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Molecular Mechanism of Scorpion Neurotoxins Acting on Sodium Channels

Insight Into Their Diverse Selectivity

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Abstract

Scorpion toxins that affect sodium channel gating traditionally are divided into α- and β-classes. They show vast diversity in their selectivity for phyletic- or isoform-specific sodium channels. This article discusses the molecular mechanism of the selectivity. Moreover, a phylogenetic tree of scorpion toxins has been constructed, which, together with the worldwide distribution of toxins and the zoogeographic dispersion of the studied genera, offers an insight into the evolution of diverse scorpion toxins.

Index Entries: Scorpion toxin; voltage-gated sodium channel; selectivity; receptor site; phylogenetic tree.

Introduction

As the key transmembrane proteins responsible for the initiation and propagation of action potentials in excitable cells, voltage-gated Na^+ channels (VGSCs) have been demonstrated to be targets for numerous natural neurotoxins *(1).* These neurotoxins can strongly alter channel function by binding to specific receptor sites, thereby representing powerful tools to study the distribution, structure, and function of VGSCs *(2).*

To date, hundreds of Na^+ channel-specific neurotoxins have been purified from different scorpion species worldwide *(3).* Traditionally, they have been divided into α- and β-classes based on their mode of action. α-toxins can prolong action potentials by slowing the inactivation of sodium current, whereas β-toxins shift the voltage dependence of activation to a more negative potential *(4).* However, recent studies

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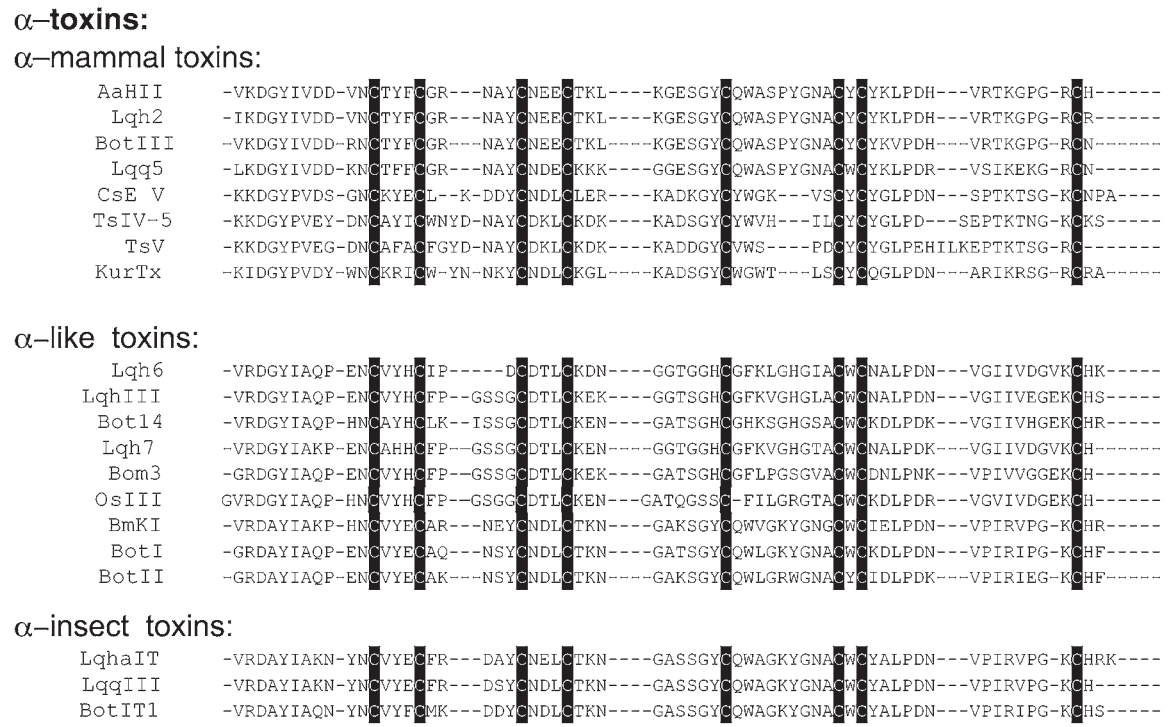

Fig. 1. Sequence alignment of toxin representatives of various pharmacological groups (*see* text) that affect voltage-gated sodium channels. They were aligned taking as reference the Cys residues (shaded by *dark gray*). Dashes indicate Gaps which were introduced to maximize similarities. The conserved *(solid line)* and the non-conserved (dashed line) disulfide bridges are depicted at the bottom. *(Figure continues)*

have revealed that there are more variants among these toxins that are able to selectively target phyletic- or isoform-specific VGSCs *(5)*.

