A single charged surface residue modifies the activity of ikitoxin, a beta-type Na+ channel toxin from Parabuthus transvaalicus

A. Bora Inceoglu1,*, Yuki Hayashida2, Jozsef Lango3, Andrew T. Ishida2 and Bruce D. Hammock1

1Department of Entomology and Cancer Research Center, 2Section of Neurobiology, Physiology and Behavior, and 3Department of Chemistry and Superfund Analytical Laboratory, University of California, Davis, CA, USA

We previously purified and characterized a peptide toxin, birtoxin, from the South African scorpion Parabuthus transvaalicus. Birtoxin is a 58-residue, long chain neurotoxin that has a unique three disulfide-bridged structure. Here we report the isolation and characterization of ikitoxin, a peptide toxin with a single residue difference, and a markedly reduced biological activity, from birtoxin. Bioassays on mice showed that high doses of ikitoxin induce unprovoked jumps, whereas birtoxin induces jumps at a 1000-fold lower concentration. Both toxins are active against mice when administered intracerebroventricularly. Mass determination indicated an apparent mass of 6615 Da for ikitoxin vs. 6543 Da for birtoxin. Amino acid sequence determination revealed that the amino-acid sequence of ikitoxin differs from birtoxin by a single residue change from glycine to glutamic acid at position 23, consistent with the apparent mass difference of 72 Da. This single-residue difference renders ikitoxin much less effective in producing the same behavioral effect as low concentrations of birtoxin. Electrophysiological measurements showed that birtoxin and ikitoxin can be classified as beta group toxins for voltage-gated Na+ channels of central neurons. It is our conclusion that the N-terminal loop preceding the alpha-helix in scorpion toxins is one of the determinative domains in the interaction of toxins with the target ion channel.

Keywords: birtoxin; ikitoxin; Parabuthus; scorpion; voltage-gated Na+ current.

The scorpion genus Parabuthus includes several species of medical importance. Among these scorpions, Parabuthus granulatus and Parabuthus transvaalicus have been suggested to be of most significance in terms of mammalian toxicity [1,2]. Symptoms associated with envenomation by Parabuthus species have been well described. These include a wide range of symptoms of neuromuscular, cholinergic and adrenergic stimulation such as restlessness, salivation, hypersensitivity to noise, defecation, unprovoked jumps, severe pain, severe convulsions, prolonged tremors and, in serious cases, death [1,2]. A conspicuous symptom not well described for the venom of other scorpions is the unprovoked jumps of experimentally envenomed animals. In our studies of the venom of P. transvaalicus, we observed unprovoked jumps in mice when sublethal doses of venom were administered to animals through either intracerebroventricular or intraperitoneal routes. Fractionation of venom and the administration of individual fractions to test animals resulted in each of the distinct symptoms being observed for a separate fraction, including one fraction that showed little toxicity but did show unprovoked jumps.

Although the general 3D structure of scorpion toxins is retained in most of the peptide toxins, with a few exceptions [3], subtle changes in primary structure result in the ability to bind to different types of ion channels. Currently, scorpion toxins affecting sodium channels are classified in several ways [4,5]. The functional classification divides these peptides as alpha, beta and insect-selective toxins, depending on the biological effect and toxin binding sites on the channel. Site 3, or alpha, toxins bind to the S3–S4 loop of domain IV and slow the decay of whole-cell current. Site 4, or beta, toxins are proposed to bind to and trap the voltage sensor of the channel and are recognized by the reduced peak amplitude of the sodium current. Insect-selective toxins are proposed to bind to overlapping sites in the corresponding insect Na+ channels, although these are subdivided as excitatory and depressant toxins. Structurally, all scorpion toxins that target sodium channels are classified into a group known as long chain neurotoxins (LCNs). These peptides are about 64–70 residues long and are stabilized by four disulfide bridges. Birtoxin (Swiss-Prot accession number P58752) is the first known exception to this structural pattern due to its slightly smaller size and the presence of only three disulfide bridges [6].

