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Molecular analysis of voltage-gated a-Na? channel toxin binding site 3 to determine key residues that confer resistance to a-scorpion toxin

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Molecular Analysis of Voltage-gated α-Na^+ Channel Toxin Binding Site 3 to Determine Key Residues that Confer Resistance to α-scorpion Toxin

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Molecular Analysis of Voltage-gated α-Na^+ Channel Toxin Binding Site 3 to Determine Key Residues that Confer Resistance to α-scorpion Toxin

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ABSTRACT

Voltage-gated Na^+ channels ($Na_v1.2$) which are proteins that posses 4 (I-IV) domains and six transmembrane (S1-S6) helices in each domain display abnormally slowed fast inactivation when an α-scorpion toxin binds to it. However, voltage gated Na^+ channels of the Mesobuthus martensii Karsch (*Mm*K) scorpion that produces α-scorpion toxins are resistant to their toxins. The previously described α-toxin binding site 3 of the *Mm*K voltage-gated Na^+ channels were examined by a comparative method using an alignment of many sodium channel protein sequences (multi-alignment) generated using the rat $Na_v1.2$ α-toxin sequence of binding site 3 as a template for the alignment. The α-toxin binding site 3 is comprised of 4 regions or segments and found in domains I and IV of the Na^+ channel. An analysis suggested that key residues exist that presumably are essential for the α-scorpion toxin binding at each of the four segments of the toxin binding site 3. The most significant mutations either altered the charge distribution and or polarity of the binding region or likely introduced changes in secondary structure (introduction of a turn). Mutations observed in the first segment of domain I S5-S6 that correspond to protein sequence positions in the rat and scorpion Na^+ channel (rat/Mmk) at K355/S339, A356/N340, -/Y341 (- refers to a missing residue), R358/P343, Y362/H347, and G371-S371/Y356 were the most significant. Significant mutations in the second segment of domain I S5-S6 were evident at K399/P384 and T400/W385. The fourth segment of domain IV S5-S6 had significant mutations at K1687/H1599, E168/R1600, and M1694/N1606. The third segment of domain IV S3-S4 was suspected of having significant mutations at E1616/A1528 and K1617/S1529. Changes in these key resides may introduce resistance to binding and action of α-scorpion toxins to the Na^+ channel. Future experimentation and or analysis will be needed to determine which of these residues are the most important for binding of the toxin to the channel.

INTRODUCTION

The properties of Na^+ channels date back to the work of Hodgkin &Huxley (1952) that described the ionic currents in the surface membrane of squid giant nerve fibers. When stimulated, voltage-sensitive Na^+ channels open to cause the upstroke of action potentials observed in nerve, skeletal muscle and cardiac muscle cells. Na^+ channels can exist in three possible voltage-dependent states, an open, closed and inactivated state. To produce action potentials Na^+ channels undergo transitions between these three states; stimulation (voltage depolarization) causes closed channels to open followed by channel inactivation. When the voltage returns to its normal resting value the channels again close. Some of the most important

ideas obtained about the function and structure of the Na^+ channels came from the use of neurotoxins that bind to and alter the channels. Recent studies have confirmed that the Na^+ channel is composed of a several protein subunits that form a complex. The most important protein subunit in the complex is the α-subunit [220-260 kDa] which contains both voltage-sensing and pore-forming pieces. The α-subunit also is accompanied by 1-2 auxiliary β-subunits [33-36 kDa] (Catterall 1984, 1995, 2000) that modify the properties of the α-subunit.

The single α-subunit of the Na^+ channel contains four homologous domains (Domains I-IV) and each domain possesses six hydrophobic transmembrane segments (S1-S6). The Na^+ channel four domains form a central pore and the S5-S6 loops of each domain play a key role in the pore structure. The S4 transmembrane segments contain a set of positively charged amino acids and act as the voltage sensor for the Na^+ channel. Movements of the voltage sensors in response to voltage changes act to cause either channel openings of closings (Catterall et al. 2007).

Goldin et al. (2000) suggested the use of a standardized nomenclatuare ($Na_v1.1$- $Na_v1.9$) for the nine voltage-gated Na^+ channel α-subunits isoforms that had been identified. Use of this nomenclataure has helped to eliminate confusion resulting from a multitude of names created to describe all the Na^+ channels discovered. Na^+ channel α-subunits display greater than 70% similarity in their amino acid sequences; however important sequence variations were identified and it was possible to further define subfamilies and subtypes of channels. (e.g., $Na_v1.1a$). The number following the subscript v (indicating the Na^+ channel is voltage dependent) indicates the gene subfamily, while number after the decimal point (1 through 9) identifies the specific channel isoform and the gene splice variant is the lower case letter.

Different receptor sites for neurotoxins that bind to Na^+ channels have been identified since the various neurotoxins alter Na^+ channel behavior in different ways. The effects produced by toxin binding include altering the ability of the Na^+ channel to open, close or inactivate as a function of voltage (Catterall 1984, 1995, 2000). Neurotoxins can also be labeled (e.g. radioactive or fluorescent) so that once bound to the Na^+ channel the site or location on protein that they interact with the channel can be identified. Since neurotoxins bind specifically to a particular location on the channel protein six distinct sites that produce identifiable changes in either the ion permeability or voltage-dependent opening or closing properties of Na^+ channels have been characterized (Cestèle S and Catterall WA, 2000). There are a number of neurotoxins produced by scorpions that alter Na^+ channels; however in this project only the α-scorpion toxins are considered. The α-scorpion toxins act to bind to Na^+ channels and act to lengthen action potentials by slowing channel inactivation. Several polypeptide toxins including sea-anemone toxins, atrachotoxins and α-scorpion toxins bind to the same site on the Na^+ channel. All these toxins are known to slow or block inactivation of the Na^+ channel upon binding to the Na^+ channel (Couraud F et al., 1978; Catterall, 1980).

