Mapping the interaction surface of scorpion β-toxins with an insect sodium channel

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Abbreviations: MC, Monte Carlo; MCM, MC-minimizations; Na+, Voltage-gated sodium channels; VSM, Voltage-sensing module; PD, Pore Domain; S1-S6, transmembrane helices in Na+, S1 and P2, Membrane-descending and -ascending helices in P-loops between S5 and S6; R1, R2, R3, K4, K5 - Basic residues in IIS4; BgNa,1-1a, derivative of BgNa,1-1
ABSTRACT. The interaction of insect-selective scorpion depressant β-toxins (LqhIT2 and Lqh-dprIT3 from Leiurus quinquestriatus hebraeus) with the Blattella germanica sodium channel, BgNa1-1a, was investigated using site-directed mutagenesis, electrophysiological analyses, and structural modeling. Focusing on the pharmacologically-defined binding site-4 of scorpion β-toxins at the voltage-sensing domain II (VSD-II), we found that charge neutralization of D802 in VSD-II greatly enhanced the channel sensitivity to Lqh-dprIT3. This was consistent with the high sensitivity of the splice variant BgNa2-1, bearing G802, to Lqh-dprIT3, and low sensitivity of BgNa2-1 mutant, G802D, to the toxin. Further mutational and electrophysiological analyses revealed that the sensitivity of the WT = D802E < D802G < D802A < D802K channel mutants to Lqh-dprIT3 correlated with the depolarizing shifts of activation in toxin-free channels. However, the sensitivity of single mutants involving IIS4 basic residues (K4E = WT << R1E < R2E < R3E) or double mutants (D802K = K4E/D802K = R3E/D802K > R2E/D802K > R1E/D802K > WT) did not correlate with the activation shifts. Using the cryo-EM structure of the Periplaneta americana channel, NaPaS, as template and the crystal structure of LqhIT2, we constructed structural models of LqhIT2 and Lqh-dprIT3-c in complex with BgNa1-1a. These models along with the mutational analysis suggest that depressant toxins approach the salt-bridge between R1 and D802 at VSD-II to form contacts with linkers IIS1-S2, IIS3-S4, IIIP5-P1 and IIIP2-S6. Elimination of this salt-bridge enables deeper penetration of the toxin into a VSD-II gorge to form new contacts with the channel, leading to increased channel sensitivity to Lqh-dprIT3.
1. Introduction

Voltage-gated sodium channels (Na,§) are responsible for the rising phase of action potentials. These channels are targets of many naturally occurring toxins [1-3]. The pore-forming α-subunit of eukaryotic Na,§ folds from a single-polypeptide chain of four homologous repeat domains. Each repeat contains a voltage-sensing module (VSM) and contributes a quarter to the central pore-forming module. Insect Na,§ are targets for various chemical insecticides, including pyrethroids [4-6]. However, continuous use of these compounds led to resistance build-up in many pest populations posing serious global problems in pest control that affects agriculture as well as human health [7-9]. Therefore, there is an urgent need to develop alternatives to conventional insecticides. One such potential alternative may arise from the study of action of natural neurotoxins that selectively affect insects [10-12]. For example, scorpion depressant β-toxins exhibit selectivity to insects and have already been shown to arrest Spodoptera littoralis growth when expressed using a baculovirus vector [13, 14].

Investigations of the action of the scorpion β-toxin Css4 have revealed its binding to the mammalian brain channel rNa1.2 at rest. Upon strong depolarization, as the IIS4 voltage sensor moves to the outward position, it is trapped by the toxin, which leads to enhanced channel activation in subsequent depolarizations [15]. Genes encoding scorpion β-toxins (e.g., LqhIT1, LqhIT2 and Lqh-dprIT3-c from Leirus quinquestriatus hebraeus) have been cloned and functionally expressed in large amounts in heterologous systems, which enabled mutagenic dissection and X-ray crystallography of LqhIT2 [16, 17]. Notably, a few representatives of toxin genes were engineered into genomes of microbial pesticides to increase their insecticidal potency [18]. Scorpion toxins can also directly translocate to insect hemolymph by fusing them with a special food carrier [19].

Cryo-EM structures of several eukaryotic channels in the apo form and in complex with different toxins are now available, e.g., [20-24]. Although these structures are useful in rationalizing numerous mutational and ligand-binding studies, including action of chemical insecticides like pyrethroids and proteinaceous toxins, they are of limited resolution and represent snapshots of certain conformations captured in channel states with presumably inactivated pore domain [25]. Therefore, mutagenesis ensued by functional assays coupled with molecular modeling may shed light on possible conformational alterations of portable channel entities that currently could not be resolved experimentally.