In this study, we have isolated, identified and characterized a variant of birtoxin, from the South African scorpion P. transvaalicus. This toxin, which we named ikitoxin, differs from birtoxin by a single amino acid. As described below, we have found that this single-residue substitution has several interesting consequences. Firstly, it dramatically decreases the effectiveness of birtoxin on intermittent and unprovoked jumping in mice. Secondly, at the doses we tested, it renders birtoxin toxic and ikitoxin not. Thirdly, despite these differences, both toxins alter the amplitude of voltage-gated Na+ current in ways that are characteristic of beta-group scorpion toxins. Structural and functional comparison with other beta toxins shows that birtoxin
and ikitoxin are the first two examples of a new group of beta toxins. These results add to a literature indicating that scorpions have expanded their palette of venoms by small modifications of genes already present.

**MATERIALS AND METHODS**

**Peptide purification**

Birtoxin was purified as described previously with the exception of the following modifications [6]. The crude venom was resuspended in solvent A (acetonitrile/H$_2$O/trifluoroacetic acid, 2 : 98 : 0.1, v/v/v) and sonicated briefly until no precipitate remained. The venom was first injected into a Michrom Magic 2002 microbore HPLC system equipped with a tapered bore C4 Magic Bullet column (4–1 mm internal diameter) and a 5μ peptide trap (Michrom Bioreources Inc., Auburn, CA, USA). A gradient of 2–65% solvent B (acetonitrile/H$_2$O/trifluoroacetic acid, 98 : 2 : 0.1, v/v/v) was generated over 15 min with a flow rate of 300 μL·min$^{-1}$. The UV absorbance trace was followed at 214 nm. Fraction P4 of the C4 separation (Fig. 1) from multiple runs was collected and injected into a Michrom C18 RP-HPLC microbore column. The 15.3 min retention time peak was collected and rerun on the same column to purify the peptide further. For ikitoxin, fraction P3 of the C4 column was collected and injected into the same microbore C18 column running at 50 μL·min$^{-1}$ with a linear gradient of 3% solvent B per minute increase for 23 min. The third major fraction was collected as ikitoxin and polished by re-running on the same column.

**Mass spectroscopy**

Mass spectra of crude venom, separated fractions and isolated peptide were analyzed off-line in a Biflex III (Bruker Daltonics, Bremen, Germany) MALDI-TOF instrument in positive ion mode as described previously [6]. External calibration was performed using angiotensin II (1046.53 Da, average) and human recombinant insulin (5808.6 Da, average) from Sigma. For analysis, matrix solutions consisting of sinapinic acid, 3,5-dimethoxy-4-hydroxyxinnaminic acid, or α-cyano-4-hydroxycinnamic acid, or z-cyano-4-hydroxycinnamic acid, were mixed in a 1 : 1 ratio with samples, spotted on the target and allowed to dry, masslynx (Micromass UK Limited, Manchester, UK) software was used for data processing and analysis.

**Edman degradation and peptide quantification**

Protein sequencing was accomplished as described previously for birtoxin [6]. Briefly, the cysteine residues of the peptide were reduced and carboxymethylated by incubating in 6 m guanidine hydrochloride, 0.1 m Tris/HCl (pH 8.3), 1 m EDTA and 20 mm dithiothreitol for 1 h at 37 °C. Iodoacetic acid was then added to a final concentration of 50 mm and incubated for an additional hour at 37 °C in the dark. Finally, approximately 900 picomoles of peptide was subjected to automated Edman sequencing for 60 cycles using a Hewlett-Packard HP GS1000 Sequence Analyzer at the Molecular Structure Facility at UC Davis. Peptides were quantified as described previously for birtoxin [6].

**Bioactivity**

Biological activity was monitored by intracerebroventricular injections of 4- to 6-week-old male Swiss–Webster mice with both fractions from the C4 separation and 0.002–4 μg purified toxin. The subject animals were monitored continuously up to 24 h, after which the symptoms faded and the mice completely recovered. Ikitoxin did not show lethality during the course of the observation period in the range of injected doses. Activity against insects was tested by injecting blowfly and cabbage looper larvae.

All animal care and experimental protocols conformed to the guidelines of the Animal Use and Care Administrative Advisory Committee of the University of California, Davis.