The location of the α-scorpion toxin binding site (neurotoxin binding site 3) was obtained by use of a photoaffinity label and subsequent channel protein cleavage with proteases to identify the residues that carried the label. Using the rat Na^+ channel ($Na_v1.2$; SCN2A_RAT) residues 317-335 and 382-400 of domain I in the extracellular loop between the S5-S6 were initial identified as part of the binding site (Tejedor and Catterall, 1988). When antibodies were bound to residues 355-371 and 382-400 of the S5-S6 loop of domain I and residues 1686-1703 of a similar loop of domain IV α-scorpion toxins were inhibited in their binding by 30-55% (IC_{50} = 0.4 - 2 μM; Thomsen and Catterall , 1989).

Mutagenesis of another extracellular loop decreased the binding affinities of α-scorpion toxins by 62-fold and 82-fold. Specifically, mutation of Glu 1613 residue (IVS3) to Arg or His blocked α-scorpion toxin binding, and suggested that toxin channel interactions at this site of nearby altered the coupling of channel activation to inactivation (Rogers et al. 1996). This later became known as the voltage-sensor trapping hypothesis, and describes how α-scorpion toxin binding to the S3-S4 loop impedes the transmembrane movement of the voltage sensor by holding it in its inward position (Catterall, 2007).

In this study, the Na^+ channel α-scorpion toxin binding site 3, specifically segments in the extracellular side of both domains I and IV, were analyzed. After aligning numerous Na^+ channel sequences by similarity residues that are presumed to be important for toxin binding, characterization was ran by analyzing their properties including size, hydrophobicity, and charge. These residue properties were then compared with comparable residues of Na^+ channels from a scorpion Na^+ channels that is unaffected by α-scorpion toxins. Using this technique, key residues crucial for toxin binding will be presumptively identified.

METHODS

Obtaining the initial sequences of Na^+ channels sharing high similarities

In all photoaffinity labeling, sequence-specific antibody, and mutagenesis studies designed to identify Na^+ channel toxin binding site three, the R_{II} rat brain Na^+ channel ($Na_v1.2$) was used (Tejedor and Catterall, 1988; Thomsen and Catterall , 1989; Catterall, 2007). Thus, the rat brain Na^+ channel $Na_v1.2$ is used here as the template channel to identify other similar Na^+ channel sequences using the blast program. The blast program derives from the initial work of Altschul et al. (1997). Most sequences used in this study were obtained through the use of ExPASy tools (http://www.expasy.org/tools/) including a blast program that identified sequences on the protein databases SWISS-PROT and TrEMBL which included channel protein splice isoforms (Boeckmann et al. 2003).

A blast was run using the rat brain Na^+ channel $Na_v1.2$ as template (accession number/title "P04775/SCN2A_RAT; Na^+ channel protein type 2 subunit - Rattus norvegicus). The blast identified 100 Na^+ channels sequences that were similar to $Na_v1.2$. These sequences were transcribed to the FASTA format and kept in an electronic file to align them using bioinformatics alignment software.

Bioinformatics software used to align the sequences and obtain phylogenetic trees

Several software programs that align and edit alignment were used. The multiple alignment software known as Jalview, that use ClustalW either locally or via the web at the EBI ClustalW server, was used to align and edit the sequences of the blast obtained from rat $Na_v1.2$ (Clamp et al., 2004). Other software (SeaView) used to align and view the sequences on PDF files was also align the sequences through an interface with the ClustalW program (Galtier et al. 1996). All software used is freely available as freeware (see references). Phylogenetic trees were obtained using Jalview.

The Editing process of the aligned sequences

The sequences obtained via blast contained repeated identical sequences (repeats) and sequence fragments of Na^+ channels, which created an alignment that contained many superfluous gaps. To minimize the gaps, all channel sequence fragments were eliminated and a second alignment was produced that had fewer gaps. To ensure that the aligned sequences possessed regions of similarity to the extracellular site three residues, the rat Na^+ channel were used as template. Thus the hypothesized critical sequence fragments identified as site three by Tejedor and Catterall (1988), Thomsen and Catterall (1989) and Catterall (2007) were used (residues 355-371, 382-400, 1613-1625, and 1686-1703; see Table I). Thus, other residues that didn't correspond in the alignment with the segments associated with receptor site three were eliminated to only obtain site three of all the sequences in the multi-alignment of Na^+ channels.

TABLE I

Rat $Na_v1.2$ site three residues used as a template to identify site three in other Na^+ channels

Domain	Segment	Residue number	Residue number corresponding to fig. 1	TOTAL
I	S5-S6	355-371	1-17	17
I	S5-S6	382-400	18-36	19
IV	S3-S4(whole)	1613-1625	37-49	13
IV	S5-S6	1686-1703	50-67	18

Programs used to get Probability calculations

Microsoft Excel was used to calculate the probabilities of the residues in the alignment using the command

$$P_{Rn} = \text{COUNTIF(range/"criteria")/N}$$

where Rn = amino acid residue n (20 possible) and N = total number of sequences

$$(P_{Alanine}) = \text{COUNTIF(B1:B54/"A")/54}$$

Final editing process

The α-scorpion toxin binding site sequences (a total of 67 residues) were sorted using phylogenetic relationships to eliminate duplicated sequences (channel sequences that conferred a 1.00 probability with each other in a computed phylogenetic tree were identified and eliminated). Using this method, repeats were detected and eliminated and reduced the total number of Na^+ channels from 100 to 41. After this final edit, the scorpion Na^+ channels were aligned. The sequence of the scorpion Na^+ channel used was from the Manchurian scorpion, *Mesobuthus martensii* Karsch (*Mm*K) (previously designated as *Buthus martensii* Karsch) via the ExPASy website. When aligned with the other channel sequences, any significant differences in residues when compared with the other aligned sequences over the region of receptor site three may yield important clues that delineate the key residues that participate in binding with the toxin. Coloration schemes were used to identify vertically aligned residues according to their hydrophilicity (green), hydrophobicity (blue), size (absence of side chain; orange), weakly ionizable (- or +; light blue), bridge forming residues (peach; cystine), probable turn predictors (yellow; proline) or charge (- purple; + red).

