preparations were immediately fixed in Bouin's solution and processed for embedding in historesin. Sections 3-5 μ m thick

were stained with hematoxylin-eosin and examined by light microscopy, using an Olympus microscope (Olympus Optical Co Ltd, Tokyo, Japan) prior to photographing. Muscle damage was quantified by counting 50 fibers (normal or damaged) in four randomly chosen, non-overlapping fields (200 fibers/section) in one section from each of five tissues (experiments) per preparation (total of 1000 fibers each for BC and PND preparations). Similarly, 1000 fibers were counted from five control experiments for each preparation. The percentage of damaged fibers was calculated as (number of damaged fibers \div total number of fibers) \times 100.

Statistical analysis

The results were expressed as the mean \pm SEM and were compared statistically using Student's unpaired *t*-test or ANOVA for repeated measures. A value of $p \leq 0.05$ indicated significance.

RESULTS

Blockade of contractile responses

M. pyrrochryptus venom produced time- and concentration-dependent blockade of contractile responses in BC and PND preparations (Figure 1A and 1B), with the former preparations being more sensitive to blockade: At venom concentrations of 5 μ g/ml and 10 μ g/ml, the time

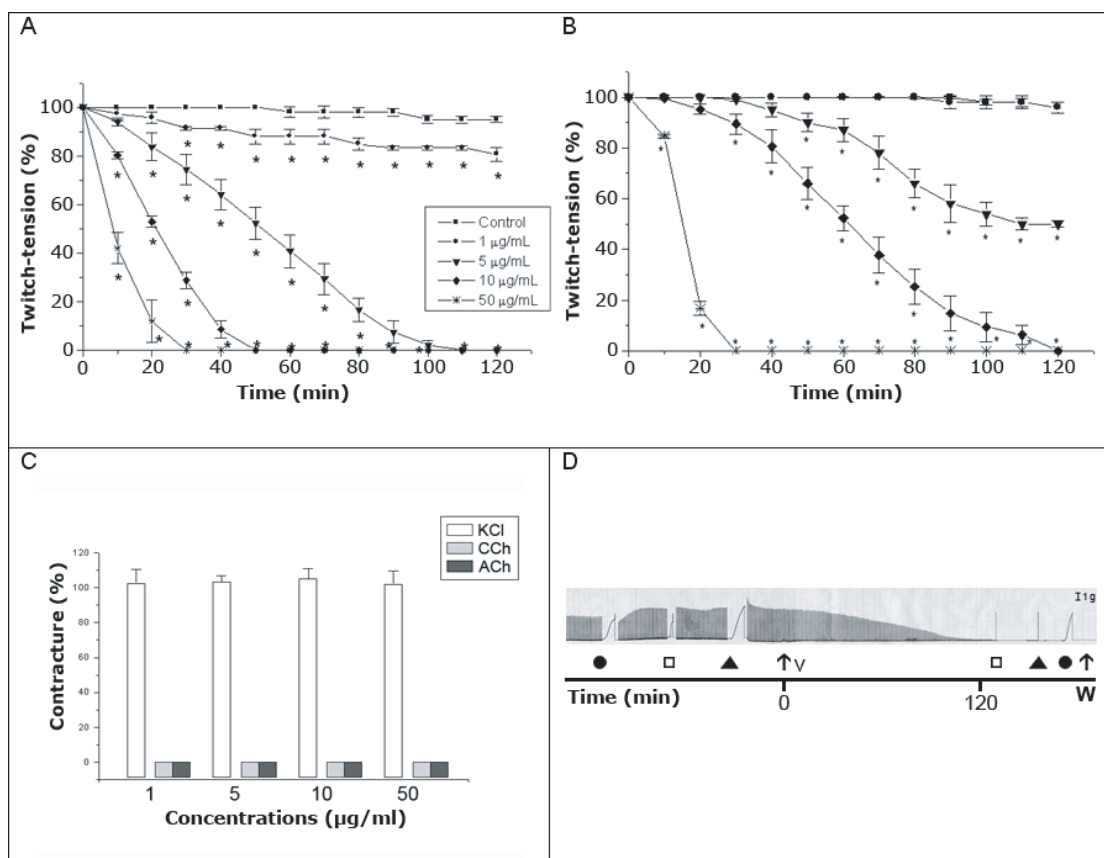


Figure 1. Concentration-dependent neuromuscular blockade caused by *M. pyrrochryptus* venom in indirectly stimulated chick biventer cervicis (A) and mouse phrenic nerve-diaphragm (B) preparations. Each point represents the mean \pm SEM of six experiments. C. Contractures of chick muscle to exogenous ACh, CCh and KCl after incubation with venom (1, 5, 10 and 50 μ g/ml). D. Recording of a chick biventer cervicis preparation incubated with *M. pyrrochryptus* venom (5 μ g/ml, arrow, 0min) showing contractures to exogenous ACh (□ 110 μ M), CCh (▲ 8 μ M) and KCl (● 20mM) before and after incubation with venom. This recording is representative of six experiments, the mean values of which are shown in panels A-C. W = wash. * $p < 0.05$ compared with control values.