**Electrophysiological measurements**

Effects of birtoxin and ikitoxin on voltage-gated Na$^+$ current were measured under voltage clamp, using whole-cell patch electrodes, in retinal ganglion cells dissociated from common goldfish (*Carassius auratus*; 9–16 cm body length). The voltage-gated Na$^+$ conductance of these cells is typical of adult vertebrate central neurons in terms of voltage-sensitivity of activation and steady-state inactivation, susceptibility to blockade by tetrodotoxin (TTX), presence of transient and persistent components, and relative permeability to Na$^+$ and Li$^+$ ions [7]. Also, these cells display E0III-segment-like immunoreactivity [8]. Cells were dissociated, identified and recorded from as described elsewhere [7,9], with two exceptions. First, an enzyme-free, low-Ca$^{2+}$ solution was used for retinal dissociations (Y. Hayashida, G. J. Partida, and A. T. Ishida; unpublished observation) to avoid the possible distortion of Na$^+$ current kinetics by exposure to proteases typically used to dissociate cells. Secondly, currents were recorded in the perforated-patch configuration [10], using amphotericin B as the perforating agent and a single-electrode voltage-clamp amplifier (SEC-05LX; npi electronic, Tamm, Germany) in discontinuous voltage-clamp mode [11]. The switching frequency and duty cycle (current injection/potential

![Fig. 1. UV trace of C4 separation of the crude venom of Parabuthus transvaalicus.](image)
Patch electrodes were pulled from borosilicate glass capillaries (Sutter Instrument Co., Novato, CA, USA) to tip resistances of 2–5 MΩ, and coated with SigmaVacat (Sigma, St Louis, MO, USA) to reduce electrode capacitance. The tip of each electrode was filled with 'pipette solution' that contained 15 mm NaCl, 140 mm CsOH, 2.6 mm MgCl2, 0.34 mm CaCl2, 1 mm EGTA and 10 mm Hepes. The pH was adjusted to 7.4 with methanesulfonic acid, and the osmolality was adjusted with sucrose to 260 mOsmol·kg⁻¹.

Pipette shanks were filled with this solution after the addition of 1/200th of a solution containing 2 mg amphotericin B (Sigma) with 3 mg Pluronic F-127 (Molecular Probes, Eugene, OR, USA) in 60 µL dimethylsulfoxide (Sigma). The control 'bath solution' contained 110 mm NaCl, 3 mm CaCl2, 30 mm tetraethylammonium chloride, 2.4 mm MgCl2, 0.1 mm CaCl2, 10 mm D-glucose and 5 mm Hepes. The pH was adjusted to 7.4 with CsOH, and the osmolality was adjusted with sucrose to 280 mOsmol·kg⁻¹. Pipette shanks were filled with this solution after the addition of 1/200th of a solution containing 2 mg amphotericin B (Sigma) with 3 mg Pluronic F-127 (Molecular Probes, Eugene, OR, USA) in 60 µL dimethylsulfoxide (Sigma). The control 'bath solution' contained 110 mm NaCl, 3 mm CaCl2, 30 mm tetraethylammonium chloride, 2.4 mm MgCl2, 0.1 mm CaCl2, 10 mm D-glucose and 5 mm Hepes. The pH was adjusted to 7.4 with CsOH, and the osmolality was adjusted with sucrose to 280 mOsmol·kg⁻¹. The combined use of these pipette and bath solutions blocked voltage-gated Ca²⁺ and K⁺ currents [7,9]. Because it is not possible to null cell capacitive currents with the amplifier used here, the Na⁺ currents given (maximum amplitudes as well as current traces) are the differences between currents recorded before and after steady-state blockade by TTX (> 9 µm). Ikitoxin, birtoxin, and tetrodotoxin were applied by the addition to the bath solution through a large bore pipette. Toxins were applied while recording from only one cell per dish, so that the birtoxin and ikitoxin effects reported here were obtained from cells that had not previously been exposed to any Na⁺ channel toxin. All toxins were applied at concentrations considered to be supersaturating, to increase the likelihood that maximal effects were observed.

Voltage-jump protocols, data acquisition and some off-line analyses were performed with the pClamp system (version 8.101, Axon Instruments). The amplifier output was analog-filtered by the two-pole Bessel filters of the amplifier [corner frequencies (fₚ) of 20 kHz for voltage and 8 kHz for current] and digitally sampled at 50 kHz. To reduce noise contained in the sampled signals, the current and voltage recordings reported here were digitally filtered off-line, using pClamp software and an eight-pole Bessel filter with the fₚ set to 4 kHz. The recording chamber was grounded via an agar bridge, and all membrane potentials were corrected for liquid junction potentials attributable to differences between the bath and pipette solution compositions. All experiments were performed at room temperature (~23 °C).

