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VdTX-1, a reversible nicotinic receptor antagonist isolated from venom of the spider *Vitalius dubius* (Theraphosidae)

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ABSTRACT

Theraphosid spider venoms can block neurotransmission in vertebrate nerve–muscle preparations *in vitro*, but few of the components involved have been characterized. In this work, we describe the neuromuscular activity of venom from the Brazilian theraphosid *Vitalius dubius* and report the purification and pharmacological characterization of VdTX-1, a 728 Da toxin that blocks nicotinic receptors. Neuromuscular activity was assayed in chick biventer cervicis preparations and muscle responses to exogenous ACh and KCl were determined before and after incubation with venom or toxin. Changes in membrane resting potential were studied in mouse diaphragm muscle. The toxin was purified by a combination of filtration through Amicon® filters, cation exchange HPLC and RP-HPLC; toxin purity and mass were confirmed by mass spectrometry. Venom caused progressive neuromuscular blockade and muscle contracture; the blockade but not the contracture was reversible by washing. Venom attenuated contractures to exogenous ACh and KCl. Filtration yielded low (LM, <5 kDa) and high (HM, >5 kDa) fractions, with the latter reproducing the contracture seen in venom but with a slight and progressive twitch blockade. The LM fraction caused reversible blockade and attenuated contractures to ACh, but had no effect on contractures to KCl. VdTX-1 (728 Da) purified from the LM fraction was photosensitive and reduced the E_{max} to ACh in biventer cervicis muscle without affecting the EC_{50}; VdTX-1 also abolished carbachol-induced depolarizations. *V. dubius* venom contains at least two components that affect vertebrate neurotransmission. One component, VdTX-1, blocks nicotinic receptors non-competitively to produce reversible blockade without muscle contracture.

1. Introduction

Spider venoms are an important source of bioactive molecules with applications in several areas of pharmacology (Rash and Hodgson, 2002). Tarantula (Theraphosidae) venoms contain a variety of peptides that selectively interact with ion channels (as blockers or modulators) and may be useful probes for elucidating structure–function relationships (Siemens et al., 2006; Dutertre and Lewis, 2010). Some theraphosid spider venoms can produce neuromuscular blockade in vertebrate nerve-muscle preparations *in vitro* (Fontana et al., 2002; Herzig and Hodgson, 2009) and, in at least one case, the venom component responsible for neuromuscular blockade has been shown to be a 33-amino acid peptide,

Abbreviations: HWTX-I, huwentoxin-I from the spider *Selenocosmia huwena*; VdTX-1, *Vitalius dubius* toxin 1.

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i.e., huwentoxin-I from the theraphosid *Selenocosmia* (now *Ornithoctonus*) *huwena* (Liang et al., 1993; Zhou et al., 1997).

Spider and wasp venoms contain (acyl)polyamines that can interact with excitatory neurotransmitter receptors, principally glutamate ionotropic receptors (Beleboni et al., 2004; Estrada et al., 2007), but also cholinergic nicotinic receptors (Anis et al., 1990; Strømgaard et al., 2005). Although tarantula venoms contain polyamines (Cabbiness et al., 1980; Skinner et al., 1990; Moore et al., 2009), the role of these components in neuromuscular blockade caused by these venoms in vertebrates is unknown. In contrast, philanthotoxins (from the digger wasp *Philanthus triangulum*) and orb web spider polyamines can cause neuromuscular blockade by blocking nicotinic channels (Rozental et al., 1989).

We have previously reported the general properties and composition of venom from the Brazilian theraphosid spider *Vitalius dubius* Mello-Leitão 1923 (Rocha-e-Silva et al., 2009a,b). In this work, we describe the neuromuscular activity of this venom and of a low molecular mass component capable of blocking vertebrate motor endplate cholinergic nicotinic receptors.

2. Material and methods

2.1. Animals

Male Swiss white mice (25–30 g) were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at UNICAMP and male HY-LINE W36 chicks (4–8 days old) were supplied by Granja Globo Aves Agrovícola Ltda (Mogi Mirim, SP, Brazil). The animals were housed at 23 ± 2 °C on a 12 h light/dark cycle with access to food and water *ad libitum*. The animal experiments described here were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 1587-1) and were in agreement with the Ethical Principles for Animal Research established by the Brazilian Society of Laboratory Animal Science (SBCAL).

