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Three structurally related, highly potent, peptides from the venom of *Parabuthus transvaalicus* possess divergent biological activity

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Abstract

The venom of South African scorpion *Parabuthus transvaalicus* contains a novel group of peptide toxins. These peptides resemble the long chain neurotoxins (LCN) of 60–70 residues with four disulfide bridges; however they are 58 residues long and have only three disulfide bridges constituting a new family of peptide toxins. Here we report the isolation and characterization of three new members of this mammal specific group of toxins. Dortoxin is a lethal peptide, bestoxin causes writhing in mice and altitoxin is a highly depressant peptide. Binding ability of these peptides to rat brain synaptosomes is tested. While the crude venom of *P. transvaalicus* enhances the binding of [^{3}H] BTX to rat brain synaptosomes none of these individual toxins had a positive effect on binding. Although the primary structures of these toxins are very similar to birtoxin, their 3D models indicate significant differences. Dortoxin, bestoxin and altitoxin cumulatively constitute at least 20% of the peptide contained in the venom of *P. transvaalicus* and contribute very significantly to the toxicity of the venom of this medically important scorpion species. Therefore the amino acid sequences presented here can be used to make more specific and effective antivenins. Possible approaches to a systematic nomenclature of toxins are suggested.

Keywords: Scorpion; Toxin; Na^+ channel; Birtoxin; Ikitoxin; Nomenclature

1. Introduction

Mining the rich pool of scorpion venoms has proved valuable in isolation of specific modulators of ion channel function and the development of targeted antivenins. Possani et al. (2000) predicted that each scorpion species may possess at least 100 peptide toxins. A conservative calculation than indicates that there may at least be 125,000 peptides from 1250 scorpion species around the world. This estimate does not include the intra-species variation, which may raise the above estimate considerably. Considering the 250 or so individual toxins currently identified, it is clear that only a small fraction of the existing structural diversity of scorpion peptide toxins have been elucidated so far.

Classification of scorpion toxins is evolving rapidly as new members are being isolated and characterized. One way to classify peptide toxins is based on their site of action. Indeed, conserved primary sequences among scorpion toxins are known to target particular types of ion channels. For example, long chain neurotoxins (LCNs) act on sodium channels, short chain neurotoxins (SCNs) act on potassium and chloride channels with the exception of Kβ toxins, which are 'long-chain' toxins of 60–64 amino acid residues with three disulfide bridges acting on potassium channels, maurocalcine-like peptides act on ryanodine sensitive calcium channels (Possani et al., 2000; Legros et al.,

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1998). Among the LCNs the peptides are classified as alpha and beta toxins. Alpha toxins bind to site 3 of the voltage gated sodium channel and prolong the inactivation phase, inhibiting sodium current inactivation. Beta toxins on the other hand bind to site 4 of the voltage gated sodium channel and induce both a shift in the voltage dependence of channel activation in the hyperpolarizing direction and a reduction of the peak sodium current amplitude (Cestèle and Catterall, 2000). Additionally, insect specific excitatory and depressant toxins also constitute different structural classes that target unique binding sites and modes of action.

Despite the abundance of knowledge about the primary structure and modes of action of scorpion toxins, few studies have addressed the structure–activity relationships of these peptides. Identification of the bioactive surfaces of toxins responsible for modulating ion channels can lead to the synthesis of non-peptidic effectors of these channels that may have beneficial uses in therapeutics or pest management. Birtoxin, from the venom of *Parabuthus transvaalicus* for example resembles LCNs, with the exception of being slightly smaller and more importantly having three disulfide bridges instead of the four disulfide bridges of other members of this group (Inceoglu et al., 2001). The primary structure of birtoxin indicated that its site of action could be the sodium channel. Indeed electrophysiological characterization of the effects of birtoxin revealed that it is a beta group peptide (Inceoglu et al., 2002).