RESULTS

Site three alignments: The domain and segments containing the important residues are shown

Since the Na^+ channels obtained from the blast are evolutionarily related (either as families of the same species or isoforms of different species) they were aligned first and then the segments corresponding to $Na_v1.2$ receptor site 3 were subsequently identified and compared. This method of analysis eliminated errors that could arise from the presence of deletion or addition mutations that would produce gaps in the alignment.

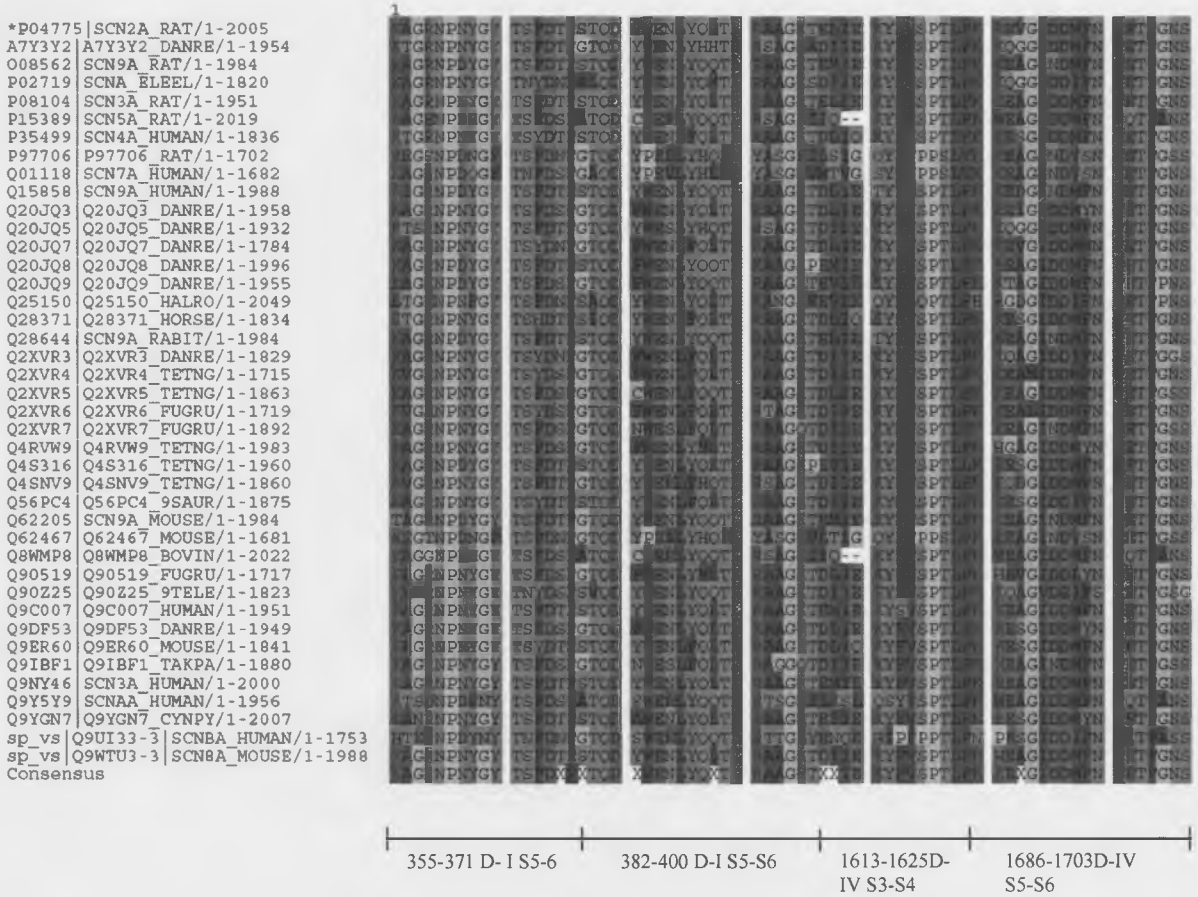

Figure 1. Alignment of the Na^+ channel binding site three for α-scorpion toxins.The alignment was obtained after elimination of repeats and virtually identical isoforms identified using phylogenetic analysis. The color key correspond to amino acid residue groups : KR,AFILMVW,NQST,HY,C,DE,P,G; which correspond to red, blue, green, light blue, beach, purple, yellow, and orange respectively.

Amino acid Residue Probabilities for receptor site 3 sequence fragments

Probabilities of charged amino acids of receptor site 3 were calculated and shown below in figure 1 with respect to their location in alignment. Charged residues can introduce electrostatic potential gradients that are important for binding of the toxin that displays and oppositely charged determinant (group).

