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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

(Notredame et al., 2000; Gouet et al., 1999). Peptide amino acid sequences are submitted to Swiss-Model automated modeling server (http://www.expasy.org/swissmod/SWISS-MODEL.html) and modeled according to previously determined NMR structures (Peitsch, 1995; Pintar et al., 1999; Cook et al., 2002; Zhao et al., 1992; Lee et al., 1994; Jablonsky et al., 1999). Results are retrieved by email.

3. Results

3.1. Bioassay driven peptide purification

Three HPLC peaks separated from the venom of *P. transvaalicus* having potent biological activity toward mammals are identified (Fig. 1). Fraction P5 is very potent against mice but shows minor insecticidal activity at >10 μg/insect using 200 mg *Trichoplusia ni* larvae indicating its mammal specificity. This fraction is well resolved on the C4 column from the preceding peak that contains birtoxin (Inceoglu et al., 2001). Further purification on a C18 microbore column results in a single major peak with shoulder peaks on both sides. These shoulders are eliminated by re-running the major peak on a phenyl microbore column and collecting the middle part of the peak. Molecular mass and purity of dortoxin is then confirmed by ESI-TOF (Fig. 2(A)). Biological activity of dortoxin from fraction P5 is assessed by injecting 4-week-old mice. This fraction shows very high toxicity to mice. Immediately after pure dortoxin is injected, the mice display hyperactivity that persists until death.

Other symptoms include tremors, convulsions, profuse salivation, lacrymation, continuous urination and vocalization. In contrast to birtoxin whose effect increases over a 10-min period following injection, dortoxin has a more rapid onset of symptoms that progress to generalized tremors and seizures with intermittent hyperactivity lasting several seconds. Death occurs after the last of the runs, which is significantly more intense and contains uncontrolled movements (e.g. jumps, whole body twitches) and urination. After death, limb muscles continue to twitch at least for another 30 s. The LD_{99} is 200 ng of peptide per 20 g mouse. The amino acid sequence of this peptide is in agreement with the MS determined molecular mass of 6644.3 Da.

Fraction P6 and purified bestoxin both cause intense writhing in injected animals. Immediately after injection these animals start displaying a unique posture with their neck twisted and the body following the neck thus completing a full turn around the body axis. With time, the severity of this behavior increases and the mice start to spin around. When manually restrained the mice do not respond to the handler. However, they involuntarily continue to writhe; first the neck twists then the forelegs and the body following to complete a turn. After 24 h post-injection the test animals still continue to writhe albeit exhausted, the rate of writhing decreases gradually to half to one turn per second. No lethality is observed at doses studied. The ED_{99} is 100 ng of peptide per 20 g mouse. Fraction P6 is further purified as for fraction P5 and

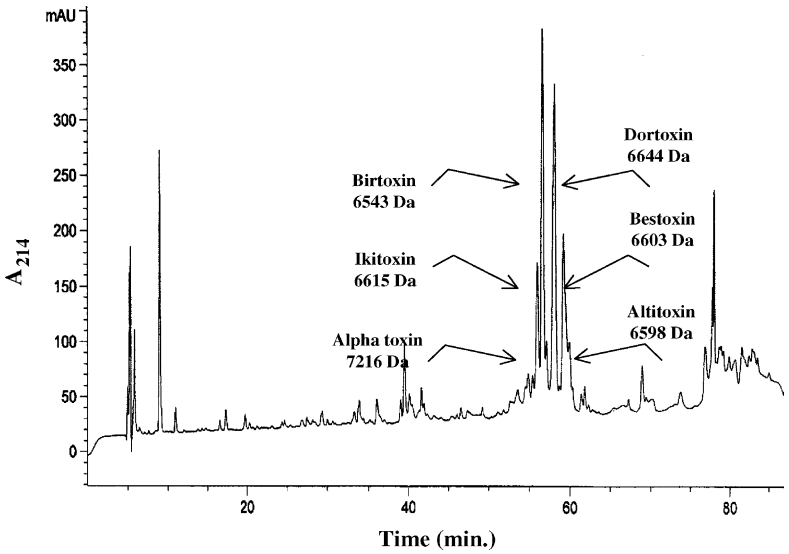

Fig. 1. Reverse phase HPLC profile of the crude venom of *Parabuthus transvaalicus*. Crude venom was injected to a Vydac C18 RP column and a linear gradient of 5–60% mobile phase was formed. Peaks were collected separately and amino acid sequences were determined as described in Section 2. Fully identified and characterized peaks are shown with assigned names and determined molecular masses.

