[Frontiers in Bioscience, Landmark, 18, 572-587, January 1, 2013]

Scorpion beta-toxins and voltage-gated sodium channels: interactions and effects

Martha Pedraza Escalona[1], Lourival D. Possani[1]

[1]Department of Molecular Medicine and Bioprocesses, Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico, Avenida Universidad, 2001, Colonia Chamilpa, Apartado Postal 510-3, Cuernavaca 62210, Mexico

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Voltage-gated sodium channel
4. General characteristics of the sodium channel scorpion toxins
4.1. Electrophysiological effects of -ScTxs on VGSCs
4.2. Voltage sensor trapping model
4.3. Structure and bioactive surface of the -ScTxs
4.3.1. Bioactive surface of anti-mammalian -ScTxs
4.3.2. Bioactive surface of anti-insect excitatory -ScTxs
4.3.3. Bioactive surface of anti-depressant -ScTxs
4.3.4. Bioactive surface of anti-mammalian/insect -ScTxs
4.3.5. Functions of Glu15 in the -ScTxs
5. Systemic effects produced by -ScTxs
6. Perspectives
7. Acknowledgements
8. References

1. ABSTRACT

Scorpion beta-toxins (beta-ScTxs) modify the activity of voltage-gated sodium (Na_v) channels, thereby producing neurotoxic effects in diverse organisms. For this reason, beta-ScTxs are essential tools not only for discriminating among different channel sub-types but also for studying the mechanisms of channel gating and the structure-function relationship involved in this process. This review considers both the structural and the functional implications of the beta-ScTxs after they bind to their receptor sites, in accord with their classification into a) anti-mammalian beta-ScTxs, b) anti-insect selective excitatory beta-ScTxs, c) anti-insect selective depressant beta-ScTxs and d) beta-ScTxs active on both insect and mammals Na_v channels. Additionally, the molecular mechanism of toxin action by the "voltage sensor trapping" model is discussed, and the systemic effects produced by these toxins are reviewed.

2. INTRODUCTION

Approximately 1500 species of scorpions, distributed among 18 distinct families, have been described in the tropical and subtropical regions of North and Sahelian Africa, Near- and Middle-East, Asia, Australia, North and South America (1). All scorpions possess a specialized venom-producing apparatus, situated in the last post-abdominal segment (the telson), which contains a pair of glands connected to a stinger (2). Scorpions use the venom for immobilizing their prey or for defending themselves against predators. Their venoms have been studied by classic biochemical separation to describe the functions and chemical structures of their constituent compounds. Scorpion venoms are complex mixtures of neurotoxins (low molecular weight proteins), cardiotoxins, hemolytic toxins, antimicrobial peptides, enzymes (such as hyaluronidase, acetylcholinesterase, phospholipase and metalloproteinases), lipids, nucleotides, mucopolysaccharides and biogenic amines (3, 4).

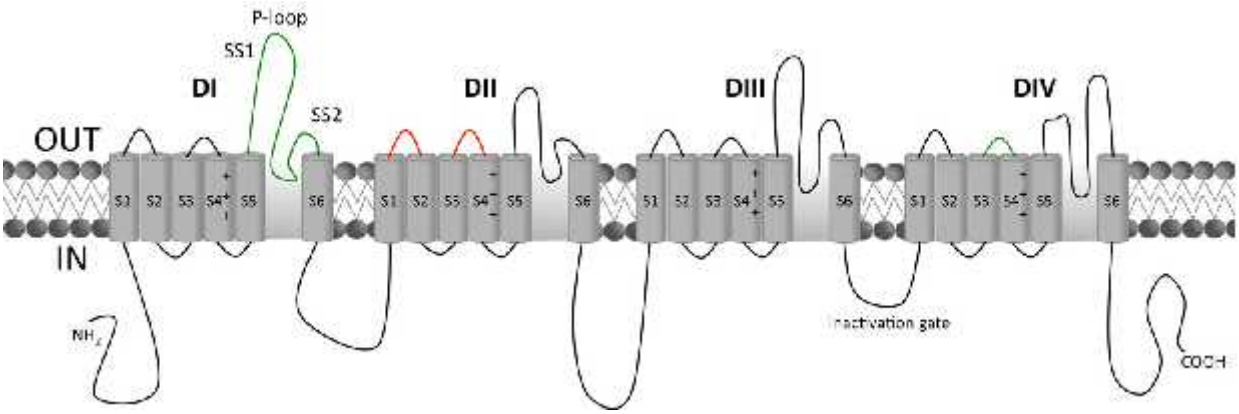

Figure 1. Schematic representation of transmembrane organization of the α-subunit from the voltage-gated sodium channel (VGSC). The α-subunit consists of four homologous domains (DI-DIV), each one containing six transmembrane alpha-helical segments (S1-S6) and one membrane re-entrant segment (P-loop) between the S5 and S6 segments, which is divided into an N-terminal (SS1) and a C-terminal (SS2) fragments. The positively charged S4 segments (+) act as the voltage sensors. The neurotoxin receptor site 3 (for α-ScTxs) includes the DIV S3-S4 extracellular loop (in green). The binding site for the β-ScTxs (site 4) involves the DII S1–S2 and S3–S4 loops (in red).

During 400 million years of evolution, selection has favoured scorpion venoms with peptides (neurotoxins) that block or modify the function of Na^+, K^+, Ca^{2+} and Cl^- ion channels located in the cell membranes of a variety of organisms. (5). These toxic peptides fold into compact disulfide bridge-rich structures that show high potency and target specificity for their receptors. These toxins are principally responsible for the symptoms displayed during the envenomation process. Thus, scorpion toxins can be classified according to their modes of action and their binding sites on different channels. The short-chain toxic peptides (consisting of 28 to 46 amino acids) interfere with the function of potassium ion channels, whereas the long-chain peptides (with 61 to 76 amino acids) modify the gating properties of the sodium channels (reviewed in (6)). Among Na_v channel-specific toxins, there are two widely characterized types, the alpha-toxins (α-ScTxs) and the beta-toxins (β-ScTxs) (7, 8), as discussed below. Whereas the interface of K^+ channel-specific toxins and their interactions with the channels have been extensively characterized (9, 10, 11), much less is known about the interaction surfaces between the Na^+ channel-specific toxins and their receptors. However, in recent years, site-directed mutagenesis studies of Na_v channels and their cognate toxins have revealed important residues involved in this interaction and have provided some evidences of their interacting surfaces (12, 13, 14). In this review, we focus on the β-ScTxs and analyze their molecular characteristics, their modes of action in voltage-gated sodium channels and the effects they produce during the envenomation process.

3. VOLTAGE-GATED SODIUM CHANNELS

Voltage-gated sodium channels (VGSCs) are transmembrane proteins that are responsible for action potential initiation and propagation in neuronal, muscular, skeletal and neuroendocrine cell types (15). Na_v channels are composed of the pore-forming α-subunit (260 kDa) and smaller accessory molecules such as β subunits (the 36-kDa β1/β3 and 33-kDa β2/β4) in mammals or TipE in insects (16, 17). While only nine distinct mammalian sodium channel α-subunits ($Na_v1.1$-1.9) have been described, more than 100 different insect sodium channels may exist due to alternative splicing and RNA editing, although only some have been reported (18). Insect sodium channels are highly conserved but have low identity when compared with mammalian sodium channels (18). The highly glycosylated α-subunits are organized into four homologous but non-identical domains (I-IV), each containing six transmembrane alpha-helical segments (S1-S6) and one membrane re-entrant segment (P-loop) between the S5 and S6 segments; the P-loops are further divided into N-terminal (SS1) and C-terminal (SS2) fragments (Figure 1) (19, 20, 21). The four domains are arrayed around a central pore that is responsible for ion selectivity and is formed by the S5 and S6 segments and the P-loops (22). The channel activation gate is composed of the voltage-sensing domain (S1-S4 segments), whereas the inactivation gate is formed by the intracellular loop-connecting domains III and IV (Figure 1) (23, 24).

Structural studies in potassium channels revealed that in response to membrane depolarization, the activation of VGSCs occurs; the channels open (open state), allowing a selective influx of Na^+ ions through the pore. From the open state, they promptly inactivate (inactive state). When the membrane potential becomes more negative (repolarization), the channels recover from inactivation, reaching a state (closed state) that can be opened by further depolarization (20, 24). All voltage-gated ion channels have voltage sensor paddle motifs (S3b-S4 units) that contain 4 to 7 repeated segments of three positively charged residues (arginines and lysines) followed by two hydrophobic residues (23). In response to channel depolarization, the S4 segment moves its positive charges towards the extracellular face of the membrane by approximately 12 Å with respect to the S3 segment (25) and 15 to 20 Å with respect to the surface of the lipid

bilayer. It also rotates counter-clockwise by 37° along its main axis, allowing activation to occur (18, 26, 27). Additionally, the surface density of the mammalian sodium channels and their biophysical properties may be modulated by the β-subunits (28). The extracellular domain of the β-subunits belongs to the cell adhesion molecule (CAM) motif family and is able to interact with members of the L1 CAM family (29), whereas the cytoplasmic side binds to ankyrin G and B proteins that connect the channel to the cytoskeleton (30).

4. GENERAL CHARACTERISTICS OF THE SODIUM CHANNEL SCORPION TOXINS

As mentioned above, the long-chain scorpion neurotoxins are classified into α- and β-ScTxs depending on the receptor binding site they recognize and their mode of action. The α-ScTxs have been classified into three groups in accordance with their host preference: a) classic α-ScTxs (highly selective for mammalian VGSCs and non-toxic to insects) such as the Aah2 toxin from *Androctonus australis* Hector; b) anti-insect α-ScTxs (highly toxic on insects) such as LqhIT from *Leiurus quinquestriatus hebraeus*; and c) α-like toxins (acting on both mammalian and insect VGSCs) such as BmKI from *Buthus martensi* (31). Rodriguez de la Vega and Possani (32) showed, by probability-based phylogenetic reconstruction, that β-ScTxs can be alternatively classified into four well-supported phylogenetic branches or subclasses: a) anti-mammalian β-toxins exclusively found in scorpions of the genus *Centruroides*, such as Cn2 from *Centruroides noxius* and Css4 from *Centruroides suffusus suffusus* (33, 34); b) β-toxins active on both insect and mammalian Na_v channels, such as Ts1 from *Tityus serrulatus* (35) and Lqhβ1 from *Leiurus quinquestriatus hebraeus* (36); c) anti-insect selective excitatory β-toxins such as AahIT from *Androctonus australis Hector* and Bj-xtrIT from *Hotentota judaica*, which induces contraction paralysis in fly larvae (37, 38, 39); and d) anti-insect selective depressant toxins, which induce flaccid paralysis upon injection, such as LqhIT2 from *Leiurus quinquestriatus hebraeus* (40, 41).