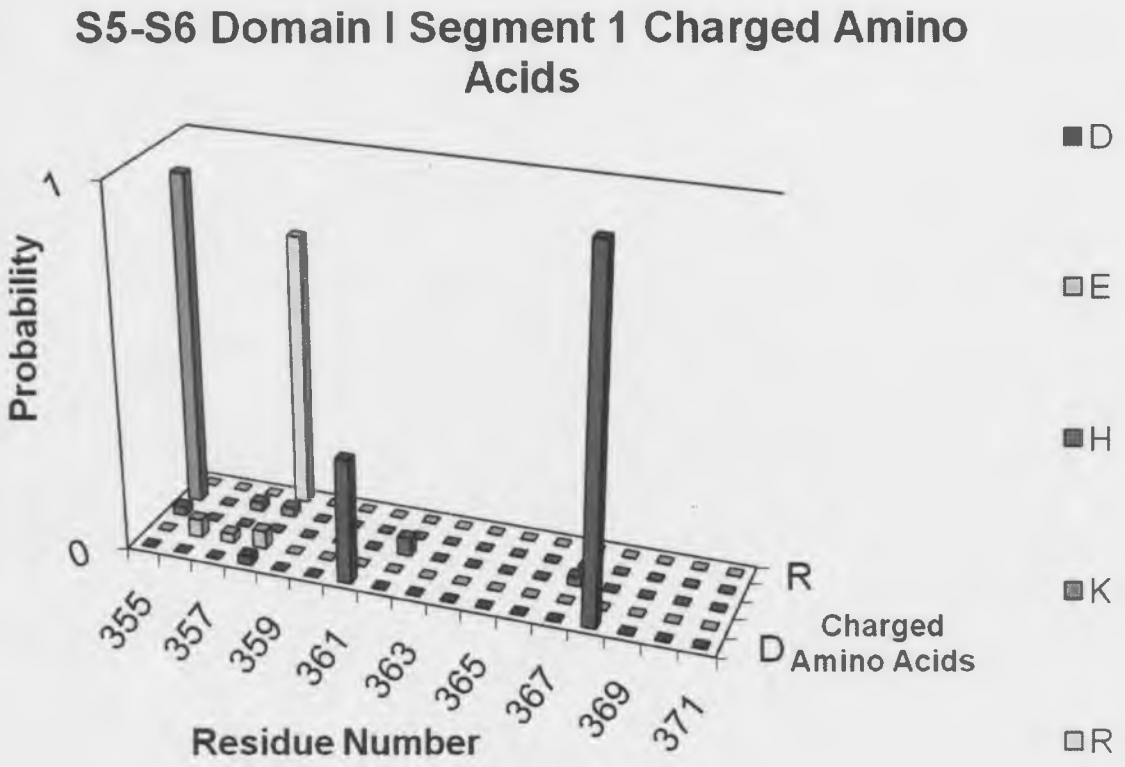

Figure 2. Probabilities of charged amino acid residues of receptor site 3, segment 1 of S5-S6 of domain I. After identifying the aligned residues that correspond to Rat $Na_v1.2$ having the range 355-371 residues (segment 1 of receptor site 3) the probabilities were obtained. Negatively charged aspartate (D) was found at positions 358 and 361 with low probability, but at 368 with probability of 1. Positively charged lysine (K) and arginine (R) was found with high probability ($P_K = 0.93$) at 355 and ($P_R = 0.76$) at 358 in the sequence alignment.

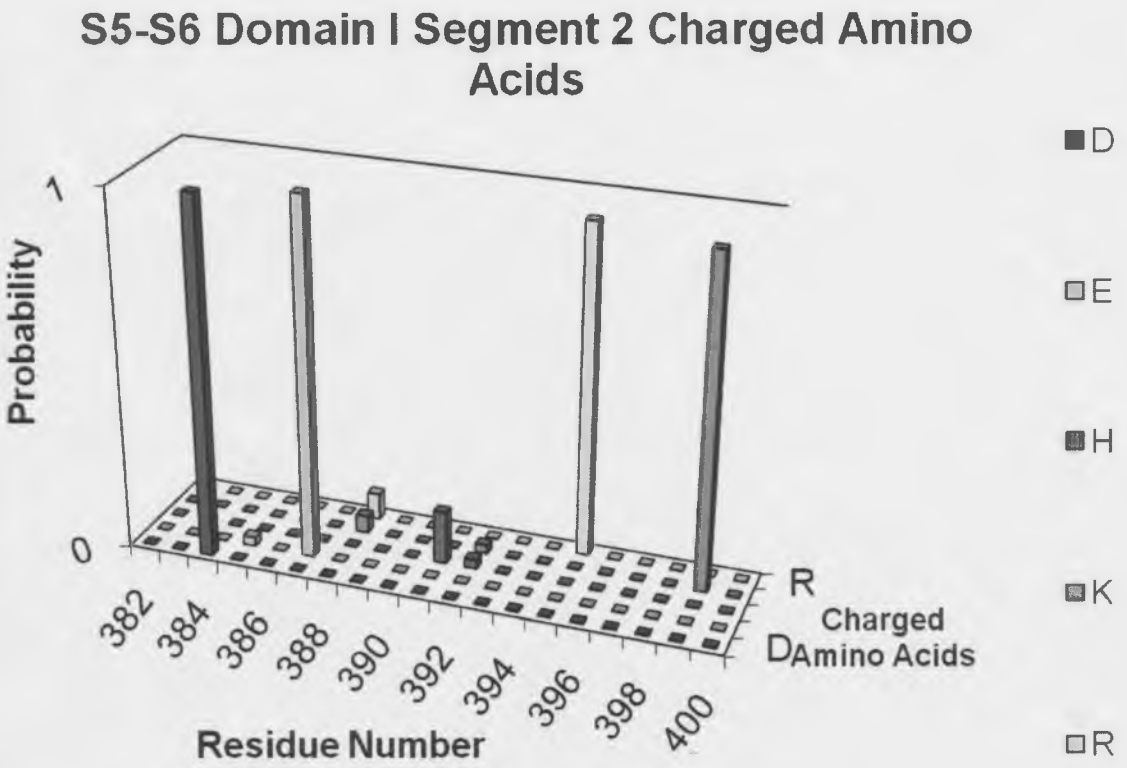

Figure 3. Probabilities of charged amino acid residues of the receptor site 3, segment 2 of S5-S6 of domain I. After identifying the aligned residues that correspond to Rat $Na_v1.2$ 382-400 residues (segment 2 of receptor site 3) the probabilities were obtained. Negatively charged aspartate (D) and glutamate (E) found with probability 1 at 384 and 387 respectively. Positively charged lysine (K) and arginine (R) were found with probabilities of ($P_K = 0.93$) at 395 and ($P_R = 0.93$) at 399.