In accordance, α-toxins have now been divided into three major groups: (a) α-mammal toxins that are highly active in mammalian brain (e.g., *Androctonus australis* Hector-II [AaHII], *Leiurus quinquestriatus hebraeus-II* [LqhII], *L.q.quinquestriatus* [LqqV]; *Centruroides sculpturatus* Ewing-V [CsEV], and *Tityus serrulatus-IV* [TsIV]); (b) α-insect toxins that are very toxic to insects but not to mammalian brain (e.g., LqhαIT and Lqq3); and (c) α-like toxins, which are potently active in both mammalian brain and insects but lack specific binding to sodium channels of rat brain synaptosomes (e.g., *Buthus martensii* Karsch-I, [BmKI], LqhIII and Lqh6) *(6,7)* (Fig. 1).

β-toxins, similarly to α-toxins, affect insects and mammals in a variety of preferences and, therefore have been divided into several groups: (a) β-mammal toxins that are specific for mammals only (e.g., *Centruroides suffusus suffusus* [Css] II and -IV, *C. noxius* Hoffmann [Cn] 2 and -3); (b) toxins active on both mammals and insects, mainly from the same genus, *Tityus* (e.g., Ts7, *T. stigmurus* [Tst1], and *T. bahiensis* [Tbs1 and -2]); (c) excitatory insect-selective toxins (e.g., AaHIT, LqhIT1, and BmKIT; (d) depressant insect-selective toxins (e.g., BmKIT2, LqhIT2, and LqqIT2); and (e) a novel group (which exhibits similar activity as group 2 β-toxins but differs much in sequences), including the four members BmKAS, BmKAS-1, AaHIT4, and Lqhβ1. For clarity, these are termed β-like toxins *(8,9)* (Fig. 1).