Molecular modeling

The SWISS-PROT VIEWER software from the EXPASY server (http://www.expasy.ch) was used to visualize and compare the effect of the substitution of a glutamic acid for a glycine on the structure and electrochemical surface of birtoxin. The mutation was introduced into the previously modeled birtoxin structure using the functions in this software for mutation, energy minimization and electrochemical surface calculation.

RESULTS

The separation obtained on the magic bullet C4 column was identical to that obtained on a Vydac analytical C4 column in one quarter of the running time using eight times less solvent (Fig. 1). Birtoxin and ikitoxin were well separated on the C4 column, whereas they have a similar retention time on the C18 column (data not shown). Therefore we purified the 6615 Da species by first separating the P3 and P4 fractions on a C4 column and then running smaller quantities of the C4-P3 fraction on the C18 column multiple times and collecting the second peak that eluted at 15.3 min. The compositions of fractions P3, P4 and their mixture were determined using mass spectroscopy. The MS results indicate the presence of species with molecular mass of 6543 Da and 6615 Da in fraction P3 and the presence of only species with molecular mass 6543 Da in fraction P4 (Fig. 2). Both peptides were then purified to more than 98% purity, as confirmed for each peptide by mass spectrometry.

The biological activity of both peptides was then compared. When administered to blowfly and cabbage looper larvae, neither toxin produced noticeable effects. In particular, the contraction and paralysis that are typically produced by excitatory or depressant insect-specific toxins were not observed, even at doses as high as 2 µg peptide per 150 mg of insect body weight. When injected into mice, ikitoxin produced some, but not all, of the effects produced by birtoxin. For example, ikitoxin and birtoxin both caused intermittent jumping. This jumping was remarkable in that, between jumps, mice displayed normal motor activity (e.g. the ability to hold on to horizontally held pencils). Ikitoxin differed from birtoxin in that it caused jumps at much higher doses (e.g. 4100 ng peptide injected per mouse, but birtoxin caused jumps at a very low dose of 3.7 ng peptide injected per mouse), and its effects were much slower in onset than those of birtoxin (effects appearing 30 min after ikitoxin injections vs. 5 min after birtoxin injections). A third difference between these toxins is that birtoxin produced convulsions, tremors, increased ventilation and, subsequently, death, whereas ikitoxin did not. Similar effects were produced by purified birtoxin and by fraction P4, and the LD₉₀ value for intracerebroventricularly
introduced birtoxin was 800 ng of peptide [6]. Figure 3 summarizes the qualitative effects of both toxins at various doses.

Full sequencing of ikitoxin showed that the only difference between birtoxin and ikitoxin is at the 23rd residue, a glycine in birtoxin and a glutamic acid residue in ikitoxin (Fig. 4). This difference of Gly23 to Glu23 agrees with the presence between birtoxin and ikitoxin is at the 23rd residue, a glycine in birtoxin and a glutamic acid residue in ikitoxin (Fig. 4). This difference of Gly23 to Glu23 agrees with the presence.

Based on their sequence homology to known toxins (Fig. 4), birtoxin and ikitoxin are expected to bind to voltage-gated Na⁺ channels. This possibility was examined by measuring the effect of these toxins on the whole-cell Na⁺ current of retinal ganglion cells (see Materials and methods). To assess the effects of birtoxin (Fig. 5A,B) and ikitoxin (Fig. 5C,D), the Na⁺ current was routinely activated by a step depolarization from a holding potential of −72 mV to a test potential of −7 mV. These voltages were used because the resting potential of these cells is normally around −70 mV, and the voltage that typically activates the maximum, whole-cell Na⁺ conductance in these cells is between −10 and 0 mV. At the times marked by the first upward arrows in Fig. 5A,C birtoxin and ikitoxin were applied at concentrations of approximately 490 nm and 195 nm, respectively. Within 2–6 min thereafter (between the times marked ‘a’ and ‘c’), the amplitude of the peak of the Na⁺ current decreased. In the cells we recorded from, the peak Na⁺ current amplitude decreased to about 65% of the control value (64–85% with 80–490 nm birtoxin, n = 3; 63–77% with 25–200 nm ikitoxin, n = 3). Application of increased toxin concentrations did not reduce the current amplitude further. The complete blockade of the remaining current by TTX (second arrow in both A,C) shows that the reduction of inward current amplitude by birtoxin and ikitoxin (A,D) is not due to activation of an outward current. In turn, these observations suggest that these concentrations of birtoxin and ikitoxin only partially block the total Na⁺ current that can be elicited in these cells.