for 50% neuromuscular blockade was 52 ± 0.9 min and 22 ± 3 min ($n=6$ each), respectively, in BC preparations; while in PND preparations, it was 110 ± 2 min and 62 ± 4 min ($n=6$ each), respectively. In contrast, there was no difference in sensitivity at the highest venom concentration ($50 \mu\text{g/ml}$: 15.1 ± 1.6 min vs 18.8 ± 2.3 min for BC and PND preparations, respectively). Treatment with neostigmine (a cholinesterase inhibitor) and 3-4-diaminopyridine (a potassium channel blocker) produced a transient reversal after 50% of venom-induced neuromuscular blockade ($n=6$ each; data not shown), followed by complete irreversible blockade after 60 min. This finding suggests that when only half of the endplates are affected by venom, some reversibility of blockade is viable.

The venom (1, 5, 10 and $50 \mu\text{g/ml}$) inhibited muscle contractions to exogenous acetylcholine (ACh, $110 \mu\text{M}$) and carbachol (CCh, $8 \mu\text{M}$) in BC preparations, but did not affect the responses to KCl (20mM) (Figure 1C and 1D).

Tetanic responses in mouse phrenic-nerve diaphragm preparations

In PND preparations exposed to tetanic stimuli (70 Hz, 0.2 ms), followed by incubation with venom ($10 \mu\text{g/ml}$, $n=6$), there was a progressive, time-dependent decrease in the amplitude of the responses (Figure 2A and 2B) more similar to that seen with α -bungarotoxin (a non-depolarizing toxin, $10 \mu\text{g/ml}$, $n=6$) than with succinylcholine (a depolarizing agent, $10 \mu\text{g/ml}$, $n=6$; data not shown).

Kinetics of inhibition of responses to exogenous acetylcholine

The incubation of BC preparations with a low venom concentration ($0.5 \mu\text{g/ml}$) for up to 30 min resulted in progressive inhibition of the contractile response to exogenous ACh (Figure 3). This finding suggested that the venom contained components that interacted with postsynaptic nicotinic receptors.

Effect of venom on directly stimulated preparations

Incubation with venom ($10 \mu\text{g/ml}$) did not significantly affect muscle twitches of curarized (d-tubocurarine, $3 \mu\text{M}$), directly stimulated PND preparations (Figure 4).

PLA₂ activity

The PLA₂ activity of *M. pyrrhocryptus* venom was 0.09 ± 0.04 U/mg, approximately one-third that of *C. d. terrificus* (South American rattlesnake) venom (0.30 ± 0.07 U/mg; $n=6$ each, $p < 0.05$). To examine whether this PLA₂ activity could contribute to the venom-induced neuromuscular blockade, experiments were done at 22°C (BC preparations) or Ca^{2+} (1.8mM) was replaced by Sr^{2+} (4.0mM) (PND preparations) in order to inhibit PLA₂ activity. These interventions did not significantly affect the venom potency and time for neuromuscular blockade, indicating that PLA₂ activity was not involved or had only a minor role in the venom-induced blockade.