Here we are reporting the discovery of three new members of the birtoxin family, each with its unique biology. These peptides are named dortoxin (lethal), bestoxin (writher) and altitoxin (depressant-lethal). Despite their close similarity to birtoxin, the 3D structural models for each of these toxins indicate sufficiently significant differences to sub-categorize these peptides into a separate subgroup.

2. Materials and methods

2.1. Peptide purification and characterization

Birtoxin, dortoxin, bestoxin and altitoxin are purified through three steps of RP-HPLC guided by murine bioassays as described previously (Inceoglu et al., 2001). Fractions P5 for dortoxin and P6 for bestoxin and P6B for altitoxin from this column are collected, then several runs are pooled and freeze dried. These fractions are then run on a Michrom C18 microbore column on a Magic 2002 Microbore HPLC system equipped with a peptide trap. The main peaks from the C18 column purifications are collected and polished by running them through a microbore phenyl column on the same system. Purity is confirmed by ESI-TOF. Mass spectra of crude venom, separated fractions and isolated peptides are analyzed off-line as described previously (Inceoglu et al., 2001). Protein sequencing and peptide quantification are accomplished in the same manner as for birtoxin (Inceoglu et al., 2001).

2.2. Bioassays

Biological activity of peptides is monitored by injecting mice and insects as described previously (Inceoglu et al., 2001). Briefly, fractions are concentrated to dryness using a Heto Speed Vac (ATR, Inc., Emeryville, CA). Dried samples are resuspended in 10 μl 20 mM ammonium acetate buffer with 1 μg/μl BSA and incubated overnight at 4 °C to insure proper refolding before injection to the test animals. Mice are anesthetized using ethyl ether and intracerebroventricular injections of peptide solutions are executed immediately. Control animals injected with BSA in buffer do not show any symptoms when recovering from anesthesia. All symptoms are observed and recorded up to 24 h post-injection. Blowfly (*Sarcophaga* spp.), and crickets (*Acheta domesticus*) were purchased from Carolina Biologicals (Burlington, NC), Cotton bollworms (*Heliothis virescens*) were obtained from USDA/ARS (Stoneville, MI) and reared on artificial diet.

2.3. Binding assays

Tritiated BTX-A-20-a-benzoate ([^{3}H] BTX-B) is purchased from Dupont-NEN (specific activity, 37.2 Ci/mmol). Binding of toxins to rat brain synaptosomes is measured by utilizing the ability of site 3 toxins to enhance the binding of batrachotoxin (Catterall et al., 1981; Little et al., 1998). Rat brain synaptosomes are prepared from two Springer-Dowley male rats as described previously (Catterall et al., 1981). A crude synaptosomal fraction is prepared by centrifuging the brain homogenate at $10{,}000 \times g$ for 10 min and taking the supernatant and centrifuging it for 1 h at $100{,}000 \times g$. For binding assays, rat brain synaptosomes (300 μg/mL) are suspended in 250 μL of binding buffer (choline chloride 130 mM, $CaCl_2$ 1.8 mM, KCl 5.4 mM, $MgSO_4$ 0.8 mM, β D-glucose 5.5 mM, HEPES 50 mM, pH 7.4, BSA 1 mg/ml) in the presence of 1 μM tetrodotoxin together with 25 nM [^{3}H] BTX-A (NEN) and appropriate concentrations of toxins. Non-specific binding is determined in the presence of 300 μM veratridine (Calbiochem) and is subtracted from the total binding to determine specific binding. The reaction is incubated for 50 min at 37 °C and terminated by filtering through GF-C glass fiber filters that are equilibrated in wash buffer (choline chloride 163 mM, $CaCl_2$ 1.8 mM, KCl 5.4 mM, $MgSO_4$ 0.8 mM, HEPES 5 mM, pH 7.4, BSA 1 mg/ml). The filtrate is then rinsed three times with cold wash buffer (Catterall et al., 1981).

2.4. Alignment analysis and homology modeling

Multiple alignment analysis is done by using the T-coffee software and visualized with ESPript software