Superimposition of current traces recorded before and after toxin application shows that neither of these toxins produced marked changes in the time course of the increase or decrease in Na⁺ current amplitude that occurs during individual depolarizations (B,D).

Figure 6 shows the effects of birtoxin (A–D) and ikitoxin (E–H) on the voltage dependence of Na⁺ current. As in Fig. 5, effects on current activation were examined in cells depolarized from a holding potential of −72 mV to test potentials ranging from −57 to +3 mV. Both toxins reduced the amplitude of the Na⁺ current peak at test potentials more positive than −37 mV, and increased it at test potentials more negative than −37 mV (A,B for birtoxin, E,F for ikitoxin). The current traces in Fig. 6 show that neither toxin produced a marked change in the Na⁺ current time course at these voltages (A,E), consistent with the results in Fig. 5.

To examine effects on steady-state inactivation, cell membrane potential was shifted as shown at the top of Fig. 6C,G. The amplitude of the Na⁺ current activated by the depolarization to −7 mV measures the fraction of total current that is available for activation after shifting the membrane potential to the ‘conditioning’ values used (ranging from −87 to −27 mV). Fits of Boltzmann distributions to plots of these amplitudes vs. the conditioning potential (so-called ‘steady-state inactivation’ plots) are shown by the solid lines in Fig. 6D,H. The conditioning potential that reduced peak amplitude to 50% of the maximum value (V_{1/2}) was −56 ± 0.3 mV in the control (n = 6), −58 ± 0.7 mV in the presence of birtoxin (n = 3, 80–490 nm), and −58 ± 0.5 mV in the presence of ikitoxin (n = 3, 25–200 nm).

The toxin effects mentioned above were similar in all six cells examined. These results are consistent with the effects of previously classified beta group scorpion toxins on voltage-gated Na⁺ channel isoforms of brain and skeletal muscle [4,5].

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**Fig. 3.** Dose–response curves of birtoxin and ikitoxin. Birtoxin is shown as open bars and ikitoxin is shown as filled bars. Peptides were injected intracerebroventricularly, with at least three animals injected for each dose. The mice were observed for 24 h, and effects were ranked between 0 and 10, 0 being no effect and 10 being lethality. The intermediate ratings are based on the strength of the symptoms observed, 5 and above is given for heavy tremors and paralysis of hind legs, 4 for moderate and occasional tremors, and below 4 for light and rare tremors. Jumping due to birtoxin and ikitoxin is indicated by ‘J⁺’. Note that jumping occurs at about a thousand-fold lower concentration for birtoxin compared to ikitoxin. Except for unprovoked jumps, ikitoxin-injected animals behave normally (full motor activity) even at the highest doses used.

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**Fig. 4.** Multiple alignment of birtoxin and ikitoxin to Neurotoxin Variant 1 from *Centruroides exilicauda* (Cse-V1). Birtoxin and ikitoxin are 98% identical to each other and Cse-V1 is 54% identical to toxins from *Parabuthus*. Note that birtoxin and ikitoxin do not possess the C-terminal residues that are commonly found in all other LCNs.
The marked differences of in vivo symptoms produced by ikitoxin and birtoxin prompted us to examine the effect of the Gly23 to Glu23 change at the molecular level. The α-helix region of birtoxin was modeled according to an NMR determined structure of CeNV1 using SWISS-PDB VIEWER as described previously [6]. According to our model, the region where Gly23 resides in birtoxin is solvent accessible (Fig. 7). This is supported by the fact that the change alters the biological activity. The surface potential calculation presentation also indicated a significant structural difference (i.e. protrusion) where the region preceding the α-helix is transformed from a neutral patch to an acidic patch.