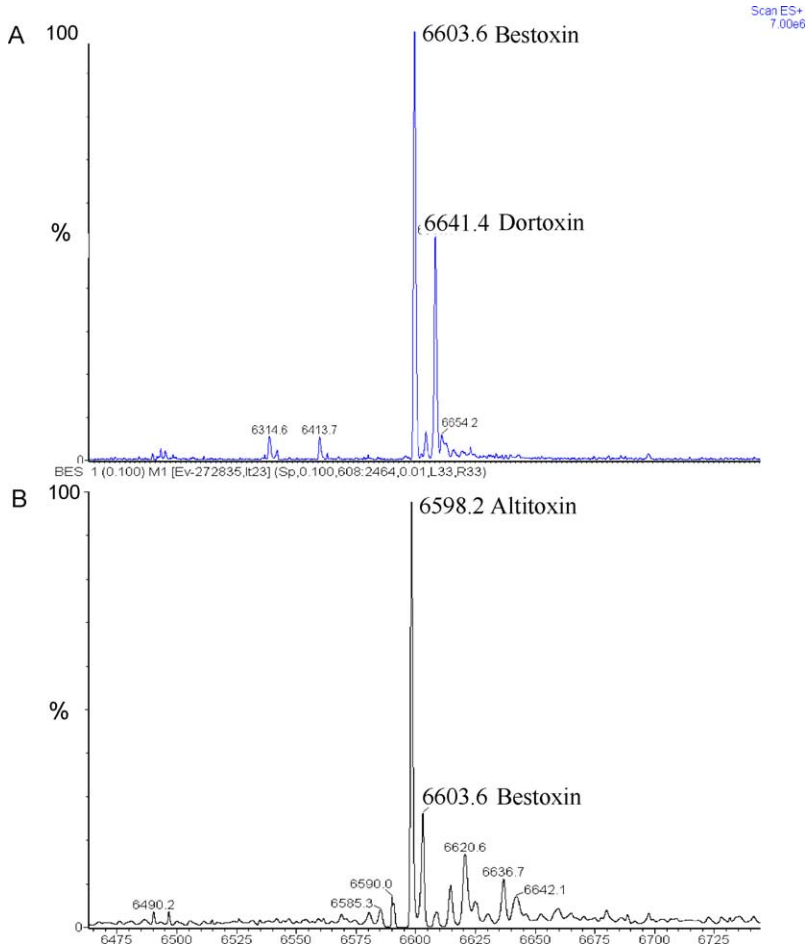

Fig. 2. ESI-MS determined molecular masses for each toxin is presented. 2A shows dortoxin and bestoxin together whereas 2B presents altitoxin and bestoxin.

submitted for amino acid sequencing after the purity is confirmed by MS. The calculated amino acid sequence is in agreement with the determined molecular mass of 6603 Da (Fig. 2(B)). The sequence of bestoxin has only two amino acid differences from the sequence of dortoxin (Fig. 3).

The shoulder peak of fraction P5 was subsequently resolved into a distinct peak with the continuous use of the C4 column. These two peaks were separated and their masses were determined by ESI-TOF. Peak A has a molecular mass of 6603 Da which is bestoxin and peak B, named altitoxin, has a molecular mass of 6599 Da.

Injection of altitoxin to mice results in a very profound state of akinesia, depression and death. The ED_{99} is 100 ng of peptide per 20 g mouse. The peptide is then submitted for amino acid sequencing. Complete sequencing shows that altitoxin has a mutation, a deletion and an insertion at the C terminus compared to bestoxin. Calculated molecular mass is in agreement with determined molecular mass of 6599 Da.

3.2. Binding assays

The crude venom of *P. transvaalicus* increases the binding of [^{3}H] BTX by 6-fold compared to 12-fold increase seen with equal concentration of venom of *Leiurus quinquestriatus* (Fig. 4(A)). In contrast none of the toxins identified from this venom (birtoxin, ikitoxin, dortoxin, bestoxin and altitoxin) cause any enhancement in the binding of [^{3}H] BTX to rat brain synaptosomes (Fig. 4(B)).