Despite of the availability of the crystal structure of LqhIT2 [16] and elucidation of key bioactive residues using directed mutagenesis and binding assays on cockroach (Periplaneta americana) neuronal membrane preparations [16], the structure of LqhIT2 in complex with a sodium channel was not attained. Consequently, the molecular mechanism underlying its action is vaguely understood. Furthermore, rather low chemical stability of scorpion β-toxins has been suggested to limit application of the cryo-EM methodology to solve the structure of a toxin-channel complex [26]. Therefore, we used a different strategy that included two stages. In the first stage, we employed mutational and electrophysiological analyses to explore the roles of six ionizable residues in VSM-II.

We first focused on the conserved negatively-charged aspartate at position 802 (D802) in the extracellular loop IIS1-S2, which was shown to be critical for gating of the BgNa,1-1a channel (from the German cockroach Blattella germanica) and in the action of pyrethroid insecticides [27]. Moreover, substitutions E779Q/C at the homologous position of the mammalian channel rNa,1.2 reduced the effect of the β-toxin Css4 [28]. Of the 69 splice variants of the BgNa, channel [29], almost all variants including BgNa,1-1 and its BgNa,1-1a derivative bear aspartate at position 802.
An exception is variant BgNa,2-1, which bears glycine at this position [30] Strikingly, BgNa,2-1 was found to be more sensitive to Lqh-dprIT3-c. Thus, in contrast to the critical role of E779 in rNa,1.2 for action of Css4, D802 in BgNa,1-1a seems to reduce the Lqh-dprIT3-c effect. To further investigate the role D802 and basic residues in the voltage-sensing segment IIS4, we generated ten mutants of BgNa,1-1a (D802A, D802K, R1E, D802K/R1E, R2E, D802K/R2E, R3E, D802K/R3E, K4E, K5E), and analyzed their gating properties and sensitivity to Lqh-dprIT3-c. Then we employed structural modeling of the BgNa,1-1a channel in the apo form and the crystal structure of LqhIT2 to construct structural models of BgNa,1-1a-LqhIT2 and BgNa,1-1a-Lqh-dprIT3-c complexes. In doing so, we were able to rationalize previous results of binding studies employing LqhIT2 and its multiple mutants [16]. Analysis of the structural models clarified the varying sensitivity of the mutant channels to depressant toxins, and shed more light on the way β-toxins affect sodium channel activation.

2. Material and Methods

2.1. Toxin production and functional analysis. Out of the eight gene variants encoding Lqh-dprIT3 in the scorpion Leiurus quinquestriatus hebraeus, variant "c" was found the most potent, and approximately 15 times more toxic than its depressant toxin homologue LqhIT2 [12]. Therefore, in all experiments of the present study we used variant Lqh-dprIT3-c, which we produced in recombinant form and analyzed as was described previously [12].

2.2. Site-directed mutagenesis. Site-directed mutagenesis of BgNa,2-1 and BgNa,1-1a (Fig. 1A) was performed by PCR using Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA), and the mutations were verified by DNA sequencing.

2.3. Expression of BgNa, channels in Xenopus oocytes. The procedures for oocyte preparation, cRNA synthesis and injection were identical to those described previously [31]. cRNA was prepared by in vitro transcription with T7 polymerase using the mMESSAGE mMACHINE high yield capped RNA kit (Ambion, Austin, TX). To enhance the expression of BgNa,2-1 and BgNa,1-1a and the mutant channels, their cRNAs (2-5 ng/oocyte) were co-injected into oocytes with tipE cRNA from Drosophila melanogaster in a 1:1 ratio [32, 33]