**DISCUSSION**

It is often difficult to assess the effect of a single peptide in a venom mixture due to the variety and interference of activities of many individual toxins. However, investigations of sublethal effects of venom or of individual fractions of that venom are more likely to result in the identification of certain peptides associated with unusual symptoms. The results presented here illustrate how behavioral observations and electrophysiological measurements may be used towards this type of identification. We have found, in particular, that while high doses of ikitoxin and birtoxin produce different behavioral effects, the effects at low concentrations of birtoxin are similar to those of ikitoxin. Although the difference in actions at some concentrations suggests that these toxins might differ in their locus or mechanism of action, the similarity of their effects at other concentrations raised the possibility that both toxins have a common mechanism of action. By assessing the effect of these toxins on current flowing through voltage-gated Na⁺ channels, we have been able to show that both toxins produce effects that are characteristic of beta group scorpion toxins. This suggests that the restricted region of the toxins that we know to be structurally different may be responsible for the marked difference in potency of the two toxins.

Previously it has been shown that beta group scorpion toxins modify current through different Na⁺ channel isoforms in at least two distinct ways. On one hand, beta group toxins shift the voltage dependence of Na⁺ channel activation toward more negative potentials, and also reduce the peak sodium current amplitude of the brain and skeletal muscle isoforms. On the other hand, these toxins reduce the current amplitude but have little effect on the voltage dependence of activation of the cardiac isoform [12,13]. The electrophysiological measurements presented here show that the effects of birtoxin and ikitoxin are like those of beta group toxins on brain and skeletal muscle cells. This leaves open the question of whether the shift in current activation or the reduction in peak amplitude is responsible for the specific behavior we have observed, and how each of these effects is produced in single Na⁺ channels. The electrophysiological measurements presented here show that birtoxin and ikitoxin partially block the whole-cell Na⁺ current at supersaturating doses, and that the portion of Na⁺ current that resisted block by ikitoxin and birtoxin could be completely blocked by the addition of tetrodotoxin (Fig. 5). The similarity of this blocking pattern to effects reported elsewhere suggests that binding of birtoxin and ikitoxin to some Na⁺ channel subunits in the cells we
recorded from may have produced the whole-cell Na⁺ current amplitude reduction, and that the binding of birtoxin and ikitoxin to at least one other subunit produced the negative shift in activation threshold [12,13]. However, on the basis of the results presented here, we can not yet exclude the possibility that birtoxin and ikitoxin differen-
tively modulated current through subtypes of Na⁺ channel in the cells we have recorded from.

Modeling of the peptides birtoxin and ikitoxin shed light on how these beta toxins might interact with their target ion channels. Our model indicates a significant change in surface potential that is correlated with a change in bioactivity in vivo. Binding of scorpion toxins to target ion channels occur through multiple interactions [14]. Numerous amino acid residues have been determined to affect binding [4]. Beta scorpion toxins classified in previous

Fig. 6. Effects of birtoxin (A–D) and ikitoxin (E–H) on the voltage dependence of Na⁺ current. A,B and C,D show the effect of birtoxin on current activation and steady-state inactivation, respectively, in one cell. E,F and G,H show the effect of ikitoxin on the same properties in a different cell. The traces in the upper row of A, C, E and G are the membrane potentials measured in discontinuous voltage-clamp mode. In A and E, the holding potentials are −72 mV, and the test potentials were increased from −57 to +3 mV, in 10-mV steps. In C and G, the test potentials are −7 mV, and the conditioning potential (100 ms duration) was increased from −87 to −27 mV in 10-mV increments. Cells were depolarized once per 12 s, at most, regardless of the protocol or test potential. The traces in the lower row of A, C, E and G are the Na⁺ currents activated by these test depolarizations. The currents in A and C were recorded before (control) and ≈4 min after the application of birtoxin (490 nm). The currents in E and G were recorded before (control) and ≈ 10 min after the application of ikitoxin (195 nm). Each trace plots the current activated by a single depolarization, after subtraction of TTX-resistant leak and capacitive current. The zero-current level in each family of traces is shown by the dashed horizontal lines. The amplitude of the peaks of these currents are plotted in B, D, F and H, respectively. B and F plot the maximum Na⁺ current amplitude, at each test potential, in the absence (filled circles) and presence (open circles) of toxin. In D and H, all current amplitudes are normalized to the maximum value obtained in each control condition, and plotted against conditioning potential. Data were fitted with Boltzmann distri-