The Second segment of the α-scorpion toxin binding site contained four strongly charged residues each occupying a single location. Negatively charged aspartate (D) and glutamate (E) were found with probability 1 at 384 and 395 respectively. Positively charged lysine (K) and arginine (R) were both found with probability of ($P_{K/R} = 0.93$) at 395 and 399 respectively (see Fig 3).The S3-S4 segment extracellular segment of domain IV had the most variable probabilities of charged residues. Residue site 1613 showed the presence of either negatively charged residues Asp ($P_D = 0.53$) or Glu ($P_E = 0.31$). The remaining residues after positively charged Lys-1617 ($P_K = 0.80$) show no charges (see Fig 4).

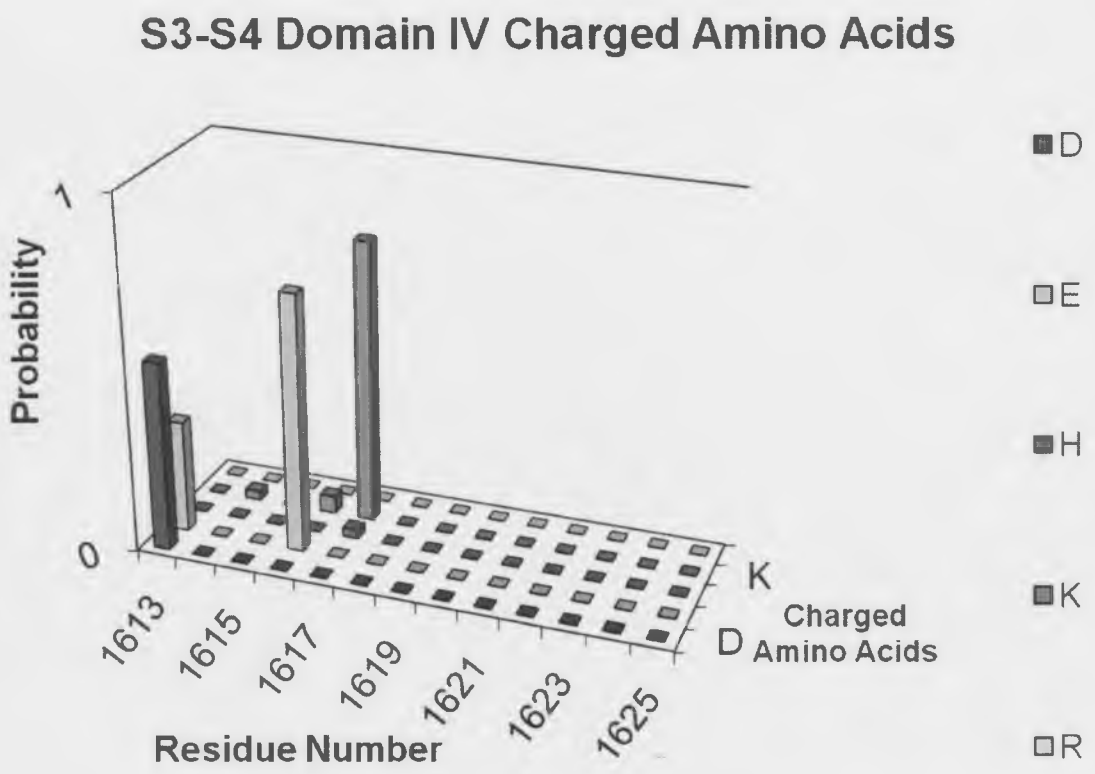

Figure 4: charged amino acids probabilities of the S3-S4 segment of domain IV (correspond to Rat $Na_v1.2$ 1613-1625). Negatively charged aspartate (D) and glutamate (E) were both found with probability of (P_D = 0.53) and (P_E = 0.31) at 1613. Glutamate also was found at 1616 with probability (P_E = 0.73). Positively charged lysine (K) was found with probability of (P_K = 0.8) at 1617.

Segment four (S5-S6 domain IV residues 1686-1702) had the most charged residues and they were located at positions 1686-1688, 1692-1693, and 1698. Most of the charged residues were negative (two Asp and Glu residues) except for two positively charged Lys residues (see Fig 5). Furthermore, Arg residues were not found in this segment, but found with low probability in the S3-S4 (.024≥ Arg ≤.17) segments.

Histidine was infrequently observed in all of the segments of the α-scorpion toxin binding site. Histidine can display a positively charge and can substitute for either Arg or Lys residues as seen in 1617, 1686, and 1687 with probabilities in the range (.024≥ His ≤.073) although the total probability of finding His was (.024≥ His ≤.1).