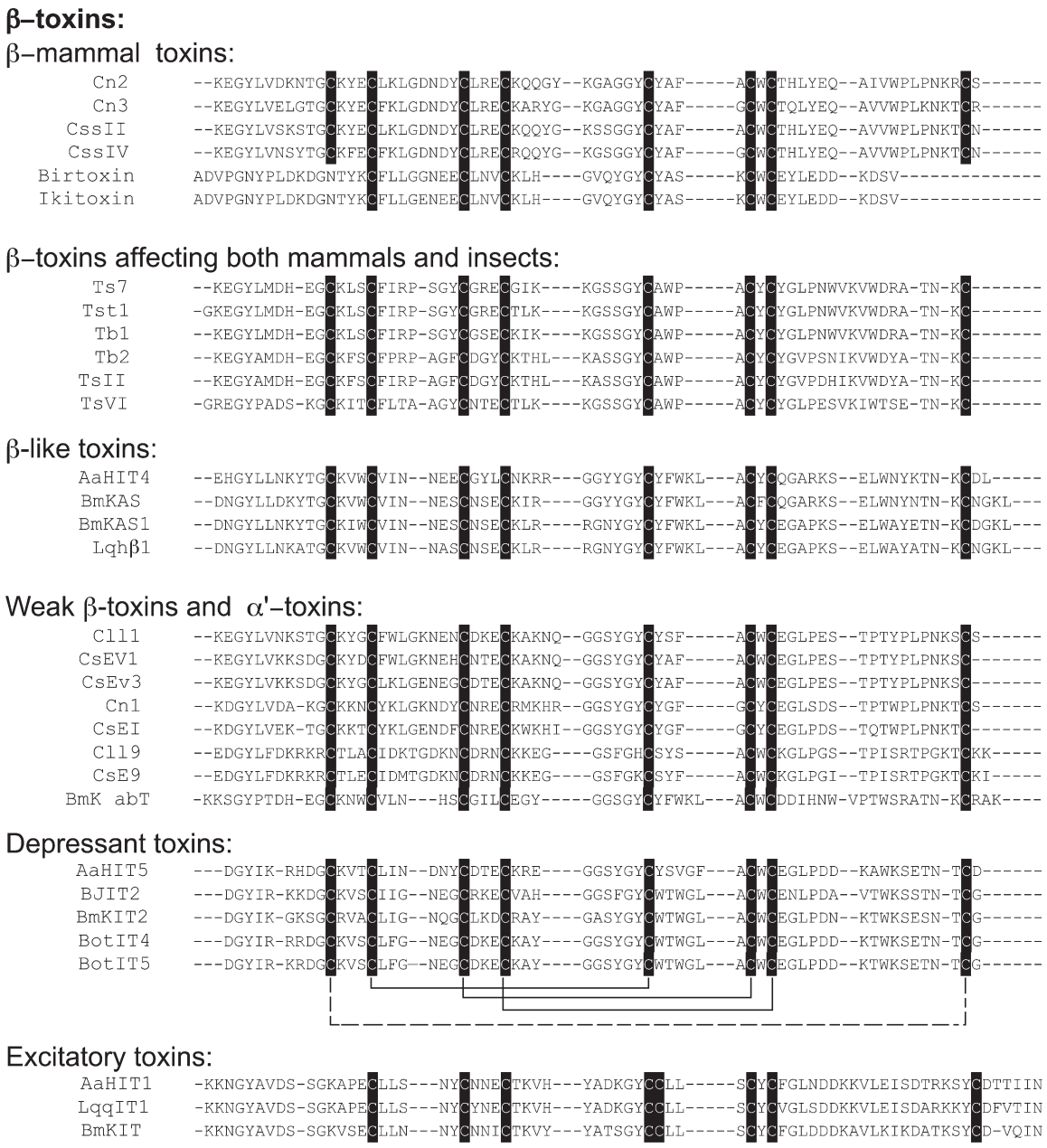

Fig. 1. *(continued)*

The variance in the selectivity of toxins is deemed to be an outcome of coevolution of the toxins and channels. In recent years, a series of Na^+ channel genes and their tissue distribution pattern have been identified in many phyla *(10)*. The difference in the selectivity of various scorpion neurotoxins may be attributed to their distinct receptor sites on diverse VGSCs.

Here, we discuss the selectivity of each group of toxins and the possible mechanisms in which they are involved.

Receptor Site on VGSCs Specific for Scorpion α-Toxins

Three groups of α-toxins are deemed to bind to a homologous or identical receptor site—namely, neurotoxin receptor site-3, inferred from the competition binding assays that all site-3 toxins (scorpion α-toxins, the sea anemone toxin ATXII, and spider δ-aracotoxins), were able to compete for the anti-insect α-toxin LqhαIT binding site on insect VGSCs *(6).* Site-directed mutagenesis has identified that they bind to the extracellular loop S3–S4 in domain 4 (IVS3-S4) through electrostatic interaction with E1613 for rNav1.2, D1612 for rNav1.5, and D1428 for rNav1.4, despite their variable selectivity *(11–13).* Interestingly, an anionic residue is always present at the corresponding position in known mammalian and insect Na^+ channel subtypes (Fig. 2).

α-like toxins are active on insect VGSCs and heterologously expressed rNav1.4 and rNav1.5 *(14–17).* However, they lack effect on heterologously expressed rNav1.2 *(14–18)* and, most likely, on rNav1.1 and rNav1.7, because the tissues expressing high density of rNav1.1 on the rat caudal regions or rNav1.7 on the peripheral nerves, respectively, were found to be insensitive to α-like toxins *(14,19).* Notably, in those α-like toxin-sensitive channels (the insect VGSCs and rNav1.4–1.5), the acidic residue (homologous to E1613 in rNav1.2) is all the same (Asp), whereas in the insensitive channels (rNav1.1, rNav1.2, and rNav1.7), it is altered to another acidic residue (Glu) (Fig. 2). Thus, the anionic residue Asp seems to determine the selectivity of α-like toxins. This idea derives support from the acquired sensitivity of double-mutant rNav1.2 E1613D · K1617T toward α-like toxins *(20).*

Based on this idea, we can infer that rNav1.6 should be sensitive to α-like toxin because it possesses an Asp, rather than Glu, at the corresponding position. Thus, this isoform rNav1.6 may mediate the toxicity of α-like toxins to mammal brain upon intracerebroventricular (ICV) injection. However, despite its high expression in brain tissues, rNav1.6 seems to be present essentially on neuronal somata but not on nerve trucks that form brain synaptosomes *(14,18).* In contrast, the main sodium channel subtypes in nerve trucks are rNav1.2 (at least 80%) and rNav1.1 (about 20%) *(21);* therefore, α-like toxins lack binding affinity for rat brain synaptosomes. This explains the discrepancy between the toxicity and pharmacological binding features of α-like toxins to mammal brain *(22).*

	--------IVS3 ~ S4-----
	1------8--------17
(fruitfly)	ILGLVLSDIIEKYFVSP$_{1710}$
(budworm)	ILSLVLSDIIEKYFVSP
(Cockroach)	ILGLVLSDIIEKYFVSP$_{1679}$
eel	IIGLLLSDIIEKYFVSP$_{1413}$
fugu	IVGMFLADLIEKYFVSP$_{1379}$
rNav1.5	IVGTVLSDIIQKYFFSP$_{1621}$
rNav1.4	IVGLALSDLIQKYFVSP$_{1437}$
rNav1.6	IVGMFLADIIEKYFVSP$_{1609}$
hNav1.7	IVGMFLADLIETYFVSP$_{1595}$
rNav1.7	IVGMFLAELIETYFVSP$_{1604}$
rNav1.1	IVGMFLAELIEKYFVSP$_{1632}$
rNav1.3	IVGMFLAELIEKYFVSP$_{1568}$
rNav1.2	IVGMFLAELIEKYFVSP$_{1622}$

Fig. 2. Sequence of the extracellular loop IVS3-S4 from phylogenetically distinct sodium channels were aligned. The channels used are insect channels from fruitfly *D. melanogaster* Para (P35500), budworm *Heliothis virescens* (A56595), cockroach *B. germanica* Para (AAC47484), and vertebrate channels from eel *E. electricus* (72012), fugu *F. rubripes* (BAA07195), rat *R. norvegicus* $Na_v1.1$ (CAA27286), $rNa_v1.2$ (CAA27287), $rNa_v1.3$ (CAA68735), $rNa_v1.4$ (CHRTM1), $rNa_v1.5$ (A33996), $rNa_v1.6$ (AAC42059), $rNa_v1.7$ (AAB50403), human *H. sapiens* $Na_v1.7$ (CAA58042). To clarify, the animals belonging to different groups are highlighted by different brackets.