2.4. Electrophysiological recording and analysis. The voltage dependence of channel activation and inactivation was measured using the two-electrode voltage clamp technique as was previously described [31]. Sodium currents were measured with an OC725C oocyte clamp (Warner Instruments, Hamden, CT) and a Digidata 1322A interface (Axon Instruments Inc., Foster City, CA). Data were sampled at 50 kHz and filtered at 2 kHz. Leak currents were corrected by P/4 subtraction. pCLAMP 10.2 software (Axon Instruments Inc.) was used for data acquisition and analysis. The maximal sodium current peak was limited to < 2.0 μA to achieve optimal voltage control. The voltage dependence of channel conductance (G) was calculated by measuring the peak current at test potentials ranging from -80 to +65 mV in 5 mV increments and divided by (V − Vrev), where V is the test potential and Vrev is the reversal potential for sodium ions. Peak conductance values were normalized to the maximal peak conductance (Gmax) and fit with the two-state Boltzmann equation: G/Gmax = (1 + exp(V − V1/2)/k)−1, where V is the potential of the voltage pulse, V1/2 is the voltage for half-maximal activation, and k is the slope factor. The voltage dependence of channel inactivation was determined by using 100 milliseconds prepulses ranging from -120 to -10 mV in 5 mV increments from a holding potential of -120 mV, followed by test pulses to -10 mV for 20 milliseconds. The peak current amplitude during the test depolarization was normalized to the maximal current amplitude and plotted as a function of the prepulse potential. Data were fitted with the two-state Boltzmann equation: I/I1/2 = (1 + exp(V − V1/2)/k)−1,
where \( I \) is the peak sodium current, \( I_{\text{max}} \) is the maximal current evoked, \( V \) is the potential of the voltage prepulse, \( V_{1/2} \) is the half maximal voltage for inactivation, and \( k \) is the slope factor.

To detect the effect of scorpion \( \beta \)-toxins, a train of conditional prepulses is required [34]. Therefore, we applied a 20 Hz train of fifty 5 ms depolarizing prepulses to 50 mV followed by a 20 ms depolarizing test pulse between -80 and -65 mV from a holding potential of -120 mV (Fig. 1B). The channel modification by Lqh-dprIT-c was determined as the percentage of channels with the voltage dependence of activation shifted to negative membrane potentials, which was derived from double Boltzmann fits of the conductance-voltage relationships. Data are presented as means ± S.D. Statistical significance was determined by one-way analysis of variance (\( p < 0.05 \)).

### 2.5. Molecular modeling

Cryo-EM structure of the Na₃PaS channel [20] was used as a template to construct a homology model of the BgNa₁-1a channel. The model of toxin Lqh-dprIT-c was constructed using the crystal structure (PDB ID: 2i61) of the homologous toxin LqhIT2 as template [16]. The models were optimized by Monte Carlo energy minimizations (MCM) [35] with all side-chain torsions randomly sampled. Both the side-chains and backbones were flexible during the MCM process. Bond angles (except those in prolines) and bond lengths were kept rigid during the calculations. Each MCM trajectory was terminated when 2,000 consecutive energy minimizations did not decrease the energy of the apparent global minimum. All calculations were performed with the ZMM program [36], which minimizes energy in the space of generalized (internal) coordinates [37]. The models were visualized using the PyMol Molecular Graphics System, version 0.99rc6 (Schrödinger, New York, NY).

Nonbonded interactions were calculated with the AMBER force field [38]. Electrostatic interactions were calculated with the distance- and environment-dependent dielectric function [36]. No specific energy terms were used for cation-\( \pi \) interactions, which were accounted for with partial negative charges at the aromatic carbons [39]. No distance cutoff was used to calculate electrostatic interactions involving ionized groups in amino acids. For other interactions the distance cutoff of 9 Å and a shifting function [40] were used.

To incorporate the experimental data in our calculations we used constraints. A distance constraint is a flat-bottom energy function that allows respective atom-atom distance to deviate the energy-free between lower and higher limits and imposes a parabolic energy penalty if the distance is beyond the limits. To maintain the template folding in the channel model we used “pin” constraints. A pin allows an alpha carbon of an amino acid residue to deviate up to 1 Å from the respective template position without a penalty and imposes a parabolic energy function to penalize larger deviations. Pins were not used to preserve the mobile toxin fold stabilized by three disulfide bridges. To bias some toxin-channel contacts we used residue-residue constraints. Such a constraint specifies two residues and the target distance between their side-chains (which was set to 5 Å). In the beginning of each cycle of energy minimization, the ZMM program selects the closest pair of atoms between the two side-chains and applies a distance constraint to these atoms so that the atom-atom contacts may switch during the MCM trajectory [41]. For all constraints, the energy penalty was calculated with the force constant of 10 kcal·mol⁻¹·Å⁻². To minimize possible bias due to the imposed constraints, each model was minimized in two stages. After the first MC-minimization with constraints, the model was refined by a second MC-minimization, in which all constraints were removed and all degrees of freedom, including backbone torsions, were allowed to vary.