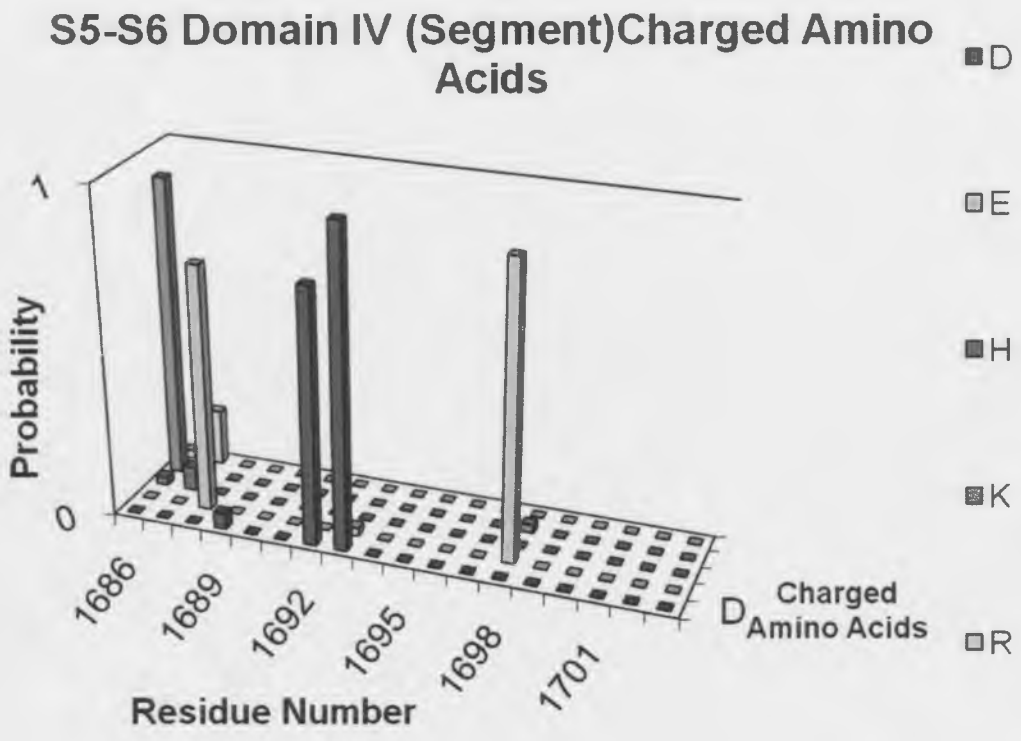

Figure 5: Charged amino acids probabilities of the S5-S6 of domain IV (corresponding to Rat $Na_v1.2$ 1686-1702 residues). Negatively charged Aspartate (D) was found with probability of ($P_D = 0.78$) at 1692 and ($P_D = 0.98$) at 1693 while Glutamate (E) was found with probability of ($P_E = 0.76$) at 1688 and ($P_E = 0.9$) at 1698. Positively charged Lysine (K) was found with a probability of ($P_K = 0.93$) at 1686 and ($P_K = 0.66$) at 1687.

Alignment of scorpion Na^+ channel residues with corresponding residues of receptor site three of the Rat $Na_v1.2$

The scorpion Na^+ channel of *Mm*K was aligned with the other voltage gated Na^+ channels (see fig. 6). Since the sequence of the Na^+ channel from *Mm*K differed in size from the rat $Na_v1.2$ the predicted residues corresponding to *Mm*K Na^+ channel receptor site three differed in their postions from those of the rat $Na_v1.2$ and are recorded in table II.

TABLE II
Scorpion Na^+ channel α-toxin predicted site

Domain	Segment	Scorpion channel Residue number *	Residue number correspond to Fig. 6	TOTAL residues
I	S5-S6	339-356	1-18	18 †
I	S5-S6	367-385	29-48	19
IV	S3-S4(whole)	1525-1537	57-70	13
IV	S5-S6	1598-1615	80-97	18

*These residue ranges are predicted after alignment with the Rat $Na_v1.2$ α-toxin binding site segments.
†18 residues are the result of the Tyr that also produced a gap on the other α-toxin binding site.

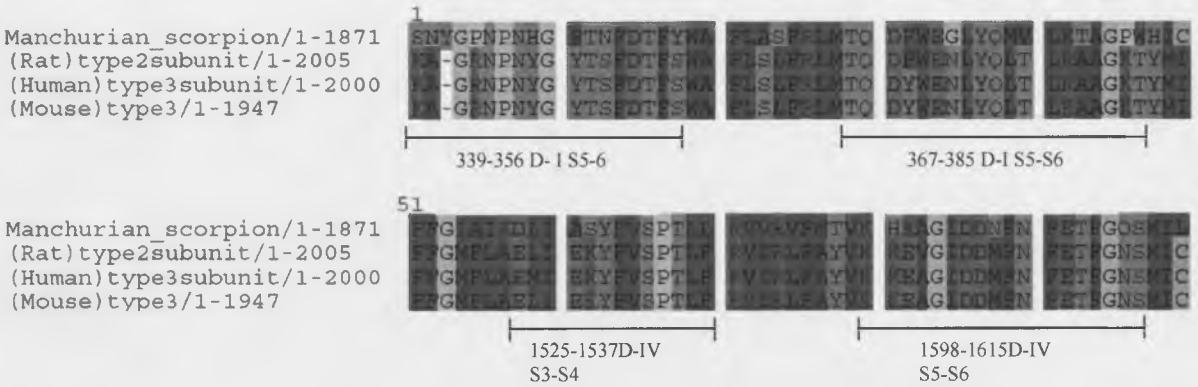

Figure 6: Alignment of the scorpion Na^+ channel of *Mesobuthus martensii* Karsch (Accession number Q67FT1) with the rat $Na_v1.2$ (Accesion number P04775) and two human Na^+ channels over the segments that form the α-toxin binding site. The scorpion Na^+ channel contains an additional residue (Tyr; Y) at the 10^{th} position of the sequence fragment and produced a gap in the multiple alignment with the other Na^+ channels. The color key for residues: KR,AFILMVW,NQST,HY,C,DE,P,G; which correspond to red, blue, green, light blue, beach, purple, yellow, and orange respectively.

Analysis of the predicted α-toxin binding site of the scorpion Na^+ channel

After alignment with the blast from the rat $Na_v1.2$, we were able to identify the MmK equivalent of the α-scorpion toxin binding site (see table II). Loret et al. (1994) suggested that α-scorpion toxins and sea anemone toxin possessed two distinct positively charged poles on the surface of the toxin that play a key role in binding with the Na^+ channel.