α-insect toxins exhibit almost the same activity as α-like toxins. Both groups are toxic to mammals and insects when injected subcutaneously and are incapable of binding to the rat brain synaptosomes *(6).* They also have a similar effect on native mammal tissues of skeletal and cardiac muscles as well as on heterologously expressed rNav1.4 and rNav1.5 *(15,16).* However, these two groups are distinct from each other in that α-insect toxins are nontoxic to mammal brain even at high concentration *(22).* This indicates the lack of effect of α-insect toxins on the isoforms rNav1.1–1.3 and rNav1.6 resulting from their high expression in rat brain.

Sequence comparison reveals that the prime important anionic residue for α-toxins is the same in insect channels and rNav1.4–1.5 (Asp). However, it is also present in rNav1.6. Therefore, the anionic residue should not be the sole determinant of the binding of α-insect toxins. Further inspection of the region showed that there are some commonalities near the extracellular end of the IVS3 individually within those sensitive and insensitive channels. The consecutive residues are Leu-Val-Leu-Ser (LVLS) in insect sodium channels, Leu-Ala-Leu-Ser (LALS) in sensitive mammal isoforms rNav1.4, and Thr-Val-Leu-Ser (TVLS) in rNav1.5. However, they are changed into Met-Phe-Leu-Ala (MFLA) in the insensitive isoforms rNav1.1–1.3 and rNav1.6 (Fig. 2). The amino acid residues neighboring the critical anionic residue appear to be involved in the binding of anti-insect α-toxins. This idea agrees with the mutagenesis of the external loop IVS3–S4 of rNav1.4, which implies that Asp1428 within this loop is most crucial for the binding of α-insect toxins and α-like toxins, whereas the charge-neutralizing mutation at the neighboring residue (Lys1432) affects the binding affinity of α-insect toxins but not α-like toxins *(13,22).* Accordingly, the receptor sites for α-like toxins and α-insect toxins are not identical but do overlap; the former is mainly determined by the anionic residue, whereas the latter involves more neighboring residues. This is consistent with the stronger toxicity of α-insect toxins toward insects and the need for a high α-like toxin concentration to inhibit the binding of α-insect toxins on insect sodium channel *(17).*

α-mammal toxins are highly toxic to mammals and specifically bind to rat brain synaptosomes, but they exhibit little toxicity for insects *(22).* Electrophysiological studies have shown that they could strongly affect mammal tissues of brain and peripheral nervous system as well as heterologously expressed rNav1.2 *(20),* rNav1.4, and rNav1.5 *(15,16)* but not hNav1.7 *(19).* However, rNav1.7, the rat homolog of hNav1.7, seems to be the target for α-mammal toxins, because the peripheral nerves that express high density of rNav1.7 were affected by α-mammal toxins but not by α-like toxins *(14).* Sequence comparison reveals that rNav1.7 and hNav1.7 are almost identical in IVS3–S4, with the exception of the acidic residue that is critical for α-toxin binding (Glu in the former vs Asp in the latter). However, according to the mutagenesis experiment done on rNav1.2 *(11,20),* this residue difference does not discern its sensitivity to α-mammal toxins. Thus, the sequence variance of the region IVS3–S4 is not sufficient to explain selective recognition of α-mammal toxins.

The smaller contribution of IVS3–S4 to the binding of α-mammal toxins is corroborated by the mutagenesis performed on rNav1.4, which implies that Asp is crucial for the binding of three groups of α-toxins; however, the mutation D1428N affects the binding affinities to a different extent: weak on LqhII and pronounced on LqhIII and LqhαIT *(22).* The molecular basis of the selectivity of α-mammal toxins may involve additional external loops—most likely the S5–S6 loops of D1 and D4, as suggested by the photo-affinity labeling and site-directed antibody experiments *(23,24).*

To date, the 3D structure of several α-toxins have been identified *(25–32).* The overall similarity is high, and the most salient differences between these structures appear at three turns that are believed to be functionally important *(31).* Therefore, these three turns may be essential for the selectivity difference among groups. Interestingly, the electrostatic charge distribution is surprisingly similar between LqhIII and

LqhαIT but not AaHII *(29).* This might correlate with the binding feature of α-mammal toxins—that is, the additional region, besides IVS3–S4, is expected to be involved. Moreover, structure comparison of BmkI with AaHII and LqhαIT showed that the specific orientation of the C-terminus mediated by the reverse turn might be relevant to the selectivity of distinct α-toxin subgroups *(33).* As discussed earlier, the selectivity of α-like toxin is determined by Asp and is abolished by Glu at the critical position within receptor site-3; the side-chain of the Glu residue may be longer and, therefore, may hinder the interaction of the C-terminus of α-like toxins with receptor site-3.