The BgNa₁-1a-toxin complex was used as the starting structure for construction of the models of toxin-channel mutants, which included VSM-II and PD-III (both elements interact...
directly with the toxin). Other parts remote from the toxin in the channel mutants were not included. It should be noted that MC-minimization of each toxin-channel complex yielded several low-energy structures, which had comparable energy characteristics. Respective figures show low-energy structures in which the maximal number of specific contacts is established between the
toxin and the channel.

The in-silico deactivation of II-VSM was computed as follows. C^ atoms of IIS4 basic residues were forced to progressively move through 21 sets of planes (5 planes for five C^ atoms of basic residues in each set), which are normal the pore axis. Two adjacent planes are distant from one another by 0.5 Å. The starting conformation for the II-VSM deactivation transit corresponded to the BgNa_1-1a model with activated VSMs, which is based on the Na_PaS cryo-EM structure. At each step of the transit, the C^ atoms of basic residues were free to move within the corresponding plane, but not to leave it. The MCM trajectory at each step of the transit was terminated when the last 200 consecutive energy minimizations did not improve the respective apparent global minimum. The starting conformation for the next transit step corresponded to the MC-minimized conformation of the previous step.

3. Results

3.1. Electrophysiological analyses of modified channels

3.1.1 Substitution G802D decreased the sensitivity of BgNa_2-1 splice variant to Lqh-dprIT3-c. Lqh-dprIT3-c has been reported to cause hyperpolarizing shifts in the voltage-dependence of activation (V_0.5) of BgNa_1-1a [34]. However, 30 nM Lqh-dprIT3-c had little effect on V_0.5 of the splice variant BgNa_2-1 that bears a glycine at position 802 rather aspartate which is found in all other channel variants (Fig. 1C, D). At higher toxin concentrations, 100 nM and 300 nM, the G-V curves exhibited two components: one with V_0.5 similar to that obtained with the toxin-free channel, and the other with V_0.5 shifted in the hyperpolarizing direction (Fig. 1C, D and Table S1). The two components likely represent unmodified and toxin-modified channels, respectively.

To investigate the role of residue 802 in the interaction of BgNa_2 channels with Lqh-dprIT3-c, we substituted the glycine for aspartate (G802D) in BgNa_2-1. As shown in Fig. 1E, the BgNa_2-1G802D mutant was less sensitive to Lqh-dprIT3-c. At 300 nM, Lqh-dprIT3-c affected only 21% of the G802D mutant channels, compared to 73% of WT BgNa_2-1 channels (Fig. 1E, F and Table S1). In addition, the G802D substitution resulted in only a -15 mV shift in the voltage-dependence of channel activation (V_0.5) (Fig. 1G).

3.1.2. Effects of substitutions D802G/A/K/E on the G-V curves and channel sensitivity to Lqh-dprIT3-c. To further examine the role of D802 in toxin action, we made multiple substitutions at position 802 in the BgNa_1-1a channel which produces more robust sodium currents in oocytes than BgNa_2-1 [29]. Substitutions D802G/A/K/E shifted the V_1/2 by +10, +11, +20 and -4 mV, respectively (Fig. 2A and Table S2), whereas none of these substitutions significantly changed the voltage-dependence of inactivation, V_1/2 (Fig. 2B and Table S2).

At 300 nM, Lqh-dprIT3-c had almost no effect on V_0.5 of the WT channel or mutant D802E (Fig. 2C, D), but rendered hyperpolarizing shifts of V_0.5 in mutants D802G and D802A (Fig. 2E, F) and especially for D802K, where V_0.5 was shifted by -57 mV (Fig. 2G). The toxin-induced shifts of V_0.5 correlated with the enhanced sensitivity of the channels to Lqh-dprIT3-c (Fig. 2H). These
results indicated that the charge at position 802 greatly influenced the effect of Lqh-dprIT$_3$-c on channel activation.