Table III
Alignment of Scorpion predicted α-toxin binding site (the first segment S5-S6 of domain I) with the multi-alignment of α-toxin binding site three regions

Position in multialignment $Na_v1.2$ number	Probability of the residue in multialignment	Postion in Scorpion channel*	Alignment match	Predicted effect of the substitution causing the resistance to α-toxins in scorpion
K-355	0.93	S-339	NO	Loss of [+] Charge
A-356	0.66	N-340	NO	Polarity gain
-	-	Y-341	NO	Polarity gain
G-357	0.90	G-342	YES	No effect
R-358	0.76	P-343	NO	Loss of [+] Charge and hydrophilicity /turn†
N-359	1	N-344	YES	No effect
P-360	1	P-345	YES	No effect
N-361	0.66	N-346	YES	No effect
Y-362	0.83	H-347	NO	[+] charge gain
G-363	0.95	G-348	YES	No effect
Y-364	0.93	F-349	NO	Polarity decrease
T-365	1	T-350	YES	No effect
S-366	0.90	N-351	NO	Tolerated‡
F-367	0.76	F-352	YES	No effect
D-368	1	D-353	YES	No effect
T-369	0.46	T-354	YES	No effect
F-370	1	F-355	YES	No effect
G-371, S-371	0.49, 0.41	Y-356	NO	Increased steric hindrance

*Scorpion Predicted Residue of the α-toxin binding site that correspond to table 2 and fig 6 of IS5-S6.
† Multiple effects may prove quite significant in altering toxin binding R→ P causes [+] charge loss and may cause turn.

When compared with rat $Na_v1.2$ and other Na^+ channels, the first segment S5-S6 of domain I of the scorpion channel α-toxin binding site showed mutations that display properties that may alter the ability of the α-toxin to bind to the channel. Table 3 shows all the mutations and describes the possible effect the change will likely introduce to the IS5-S6 first segment of the scorpion Na^+ channel. The scorpion Na^+ channel IS5-S6 first segment expresses P-343 that corresponds to R-358 of the multi-alignment which is a major mutation since a hydrophilic, positively charged, arginine is replaced by a residue that typically correlates with a turn and lacks any charge. Other mutations in this segment of the scorpion site three binding region show charge reversals or loss of charge and may also significantly reduce toxins binding.

Table IV
Alignment of Scorpion predicted α-toxin binding site (the second segment S5-S6 of domain I) with the multi-alignment of α-toxin binding site three regions

Position in multi-alignment $Na_v1.2$ number	Probability of the residue in multi-alignment	Position in Scorpion channel*	Alignment match	Predicted effect of the substitution causing the resistance to α-toxins in scorpion
T-382	0.88	T-367	YES	No effect
Q-383	1	Q-368	YES	No effect
D-384	1	D-369	YES	No effect
Y-385	0.54	F-370	NO	Loss of polarity
W-386	0.93	W-371	YES	No effect
E-387	1	E-372	YES	No effect
N-388	0.73	G-373	NO	Loss of polarity /Loss of steric hindrance
L-389	1	L-374	YES	No effect
Y-390	0.71	Y-375	YES	No effect
Q-391	0.80	Q-376	YES	No effect
L-392	0.54	M-377	NO	Tolerated
T-393	0.93	V-378	NO	Decreased hydrophilicity
L-394	1	L-379	YES	No effect
R-395	0.93	R-380	YES	No effect
A-396	0.80	T-381	NO	Increased hydrophilicity
A-397	0.85	A-382	YES	No effect
G-398	1	G-383	YES	No effect
K-399	0.93	P-384	NO	Loss of charge [+]/ Possible turn gain†
T-400	0.71	W-385	NO	Hydrophilicity loss/ Increased steric hindrance‡

*Scorpion Predicted Residue of the α-toxin binding site that correspond to table 2 and fig 6 of IS5-S6.
†Multiple effects may prove quite significant in altering toxin binding K→ P causes [+] charge loss and may cause turn.
‡Multiple effects may prove quite significant in altering toxin binding T→ W (hydrophilic residue replaced by hydrophobic one; Tryptophan is largest residue—so size change may prove significant)

The second segment of the S5-S6 of domain I also showed several substitutions which may well contribute to the loss of toxin binding of the α-scorpion toxin. A fairly significant substitution of P [residue 384] of the *Mm*K Na^+ channel for a K [residue 399 in $Na_v1.2$; corresponds to 384 in *Mm*K causes a loss of [+] charge and may alter the secondary structure of the protein at this location by introducing a tight turn. Similarly the substitution of W in *Mm*K at position 385 residue for T [residue 400 in $Na_v1.2$] introduces a large hydrophobic residue into the protein at a location where a hydrophilic residue existed previously in $Na_v1.2$ or other similar channels in the multi-alignment.

The extracellular loop of the α-scorpion toxin binding site, Domain IV S3-S4, of the predicted scorpion Na^+ channel showed only three mutations that are believed to decrease α-toxins binding affinity. E-1616 residue of the blast pertaining to the rat $Na_v1.2$ was found to be substituted to A-1528 loosing the charge and the hydrophilic effect of Glu. Another charge loss was evident between the substitution of K-1617 by S-1529. However, the last substitution was a conformational turn loss by the substitution of F-1625 by L-1537 (see table V).

Table V

Alignment of Scorpion predicted α-toxin binding site (loop S3-S4 domain IV) with the multi-alignment of α-toxin binding site three regions.