	--------IIS3 ~ S4---
	1------8-----17
[scorpion]	ELCGENVSLPGLSV$_{695}$
(fruitfly)	ELGLEGVQ--GLSV$_{907}$
(cockroach)	ELGLEGVQ--GLSV$_{871}$
(beetle)	ELGLEGVQ--GLSV$_{635}$
[rNav1.5]	ELGLSRMG--NLSV$_{807}$
rNav1.4	ELGLANVQ--GLSV$_{661}$
rNav1.1	ELGLANVE--GLSV$_{857}$
rNav1.3	ELGLANVE--GLSV$_{800}$
rNav1.2	ELGLANVE--GLSV$_{848}$

Fig. 3. Comparison of amino acid sequences within the extracellular loop IIS3-S4 in several phylogenetically distinct channels. Some are already listed in Fig. 2, with the addition of scorpion sodium channel and beetle channel *Leptinotarsa decemlineata* (AAD22957).

Receptor Site on VGSCs Specific for Scorpion β-Toxins

Antimammal β-toxins bind to receptor site-4 of the mammal VGSCs and induce both a shift in the voltage dependence of activation to a more negative membrane potential and a reduction of the peak sodium current amplitude *(2).* The activity of them, similarly to the differential effect of α-toxins among distinct channel isoforms, has been demonstrated to be much weaker on expressed rNav1.5 compared with rNav1.2 and rNav1.4 *(34,35).* A molecular approach to the selectivity issue of β-toxins, using a series of domain-replacing between rNav1.5 and rNav1.4 and extracellular loop-exchanging between rNav1.5 and rNav1.2, indicate that four extracellular loops (IS5–SS1, IIS1–S2, IIS3–S4, and IIIS5–S6) are involved in the binding of β-toxins—most pronouncedly, the external loop IIS3–S4 *(34,35).* Indeed, a single-point mutation (G845N) in IIS3–S4 of rNav1.2 strongly decreased the affinity for β-toxin binding.

It is believed that the local conformation of IIS3–S4 may be important for the interaction of β-toxins, whereas G845N replacement could induce conformational change and thus prevent the binding of β-toxins *(35).* This idea is consistent with the newly cloned scorpion sodium channel (Genebook™ accession number AY322171) which exhibits a unique feature in region IIS3–S4 compared to all known vertebrate and insect sodium channels (two residues are uniquely inserted in IIS3–S4; Fig. 3). This insertion, especially when one is Pro, is believed to disrupt the local conformation and contribute to the adaptive insensitivity of scorpion sodium channel to β-toxins (unpublished results).

Much less information is available regarding the molecular aspects of the interaction between insect-specific β-toxins and insect sodium channels. This may be attributable to the difficulty of expressing insect sodium channels in heterologous systems *(36).* A recent study was initiated using the chimeric channel of rNav1.2, in which DII was replaced by that of the *Drosophila* para channel and analyzed following expression in *Xenopus* oocytes. Notably, similarly to *Drosophila* Para but unlike rNav1.2, the chimera gained sensitivity to excitatory toxin AaHIT, highlighting that DII dictates the selectivity of excitatory toxin *(36).* This is in accordance with the key role of counterpart mammal channel DII in the binding of antimammal β-toxins and the typi-

fication of β-toxin activity of excitatory toxins on insect sodium channels *(22,34,35).* Moreover, the β-toxin TsVII (which is active to either mammals or insects and is capable of binding with high affinity for either mammal or insect sodium channels) competitively inhibits the binding of the excitatory toxins to insect neuronal membranes *(37),* further corroborating that excitatory toxins bind to the equivalent of receptor site-4 on insect sodium channels.

Sequence comparison has revealed that the IIS3–S4 loop is highly similar in insect Para and β-mammal toxin-sensitive VGSCs, and the residue Gly, which is critical for the binding of antimammal β-toxins, is well-conserved in them (Fig. 3). Therefore, Gly at the homologous position is also believed to be crucial for the interaction of excitatory toxins with insect sodium channels. On the other hand, there are subtle, but probably important, difference between insect Para and mammalian channels (Fig. 3). Two consecutive residues (Glu and Gly) near the extracellular end of the IIS3 are well-conserved among insect Para channels and different from mammalian channels. Moreover, Pro782 located in IIS1–S2 of rNav1.2, which also plays a role in binding of β-toxins, is conserved among mammalian isoforms that are sensitive to antimammal β-toxins but is altered to Asp or Asn in insect sodium channels (data not shown). Altogether, these two structural features may be vital in discerning the selectivity of β-toxins to mammalian or insect sodium channels.