2.2. Materials

Venom was collected from male and female *V. dubius* by electrical stimulation (Rocha-e-Silva et al., 2009b) into Eppendorf tubes covered with Parafilm® to avoid contamination with saliva. All reagents and salts for organ bath solutions were purchased from JT Baker (Center Valley, PA, USA) and drugs were from Sigma Chemical Co. (St. Louis, MO, USA) or JT Baker.

2.3. Chick biventer cervicis preparation

Male chicks were killed with isoflurane inhalation and the biventer cervicis muscles were removed (Ginsborg and Warriner, 1960) and mounted under a tension of 1 g/cm in a 5 mL organ bath containing warmed (37 °C), aerated (95% O_2 + 5% CO_2) Krebs solution of the following composition (in mM): NaCl 118.7, KCl 4.7, $CaCl_2$ 1.8, $NaHCO_3$ 25, $MgSO_4$ 1.17, KH_2PO_4 1.17 and glucose 11.65, pH 7.5. A bipolar platinum ring electrode was placed around the tendon within which runs the nerve trunk supplying the muscle. Field stimulation was done with a Grass S48 stimulator (0.1 Hz, 0.2 ms, 4–6 V). Muscle contractions and contractures were recorded isometrically via a force-displacement transducer coupled to a PowerLab ML866/P digital myographic system (ADInstruments Pty. Ltd., Sydney, Australia). Contractures to exogenously applied acetylcholine (ACh, 110 μM) and KCl (20 mM) were obtained in the absence of field stimulation prior to treatments and at the end of the experiment, as a test for the presence of myotoxic and neurotoxic activities (Harvey et al., 1994). The preparations were allowed to stabilize for at least 20 min before the addition of ACh or KCl. Venom was added to the bath and changes in the contractile responses were monitored for 60 min for crude venom and up to 120 min for fractions. The changes in twitch-tension are expressed as a percentage of the basal (−5 min) values prior to incubation with venom.

Cumulative concentration–response curves to exogenous ACh were obtained before and after incubation with purified toxin. The protocol consisted of first obtaining a concentration–response curve in the absence of toxin and then incubating indirectly stimulated preparations with toxin until complete blockade of the contractile responses, after which electrical stimulation was stopped and a new concentration–response curve to ACh was obtained in the presence of toxin. Repeated curves without toxin were performed as control for tissue fatigue.

2.4. Measurement of the membrane resting potential

The membrane resting potential was recorded from mouse diaphragm muscle (Bülbring, 1946) using conventional microelectrode techniques (Ling and Gerard, 1949; Fatt and Katz, 1951). The dissected muscle was mounted in a lucite chamber containing aerated (95% O_2 + 5% CO_2) Tyrode solution (composition, in mM: NaCl 137; KCl 2.7; $CaCl_2$ 1.8; $MgCl_2$ 0.49; NaH_2PO_4 0.42; $NaHCO_3$ 11.9 and glicose 11.1, pH 7.0) at 37 °C. The resting potential of up to eight fibers in each muscle was recorded using glass microelectrodes filled with 3 M KCl (resistance 10–20 MΩ) and positioned within the muscle fiber. All recordings were displayed on a Tektronix oscilloscope. To examine the influence of the toxin on carbachol-induced membrane depolarization, the membrane resting potential was measured followed by the addition of carbachol (68 μM) and 15 min later the membrane potential was measured again. Subsequently, the preparation was washed, the resting potential was checked and toxin (110 μM) was added for 15 min. At the end of this incubation carbachol was added (without washing the preparation) and the membrane potential was measured after 15 min.

2.5. Purification of a low molecular mass toxin (VdTX-1)

A low molecular mass fraction of the venom was initially obtained by filtering venom (10 mg dissolved in distilled water) through a 5 kDa nominal cut-off Amicon® filter (Millipore, Billerica, MA, USA) by centrifugation. The resulting fractions were referred to as the LM (low-mass; <5 kDa) and HM (high-mass; >5 kDa) fractions and both were tested for neuromuscular activity in biventer cervicis preparations. The LM fraction was subsequently