Position in multialignment $Na_v1.2$ number	Probability of the residue in multialignment	Postion in Scorpion channel*	Alignment match	Predicted effect of the substitution causing the resistance to α-toxins in scorpion
D-1613	0.54	D-1525	YES	No effect
I-1614, L-1614	0.39, 0.29	L-1526	YES	No effect
I-1615	0.93	I-1527	YES	No effect
E-1616	0.73	A-1528	NO	[-] Charge loss/hydrophilic effect loss†
K-1617	0.80	S-1529	NO	[+] Charge loss
Y-1618	0.95	Y-1530	YES	No effect
F-1619	0.90	F-1531	YES	No effect
V-1620	0.88	V-1532	YES	No effect
S-1621	0.88	S-1533	YES	No effect
P-1622	1	P-1534	YES	No effect
T-1623	0.93	T-1535	YES	No effect
L-1624	1	L-1536	YES	No effect
F-1625	0.90	L-1537	NO	Loss of steric hindrance

*Scorpion Predicted Residue of the α-toxin binding site that correspond to table 2 and fig 6 of IVS3-S4.
† Multiple effects may prove quite significant in altering toxin binding K→ P causes [+] charge loss

Table VI

Alignment of Scorpion predicted α-toxin binding site (last segment on S5-S6 of domain IV) with the statistical prediction of the blast collective α-toxin binding site

Position in multialignment $Na_v1.2$ number	Probability of the residue in multialignment	Postion in Scorpion channel*	Alignment match	Predicted effect of the substitution causing the resistance to α-toxins in scorpion
K-1686	0.93	K-1598	YES	No effect
K-1687	0.66	H-1599	NO	[+] charge loss (may be tolerated)†
E-1688	0.76	R-1600	NO	Charge reversal [(-) → (+)]
A-1689	0.59	A-1601	YES	No effect
G-1690	0.90	G-1602	YES	No effect
I-1691	0.95	I-1603	YES	No effect
D-1692	0.78	D-1604	YES	No effect
D-1693	0.98	D-1605	YES	No effect
M-1694	0.76	N-1606	NO	Hydrophilic and polarity gain
F-1695	0.78	F-1607	YES	No effect
N-1696	0.98	N-1608	YES	No effect
F-1697	1	F-1609	YES	No effect
E-1698	0.90	E-1610	YES	No effect
T-1699	1	T-1611	YES	No effect
F-1700	1	F-1612	YES	No effect
G-1701	0.85	G-1613	YES	No effect
N-1702	0.80	Q-1614	NO	May be tolerated‡
S-1703	0.98	S-1615	YES	No effect

*Scorpion Predicted Residue of the α-toxin binding site that correspond to table 2 and fig 6 of IVS5-S6.
† His produces a weak positively charge at pH's less than ~ 6.0 while Lys is always positively charged.
‡Both Asn and Gln are polar, hydrophilic, and neutral.

Rogers et al. (1996) reported that rat $Na_v1.2$ mutation of both Y-1618 and F-1619 with Ala did not affect the binding of the α-scorpion toxin suggesting both residues to be oriented toward the channel protein and not involved in the toxin binding. This is confirmed here through the presence of these residues (equivalent to Y-1530 and F-1531) in the scorpion Na^+ channel suggesting no participation in the binding to the toxin (see table V).

The last segment of the α-toxin binding site, domain IV S5-S6, also confirmed key residues that could potentially alter the binding affinity of the α-scorpion toxin. The E-1688 was substituted by R-1600 in scorpion protruding a positive charge instead of negative. This positive charge has the ability to repel the positive charges found in α-toxins believed to aid the binding of the toxin (Loretet al., 1994). The other substitution observed to provide Hydrophilic effect and polarity gain is evident in N-1606 from M-1694 (see table VI).

DISCUSSION

The scorpion *Mm*K possesses Na^+ channels that are resistant to its own toxins. This presumably occurred by evolutionary mutations to a precursor ancestoral gene of the Na^+ channel. This work was designed to identify presumptive α-toxin binding regions (receptor site three) of the Na^+ channel that possess mutations that prevent binding of the toxin. A comparison of the residues of receptor site three in Na^+ channels from the multi-alignment and the scorpion suggest a number of mutations may play an important role in conferring toxin resistance to the Na^+ channel. Below the mutations that were seen will be discussed in order of significance.

The first segment of Domain I S5-S6 receptor site three (see Table III) showed a number of mutations that may well play a key role in introducing toxin resistance in *Mm*K. Three mutations in a row appeared in the beginning of this segment of receptor site three: K355/S339, A356/N340, and -/Y341. The first mutation caused a loss of [+] charge while the other two caused the region to become more polar. However, the third mutation (-/Y341) introduced an additional residue into the sequence of the scorpion channel. Also at a neighboring residue R358 was changed to a proline (P343) and this may well have altered the shape of the secondary structure of this region because the presence of prolines correlates with turns. Also four residue positions forward Y362/H347 likely introduced a positive charge at this location. Also at the end of the segment G371-S371/Y356 introduced steric hindrance to the protein segment. The sum of all the first five mutations is quite significant because they are localized to a short stretch of the protein segment.

The second segment of Domain I S5-S6 receptor site three (see Table IV) possessed changes that also may play a significant role in the development of toxin resistance. K399/P384 introduced a possible turn and a loss of [+] charge at the end of the segment which could alter the secondary structure of the protein and lead to decreased toxin binding. In addition the next residue T400 is altered to W385 which caused a loss of hydrophilicity and introduced steric hindrance which likely will alter the protein's secondary structure at this location.

The fourth segment of Domain IV S5-S6 receptor site three (see Table VI) possessed a [+] charge loss K1687/H1599 and a charge reversal [(-)/(+)], E168/R1600 that may well significantly alter the properties of this segment of the receptor site to alter toxin binding. Charge reversals typically are not tolerated well in important regions of the protein. Also the change of M1694/N1606 introduced a residue that was more polar and hydrophilic.

Changes in the third segment of Domain IV S3-S4 receptor site three (see Table V) may also play a significant effect in altering the binding of the toxin to the site. Two charge losses were seen in neighboring positions E1616/A1528 [-], charge loss, and K1617/S1529, a [+] charge loss. If these two charges produced an electrostatic potential surface that was important for binding, then their loss would be significant, and reduce or eliminate the binding of the toxin.

Two future methods may be used to test whether the mutations identified in this paper play key roles in toxin binding. An exhaustive analysis of how various toxins bind to various

Na^+ channel isoforms would be quite valuable in providing insight into key residues. However, there is little published data available to conduct this type of analysis. The alternative method is to create channels and or toxins with appropriate mutations to test whether particular mutations prove to eliminate binding.

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