4.1. Electrophysiological effects of -ScTxs on voltage-gated sodium channels

The VGSCs can be distinguished as targets by the selective action of several classes of compounds; they were first identified by the use of antibodies and later by site-directed mutagenesis. A large variety of neurotoxins from the venoms of different organisms can modulate their activity, which occurs through their interaction with seven different toxin-binding sites located into the α-subunits (6, 42, 43, 44). Among the activities that can be modulated by toxins, long-chain scorpion toxins can modify the gating mechanisms of sodium channels (4, 32, 45). The α-ScTxs, found in the venoms of Old World scorpions, interact with channel receptor site 3 located in the S3-S4 extracellular loop in domain IV and in the S5-S6 extracellular linker domain I of Na_v channels (Figure 1), a target shared by sea-anemone toxins and some spider toxins (7, 8, 42, 46, 47). This interaction induces prolonged action potentials by inhibiting the outward translocation of the S4 segment during depolarization, slowing the fast inactivation process of the Na^+ current and generating a persistent current component (6, 48, 49). The venoms of New World scorpions, especially from the genus *Centruroides*, are the major source of β-ScTxs. Upon binding to receptor site 4, these toxins induce a reduction of the peak sodium current amplitude and shift the voltage dependence of Na_v channel activation towards a more hyperpolarized membrane potential (50, 51). With exception of the Ts1 toxin from *Tityus serrulatus* (52, 53, 54), the negative shift occurs after a strong but short depolarizing prepulse that resembles the depolarization during an action potential spike. By following these electrophysiological effects and using chimeric constructions of sodium channels, the location of the receptor site 4 was identified. Cestèle *et al.* (13, 55), using the Css4 toxin from *Centruroides suffusus suffusus*, found that this neurotoxin binds to receptor site in the extracellular loops connecting transmembrane segments S3 and S4 (S3-S4 linker) and the S1 and S2 segments (S1-S2 linker) in domain II of brain $Na_v1.2$ channels; the Css4 toxin also binds lesser to the S5-SS1 loop of domain I and the SS2-S6 loop of domain III (55). Additionally, using chimeric constructions of the four S3b-S4 paddle motifs of $rNa_v1.2$ and fluorescence spectroscopy binding analysis, Campos *et al.* (52) and Bosmans *et al.* (56) found that the Ts1 toxin from *Tityus serrulatus* not only binds the S3-S4 linker of the domain II but also interacts with the S3-S4 linker of domain III, where this interaction has an allosteric effect in the activation on the voltage sensors of domains I and IV of $Na_v1.2$ channels; whereas Leipold *et al.* (12) showed that Tz1 toxin from *Tityus zulianus* binds mainly the SS2 pore region in the domain III of $Na_v1.4$ channels. Surprisingly, it was revealed that β-ScTxs could interact with multiple motifs in the same channel and possess very high specificity, even among toxins belonging to the same subclass. Recently, another electrophysiological effect produced by β-ScTxs was identified. Schiavon *et al.* (57, 58) found that several toxins from the genus *Centruroides* (Cn2 and Cn8 from *Centruroides noxius*, Cll1 and Cll2 from *Centruroides limpidus limpidus* and CssII from *Centruroides suffusus suffusus*) induce a brief current upon repolarization to -60 mV (resurgent current) in HEK cells transfected with the human $Na_v1.6$ channel. It was suggested that this phenomenon occurs predominantly when $Na_v1.6$ channels in the open state are blocked by particles other than the inactivation gate, preventing normal inactivation. Upon repolarization at potentials more positive than -80 mV, the blocked channels transiently open and produce this type of current (59).

4.2. Voltage sensor trapping model

After it was found that the S3-S4 and the adjacent S1-S2 extracellular loops of domain II are the principal components of the receptor site for binding to the β-ScTxs, a voltage sensor trapping model with three steps was proposed to explain the electrophysiological effects produced (13, 55, 60, 61). This model encompasses several possible molecular mechanisms. In the first step, the toxin binds to its receptor site in the inactivated state of the voltage sensor, which produces an extended period of inactivation at negative membrane potentials (62). As a result, a toxin concentration-dependent reduction of the

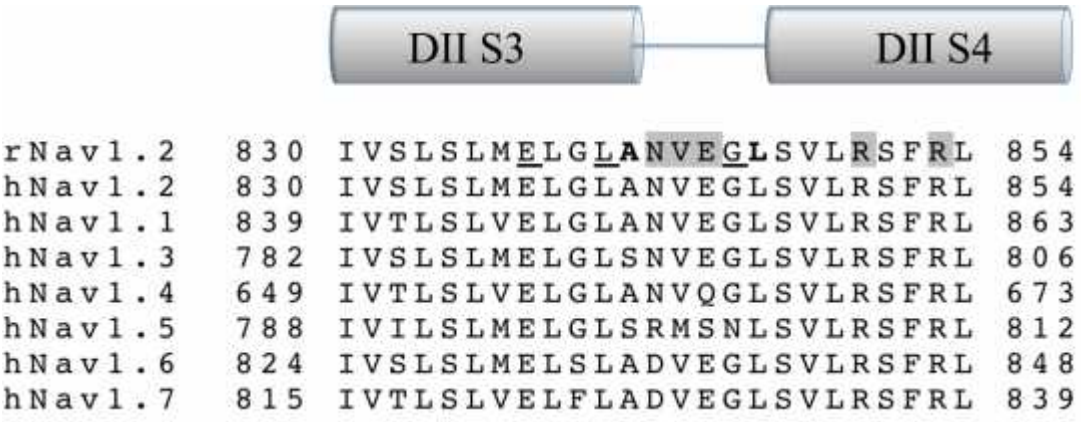

Figure 2. Amino acid sequences alignment of S3-S4 linker of sodium channel domain II. Crucial residues of neurotoxin receptor site 4 are marked in $rNa_v1.2$ in accordance with experimental analyses (reported in 13, 55, 60, 64) and compared with human VGSC that also been used for determination of the toxin activity. Those residues that are involved in the toxin binding affinity and whose substitutions prevent the voltage sensor trapping are underlined (Glu837, Leu840, Gly845). Residues whose substitutions increase the voltage sensor trapping are shallowed (Asn842, Val843, Glu844, Arg850 and Arg853), whereas those whose substitutions that reduce the toxin capacity for the voltage sensor trapping are in bold (Ala841 and Leu846).

peak current under voltage-clamp conditions is observable (13, 55). During the second step, a strong depolarization (induced by a short prepulse) activates the voltage sensor, and segment S4 moves outward and rotates as described above, exposing amino acids that are new target sites for the toxin. In the third step, the activated conformation of the voltage sensor is trapped by tightly bound toxin in a unimolecular concentration-independent reaction, in accordance with the experiments of Cestèle *et al.* 2006 (13). Upon repolarization of the cell membrane, the trapped voltage sensors remain "pre-activated", thereby requiring less activation energy in subsequent depolarizations and shifting the activation threshold to more hyperpolarized potentials (13, 55, 63). Additionally, Schiavon *et al.* (57) hypothesized that those voltage sensors that remain "pre-activated", especially in sodium channels such as $Na_v1.6$, could transiently open and produce resurgent currents when repolarization occurs at a more positive potential than -80 mV. Thus, the presence of resurgent currents is also supported by the voltage sensor trapping model.

Several interesting reports have shown that specific mutations distributed throughout the receptor binding site on $Na_v1.2$ channels can change the Css4 toxin-binding affinity and modulate the voltage sensor trapping mechanism. First, a Gly845Asn substitution, located in the DII/S3-S4 linker (Figure 2), decreased the toxin-binding affinity by 13-fold and completely prevented voltage-sensor trapping, even when high concentrations of toxin were tested; the effect of the toxin on peak-current was retained, although with lower affinity (55). Furthermore, residues located in close proximity to Gly845 represent a functional hotspot with high-relevance (Figure 2). For instance, mutations neutralizing the two outermost arginines gating charges located in the S4 segment, Arg850Gln and Arg853Gln, or substituting the residues Ans842Arg, Val843Ala and Glu844Asn strongly enhanced voltage sensor trapping (13, 60, 61, 63, 64). This finding is probably explained by more favourable outward movement through the membrane electric field even in the absence of a depolarizing prepulse, as in the case of Arg853Gln and Leu854Cys (13, 60). Moreover, substitutions in Glu837 (by Gln, Arg and Cys) and Leu840 (by Cys) reduced the toxin binding affinity and abolished its effect on activation, whereas substitutions of Ala841 (by Asn) and Leu846 (by Ala) reduced the capacity of the toxin for voltage sensor trapping (13, 63, 64). All of these results suggest that the entire DIIS3-S4 extracellular loop is directly involved with both toxin binding affinity and with functional effects (see Figure 2). Additional studies showed that substitutions of Glu779 and Pro782, located in the DII/S1-S2 extracellular loop, also reduce the toxin binding affinity (13).

4.3. Structure and bioactive surface of the β-ScTxs

Despite their diversity, all of the β-ScTxs share an essential three-dimensional (3D) α/β scaffold, according to the structures reported in the Protein Data Bank (53, 65, 66, 67). The scaffold comprises an α-helix and three or four-stranded anti-parallel β-sheets (βαββ core) that are connected by solvent-exposed irregular loops and stabilized by four spatially conserved disulfide bridges. In the majority of the 3D structures, two elongated grooves appear at the intersection of the core and the N and C-terminal regions: the first groove precedes the α-helix (N-groove) and the other groove is located in the penultimate loop of the C-tail (C-groove) (Figure 3B). An extensive array of studies has suggested four common functional sections of the β-ScTxs: i) a central "pharmacophore region" involved in the receptor binding site and composed of a negatively charged residue (Glu28 in Css4, Glu26 in Lqhβ1, Glu30 in Bj-xtrIT and Glu24 in LqhIT2) located in the α-helix that is flanked by several solvent-exposed hydrophobic residues (41, 66, 68, 69, 70); ii) a "solvent-exposed aromatic cluster" that is critical for activity and is mainly located in the β_2 and β_3 strands (69, 70); iii) some residues located in the centre of the N-groove region that are involved in voltage sensor trapping (13, 61, 69); and iv)

-scorpion toxin interacting surfaces

Table 1. Motifs of beta-ScTxs subclasses

-ScTxs subclass	Motif	Hits	Function experimentally determined
Anti-mammalian	KxGYxVx(4)GCKxxCxxLGxNxxCxxECx(9)GYCYxFxCxCxxLx(7)PLxxKxC	28	11
Anti-insect excitatory	KKxGxxxDxxGKxxECx(4,9)YCxxxCTKVxYAxxGYCCxxxCYCxGLxDDKx(9)KxxCD	12	6
Anti-insect depressant	DGY[IP][KR]x(2)[DNS]GC[KR]x[ADS]Cx(2,3)Nx(2,3)Cx(3)Cx(3)G[AG]x[FY]GYCW[AGT]WGLACWC[EQ][GN]LP[ADE]	27	18
Anti-mammalian/ insect	GCK[FLV]xC[FV][IP][NR][NP][AES][EGS]x[CGN]	11	7

The hits are the numbers of sequences of toxins that contain the submitted pattern obtained by including the information list of the UniProt database using ScanProsite (62). Amino acids are represented by the one letter code. The letter "x" means any amino acid, and the numbers between parentheses mean the number of residues in that particular space of the sequence. The anti-mammalian and anti-insect excitatory subclasses were taken from Tan *et al.* (76). The last two sub-classes are from this communication. Those hits of toxins with experimentally determined function are shown in the last column.