Depressant toxins bind to two noninteracting binding sites (a high- and low-affinity binding site) on insect neuronal membranes, in contrast to excitatory toxins that bind to a single class of receptor sites *(37–43).* Competitive binding experiments between these two groups revealed that depressant toxins completely inhibit the binding of excitatory toxins, whereas excitatory toxins compete only for the high-affinity binding site of depressant toxins *(41,42).* It seems receptor site-4, the receptor for excitatory toxins, is the right high-affinity receptor only for depressant toxins. As to the low-affinity binding site for depressant toxins, it remains unidentified. However, the competition of depressant toxin BmKIT2 for the binding of α-like toxin BmKI on cockroach synaptosomes indicates that the low-affinity binding site of depressant toxin may be related to receptor site-3 on insect sodium channels *(7).*

β-like toxins resemble β-mammal toxins as well as depressant toxins in both sequence and activity *(8).* The molecular nature of their specificity remains unknown, but the binding studies may provide some clues. β-like toxins bind specifically to a single class of receptor sites on mammal sodium channels *(9).* Competitive binding assays found that β-like toxins could strongly compete with β-mammal toxins for binding to rat brain synaptosomes *(8,9,44).* Thus, it suggests that β-like toxins are capable of binding to receptor site-4 of mammal VGSCs. Interestingly, β-like toxins were also reported to be capable of inhibiting the binding of α-mammal toxins on rat brain synaptosomes *(8,44).* This may mean that they could also recognize the region related to receptor site-3. On the other hand, β-like toxins could bind to insect synaptosomes with high affinity as well. The binding site of β-like toxins on insect channels is believed to involve receptor site-4, as deduced from their strong competitive inhibition of excitatory and depressant toxins for binding to insect synanptosomes *(8,9,44).* Additionally, similarly to its competitive ability to inhibit the binding of α-mammal toxins to rat brain synaptosomes, BmKAS could inhibit the binding of BmKI to insect synaptosome *(7).* Altogether, we hypothesized that the receptor site for β-like toxins may involve two regions: receptor site-4 and receptor site-3; this is somewhat similar to the recognition site of depressant toxins on insect VGSCs. This finding is not surprising, considering the relative high sequence similarity between these two group toxins (Fig. 4). It must be noted that there is only one binding site on mammalian brain or insect synaptosomes for β-like toxins, and this site is distinct from the two noninteracting binding sites for depressant toxins on insect synaptosomes.