### 3.1.3. Effects of substitutions R1E, R2E, R3E, K4E and K5E on BgNa$_{1.1a}$ sensitivity to Lqh-dprIT$_3$-c.
To explore possible interactions of D802 with basic residues in IIS4, we generated BgNa$_{1.1a}$ mutants, R1E, R2E, R3E, K4E and K5E, and analyzed their electrophysiological properties and sensitivity to Lqh-dprIT$_3$-c. In toxin-free channels, R1E and R2E rendered a -5 mV shift in $V_{0.5}$, whereas R3E and K5E rendered a shift of +8 and +4 mV in $V_{0.5}$, respectively (Fig. 3 and Table S2). We then examined the sensitivity of the mutants to 300 nM Lqh-dprIT$_3$-c, the concentration that had no effect on the WT channel BgNa$_{1.1a}$. In channel mutants R1E, R2E and R3E the toxin shifted the conductance curves in the hyperpolarizing direction (Fig. 3B-F), but had very little or no impact on the conductance curves of channel mutants K4E or K5E, respectively (Fig. 3E, F). The hyperpolarizing shifts of the conductance curves qualitatively correlated with the percentage of toxin-modified channels (cf. Figs. 3A and 4F).

### 3.1.4. Effects of double substitutions D802K/R1E, D802K/R2E, D802K/R3E, D802K/K4E and D802K/K5E on the channel sensitivity to Lqh-dprIT$_3$-c.
To further investigate how putative interactions between D802 and the basic residues in II-VSM affect the action of Lqh-dprIT$_3$-c, we generated four BgNa$_{1.1a}$ double mutants and measured the voltage dependence of activation in the absence (Fig. 4A) or presence (Fig. 4B-F) of the toxin. While substitution D802K resulted in a +20 mV shift of $V_{0.5}$ (Fig. 2A), double substitution D802K/R1E resulted in a -4 mV shift of $V_{0.5}$ (Fig. 4A and Table S2), likely reversing the effect of D802K, and retaining the effect of R1E on $V_{0.5}$. In comparison to the WT channel BgNa$_{1.1a}$, the double substitutions D802K/R2E, D802K/R3E and D802K/K4E rendered +7.4 mV, +9.3 mV and +20 mV shifts of $V_{1/2}$, respectively (Fig. 4A). The voltage-dependence of activation of the D802K/K4E channels was similar to that of the D802K channel mutant (Fig. 4A). In the presence of 300 nM Lqh-dprIT$_3$, 100% of D802K, D802K/R3E and D802K/K4E channels were affected (Fig. 2H), whereas only 36% of the D802K/R1E channels and 78% of the D802K/R2E channels were affected (Fig. 4F). The percentage of toxin-modified D802K/R1E channels was similar to that of R1E channels (37%) (Fig. 4F; Table S3). Notably, toxin-induced hyperpolarizing shifts of the conductance curves in all of the double channel mutants qualitatively correlated with the percentages of toxin-modified channels.

### 3.1.5. Effects of substitutions on the kinetics of toxin-channel interactions.
A depolarizing prepulse was required for measuring the Css4 effect on rNa$_{1.2}$ channels [15, 28], suggesting that the toxin trapped IIS4 in its activated outward state. However, a train of short depolarizing prepulses was required for measuring the effect of Lqh-dprIT$_3$-c on the cockroach channels. Moreover, the Lqh-dprIT$_3$-c effect on channel activation increased with the number of prepulses [34]. To examine how the charge reversal in single and double channel mutants altered the kinetics of the toxin-induced effect, we applied a single depolarizing prepulse to -10 mV of increasing durations (1-2000 ms) and then repolarized to -120 mV for 50 ms and measured the channel activation following a test pulse to -60 mV, at which only toxin-modified channels were activated [34]. As shown in Fig. S1A, the growing duration of the depolarizing prepulse increased the sodium current in the presence of Lqh-dprIT$_3$-c measured at -60 mV. Lqh-dprIT$_3$-c at two different concentrations instigated similar modifications of the BgNa$_{1.1a}$ channel, indicating that the rate of toxin-induced channel modification was concentration-independent (Fig. S1A). Unexpectedly,
a much shorter prepulse duration was sufficient to modify channel mutants R1E and D802K/R1E compared to BgNa.1-1a and channel mutant D802K (Fig. S1B).