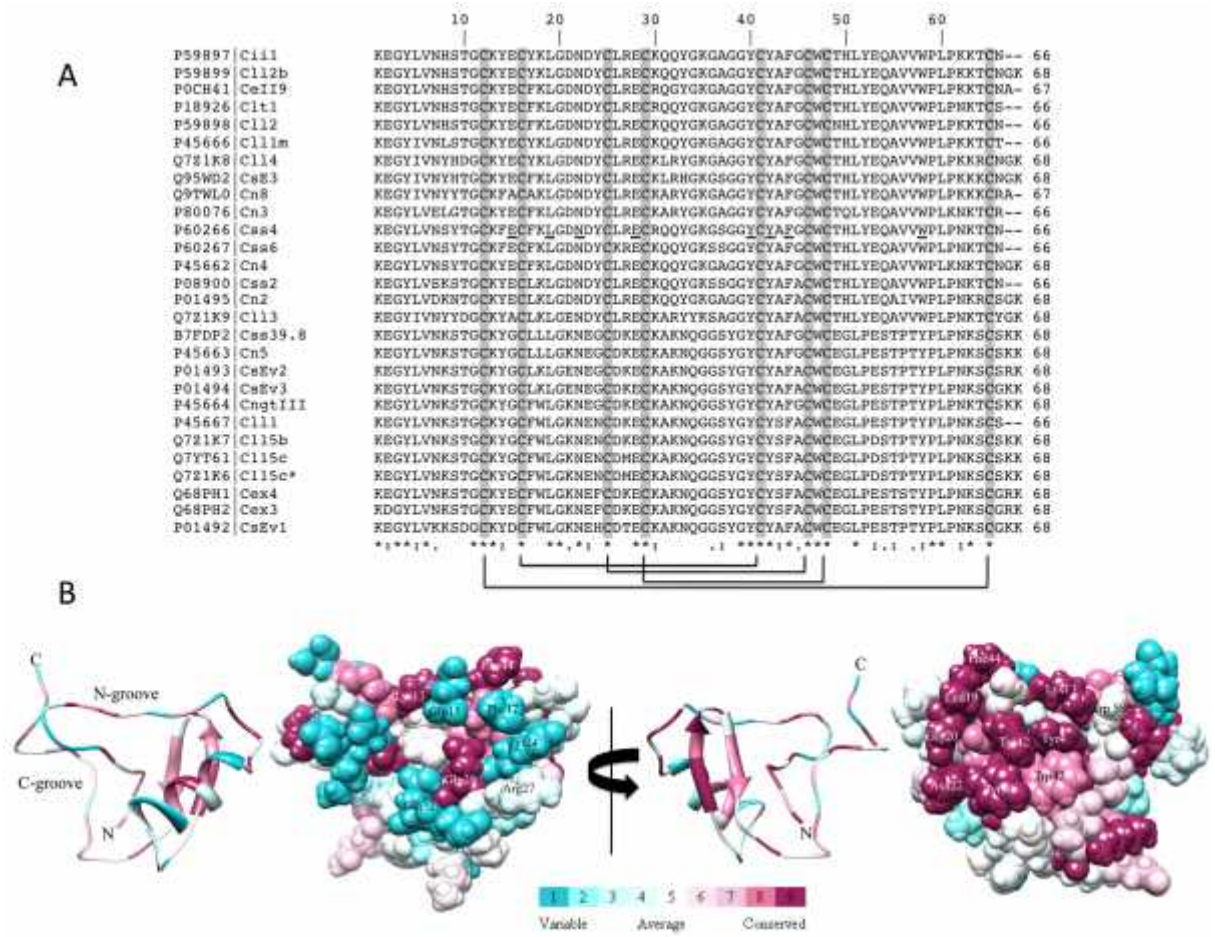

Figure 3. Analysis of the functional regions in anti-mammalian β-ScTxs. A) Multiple sequences alignment of the anti-mammalian β-ScTxs. The alignment, using ClustalW, was performed with 28 sequences reported in the Uniprot database in accordance with its motif (Table 1), with the accession numbers followed by abbreviated names. Cystein residues are shallowed, the pattern of disulfide bridges is indicated in the low part of the figure. Furthermore, residues of the Css4 toxin involved in directed-site mutagenesis are underlined (70). B) ConSurf analysis for the 3D structure of the anti-mammalian β-ScTxs is presented using a space-filled and ribbon diagrams. The amino acids are colored by their conservation grades using the color-coding bar, with cyan-through-dark magenta indicating variable-through-conserved. The figure reveals some functionally important regions that are highly conserved in this subclass of β-ScTxs. The run was carried out using the PDB 1CN2 (anti-mammalian β-ScTx, Cn2) (67). Some residues are labeled in accordance with the Cn2 numbering. The picture was generated using the UCSF Chimera (80) script output by ConSurf (79) and supervised manually. The two views are shown rotated every 180°.

-scorpion toxin interacting surfaces

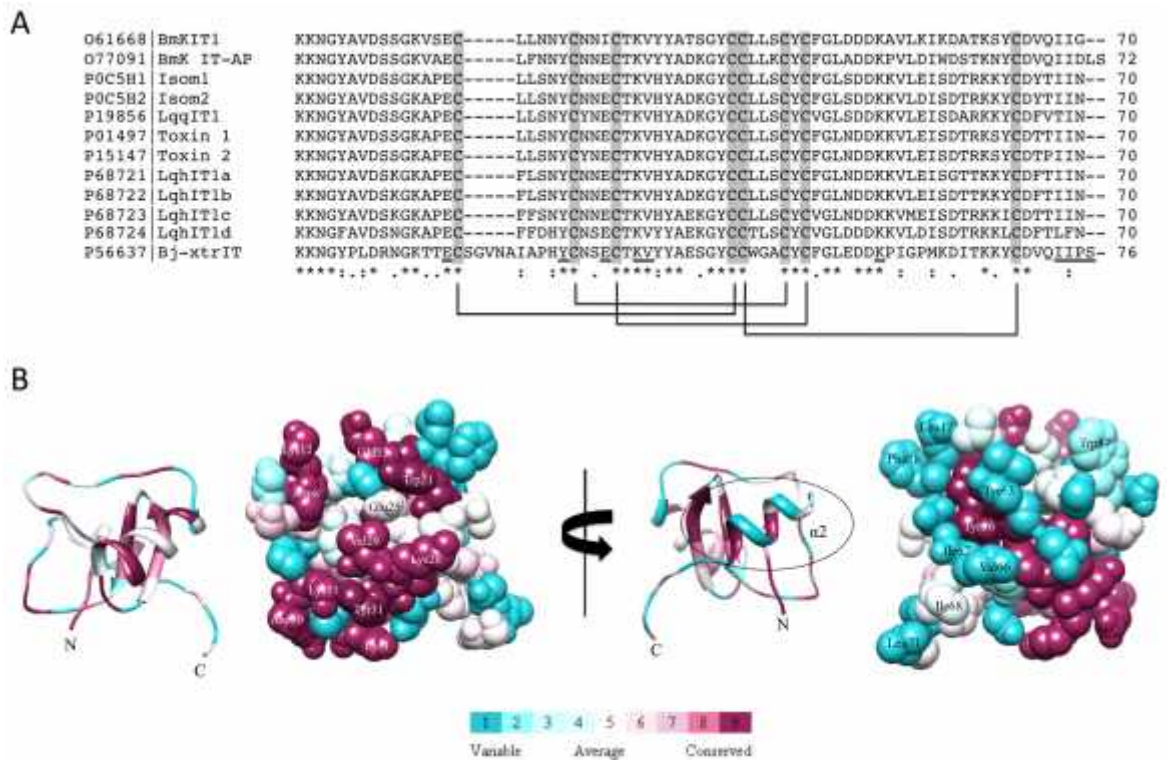

Figure 4. Analysis of the functional regions in anti-insect excitatory β-ScTxs. A) Multiple sequences alignment of the anti-insect excitatory β-ScTxs. The alignment, using ClustalW, was performed with 12 sequences reported in the Uniprot database in accordance with its motif (Table 1), with the accession numbers followed by abbreviated names. Cystein residues are shallowed and the pattern of disulfide bridges is indicated in the low part of the figure. Furthermore, residues of the Bmk IT-AP and Bj-xtrIT involved in directed-site mutagenesis are in underlined. B) ConSurf analysis for the 3D structure of the anti-insect excitatory β-ScTxs. The amino acids are colored by their conservation grades using the color-coding bar, with cyan-through-dark magenta indicating variable-through-conserved. The run was carried out using the PDB 1T0Z (anti-insect excitatory β-ScTx, BmK IT-AP (65) and the alignment made in ClustalW. Some residues are labeled in accordance with the Bmk IT-AP numbering. The two views are shown rotated every 180°.

C-terminal residues that increase the binding affinity to the receptor binding site (66, 72, 73, 74). Nevertheless, the structural differences among these toxins are located in variable loops connecting the main secondary structure elements of the toxin core (75) and in the arrangement of the C-tail, which is confined mainly by the fourth disulfide bridge; this is where anti-insect excitatory β-ScTxs contain an additional α-helix (Figure 4B)(65). Moreover, the relatively small size of these toxins magnifies the potential differences, even among a single subclass, in the surface topology derived from the primary sequence and from conformational changes. Therefore, each toxin has particular patterns for the location of functional regions, which explains the high selectivity for a specific target. For instance, solvent-exposed aromatic cluster has been identified in the loop connecting the β2 and β3 strands of the anti-mammalian Css4 toxin (Figure 3B, right), whereas such cluster is located in the C-terminal region of the anti-insect excitatory Bj-xtrIT toxin (Figure 4B, right) (65, 69, 73).

To identify the functional regions of the β-ScTxs, Tan *et al.* (76) established anti-insect excitatory and anti-mammalian β-ScTxs motifs (Table 1). Using a similar strategy, we have generated anti-insect depressant and anti-mammal/insect β-ScTxs motifs (Table 1). A consensus sequence for each motif was produced in ScanProsite (77), using toxins reported in the UniProt database (http://www.uniprot.org/). Each motif was confirmed in the PATTINPROT web server, without mismatches and with 100% similarity (78). Table 1 shows the different motifs and the total hits number that present such pattern. Furthermore, a 3D pattern was created by the Consurf server (79) and visualized in UCSF Chimera (80), taking into account the alignment of sequences for each motif and at least one structure reported in the Protein Data Bank for each subclass: 1CN2 (anti-mammalian β-ScTxs, Cn2) (67), 2I61 (anti-insect depressant β-ScTxs, LqhIT2) (66), 1NPI (anti-mammal/ insect β-ScTxs, Ts1) (53) and 1T0Z (anti-insect excitatory β-ScTxs, BmK IT-AP) (65). The 3D functional motifs for each subclass of β-ScTxs will be discussed in greater detail below.