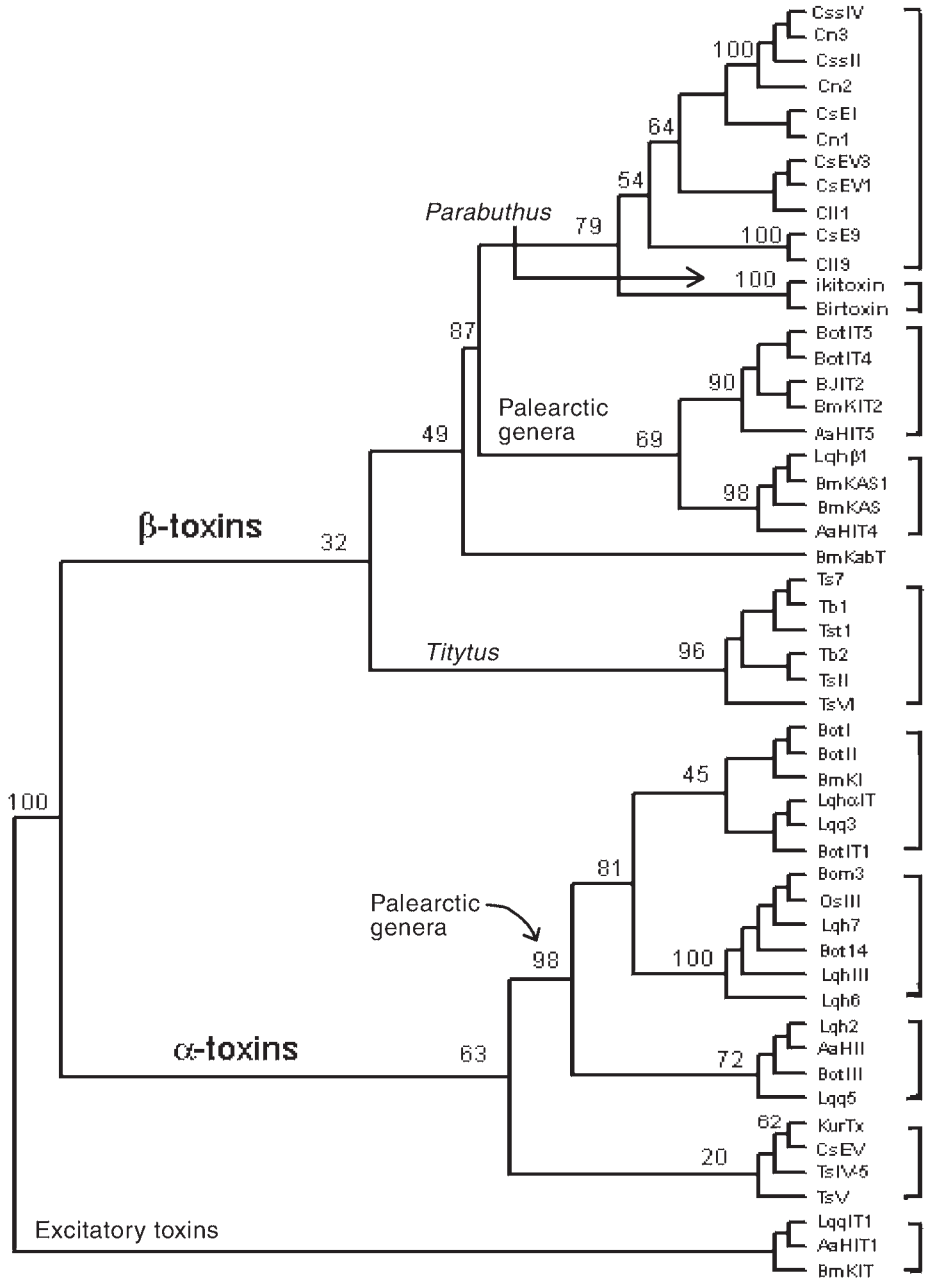

Fig. 4. Phylogenetic tree of scorpion toxins specific for sodium channels. Fifty-two aligned sequences from Fig. 1 were analyzed by maximum-parsimony method, using the PROPARS program of the PHYLIP package. A consensus tree was generated following bootstrap analysis and the final diagram was plotted using the TREE-VIEW program. The number at the nodes indicates the bootstrap value for 100 replications. The tree was rooted using the outgroup excitatory toxins.

Most likely, the two regions form one binding site for β-like toxins but are different in their contribution to the binding. Receptor site-4 is crucial because β-like toxins could strongly inhibit the binding of β-mammal toxins to rat brain synaptosomes or excitatory and depressant toxins to insect synaptosomes and vice versa *(9),* whereas receptor site-3 appears to play only a minor role and is not important for the binding of β-like toxins. Therefore, β-like toxins could inhibit the binding of α-mammal toxin to rat brain synaptosomes and BmKI to insect synaptosomes, whereas BmKI could not inhibit the binding of β-like toxins (BmKAS) *(9).*

There are some β-toxins that display little toxicity, and some even exhibit the activity of α-toxins (Fig. 1). For example, BmKabT could increase the peak sodium current and slow the inactivation phase of sodium channels on rat dorsal root ganglion neurons, behaving similarly to α-toxins despite its high sequence similarity with β-toxins *(45).* Another toxin, Cll9 (which is isolated from *Centruroides limpidus limpidus*), showed little toxicity against insects, crustaceans, or mice upon intraplantar administration *(46).* Electrophysiological recording shows that Cll9 could partially inhibit the Na^+ currents in a reversible manner and slow the inactivation of Na^+ currents in (cultured) rat peripheral ganglia-like α-toxins *(46).* However, detailed analysis found that Cll9 was distinct from the three groups of α-toxins mentioned earlier in that it lacked effect on heterologously expressed rNav1.4 *(46).* CsEv1 to CsEv3, three toxins isolated from the same genus *Centruroides* as Cll9, are weakly active toxins and have also been poorly characterized with α-type activity *(5,47).* However, their 3D structures differ considerably from CsE-V, an α-mammal toxin isolated from the same species *(48–50),* which has more sequence and structural homology with old-world α-toxins *(26).*

Several other 3D structures of β-toxins, such as TsVII, Cn2, Bj-xtrIT, and AaHIT1, have been determined by X-ray crystallography or nuclear magnetic resonance *(51–54).* Structural alignment of the various α- and β-toxins suggests that a key distinguishing feature between the two classes is the length of the loop between the second and third β-strands *(51).* The 3D structures of excitatory toxins reveal a displacement of the fourth disulphide bridge compared to other Na^+ channel-specific scorpion toxins *(52).* Moreover, the C-terminal extension that is typical for this group of toxins is folded into an additional α-helix that contributes to their selectivity for insect VGSCs *(55).* Despite the structural differences between excitatory toxins and other β-mammal toxins, a "hot spot" and an adjacent nonpolar region are spatially conserved between them, indicating that they may constitute a putative "pharmacophore" involved in the interaction of β-toxins with receptor site-4 on VGSCs *(56).*

Evolution of Scorpion Toxins: How Their Selectivity Come About

To date, all Na^+ channel-specific scorpion toxins have been purified only from species belonging to the family Buthidae *(5).* Most of them are 60 to 76 amino acid polypeptides stabilized by four disulfide bridges, with the exception of two toxins, birtoxin and ikitoxin, which contain only 58 residues and three disulfide bridges *(57,58).* Three of the four disulfide bridges, are conserved and buried with α/β-scaffold, whereas the one is conserved in all but displaying a shift in the excitatory toxins *(52).* Moreover, the excitatory toxins have longer sequence lengths (70–76 residues) than others (60–70 residues) (Fig. 1).