Creatine kinase (CK) release

Incubation with *M. pyrrhocryptus* venom ($5 \mu\text{g/ml}$) for up to 120 min did not significantly alter the release of CK by BC preparations when comparing with the corresponding controls (Figure 5).

Light microscopy

Histological analysis of control BC and PND preparations showed normal muscle morphology with little fiber damage ($1.2 \pm 0.5\%$ in BC and $1.1 \pm 0.7\%$ in PND). Incubation with the venom ($50 \mu\text{g/ml}$) did not significantly alter this basal damage ($4.3 \pm 1.5\%$ and $3.5 \pm 0.8\%$ for BC and PND preparations, respectively; $n=5$ each in all cases).

Neutralization by commercial *Micrurus* antivenom and specific antivenom

Pre-incubation (30 min at 37°C) of *M. pyrrhocryptus* venom ($10 \mu\text{g/ml}$) with commercial *Micrurus* antivenom in the proportion recommended by the manufacturer, or with specific

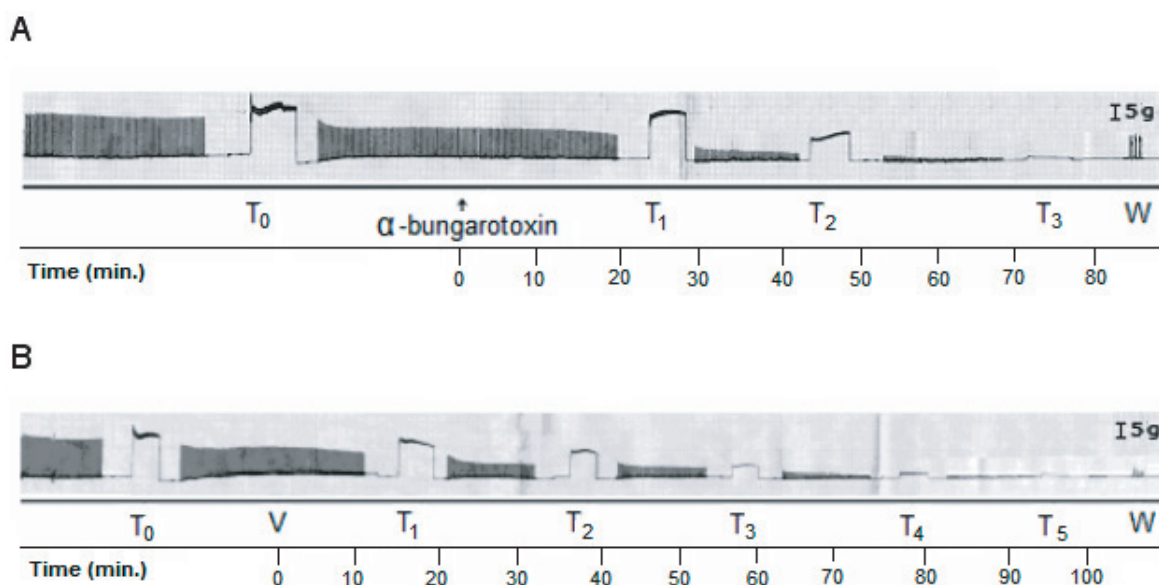


Figure 2. Comparison of the responses of mouse phrenic nerve-diaphragm preparations to indirect tetanic stimulation (T_1 , T_2 and T_3 ; 70 Hz, 0.2 ms) in the presence of (A) α -bungarotoxin and (B) *M. pyrrhocryptus* venom (V). Note that the tetanic response after incubation with *M. pyrrhocryptus* venom was similar to that seen after exposure to α -bungarotoxin ($n=6$ each).