4.3.1. Bioactive surface of anti-mammalian -ScTxs

One subclass of β-ScTxs, from 66 to 68 amino acids in length and exclusively found in scorpions of genus

Centruroides, is highly toxic to mammals and modulates the activation of mammalian brain sodium channels. These toxins mainly interact with the rat $Na_v1.1$, $Na_v1.2$, $Na_v1.4$ and $Na_v1.6$ channels, but they have no effect on the $Na_v1.5$ channels (54, 55). There are currently 28 sequences reported in the Uniprot database with this pattern (Figure 3A). Site-directed mutation studies conducted with the Css4 toxin from *Centruroides suffusus suffusus* showed that the pharmacophore region of this toxin comprises Glu28 flanked by Phe17, Tyr24, Arg27 and Gln32 (70). This functional region is present in all the anti-mammalian β-ScTxs, where the unique highly conserved residue is the Glu28 (see Figure 3B, left); the importance of this residue is confirmed by reduction in the receptor binding affinity when Glu28 is substituted in Css4 toxin (70). The substitutions Phe17Ala, Tyr24Ala and Gln32Ala also decreased the binding affinity, as did neutralization of the positive charge at position 27 (70). The alignment in the Figure 3A shows that the residues located at position 17 maintain their hydrophobic profile and the predominantly residue in position 24 is Tyr, whereas residues located at positions 27 and 32 show high variability, suggesting that these natural modifications could be involved in selectivity for different sodium channel isoforms. For example, Css4 induces a prepulse-dependent negative shift on human Nav1.1, Nav1.2 and Nav1.6 channels, while Cn2 only affects human Nav1.6 channels (57); this differential activity warrants further investigation. However, other residues such as Leu19, Asn22 (located in the loop preceding the α-helix), Tyr40, Tyr42 and Phe44 (located in β2 and β3) are highly conserved in all of the sequences (see Figure 3B, right). These residues form a hydrophobic cluster that is critical for the binding affinity in the Css4 toxin and are present in all of the anti-mammalian β-ScTxs (70). Furthermore, additional mutagenic studies have revealed the importance of Glu15 for the toxic activity of this subclass of toxins because substitution with Arg inhibited voltage sensor trapping by the Css4 toxin (70, 81). Nevertheless, this position is not conserved among these toxins, although nearby residues (Lys13 and Phe44) are completely conserved (Figure 3B, left). These results indicate that small changes in the nature of this region could affect the capacity of the toxin to produce the toxic effect (see section 4.3.5). The residues involved in the structural integrity of the Css4 toxin, such as Tyr4, Trp47 and Lys63 (70), are also highly conserved in all the anti-mammal β-ScTxs (see Figure 3B, right), which corroborates their importance in the bioactive surface.

Recently, Canul-Tec *et al.* (82) reported for the first time the 3D X-ray diffraction structure of a crystalline complex between toxin Cn2 of *Centruroides noxius* Hoffmann and a neutralizing fragment of human antibody that recognizes the toxin with nanomolar affinity. They found that two segments, Lys13 to Leu19 and Tyr42 to Ala 45, which are located around the α-helix and the region that connects β_2 with β_3, form the central region of the Cn2 epitope (82). Most of these residues are essential for sodium channel binding, as already discussed above, and are highly conserved in anti-mammalian β-ScTxs. This result may explain the neutralization capacity of the antibody for Cn2, and the possibility to broad action against other toxins of this subclass.

Finally, some reports indicate the importance of the C-terminal region in the receptor binding affinity. Cohen *et al.* (70) found that the substitution Trp58Ala had a detrimental effect on the receptor binding affinity; the aromatic profile of this position is conserved throughout the anti-mammalian β-ScTxs (Figure 3A). Estrada *et al.* (83) found that the amidated C-terminal in Css2 toxin has an important role in the interaction with its receptor, since the recombinant C-terminal carboxylated form decreased the receptor-binding affinity by 15-fold. Surprisingly, the affinity can be recovered by the addition of positive charges at this region (72).

4.3.2. Bioactive surface of anti-insect excitatory -ScTxs

The anti-insect excitatory β-ScTxs are composed of 70-76 amino acids, where one of their disulfide bridges has a different organization pattern compared to other long-chain toxins (see the alignment in Figure 4) (73). These toxins induce contraction paralysis in blowfly larvae caused by repetitive activity of motor nerves resulting from the activation of sodium currents at a more negative membrane potential (39, 73). When anti-insect excitatory β-ScTxs are structurally compared with anti-mammalian β-ScTxs, the former have besides an additional one-and-half turn α-helix in the C-terminal residues (α2) (Figure 4B, right). The Bj-xtrIT toxin from the Israeli black scorpion *B. judaicus* contains an additional five-residue α-helix (α0) formed by Ser-Gly-Val-Asn-Ala (in positions 17-21) after the first conserved Cys16 (Figure 4A) (65); this α-helix has been identified as a specific segment in the receptor binding site of this toxin (69). Despite these variations, the most critical residues implicated both in structural integrity and function adopt conformations similar to the other β-ScTxs.

Site-directed mutagenesis studies showed that the "pharmacophore region" comprises Glu25 flanked by hydrophobic residues such as Tyr21 and Val29 for the Bmk IT-AP toxin and Glu30 surrounded by Val19, Asn20, Tyr26 and Val34 in the case of the Bj-xtrIT toxin (68, 69). Substitutions at the Glu30 of the Bj-xtrIT toxin to Gln, Leu, Arg or Asp severely reduced the binding affinity but only moderately reduced toxicity (39). Similarly, substitutions in Tyr26 and Val34 (or Tyr21 and Val29 in the Bmk IT-AP sequence) strongly affected the binding affinity (69). The complete "pharmacophore region" of anti-insect excitatory β-ScTxs is highly conserved (see Figure 4B, left), with the exception of the BmKIT1 toxin, which contains Ile at position 25. Interestingly, in close proximity to Val29 in Bmk IT-AP (Val 34 in Bj-xtrIT), there are five highly conserved residues (Lys1, Lys28, Tyr31, Asp50 and Lys51) (Figure 4B, left); two of them, Lys28 and Lys51, are involved in the activity of the toxin (39). Substitution with Arg, Ala or Phe of the highly conserved residue Glu15, which is exposed to the solvent with the highly conserved Lys12 in the N-groove (Figure 4B, left), had a detrimental effect on the toxicity to insects but not on the binding affinity; however, substitution of Glu15 to Gln had a detrimental effect on the binding affinity (39, 70). This controversial result will be further discussed in section 4.3.5. Additionally, mutations in the hydrophobic cluster located in the C-terminal region of the Bj-xtrIT toxin

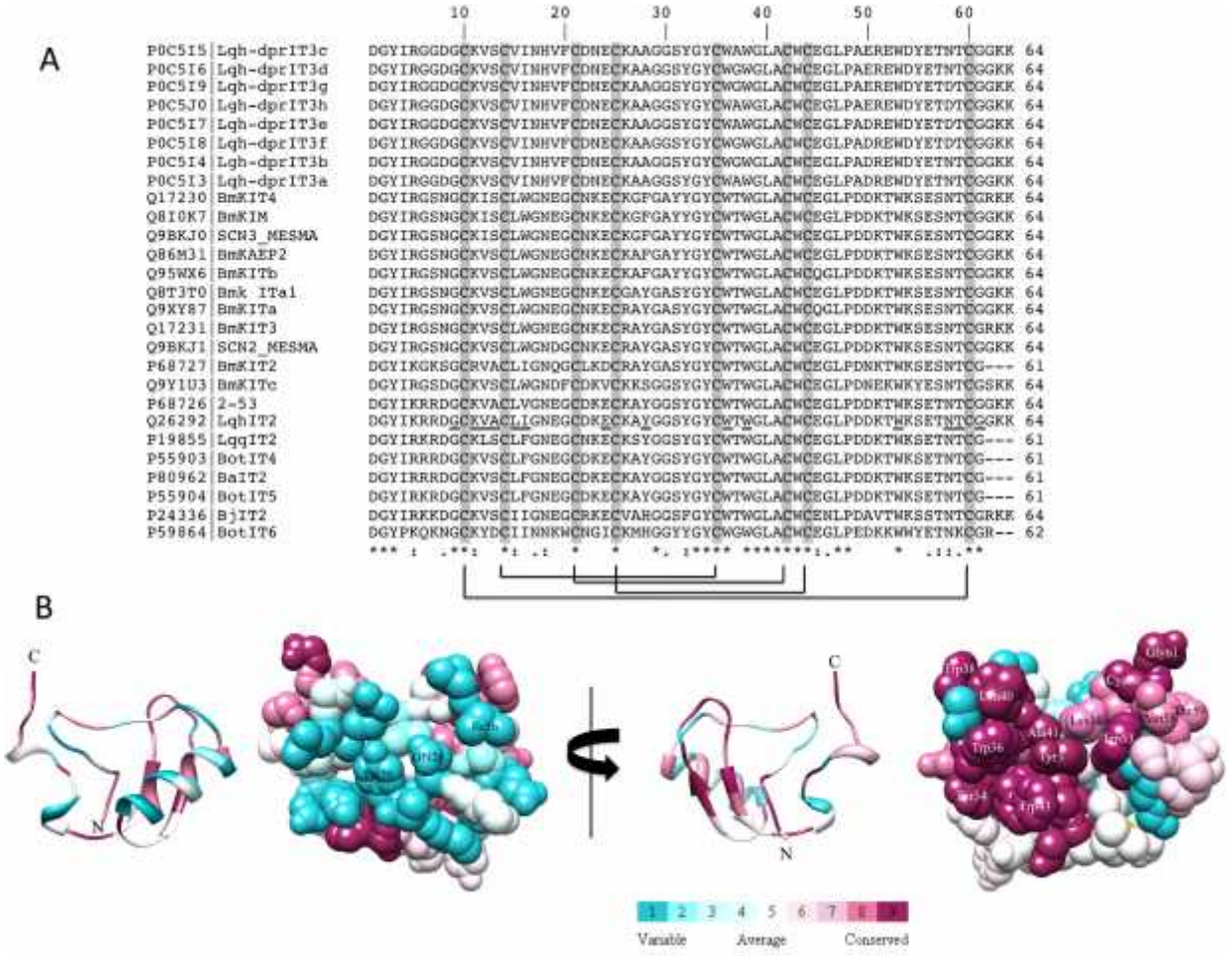

Figure 5. Analysis of the functional regions in anti-insect depressant β-ScTxs. A) Multiple sequences alignment of the anti-depressant β-ScTxs. The alignment, using ClustalW, was performed with 27 sequences reported in the Uniprot database in accordance with its motif (Table 1), the accession numbers followed by abbreviated names are showed. Cystein residues are shallowed, the pattern of disulfide bridges is indicated in the low part of the figure. Furthermore, residues of the LqhIT2 toxin involved in directed-site mutagenesis are underlined. B) ConSurf analysis for the 3D structure of the anti-insect depressant β-ScTxs. The run was carried out using the PDB 2I61 (anti-insect depressant β-ScTx, LqhIT2) (66) and the alignment made in ClustalW. Some residues are labeled in accordance with the LqhIT2 numbering. The two views are shown rotated every 180°.

(Val71, Gln72, Ile73 and Ile74) (Figure 4B, right) had a strong effect on its toxicity (73), these residues are variable among anti-insect excitatory β-toxins, although maintain a hydrophobic profile. This site is a bioactive surface specific for this subclass of toxins.

4.3.3 Bioactive surface of anti-insect depressant β-ScTxs

Anti-insect depressant β-ScTxs induce flaccid paralysis upon injection into insects and suppress evoked action potentials in isolated axonal preparations due to strong depolarization (84). The depressant β-ScTxs (61-64 amino acids in length) competitively inhibit the binding of anti-insect excitatory β-toxins to insect neuronal Na_v channels (68). Structural and mutagenesis studies conducted with the LqhIT2 toxin from *Leiurus quinquestriatus hebraeus* based on toxicity to blowfly larvae assigned the Glu24 flanked by Ile16 and Tyr28 as the "pharmacophore region" because substitutions in these residues (Glu24Arg, Ile16Ala and Tyr28Ala) had detrimental effects on the apparent binding affinity (66). Surprisingly, Glu24Ala/Asn substitutions abolished the activity of two anti-insect depressant β-toxins, LqhIT2 and Lqh-dprIT3, on cockroach neuronal membranes and *Drosophila melanogaster* ($DmNa_v1$) channels; however, these recombinant toxins showed a markedly increased affinity for receptor site 4 on rat skeletal muscle sodium channels when a long prepulse was applied (14). This remarkable result may indicate that a single amino acid change can affect the specificity of these toxins.