3.2. Structural modeling of channel-toxin interactions

3.2.1. Modeling of BgNa.1-1a structure. The homology model of the BgNa.1-1a channel was constructed using as template the cryo-EM structure of the NaPaS channel [20]. The sequences of NaBg1-1a and NaPaS (as well as that of Na.1.2) align well without indels in the II-VSM and PD-III regions (Fig. S2), that interact with the spider toxin Dc1a in the cryo-EM structure of the NaPaS-Dc1a complex [21], or that were proposed to interact with the toxin in the models of Na.1.2-Css4 complex [42, 43]. The BgNa.1-1a structure was energy-minimized in two steps. In the first step, backbones were kept rigid and the energy was MC-minimized to release clashes involving BgNa.1-1a residues that differ from residues in sequentially matching positions of NaPaS. In the second step, the energy was MC-minimized with all degrees of freedom allowed to vary. D802 at the extracellular end of IIS1 was found to form a salt-bridge with IIR1, the outermost arginine of the voltage sensor IIS4. Analogous salt bridges appear in cryo-EM structures of Na.1.4 [44], Na.1.5 [25], and KIIIA-bound Na.1.2 [45], where glutamate homologous to D802 forms a salt-bridge with IIR1.

3.2.2. Docking LqhIT2 onto BgNa.1-1a. We first docked LqhIT2, whose crystal structure and all bioactive residues were elucidated [16]. Since experimental data on specific contacts between the toxin and channel residues, which could facilitate the toxin docking are lacking, we relied on previous work, proposing that scorpion β-toxins bind between the activated II-VSM and PD-III [15, 28, 46], to set up a starting orientation of LqhIT2 at the channel. The studies by Catterall and co-authors revealed residues in rNa.1.2 involved in binding of the scorpion β-toxin Css4, leading to an insinuated model of the channel-toxin complex [15, 28, 42, 43, 46]. According to the model, the wedge-shaped toxin binds between extracellular loops IIS1-S2 and IIS3-S4 with the hydrophobic residues F44 (at the turn between the toxin beta-strands) and L19 (at the apex of the loop proximal to the toxin helix) penetrating most deeply into the II-VSM gorge, thus approaching the salt-bridge between E779 (homolog of D802 in Lqh-dprIT3-c) and IIR1. Polar residues at the toxin C-end were suggested to bind to polar residues in loop IIP2-S6 [43]. The sequence alignment between Css4 and LqhIT2 involves a few insertions-deletions (Fig. S3), yet a 3D alignment of LqhIT2 and the Css4 model [42], which was based on the crystal structure of scorpion toxin CsE-v2 (PDB ID: 1JZA), has shown unambiguously a similar fold (Fig. S4). This has suggested that the binding orientation of LqhIT2 on BgNa.1-1a may be similar to that of Css4 on rNa.1.2. Furthermore, although the sequence of the spider gating-modifying toxin Dc1a is very different from that of scorpion β-toxins, there are similar features in the Dca1 interaction with NaPas [21] and Css4 interaction with Na.1.2 [43]. First, F48 in Dc1a and F44 in Css4 are located at the apex of the loop between two beta-strands and in both complexes these phenylalanines penetrate deeply into the II-VSM gorge, wedging between R1 and the analog of D802. Second, both toxins bind to several polar residues in the channel extracellular loop IIS5-S6.

LqhIT2 lacks phenylalanines, but has three functionally important tryptophans (Fig. S3 and Table 1). The side-chain of W38 is located at the apex of the loop between two beta-strands, analogously to F44 in Css4 (Figs. S3, S4). Besides W38, the side-chain of another functionally important residue, N18 (Table 1), is located at the same basal side of the toxin (Fig. 5A). The surface opposite to the basal side is exposed to the solvent and lacks functionally important residues (Fig. 5B), and so most likely it does not interact with the channel. Two regions between...
the basal and solvent-exposed sides bear several functionally important residues: W36, I16, K23 and E24 (Table 1 and Fig. 5 C,D).

We placed LqhIT2 above II-VSM and next to IIIS5-P1 with the basal side down, and imposed a residue-residue distance constraint to force W38 of the toxin into the channel gorge to approach R1. Then we sampled multiple orientations of the toxin along axis C’-W38 --- C’-A49 and MC-minimized each sampled structure. In these computations, the C’-atoms of the channel were pinned (see section S1 in the Supplemental data), the toxin backbone was rigid, and side-chains of both the channel and toxin were flexible. Among several predicted structures we selected the one where functionally important toxin residues established specific contacts (salt-bridges, cation-π, hydrophobic, or knob-into-hole) with channel residues, whose homologs are presumably involved in binding of Css4 to rNa1.2 or/and are in direct contact in the cryo-EM structure of the Na,PaS-Dc1a complex (Table 1). Moreover, in the selected structure, a few polar residues of the toxin approach polar residues of the channel, but do not establish specific contacts, suggesting potential H-bonds. Since the AMBER force field lacks H-bond strengthening functions, which are used in the Rosetta force field [47], we imposed distance constraints to bias the potential toxin-channel H-bonds, removed all other constraints, MC-minimized the structure and arrived at an energetically preferable complex (Fig. 6). Subsequent MC minimization without any constraints has confirmed the complex stability.