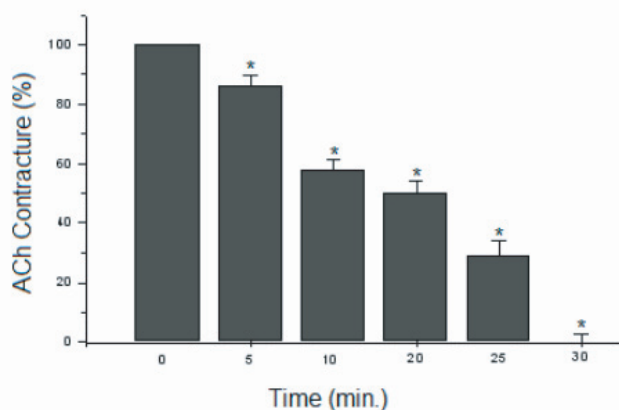


Figure 3. Kinetics of the blockade of contractures to exogenous ACh in the presence of *M. pyrrhocryptus* venom (0.5 μg/ml) in chick biventer cervicis preparations. Note the progressive decrease in the responses over time. Each bar represents the mean ±SEM of six experiments. *p<0.05 compared with the response before venom addition (0min) (n=5 for each interval).

antivenom at a venom:antivenom ratio of 1.5mg of venom to 1.0ml of antivenom, totally abolished the venom-induced neuromuscular blockade in both preparations (Figure 6).

DISCUSSION

M. pyrrhocryptus venom caused irreversible, time- and concentration-dependent neuromuscular blockade of muscle twitches in BC and PND preparations, with the former preparations being more sensitive than the latter. The greater sensitivity of BC preparations was probably related to differences in the innervation of these two preparations, with avian muscle having both focally- and multiply-innervated fibers that can respond to electrical stimulation or exogenous nicotinic agonists (Vital Brazil, 1980; Hodgson and Wickramaratna, 2002). These findings agree with reports for other *Micrurus* venoms, such as *M. altirostris* (Abreu et al, 2008), *M. dumerilii carinicauda* (Serafim et al, 2002), *M. frontalis* (Vital Brazil et al, 1976; Vital Brazil et al, 1977; Vital Brazil and Vieira, 1996), *M. lemniscatus carvalhoi* (Cecchini et al, 2005), *M. nigrocinctus* (Goularte et al, 1995) and *M. spixii* (Vital Brazil et al, 1995).

The venom inhibited contractures to exogenous ACh and CCh, indicating a predominantly postsynaptic action through the blockade of cholinergic nicotinic receptors,

as also suggested for other *Micrurus* venoms (Goularte et al, 1995; Serafim et al, 2002, Abreu et al, 2008). The time-dependent blockade of the responses to exogenous ACh shown in Figure 3 can only be properly understood when compared with Figure 1A, which shows venom-induced neuromuscular blockade in chick BC preparations. In Figure 3, a venom concentration of 0.5 μg/ml produced complete blockade of the responses to exogenous ACh within 30min. In contrast, within a similar time frame of 30min, there was <10% blockade in indirectly stimulated preparations incubated with the double venom concentration (1 μg/ml). These findings agree with the well-known existence of two populations of nicotinic receptors in BC preparations (Chang et al, 1977; Hodgson and Wickramaratna, 2002), *i.e.*, one population that is extrajunctional and responds well to exogenous ACh but is rapidly blocked by venom neurotoxins, and another located in the motor endplate that responds to nerve stimulation and is less susceptible to rapid blockade by neurotoxins (possibly because of difficulties related to toxin diffusion into the synaptic cleft). They also agree with studies for other neurotoxins that discriminate between these two populations (Chang et al, 1973; Chang and Su, 1975). The ability of toxins to distinguish between these two receptor populations could be exploited to provide a simple, sensitive assay for screening neurotoxins and nicotinic cholinergic agonists.

The postsynaptic action of *M. pyrrhocryptus* venom was also indicated by the absence of fade in the tetanic response to indirect stimulation at 70Hz. This tetanic pattern was more similar to that produced by α-bungarotoxin (a non-depolarizing toxin) than by succinylcholine (a depolarizing agent) (Gallici and Oliveira, 1994; Serra and Oliveira, 2006).