3.3. Structural analysis

Homology modeling gave us an opportunity to compare the structures of all five toxins isolated from this venom for the first time. Birtoxin and ikitoxin form a structural group whereas dortoxin, bestoxin and altitoxin have significantly different structure from the first group despite the sequence homology between all of the toxins. This seems to be due to the difference in the surface electrostatic potential of these

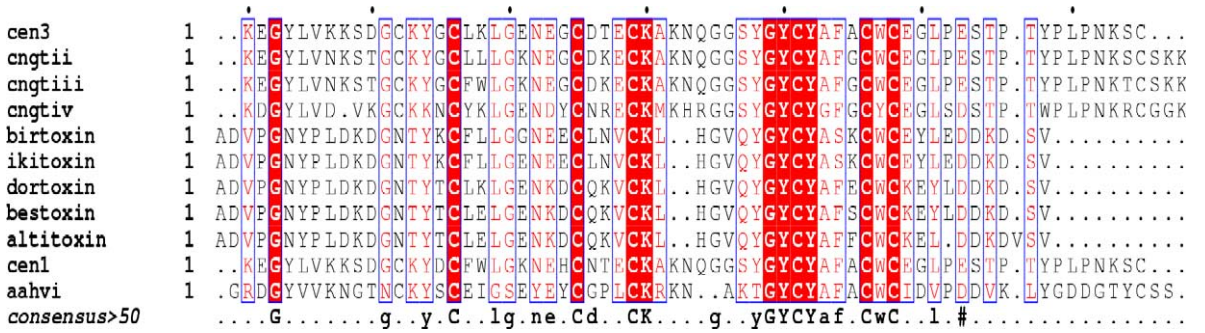

Fig. 3. Sequences of presented peptides are compared with known toxins. Multiple alignment is done using the T-coffee software and visualized using ESPript software both located on the Expasy proteomics server (www.expasy.ch). A consensus of all analyzed sequences is given at the bottom. The aligned toxins are cen3; *Centruroides exilicauda* neurotoxin 3, cngtii; *Centruroides noxius* toxin II, cngtiii; *Centruroides noxius* toxin III cngtiv; *Centruroides noxius* toxin IV, cen1; *Centruroides exilicauda* neurotoxin 1, aahvi; *Androctonus australis* neurotoxin VI.

two groups of peptides. More specifically birtoxin and ikitoxin have α-helices that do not contain the typical basic residues; instead they begin with two acidic residues. In contrast to this dortoxin, bestoxin and altitoxin have α-helices that are lined up with basic residues, typical of known scorpion toxins, all protruding from the helix. In addition, all five toxins uniquely have localized positively and negatively charged surfaces. Moreover, the amino acid differences between the toxins are concentrated to these charged regions, indicating their functional importance.

4. Discussion

Novel peptides with unique biology are isolated and characterized from the venom of South African scorpion *P. transvaalicus*. These peptides can be included in the group of LCNs. However they possess three disulfide bridges instead of the usual four disulfide bridges of other members of the group and they are uniquely shorter than other LCNs. Therefore, these peptides should be included in a new sub-structural group of scorpion toxins. We predict that the majority of the toxicity of *P. transvaalicus* venom to mammals is due to these peptides. Dortoxin and altitoxin are lethal at about 200 ng each, whereas bestoxin and ikitoxin although potent are non-lethal and birtoxin is lethal at low microgram quantities. Possible synergism among these peptides may increase their cumulative effect significantly. Therefore an antibody directed towards these peptides is expected to reduce most of the toxicity of this venom. Among these peptides birtoxin and ikitoxin are of the beta group toxins. These two toxins do not enhance the binding of [^{3}H] BTX to rat brain synaptosomes, consistent with their action on voltage gated Na^+ current (Inceoglu et al., 2002). Dortoxin, bestoxin and altitoxin also do not enhance the binding of [^{3}H] BTX to rat brain synaptosomes. This could be explained in several different ways. One possibility is that these toxins could be acting on K^+ channels rather than voltage gated Na^+ channels. Another possibility is that they could belong to the beta group of toxins that act on Na^+