butions (solid lines). A and E show that, in the presence of birtoxin and ikitoxin, the Na⁺ currents activated by small depolarizations (e.g. to −47 mV) are larger than the respective control currents, but that currents activated by larger depolarizations are reduced by both toxins. The Na⁺ current that resists inactivation at membrane potentials more positive than −37 mV (D) is consistent with the increase in persistent current amplitude at all test potentials (A). In C, D, G and H, the traces of control currents activated from −87 and −77 mV overlap, as do those of the currents activated from the same voltages after exposure to each toxin. The traces of currents activated from −37 and −27 mV in control solution overlap (C), as do those activated from the same voltages in birtoxin (C) and in ikitoxin (G).
studies are known to bind to neurotoxin receptor site 4 of the voltage-gated Na\(^+\) channel [5]. Recently their mechanism of action at the molecular level has become more apparent. It is hypothesized that the voltage sensor of Na\(^+\) channels moves outwardly when the channel is activated. A mechanism proposed to explain the shift in current activation is that beta toxins bind to this region, specifically to freshly exposed amino acid residues, and trap the voltage sensor of the channel in the activated position [13].

It has been suggested that in protein–protein interactions at least six parameters including solvation potential, residue interface propensity, hydrophobicity, planarity, protrusion and accessible surface area are important determinants of binding [15]. According to our model, the Gly23 to Glu23 change in ikitoxin renders the region more exposed to the solvent, less hydrophobic, less planar, more protruded, and with a larger accessible surface area compared to Gly23 of birtoxin (Fig. 4). In ikitoxin the presence of Glu23 charge preceding the \(\alpha\)-helix modifies the activity of this toxin in a unique way to result in reduced potency in mice.

The C-termini of scorpion toxins are hypothesized to be responsible for a significant portion of their toxicity. Gurevitz et al. [16] stated that the C-termini are the most divergent regions of the scorpion toxins. However, in many cases the N-terminal loop comprised of amino acids 10–25 preceding the conserved \(\alpha\)-helix has also been associated with changes in toxicity. For example a monoclonal antibody against a synthetic peptide of residues 5–14 of Cn2 from Centruroides noxius was able to neutralize the toxicity of this toxin [17]. Also Moskowitz et al. [18] showed that depressant and excitatory insecticidal toxins have a variable region located in the 12–20loop, preceding the \(\alpha\)-helix responsible for a change of mode of action from excitatory to depressant. A change in activity associated with this particular loop is again observed in the case of birtoxin and ikitoxin. Moskowitz et al. [18] cautioned that minor changes in primary structure can lead to major changes in mode of action and that groups of toxins based on length or apparent identity in sequence may not necessarily reflect the biological effects of the toxins. Indeed, Zilberberg et al. [19] reported that single-residue mutations can shift the phylogenetic specificity of an alpha toxin by forming toxins that are either more or less toxic to insects than to mammalian species.

Here we presented an example of protein diversification that yields a quite different bioactivity with a potential behavioral advantage to the scorpion. It is evident that there is a great diversity in scorpion toxins. However, the exact mechanism(s) of diversifying peptide toxins is yet to emerge. Clearly, making small changes in peptide sequences is a mechanism to increase diversity. For example, for ikitoxin, the mutation seems to be a single base change of guanidine to adenosine because glutamic acid is encoded by GAA or GAG and glycine is encoded by GGA or GGG codons, which differ only by an adenosine base. However, scorpion venom contains a wide range of toxins including ones that have different structural folds. Some of these affect even intracellular channels such as the ryanodine-sensitive calcium channel activators maurocalcine [3] and imperatoxin A [20]. This indicates that small changes in sequence are accompanied with other possible mechanisms such as C-tail wiggling [16] and position-specific deletion of long chain neurotoxins to obtain short chain neurotoxins [21]. The discovery of ikitoxin, a nonlethal birtoxin-like peptide with a single residue difference but a significant change in bioactivity, indicates that research on toxins will continue to increase our understanding of how ion channels work and provide the basis for designing pharmaceuticals with broad or specific activity and differences in potency.

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