According to the alignment using 27 sequences from this subclass of toxins (Figure 5A), Glu24 is conserved in almost 90% of the sequences; the exceptions are the BmKIT2 toxin with Asp, the BmKITc toxin with Val and the BotIT6 toxin with Ile. In the region surrounding the negative charged residue in the α-helix,

hydrophobic and aromatic residues occupy position 16, where Ile and Trp predominate (Figure 5A and 5B, left). The residues located at position 28 are principally hydrophobic and aromatic (Ala, Phe, Tyr); only three sequences contain polar or charged residues. These results show that the hydrophobicity of the pharmacophore region is preserved, although a detailed experimental analysis of the functional changes produced by natural variations might explain alterations in the binding affinity and selectivity for the receptor site.

Nevertheless, two important regions are highly conserved in all of the anti-insect depressant β-ScTxs. The first is located in the C-terminal region near the N-groove and comprises Gly9, Lys11, Trp53, Asn58, Thr59 and Gly61 (Figure 5B, right). Site-directed mutations in LqhIT2 toxin showed the importance of these residues in the receptor binding-site (66). Interestingly, Trp53 and Trp58 participate in the formation of one cavity, which might be occupied by a polar or negatively charged side chain of the receptor. The substitutions Trp53Val and Asn58Ala reduced the binding affinity by several orders of magnitude, which could define both residues as forming a specific bioactive surface in this subclass of toxins (66). The second highly conserved region is a very hydrophobic patch that includes Tyr3, Tyr34, Trp36, Trp38, Leu40 and Trp43 (Figure 5B, left). Mutagenic studies established that substitutions in Trp36 (Ala) and Trp38 (Ala) have strong effects on the binding affinity because these residues are involved in the structural integrity of the anti-depressant β-ScTxs. In contrast, the mutants Tyr3Phe and Tyr34Ala showed little influence on the receptor binding affinity (66). The highly conserved residues that have not yet been studied may represent excellent targets for future mutagenic analysis.

The residue involved with voltage sensor trapping by this subclass of toxins, Ala13, is located in the middle of the N-groove. Although the alignment shows that this residue is not conserved in the anti-insect depressant β-ScTxs (Figure 5A), it has a unique role in toxic activity because the substitution Ala13Trp in the LqhIT2 toxin increased its ability to shift the voltage dependence of activation in $DmNa_v1$ channels (66).

4.3.4. Bioactive surface of anti-mammalian/insect -ScTxs

Anti-mammalian/insect long-chain neurotoxins have the capacity to compete with both anti-mammalian β-ScTxs and anti-insect excitatory and depressant β-ScTxs (68, 85, 86). This property indicates that such toxins recognize common features of the receptor sites of insect and mammalian sodium channels. Among these toxins, Ts1 (also called Ts7 or TsVII or Tsγ) from *Tityus serrulatus* has unique characteristics. It does not require a depolarizing prepulse to completely affect the sodium channel, and it can interact with multiple channel paddle motifs (52, 56). Structural analysis of this toxin shows some regions in common with other β-ScTxs (53). These regions include the pharmacophore region, composed of Glu26 flanked by the hydrophobic residues Leu13, Tyr22 and Ile29 (Figure 6B, left); the solvent-exposed aromatic cluster, including Tyr4, Tyr36, Trp39, Tyr43 and Tyr45; and the Trp54 in the C-terminal region that resembles Trp58 in the anti-mammalian β-ScTxs (Figure 6B, right). In accordance with the sequence alignment, the pharmacophore region is highly variable in this subclass (see Figure 6A). Nevertheless, a highly conserved region enriched for aromatic and hydrophobic residues, including Tyr4, Ile17, Tyr 36, Ala41, Trp 39, and to a lesser degree Tyr43 and Tyr45, is observed in all of the analyzed sequences (see Figure 6B, right). The fact that this hydrophobic patch is conserved in the majority of β-ScTxs may implicate this region in the recognition of both insect and mammalian sodium channels. Near the hydrophobic region, there is a very highly conserved cavity formed by Trp54 and Lys12, whose importance in Ts1 toxicity has been confirmed (see Figure 6B, right) (87).

4.3.5. Functions of Glu15 in the -ScTxs

Previously, it was shown that Glu15, located in the middle of the N-groove (Figure 3B, left), has a unique role in the toxic activity of the β-ScTxs, as its substitution with a positively charged residue (Glu15 by Arg or Gln) retains almost normal binding affinity to neuronal preparations but can indeed abolish both the anti-mammalian toxic activity of Css4 and Css2 from *C. suffusus suffusus* (70, 82) and the anti-insect toxicity of Bj-xtrIT from *B. judaicus* (57). Recently, Karbat *et al.* (61) revealed that the Glu15Arg mutant of Css4 has a very weak voltage sensor trapping activity that is rapidly reversed upon repolarization. For this reason, it was proposed that Glu15 interacts with the voltage sensor when it emerges from the membrane upon depolarization, and only at this point is the voltage sensor trapped (61, 71). Furthermore, some results indicate that the identity of the residue located at position 15 can also modulate the binding affinity and selectivity towards sodium channels. It was observed that the majority of substitutions in this residue, with the exception of Arg, increase the affinity of the toxin for sodium channels (61, 66). For example, the mutant Glu15Ala, in Css4 as appears in the LqhIT2 toxin, maintains the negative shift and a higher binding affinity for rat brain neuronal membranes (66). This finding may explain why there are many toxins with high toxicity that do not contain a positively charged residue at this position (36, 66, 88, 89). This unexpected result suggests that the Glu15 has been conserved throughout evolution because it is crucial for toxic activity despite its negative effect on toxin affinity. Surprisingly, the Glu15Ala mutant of Css4 can induce contraction paralysis in blowfly larvae and shift the activation threshold to more hyperpolarized potentials in $DmNa_v1$ channels, where it is normally inactive (61, 66). Additionally, the broad effects of this residue on both activity and binding affinity might be due to rearrangements that are induced in the N-groove region during the interaction with receptor site 4. Recently, this possibility was bolstered by the findings of Canul-Tec *et al.* (82), who reported that the Cn2 toxin undergoes significant rearrangements in Lys13 and in the segment between residues Lys18 and Asp21 after interaction with its ligand-antibody.

-scorpion toxin interacting surfaces

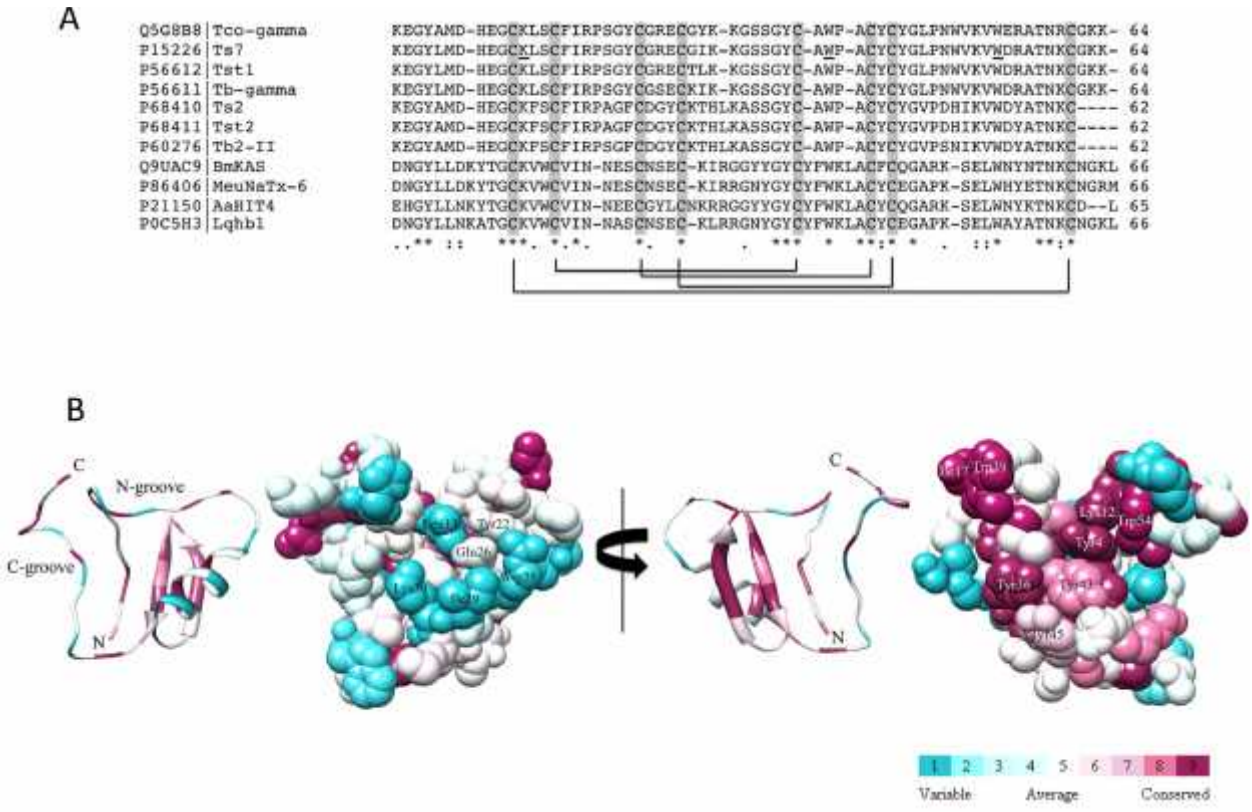

Figure 6. Analysis of the functional regions in anti-mammalian/insect β-ScTxs. A) Multiple sequences alignment of the anti-mammalian/insect β-ScTxs. A Clustal W alignment was performed with 11 sequences reported in the Uniprot database in accordance with its motif (Table 1), the accession numbers followed by abbreviated names are showed. Cystein residues are shallowed, the pattern of disulfide bridges is indicated in the low part of the figure. Furthermore, residues of the Ts1 (Ts7) toxin involved in binding affinity by chemical modifications experiments are underlined (87). B) ConSurf analysis for the 3D structure of the anti-insect depressant β-ScTxs. The run was carried out using the PDB 1NPI (anti-mammal/ insect β-ScTx, Ts1) (53) and the alignment made in ClustalW. Some residues are labeled in accordance with the Ts1 (Ts7) numbering. The two views are shown rotated every 180°.

5. SYSTEMIC EFFECTS PRODUCED BY -ScTxs

Approximately 50 species of scorpions from Buthidae family generate venom with adverse clinical effects in humans (1). The severity of the systemic effects produced by these species is dependent on the composition and potency of that particular venom, and the conditions of the patient, where children under 5 years old and elderly people are more affected (1, 4). Although, there are many components in the scorpion venom, the neurotoxins are the principal responsible for the symptoms in the envenomation process, due to persistent depolarization produced in autonomic nerves resulting in massive release of neurotransmitters that causes cascades of physiological effects (90). The clinical manifestations following scorpion stings, clearly demonstrated a dysfunction of the peripheral rather than the central nervous system (91). Initially, scorpions envenomated victims have a transient excess of cholinergic tone at the neuromuscular junction resulting in hyper-salivation, lachrymation, urinary incontinence, defecation, gastroenteritis, and emesis also high arterial blood pressure, that in some cases causes cerebral damage, edema and ischemia (92-94); the subsequent norepinephrine release causing tachycardia, hypertension, hyperpyrexia, myocardial depression, and pulmonary edema (94). Additionally, signs like mydriasis, nystagmus, dysphagia, restlessness, confusion, agitation and ataxia are due to either the release of catecholamine from the adrenal and noradrenergic nerve terminals or the release of acetylcholine from postganglionic parasympathetic neurons (95).