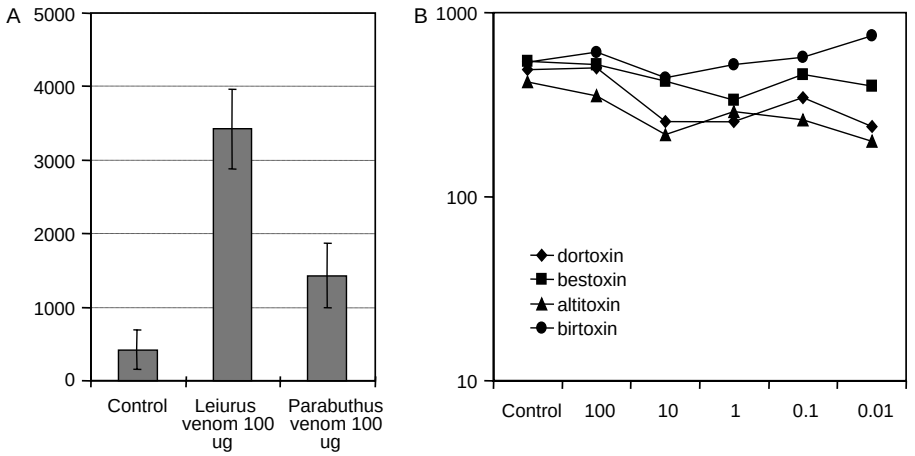

Fig. 4. The ability of the venom and purified peptides of *P. transvaalicus* to enhance the binding of [^{3}H] BTX was tested and compared to that of *L. quinquestriatus* (4A). Although the crude venom enhanced the binding of [^{3}H] BTX none of the purified peptides had any effect (4B).

channels. Alternatively, they may have different binding sites on the voltage gated Na^+ channel or specificity towards a sub-type of ion channel that may not be expressed in the rat brain. Multiple sequence alignment implies that dortoxin bestoxin and altitoxin are more related to beta scorpion toxins in primary structure. However, caution should be taken when making this assumption. Ultimately, electrophysiological measurements have to be taken into account to classify these peptides.

As suggested by Olivera (1997), rapid mutation of venom peptides would be an optimum evolutionary strategy when prey, predators, and competitors change very rapidly due to a sudden climate change or a geological catastrophe. Special mechanisms then may have evolved that accelerate the generation of new venom peptides such as frequent point mutations of the toxin encoding sequence, for example as seen at the C-termini of scorpion toxins. It should be noted however that the disulfide bridge framework is highly conserved in all scorpion peptides. In spite of the lack of the wrapper disulfide in birtoxin-like peptides the other three disulfide bridges are still well conserved (Inceoglu et al., 2001).

The fact that birtoxin-like peptides are highly conserved but produce distinct in vivo effects strongly reiterates that scorpions-like other venomous animals employ a combinatorial library strategy to evolve new peptides in their venoms. Although in the venom mixture multiple peptides that bind to the same target site seem to coexist, it is hypothesized that, in vivo, no functional interference occurs between these potentially antagonistic venom components. Therefore it is possible to speculate that each one of these three peptides may be binding to a different channel subtype, thus displaying a range of activities.

Homology model of the birtoxin-like peptides illuminate an interesting aspect of these peptides. Their surfaces have distinct negatively and positively charged regions. This is quite striking because both charged regions are concentrated into separate domains, which are highly correlated with the amino acid differences between toxins. The differences observed in toxicosis among these peptides may stem from targeting different channel sub-types and/or differentially modulating their channel targets. In any case further characterization, including solution structures and electrophysiological effects, will improve our understanding of how exactly these peptides interact with their targets and the basis of their selectivity.

With the ever-increasing number of new toxins identified there is a need in the field to classify and unify the nomenclature of these peptides (Possani et al., 1999). As with other peptides and proteins one could name scorpion toxins based on their mechanism of toxicity, their specificity, their physical properties or other systems. Some toxins are named for the species they were isolated from (Lqh), their specificity (IT for insect toxin) and a number such as LqhIT2. The system is systematic, and has high information content, but raises ambiguities such as how to deal with a peptide toxic to highly divergent organisms or with similar peptides from different scorpion species. We have been less systematic in naming toxins from *P. transvaalicus*. As the numbers of known toxins increases none of the above systems are sustainable. A systematic procedure for naming toxins must be able to handle the rapidly expanding numbers of identical toxins as well as show phylogenic relationships. The system also should be based on a consensus of scientists in the field. For example, a system emphasizing the gene or peptide sequence to form sub-families, families and clans rather than function or organism as achieved with cytochrome P450 area may serve as an illustrative model for this purpose (Nelson et al., 1996).

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