To better understand the evolution of the different groups of toxins, we constructed a phylogenetic tree that used a limited number of representative toxins from each pharmacological group. As shown in Fig. 4, the excitatory toxins share low similarity with other groups of toxins and are used as outgroups to yield the rooted phylogenetic tree.

α-toxins have been observed to form a monophyletic group (bootstrap 63%), whereas β-toxins from *Tityus, Centruroides, Parabuthus,* and others are outside this clade. Within the clade of α-toxins, a very strong support is

demonstrated for α-toxins from *Buthus, Androctonus, Leiurus, and Orthochirus* (98%), suggesting that these four genera are phylogenetically close. This is consistent with their biogeographical distribution in that they are very typical for the Palearctic desert (North Africa, Middle East, and Asia) and the phylogeny of Buthidae is determined based on 16S rRNA mitochondrial DNA, which demonstrates a monophyletic Palearctic group of 13 genera *(59).* Furthermore, two sister clades emerge within the clade of α-toxins from Palearctic genera. One contains the α-mammal toxins and the other contains α-insect and α-like toxins (Fig. 4), indicating that the ancestral Palearctic scorpion should have developed the archetype of existing pharmacological distinct groups of α-toxins before its divergence into different genera. This is supported by the fact that each group of α-toxins has been purified or cloned from *Buthus, Androctonus,* and *Leiurus,* which have been well-studied *(5,60).* In contrast, only few α-mammal toxins and no α-insect or α-like toxins have been isolated from *Centruroides, Tityus,* and *Parabuthus* genera *(5,61).* Therefore, α-mammal toxins should be the original α-toxins that present in the ancestral Buthidae scorpions, and the separation of α-toxins may have evolved during the aridification of the Palearctic in the Tertiary Period, which facilitated the radiation of scorpions. In addition to the radiation, scorpions had to deal with the novel environmental pressure; α-mammal toxins alone may not have been sufficient for them to catch and defend. In response, they evolved and gained α-like toxins and α-insect toxins that are effective for insects, mammals, and some other vertebrates. Evolutionary analysis has also suggested that there was an accelerated evolution in the α-toxins that were isolated from *Buthus* genus, indicating positive selection pressure caused by environmental context *(62).*

Although the grouping of β-toxins (not including excitatory toxins) into one monophyletic group is weakly supported (32%), there are two strongly supported separated clades. The first clade is composed of β-toxins, all of which belong to *Tityus* species (96%). The second clade is composed of the β-toxins (not including the atypical β-toxin BmKabT) from *Centrutoides, Parabuthus,* and Paleartic genera (87%). This provides the evidence that *Tityus* diverged from the ancestral Buthidae preceding other genera. When the biogeographical distribution pattern of these genera are considered, the most surprising finding is that the *Centruroides* (North and Central America) is distant from *Tityus* (South America) and, indeed, is closely related to *Parabuthus* (South Africa) and Palearctic genera. A very plausible explanation is the Continental Drift Theory. The Buthidae scorpions originated approximately 350 million years ago (MYA) and were physically separated above 150 MYA upon the splitting of the paleocontinent into "Laurussia" (North America, North Africa, Europe, and Asia) and "Gondwana" (South America and Africa). After the separation of continents, the ancestral Buthidae developed into *Tityus* in South America, whereas in Laurussia, it gave rise to the *Centruroides, Parabuthus,* and Palearctic genera. The *Parabuthus* genus is most likely the result of zoogeographic migration of the Buthidae from Laurussia land into South Africa in more recent times because the β-toxins from *Centrutoides* and *Parabuthus* further form a sister class that is strongly supported (79%). The other sister class contains the β-toxins from the Palearctic genera, which could be divided into two distinct separate groups: depressant toxins (90%) and β-like toxins (98%). The timing of the divergence of these two groups seems to be concurrent with the divergence of α-toxins in the Palearctic genera discussed earlier.

Based on the previous discussions, the phylogenetic tree sustains reliability because the clades or sister clades correlate well with the pharmacological properties of different groups and offers insight into the evolution of them. However, the origin of the toxins remains unknown. Some authors previously suggested that β-like toxins, such as AaHIT4 and BmKAS, may have been the origin deduced from the pharmacological properties of this group *(59,62).*

However, our phylogenetic analysis revealed that this was not true because of their phylogenetic position. Some authors also believed that the two short β-mammal toxins (i.e., birtoxin and ikitoxin) might have been the origin of long-chain toxins *(3,8).* In fact, as shown in the phylogenetic tree, they are unlikely to be the origin, and their peculiar structures most likely resulted from the partial deletion of original long-chain β-toxins, similarly to the previously reported position-specific deletion of original long-chain scorpion toxins *(63).*

In conclusion, natural selective pressure may play an important role in the evolution of distinct group toxins. The diverse selectivity may be consistent with the major differences in the life history and ecology of scorpions between different regions. Biogeographical distribution patterns and functional characteristics of these scorpion toxins could help explain the evolution of scorpion toxins, which, together with the zoogeographical dispersion of the studied genera, may offer insight into the evolution of scorpions.

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