Various interesting studies have shown that the long-chain scorpion neurotoxins promote release of different autonomic neurotransmitters. For instance, the Tityustoxin, an α-ScTx from the venom of the scorpion *Tityus serrulatus*, induces release of dopamine in rat brain prefrontal cortical slices (96), whereas the Makatoxin I from *Buthus martensi* Karsch induces the release of noradrenalin (97). In the case of β-ScTxs, several studies carried out with the Ts1 toxin showed that it induces an increase in the overall sodium and calcium ion internal concentrations even at low doses (98), causing a significant increment of the presynaptic release of neurotransmitters,

which includes gamma-amino butyric acid (99), acetylcholine (100), dopamine (101) and glutamate (98). This toxin also activates calcium channels, increasing the uptake of Ca^{2+} in synaptosomes (102). The obtention of more information in this area will permit to compare the possible different effects between α- and β-ScTxs. Furthermore, it has been observed that multiple organ dysfunctions are produced by inflammatory response towards different β-ScTxs. For example, *in vitro* and *in vivo* studies performed with Ts1 and Cn2 showed an increment of several proinflammatory cytokines (in shorter times of exposition) and anti-inflammatory cytokines (after longer times of application) (103-106). The induction of phagocytosis and vacuoles formation, the decoy of spreading and fluctuations of the internal calcium concentrations are also observed (107). Nevertheless, many secondary events following the scorpion envenomation suggest a direct effect produced by toxins or other components into different organs, such as heart, kidneys, lungs and secretory glands (108-111). For example, the *Tityus serrulatus* venom induced cardiovascular manifestations including high blood pressure accompanied by arrhythmias in atrio-ventricular conduction blockade that may lead to cardiovascular collapse due to myocardial involvement (112). It was suggested that "antarease", a metalloprotease purified from *Tityus serrulatus* venom decreased the pancreatic secretion by cleavage of vesicle-associated membrane proteins (113).

It seems well established that the primary molecular events taking place after a scorpion sting is the recognition of ion-channels, which causes a cascade of physiological effects, discussed above. However, it is also clear that secondary molecular events are associated to the physiological effect of scorpion toxins, which are not fully characterized yet, and need more research to fully clarify these intoxication symptoms caused by scorpion venom components.

6. PERSPECTIVES

The electrophysiological effects produced by β-ScTxs in VGSCs are independent because of different kinetic patterns (58); this finding suggests that the β-ScTxs make use of different structural segments to achieve their functions. These critical bioactive surfaces are derived from different regions of the primary sequence, where we have emphasized the presence of several highly conserved aromatic residues implicated in both the structural integrity and the binding affinity of the toxins. These residues may explain the ability of the β-ScTxs to compete for receptor site 4 of different sodium channels, although the interaction among each toxin subclass with subtypes of sodium channels must be determined. Furthermore, the high variability surrounding the Glu in the pharmacophore region located in the centre of the α-helix suggests that these natural modifications may be involved in the selectivity for different sodium channels. We have shown that small differences in the surface topology can influence the degree of toxin-channel interaction, which involves a combination of electrostatic, hydrophobic and hydrogen-bonding interactions; there is also the possibility that the β-ScTxs have an induced fit after their interaction with sodium channels. These changes allow toxins to differentiate among sodium channel sub-types and to expand their repertoire of functional actions based on the voltage sensor trapping model. Moreover, *in silico* studies of docking between the Css4 toxin and $Na_v1.2$ showed that the toxin-interacting residues fit tightly into the crevice between the S1-S2 and S3-S4 helical hairpins of domain II of the sodium channel (13, 64). These results revealed putative close interactions that have been confirmed by mutagenesis studies to be important for toxin binding and action. Analyses of the conserved regions have been undertaken, and some specific regions of each subclass of toxins have been studied, but many studies of substitutions in the loops and turns are still needed to understand their roles in toxin function. The use of double mutants, in both channels and toxins, will surely allow further identification and confirmation of interacting surfaces. With the arrival of the 'omic' era, transcriptomic and proteomic studies will certainly shed light on the composition and diversity of toxins present in venoms from different scorpion species. These new techniques will identify numerous components, including those with unknown functions, and will help advance our understanding of the complicated mechanism of intoxication that occurs during envenomation after a scorpion sting. It has been demonstrated that scorpion stings, if treated with anti-venoms (horse $F(ab)'_2$ fragments) immediately after the accident, have ensured 100% recovery; after longer periods (more than 2 hours) of exposure to the effects of scorpion venoms, survival is uncertain, even if anti-venom is eventually used (114). These findings suggest that after the primary events, which are well defined by the direct contact of toxins with the ion channels, other secondary molecular events occur causing irreversible biological alterations of the scorpion sting victim. These secondary events need to be studied by the novel "omic" techniques and approaches, which will certainly open new avenues for future research in this area.

7. ACKNOWLEDGMENTS

The authors thank Dr. Rita Restano-Cassulini for critically reading of the manuscript. The fellowship received by MPE and the grant IN204110 given to LDP laboratory from the *Dirección General de Asuntos del Personal Académico* – UNAM is greatly acknowledged. Similarly, support received from the *Instituto Bioclón S.A. de C.V.* is recognized by the authors.

8. REFERENCES

1. Chippaux J.P. and M. Goyffon. Epidemiology of scorpionism: a global appraisal. *Acta Trop*. 107, 71-9 (2008)

2. Zeh D.W. The Biology of Scorpions. Polis G. A. Ed. Stanford University Press, Stanford, CA (1990)

3. Possani L.D., B. Becerril, M. Delepierre, J. Tytgat. Scorpion toxins specific for Na+-channels. *Eur J Biochem* 264, 287-300 (1999)

4. Tuuri R.E. and S. Reynolds. Scorpion envenomation and antivenom therapy. *Pediatr Emerg Care* 27, 667-72 (2011)

5. Brownell P., G.Polis. Scorpion Biology and Research-Introduction. Ed. Oxford Univiversity Press, New York (2001)

6. Catterall W.A., S. Cestèle, V. Yarov-Yarovoy, F.H. Yu, K. Konoki, T. Scheuer. Voltage-gated ion channels and gating modifier toxins. *Toxicon* 49, 124-41 (2007)

7. Jover E., F. Couraud, H. Rochat. Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. *Biochem Biophys Res Commun* 95, 1607-14 (1980)

8. Couraud F., E. Jover, J.M. Dubois, H. Rochat. Two types of scorpion receptor sites, one related to the activation, the other to the inactivation of the action potential sodium channel. *Toxicon* 20, 9-16 (1982)

9. Imredy J. P. and R. MacKinnon. Energetic and structural interactions between delta-dendrotoxin and a voltage-gated potassium channel., *J. Mol. Biol 296*, 1283-1294 (2000)

10. Ruta V., R. MacKinnon. Localization of the voltage-sensor toxin receptor on KvAP. *Biochemistry* 43, 10071-9 (2004)

11. Rodríguez de la Vega R. C., E. Merino, B. Becerril, L. D.Possani. Novel interactions between K^+ channels and scorpion toxins. *Trends in Pharmacological Sciences* 24,222-227 (2003).

12. Leipold E., A. Hansel, A. Borges, S.H. Heinemann. Subtype specificity of scorpion beta-toxin Tz1 interaction with voltage-gated sodium channels is determined by the pore loop of domain 3. *Mol Pharmacol* 70, 340-7 (2006)

13. Cestèle S., V. Yarov-Yarovoy, Y. Qu, F. Sampieri, T. Scheuer, W.A. Catterall. Structure and function of the voltage sensor of sodium channels probed by a beta-scorpion toxin. *J Biol Chem* 281,21332-44 (2006)

14. Cohen L., Y. Troub, M. Turkov, N. Gilles, N. Ilan, M. Benveniste, D. Gordon, M. Gurevitz. Mammalian skeletal muscle voltage-gated sodium channels are affected by scorpion depressant "insect-selective" toxins when preconditioned. *Mol Pharmacol* 72, 1220-7 (2007)

15. Diaz D., D.M. Delgadillo, E. Hernández-Gallegos, M.E. Ramírez-Domínguez, L.M. Hinojosa, C.S. Ortiz, J. Berumen, J. Camacho and J.C. Gomora. Functional expression of voltage-gated sodium channels in primary cultures of human cervical cancer. *J Cell Physiol* 210, 469-78 (2007)

16. Catterall W.A., A.L. Goldin, S.G. Waxman. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev* 57, 397-409 (2005)

17. Dong K. Insect sodium channels and insecticide resistance. *Invert Neurosci* 7, 17-30 (2007)

18. King G.F., P. Escoubas, G.M. Nicholson. Peptides toxins that selectively target insect $Na_{(V)}$ and $Ca_{(V)}$ channels. *Channels (Austin)* 2, 100-16 (2008).

19. Noda M., S. Shimizu, T. Tanabe, T. Takai, T, Kayano, T. Ikeda, H. Takahashi, H. Nakayama, Y. Kanaoka, N. Minamino. Primary structure of Electrophorus electricus sodium channel deduced from cDNA sequence. *Nature* 312, 121-7 (1984)

20. Duclohier H. Structure-function Studies on the voltage-gated sodium channel. *Biochim Biophys Acta*. 1788, 2374-9 (2009)

21. Cestèle S., W.A. Catterall. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. *Biochimie* 82, 883-92 (2000)

22. Ragsdale D.S., J.C. McPhee, T. Scheuer, W.A. Catterall. Molecular determinants of state-dependent block of Na+ channels by local anesthetics. *Science* 265, 1724-8 (1994)

23. Stühmer W., F. Conti, H. Suzuki, X.D. Wang, M. Noda, N. Yahagi, H. Kubo, S. Numa. Structural parts involved in activation and inactivation of the sodium channel. *Nature* 339, 597-603 (1989)

24. West J.W., D.E. Patton, T. Scheuer, Y. Wang, A.L. Goldin, W.A. Catterall. A cluster of hydrophobic amino acids residues required for fast Na(+)-channel inactivation. *Proc Natl Acad Sci U S A*. 89,10910-4. (1992)

25. Broomand A. and F. Elinder. Large-scale movement within the voltage-sensor paddle of a potassium channel-support for a helical-screw motion. *Neuron* 59, 770-7 (2008)

26. Lewis A., V. Jogini, L. Blachowicz, M. Lainé, B. Roux. Atomic constraints between the voltage sensor and the pore domain in a voltage-gated K+ channel of known structure. *J Gen Physiol* 131, 549-61 (2008)

27. Catterall W.A. Ion channel voltage sensors: structure, function, and pathophysiology. *Neuron* 67, 915-28 (2010)

28. Qu Y., R. Curtis, D. Lawson, K. Gilbride, P. Ge, P.S. DiStefano, I. Silos-Santiago, W.A. Catterall, T. Scheuer. Differential modulation of sodium channel gating and persistent sodium currents by the beta1, beta2 and beta3 subunits. *Mol Cell Neurosci* 18, 570-80 (2001)