Micrurus venoms are rich in PLA₂ (Aird and da Silva, 1991; da Silva and Aird, 2001; Cecchini et al, 2005; Tanaka et al, 2010) that may contribute to the biological activities of these venoms (Alapé-Girón et al, 1996b; Francis et al, 1997; Oliveira et al, 2008). As shown here, *M. pyrrhocryptus* venom had low activity when compared with that of *C. d. terrificus*. The finding that reducing the temperature of the experiment from 37°C to 22°C (Goularte et al, 1995; Rodrigues-Simioni et al, 2004) or the substitution of Ca²⁺ by Sr²⁺ (Rodrigues-Simioni et al, 1995; Ponce-Soto et al, 2009) to attenuate PLA₂ activity did not significantly alter the neuromuscular blockade indicated that this enzymatic activity was not a major contributor to venom-induced blockade.

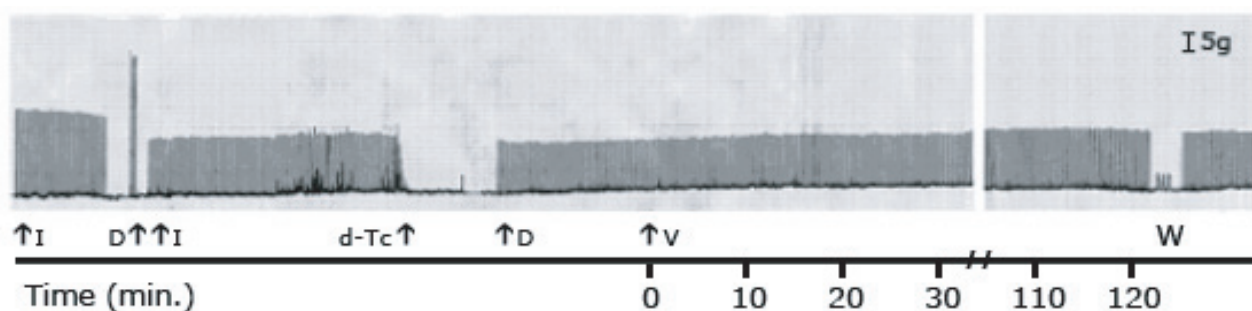


Figure 4. Myographic recording of mouse phrenic nerve-diaphragm contractions in response to *M. pyrrhocryptus* venom (V, 10 μg/ml) under direct (D) and indirect (I) stimulations. The preparation was previously treated with 3 μM d-tubocurarine (d-Tc) to inhibit any response of presynaptic origin (W = wash, n=6).

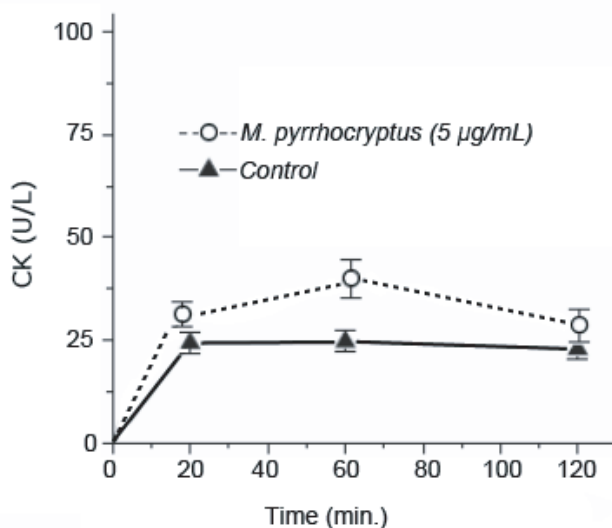


Figure 5. Creatine kinase (CK) released from chick biventer cervicis preparations incubated with *M. pyrrochryptus* venom (5µg/ml). The venom caused no significant release of CK at 120min incubation when compared with control preparations. The points are the mean \pm SEM of six experiments.