29. Leterrier C., A. Brachet, M.P. Fache, B. Dargent. Voltage-gated sodium channel organization in neurons: protein interactions and trafficking pathways. *Neurosci Lett* 486, 92-100 (2010)

30. Kaczmarek L.K. Non-conducting functions of voltage-gated ion channels. *Nat Rev Neurosci* 7, 761-71 (2006)

31. Gordon D., I. Karbat, N. Ilan, L. Cohen, R. Kahn, N. Gilles, K. Dong, W. Stühmer, J. Tytgat, M. Gurevitz. The

differential preference of scorpion alpha-toxins for insect or mammalian sodium channels: implications for improved insect control. *Toxicon* 49, 452-472 (2007)

32. Rodríguez de la Vega R.C., L.D. Possani. Overview of scorpion toxins specific for Na+ channels and related peptides: biodiversity, structure-function relationships and evolution. *Toxicon* 46, 831-44 (2005)

33. Vazquez A., J.V. Tapia, W.K. Eliason, B.M. Martin, F. Lebreton, M. Delepierre, L.D. Possani, B. Becerril. Cloning and characterization of the cDNAs encoding Na+ channel-specific toxins 1 and 2 of the scorpion *Centruroides noxius* Hoffman. *Toxicon* 33, 1161-70 (1995)

34. Martin M.F., L.G. García y Pérez, M. el Ayeb, C. Kopeyan, G. Bechis, E. Jover, H. Rochat. Purification and chemical and biological characterizations of seven toxins from the Mexican scorpion, *Centruroides suffusus suffusus. J Biol Chem* 262, 4452-9 (1987)

35. Possani L.D., B.M. Martin, I. Svendsen, G.S. Rode, B.W. Erickson. Scorpion toxins form Centruroides noxius and Tityus serrulatus. Primary structures and sequence comparison by metric analysis. Biochem 229, 739-50 (1985)

36. Gordon D., N. Ilan, N. Zilberberg, N. Giles, D. Urbach, L. Cohen, I. Karbat, O. Froy, A. Gaathon, R.G. Kallen, M. Benveniste, M. Gurevitz. An 'Old World' scorpion beta-toxin that recognizes both insect and mammalian sodium channels. Eur J Biochem 270, 2663-70 (2003)

37. Pelhate M. and E. Zlotkin. Actions of insect toxin and other toxins derived form the venom of the scorpion Androctonus australis on isolated giant axons of the cockroach (Periplaneta americana). J Exp Biol 97, 67-77 (1982)

38. Zlotkin E., D. Kadouri, D. Gordon, M. Pelhate, M.F. Martin, H. Rochat. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. *Arch Biochem Biophys* 240, 877-87 (1985)

39. Froy O., N. Zilberberg, D. Gordon, M. Turkov, N. Gilles, M. Stankiewicz, M. Pelhate, E. Loret, D.A. Oren, B. Shaanan, M. Gurevitz. The putative bioactive surface of insect-selective scorpion excitatory neurotoxins. *J Biol Chem* 274, 5769-76 (1999)

40. Zuo X.P., Y.H. Ji. Molecular mechanism of scorpion neurotoxins acting on sodium channels: insight into their diverse selectivity. *Mol Neurobiol* 30, 265-78 (2004)

41. Gurevitz M., I. Karbat, L. Cohen, N. Ilan, R. Kahn, M. Turkov, M. Stankiewicz, W. Stühmer, K. Dong, D. Gordon. The insecticidal potencial of scorpion beta-toxins. *Toxicon.* 49, 473-89 (2007)

42. Catterall W.A. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels. *Neuron* 26, 13-25 (2000)

43. Dutertre S., R.J. Lewis. Use of venom peptides to probe ion channel structure and function. *J Biol Chem* 285, 13315-20 (2010)

44. Restrepo-Angulo I., A. De Vizcaya-Ruiz, J. Camacho. Ion channels in toxicology. *J Appl Toxicol* 30, 497-512 (2010)

45. Possani L.D., E. Merino, M. Corona, F. Bolivar, B. Becerril. Peptides and genes coding for scorpion toxins that affect ion-channels. *Biochimie* 82, 861-8 (2000)

46. Wheeler K.P., D.D. Watt, M. Lazdunski. Classification of Na channel receptors specific for various scorpion toxins. *Pflugers Arch* 397, 164-5 (1983)

47. Roger J.C., Y. Qu, T.N. Tanada, T. Scheuer, W.A. Catterall. Molecular determinants of high affinity binding of alpha-scorpion toxin and sea anemone toxin in the S3-S4 extracellular loop in domain IV of the Na^+ channel alpha subunit. *J Biol Chem* 271, 15950-15962 (1996)

48. Bosmans F., J. Tytgat. Voltage-gated sodium channel modulation by scorpion alpha-toxins. *Toxicon* 49, 142-58 (2006)

49. Ulbricht W. Sodium channel inactivation: Molecular determinants and modulation. *Physiol Rev* 85, 1271-1301 (2005)

50. Cahalan M.D. Modification of sodium channel gating in frog myelinated nerve fibres by Centruroides sculpturatus scorpion venom. *J Physiol* 244, 511-34 (1975)

51. Meves H., N. Rubly, D.D. Watt. Effect of toxins isolated from the venom of the scorpion Centruroides sculpturatus on the Na currents of the node of Ranvier. Pflugers Arch 393, 56-62 (1982)

52. Campos F.V., B. Chanda, P.S. Beirão, F. Bezanilla. Beta-scorpion toxin modifies gating transitions in all four voltage sensors of the sodium channel. *J Gen Physiol* 130, 257-68 (2007)

53. Pinheiro C.B., S. Marangoni, M.H. Toyama, I. Polikarpov. Structural analysis of *Tityus serrulatus* Ts1 neurotoxin at atomic resolution: insights into interactions with Na+ channels. *Acta Crystallogr, Sect D* 59, 405-415 (2003)

54. Marcotte P., L.Q. Chen, R.G. Kallen, M. Chahine. Effects of *Tityus serrulatus* scorpion toxin gamma on voltage-gated Na+ channels. Circ Res 80, 363-9 (1997)

55. Cestèle S., Y. Qu, J.C. Rogers, H. Rochat, T. Scheuer, W.A. Catterall. Voltage sensor-trapping: enhanced activation of sodium channels by beta-scorpion toxin bound to the S3-S4 loop in domain II. *Neuron* 21, 919-31 (1998)

56. Bosmans F., M.F. Martin-Eauclaire, K.J. Swartz. Deconstructing voltage sensor function and pharmacology in sodium channels. *Nature* 456, 202-8 (2008)

57. Schiavon E., T. Sacco, R.R. Cassulini, G. Gurrola, F. Tempia, L.D. Possani, E. Wanke. Resurgent current and voltage sensor trapping enhanced activation by a beta-scorpion toxin solely in Nav1.6 channel. Significance in mice Purkinje neurons. *J Biol Chem* 281, 20326-37 (2006)

58. Schiavon E., M. Pedraza-Escalona, G.B. Gurrola, T. Olamendi-Portugal, G. Corzo, E. Wanke, L.D. Possani. Negative-shift activation, current reduction and resurgent currents induced by β-toxins from *Centruroides scorpions* in sodium channels. *Toxicon* 59, 283-293 (2012)

59. Cannon S.C. and B.P. Bean. Sodium channels gone wild: resurgent current from neuronal and muscle channelopathies. *J Clin Invest* 120, 80-3 (2010)

60. Cestèle S., T. Scheuer, M. Mantegazza, H. Rochat, W.A. Catterall. Neutralization of gating charges in domain II of the sodium channel alpha subunit enhances voltage-sensor trapping by a beta-scorpion toxin. *J Gen Physiol* 118, 291-302 (2001)

61. Karbat I., N. Ilan, J.Z. Zhang, L. Cohen, R. Kahn, M. Benveniste, T. Scheuer, W.A. Catterall, D. Gordon, M. Gurevitz. Partial agonist and antagonist activities of a mutant scorpion beta-toxin on sodium channels. *J Biol Chem* 285, 30531-8 (2010)

62. Kaneda M., Y. Oyama, Y. Ikemoto, N. Akaike. Scorpion toxin prolongs an inactivation phase of the voltage-dependent sodium current in rat isolated single hippocampal neurons. *Brain Res* 487, 192-5 (1989)

63. Zhang Z., I. Karbat, L. Cohen, T. Scheuer, D. Gordon, M. Gurevitz, W. Catterall. Mapping the -scorpion toxin receptor site on voltage-gated sodium channels. *Biophys J* 98, 108a-109a (2010)

64. Zhang J.Z., V. Yarov-Yarovoy, T. Scheuer, I. Karbat, L. Cohen, D. Gordon, M. Gurevitz, W.A. Catterall. Structure-function map of the receptor site for β-scorpion toxins in domain II of voltage-gated sodium channels. *J Biol Chem* 286, 33641-51 (2011)

65. Li C., R.J. Guan, Y. Xiang, Y. Zhang, D.C. Wang. Structure of an excitatory insect-specific toxin with an analgesic effect on mammals from the scorpion *Buthus martensii* Karsch. *Acta Crystallogr D Biol Crystallogr* 61, 14-21 (2005)

66. Karbat I., M. Turkov, L. Cohen, R. Kahn, D. Gordon, M. Gurevitz, F. Frolow. X-ray structure and mutagenesis of the scorpion depressant toxin LqhIT2 reveals key determinants crucial for activity and anti-insect selectivity. *J Mol Biol* 366, 586-601 (2007)

67. Pintar A., L. D. Possani, M. Delepierre. Solution structure of toxin 2 from *Centruroides noxius* Hoffmann, a beta-scorpion neurotoxin acting on sodium channels. *J Mol Biol* 287, 359-367 (1999)

68. Gordon D., H. Moskowitz, M. Eitan, C. Warner, W.A. Catterall, E. Zlotkin. Localization of receptor sites for insect-selective toxins on sodium channels by site-directed antibodies. *Biochemistry* 31, 7622-8 (1992)

69. Cohen L., I. Karbat, N. Gilles, O. Froy, G. Corzo, R. Angelovici, D. Gordon, M. Gurevitz. Dissection of the functional surface of the anti-insect excitatory toxin illuminates a putative “hot spot” common to all scorpion beta-toxins affecting Na+ channels. *J Biol Chem* 279, 8206-11 (2004)

70. Cohen L., I. Karbat, N. Gilles, N. Ilan, M. Benveniste, D. Gordon, M. Gurevitz. Common features in the surface of scorpion beta-toxins and elements that confer specificity for insect and mammalian voltage-gated sodium channels. *J Biol Chem* 280, 5045-53 (2005)

71. Karbat I., F. Frolow, O. Froy, N. Gilles, L. Cohen, M. Turkov, D. Gordon, M. Gurevitz. Molecular basis of the high insecticidal potency of scorpion alpha-toxins. *J Biol Chem* 279, 31679-86 (2004)

72. Estrada G., R. Restano-Cassulini, E. Ortiz, L.D. Possani, G. Corzo. Addition of positive charges at the C-terminal peptide region of CssII, a mammalian scorpion peptide toxin, improves its affinity for sodium channels Nav1.6. *Peptides* 32, 75-9 (2011)

73. Oren D.A., O. Froy, E. Amit, N. Kleinberger-Doron, M. Gurevitz, B. Shaanan. An excitatory scorpion toxin with a distinctive feature: an additional alpha helix at the C terminus and its implications for interaction with insect sodium channels. *Structure* 6, 1095-1103 (1998)

74. Gurevitz M., D. Gordon, S. Ben-Natan, M. Turkov, O. Froy. Diversification of neurotoxins by C-tail 'wiggling': a scorpion recipe for survival. *FASEB J* 15, 1201-5 (2001)

75. Cohen L., N. Lipstein, I. Karbat, N. Ilan, N. Gilles, R. Kahn, D. Gordon, M. Gurevitz. Miniaturization of scorpion beta-toxins uncovers a putative ancestral surface of interaction with voltage-gated sodium channels. *J Biol Chem* 283, 15169-76 (2008)

76. Tan P.T.J., S. Ranganathan, V. Brusic. Deduction of functional peptide motifs in scorpion toxins. *J Peptide Sci* 12, 420-427 (2006)

77. de Castro E., C.J.A. Sigrist, A. Gattiker, V. Bulliard, P.S. Langendijk-Genevaux, E. Gasteiger, A. Bairoch, N. Hulo. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. *Nucleic Acids Res* 34, W362-W365 (2006)

78. Combet C., C. Blanchet, C. Geourjon, G. Deléage. NPS@: network protein sequence analysis. Trends Biochem Sci 25, 147-50 (2000)

79. Glaser F., T. Pupko, I. Paz, R.E. Bell, D. Bechor-Shental. E. Martz, N. Ben-Tal. Consurf: identification of

functional regions in proteins by surface-mapping of phylogenetic information. *Bioinformatics* 19, 163-4 (2003)

80. Pettersen E.F., T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin. UCSF Chimera—a visualization system for exploratory research and analysis. *J Comput Chem* 25, 1605-12 (2004)

81. Hernández-Salgado K., G. Estrada, A. Olvera, F.I. Coronas, L.D. Possani, G. Corzo. Heterologous expressed toxic and non-toxic peptide variants of toxin CssII are capable to produce neutralizing antibodies against the venom of the scorpion *Centruroides suffusus suffusus. Immunol Lett* 125, 93-9 (2009)

82. Canul-Tec J.C., L. Riaño-Umbarila, E. Rudiño-Piñera, B. Becerril, L.D. Possani, A. Torres-Larios. Structural basis of neutralization of the major toxic component from the scorpion *Centruroides noxius* Hoffmann by a human-derived single-chain antibody fragment. *J Biol Chem* 286, 20892-900 (2011)

83. Estrada G., B.I. García, E. Schiavon, E. Ortiz, S. Cestèle, E. Wanke, L.D. Possani, G. Corzo. Four disulfide-bridged scorpion beta neurotoxin CssII: heterologous expression and proper folding *in vitro*. *Biochim Biophys Acta* 1770, 1161-8. (2007)

84. Strugatsky D., N. Zilberberg, M. Stankiewicz, N. Ilan, M. Turkov, L. Cohen, M. Pelhate, N. Gilles, D. Gordon, M. Gurevitz. Genetic polymorphism and expression of a highly potent scorpion depressant toxin enable refinement of the effects on insect Na channels and illuminate the key role of Asn-58. *Biochemistry* 44, 9179-87 (2005)

85. Barhanin J., M. Ildefonse, O. Rougier, S.V. Sampaio, J.R. Giglio, M. Lazdunski. *Tityus* gamma toxin, a high affinity effector of the Na+ channel in muscle, with a selectivity for channels in the surface membrane. *Pflugers Arch* 400, 22-7 (1984)

86. De Lima M.E., M.F. Martin, C.R. Diniz, H. Rochat. *Tityus serrulatus* toxin VII bears pharmacological properties of both beta-toxin and insect toxin from scorpion venoms. *Biochem Biophys Res Commun* 139, 296-302 (1986)

87. Hassani O., P. Mansuelle, S. Cestèle, M. Bourdeaux, H. Rochat, F. Sampiere. Role of lysine and tryptophan residues in the biological activity of toxin VII (Ts gamma) from the scorpion *Tityus serrulatus. Eu J Biochem* 260, 76-86 (1999)

88. Borges A., M.J. Alfonzo, C.C. García, N.J. Winand, E. Leipold, S.H. Heinemann. Isolation, molecular cloning and functional characterization of a novel beta-toxin from the Venezuelan scorpion, *Tityus zulianus. Toxicon* 43, 671-84 (2004)

89. Bosmans F., M.F. Martin-Eauclaire, J. Tytgat. The depressant scorpion neurotoxin LqqIT2 selectively modulates the insect voltage-gated sodium channel. *Toxicon* 45, 501-7 (2005)

90. Gwee M.C.E., S. Nirthanan, H.E. Khoo, P. Gopalakrishnakone, R.M. Kini, L.S. Cheah. Autonomic effects of some scorpion venoms and toxins. *Clin Exp Pharmacol Physiol* 29, 795-801 (2002)

91. Clot-Faybesse O., C. Devaux, H. Rochat, R. Guieu. *In vivo* neurotoxicity of *Androctonus australis* hector scorpion venom: evidence that the supra-thoracic nervous system is not implicated in the clinical manifestations. *Toxicon* 39, 1003-7 (2001)

92. Bahloul M., A. Chaari, N. Khlaf-Bouaziz, L. Hergafi, H. Ksibi, H. Kallel, A. Chaari, H. Chelly, C. Ben Hamida, N. Rekik, M. Bouaziz. Gastrointestinal manifestations in severe scorpion envenomation. *Gastroenterol Clin Biol* 29, 1001-5 (2005)

93. Goyffon M., M. Vachon, N. Broglio. Epidemiological and clinical characteristics of the scorpion envenomation in Tunisia. *Toxicon* 20, 337-44 (1982)

94. Ismail M. The scorpion envenoming syndrome. *Toxicon* 33, 825-58 (1995)

95. Massensini A.R., T. Moraes-Santos, M.V. Gomez, M.A. Romano-Silva. Alpha and beta-scorpion toxins evoke glutamate release from rat cortical synaptosomes with different effects on [Na+]i and [Ca2+]i. *Neuropharmacology* 37, 289-97 (1998)

96. Fernandes V.M., A.R. Massensini, M.A. Prado, M.A. Silva, T. Moraes-Santos, M.V. Gomez. Effects of alpha-scorpion toxin, tityustoxin on the release of [3H] dopamine of rat brain prefrontal cortical slices. *Neurochem Int* 44, 91-97 (2004)

97. Gong J.P., R.M. Kini, M.C.E. Gwee, P.Gopalakrishnakone, M.C.M. Chung. Makatoxin I, a novel toxin isolated from the venom of the scorpion *Buthus martensi* Karsch, exhibits nitrergic actions. *J Biol Chem* 272, 8320-4 (1997)

98. Akaike H., M.C. Shin, C. Kubo, N. Akaike. Effects of scorpion toxin on excitatory and inhibitory presynaptic terminals. *Toxicology* 264, 198-204 (2009)

99. Fernandes V.M., R. Nicolato, T. Moraes-Santos, R.S. Gomez, M.A. Prado, M.A. Romano-Silva, M.V. Gomez. Beta-scorpion toxin induces the release of gamma-[3 H]aminobutyric acid in rat brain slices. *Neuroreport* 12, 2911-3 (2001).

100. Falqueto E.B., A.R. Massensini, T. Moraes-Santos, M.V. Gomez, M.A. Romano-Silva. Modulation of Na+-channels by neurotoxins produces different effects on [3H]Ach release with mobilization of distinct Ca2+-channels. *Cell Mol Neurobiol* 22, 819-26 (2002)

101. Fernandes V.M., M.A. Romano-Silva, D.A. Gomes, M.A. Prado, T.M. Santos, M.V. Gomez. Dopamine release evoked by beta scorpion toxin, Tityus gamma, in prefrontal cortical slices is mediated by intracellular calcium stores. *Cell Mol Neurobiol* 24.757-67 (2004)

102. Miranda D.M., M.A. Romano-Silva, E. Kalapothakis, C.R. Diniz, M.N. Cordeiro, T. Moraes-Santos, L. De Marco, M.A. Prado, M.V. Gomez. Spider neurotoxins block the beta scorpion toxin-induced calcium uptake in rat brain cortical synaptosomes. *Brain Res Bull* 54, 533-6 (2001)

103. Petricevich V.L. Scorpion venom and the inflammatory response. *Mediators Inflamm*. 2010, 903295 (2010)

104. Petricevich V.L., A. Hernández Cruz, F.I. Coronas, L.D. Possani. Toxin gamma from *Tityus serrulatus* scorpion venom plays an essential role in immunomodulation of macrophages. *Toxicon* 50, 66-75 (2007)

105. Petricevich V.L. Balance between pro- and anti-inflammatory cytokines in mice treated with *Centruroides noxius* scorpion venom. *Mediators Inflamm*. 2006, 54273 (2006)

106. Lebrun I., V.L. Petricevich. Immunomodulatory effects of the *Tityus serrulatus* venom on murine macrophage functions *in vitro*. *Mediator Inflamm*. 2005, 39-49 (2005)

107. Petricevich V.L., E. Reynaud, A.H. Cruz, L.D. Possani. Macrophage activation, phagocitosis and intracellular calcium oscillations induced by scorpion toxins from *Tityus serrulatus*. *Clin Exp Immunol* 154, 415-23 (2008)

108. Corrêa M.M., S.V. Sampaio, R.A. Lopes, L.C. Mancuso, O.A. Cunha, J.J. Franco, J.R. Giglio. Biochemical and histopathological alterations induced in rats by *Tityus serrulatus* scorpion venom and its major neurotoxin tityustoxin-I. *Toxicon* 35, 1053-67 (1997)

109. Daisley H., D. Alexander, P. Pitt-Miller. Acute myocarditis following *Tityus trinitatis* envenoming: morphological and pathophysiological characteristics. *Toxicon* 37, 159-65 (1999)

110. Gueron M., R.J. Adolph, I.L. Grupp, M. Gabel, G. Grupp, N.O. Fowler. Hemodynamic and myocardial consequences of scorpion venom. *Am J Cardiol*. 45, 979-86 (1980)

111. Teixeira A.L., B.F. Fontoura, L. Freire-Maia, C.R. Machado, E.R. Camargos, M.M. Teixeira. Evidence for a direct action of *Tityus serrulatu*s scorpion venom on the cardiac muscle. *Toxicon* 39, 703-9 (2001)

112. Gueron M., R. Ilia, S. Sofer. The cardiovascular system alter scorpion envenomation. *J Toxicol Clin Toxico*. 30, 245-58 (1992)

113. Fletcher P.L., M.D. Fletcher, K. Weninger, T.E. Anderson, B.M. Martin. Vesicle-associated membrane proteína (VAMP) cleavage by a new metalloprotease from the Brazilian scorpion Tityus serrulatus. *J Biol Chem* 285, 7405-116 (2010)

114. Dehesa-Dávila M., L.D. Possani. Scorpionism and serotherapy in Mexico. *Toxicon* 32, 1015-8 (1994)

Key Words: Beta-Scorpion Toxin; bioactive surface; Voltage-Gated Sodium Channel; Voltage Sensor Trapping Model, Review

Send correspondence to: Martha Pedraza Escalona, Instituto de Biotecnologia, UNAM, Avenida Universidad, 2001,Colonia Chamilpa, Apartado Postal 510-3, Cuernavaca 62210, Mexico, Tel: 52 77 73 29 16 69, Fax: 52 77 73 17 23 88, E-mail: mapedmx@ibt.unam.mx