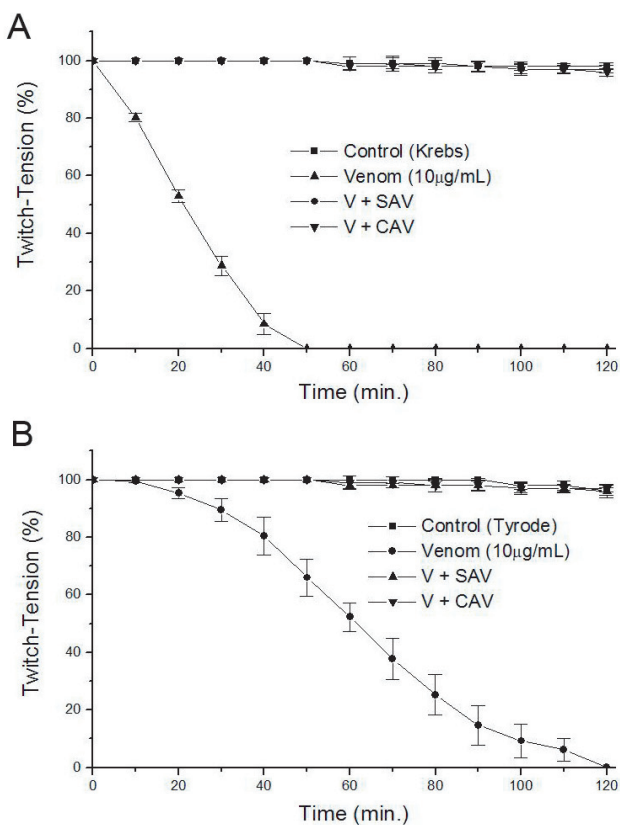


Figure 6. Neutralization by commercial antivenom (CAV) and specific antivenom (SAV) of the neuromuscular blockade caused by *M. pyrrochryptus* venom (10µg/ml) in avian biventer cervicis (A) and mouse phrenic nerve-diaphragm (B) preparations. Negative control preparations were incubated with Krebs (A) or Tyrode (B) solution alone. The points are the mean \pm SEM of six experiments ($p < 0.05$).

Previous work (Dokmetjian et al, 2009) has shown that *M. pyrrochryptus* venom is neither hemorrhagic nor myotoxic. This finding was confirmed here by the lack of effect on muscle responses to exogenous KCl and to direct

stimulation in curarized preparations, the unaltered CK release, and the absence of tissue damage seen in histological analysis. In contrast, the intramuscular injection of *Micrurus* venoms in mice and rats results in myotoxicity, seen morphologically and through an increase in serum CK levels (de Roodt AR, 2002). Myonecrosis has been also reported for several coral species (Gutiérrez et al, 1983; Gutiérrez et al, 1986, Gutiérrez et al, 1992; Goularte et al, 1995). These discrepant results may be related to the different animal models (*in vitro* vs *in vivo*) and the venom concentrations used.

Commercial Brazilian *Micrurus* antivenom raised against *M. corallinus* and *M. frontalis* venoms and specific antivenom effectively neutralized the neurotoxicity of *M. pyrrochryptus* venom *in vitro*. This finding suggests that it is not necessary to include *M. pyrrochryptus* venom in the pool of *Micrurus* venoms used for antivenom production. This conclusion differs from findings for *M. altirostris*, another species originally classified as a subspecies of *M. frontalis*, for which commercial Brazilian antivenom showed little neutralization of the lethality (Moraes et al, 2003) and neuromuscular activity (Abreu et al, 2008) of the venom. In fact, various studies have suggested the need to include additional *Micrurus* species in the pool of venoms used in immunization protocols in order to improve the neutralization capacity of commercial antivenoms (Higashi et al, 1995; de Roodt et al, 2004; Tanaka et al, 2010).

CONCLUSIONS

M. pyrrochryptus venom produced neuromuscular blockade through a predominantly postsynaptic action that was effectively neutralized by commercial and specific antivenoms. The neutralization of neurotoxicity observed here suggests that these antivenoms may be useful in treating humans envenomed by this species.

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STATE OF COMPETING INTERESTS

None declared.

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