3.2.3. Contacts of LqhIT2 with BgNa1.1a. Specific toxin-channel contacts in the complex are listed in Table 1 and are shown in Figures 7A-E. Hydrophobic toxin-channel contacts are shown in Figure 7A,B. For example, the highly important toxin residue I16 (Table 1) forms hydrophobic contacts with the channel residue L869 and with the methylene group of S870, both in the IIIS3-S4 loop (Fig. 7A). Leucine residues in rNa1.2 and Na,PaS, which are in the sequentially matching positions with L869 of BgNa1.1a (Fig. S2), form hydrophobic contacts with Css4 and Dc1a, respectively (Table 1). L15 forms hydrophobic contacts with L1493 in linker IIIP2-S6 (Fig. 7A), whose Na,PaS homolog forms a hydrophobic contact with Dc1a (Table 1). On the opposite side of the toxin, two small residues, G39 and A13, establish hole-into-knob contacts, respectively, with M806 in loop IIIS1-S2 and L1493 in linker IIIP2-S6 (Fig. 7B). The homolog of M806 in Na1.2 is involved in binding of Css4 (Table 1).

The toxin-channel complex is stabilized by several H-bonds that involve functionally important toxin residues. For example, Q867 in the IIIS3-S4 loop accepts an H-bond from K23 and donates an H-bond to E19 (Fig. 7A,C). N58 is H-bonded to D1443 in loop IIIS5-P1 (Fig. 7A), in agreement with the fact that substitution N58D strongly diminished the toxin binding (Table 1) likely due to electrostatic repulsion between the two acidic residues, N58D and D1443. E24 forms a salt-bridge with K1495 in linker IIIP2-S6 (Fig. 7A), consistent with the reduced binding of toxin mutant E24R, likely due to repulsion between the two basic residues, E24R and K1495. K11 forms a salt bridge with D1443 (Fig. 7A) explaining the weaker binding of mutant K11A probably due to the abolished salt bridge (Table 1). W53 donates an H-bond to N1445 (Fig. 7D) and substitution W53V eliminates the H-bond, which explains the dramatic decrease in toxin binding to Periplaneta Americana neuronal membrane preparation (Table 1).

Two cation-π contacts are seen in the toxin-channel structural model. W38 penetrates most deeply into the II-VSM gorge, forms hydrophobic contacts with a number of channel residues and a weak π-cation contact with R1, which is salt-bridged with D802 (Fig. 7E). Another functionally important tryptophan, W36, forms a π-cation contact with K808 (Fig. 7E).
LqhIT2 has two categories of ionizable residues. The first category comprises K11, K23, and E24 whose substitutions dramatically (over 100-fold) changed the LqhIT2 affinity for the neuronal membrane preparation of Periplaneta Americana (Table 1). In our model, these residues form specific contacts with the channel (as described above). The second category comprises 14 residues (D1, K5, R6, R7, D8, E19, D22, K26, E45, D49, D50, K51, K54 and E56) whose substitutions have either mild (< 25-fold) or small (< 5-fold) impact on toxin binding to the cockroach channel. Remarkably, all these residues are exposed to the aqueous environment, enabling hydration of the channel-bound toxin (Fig. 7F,G).

3.2.4. Docking Lqh-dprIT3-c onto BgNa1-1a. The structural model of Lqh-dprIT3-c was built using the crystal structure of LqhIT2 [16]. The amino acid sequences of LqhIT2 and Lqh-dprIT3-c align well with no insertions/deletions, except for some mismatches (Fig. S3). In particular, the functionally important LqhIT2 residues K23 and A13 are substituted in Lqh-dprIT3-c by N23 and S13, respectively (Table 1). Less functionally important LqhIT2 residues include G17 and L15 (Table 1). Among the LqhIT2 residues, which are located at the solvent-exposed side (Fig. 5B and 7F,G), mismatches with Lqh-dprIT3-c are more common (Table 1). The structure of Lqh-dprIT3-c was MC-minimized using the HotGrid sampling of side-chain conformations [48] ending up with high similarity to the crystal structure of LqhIT2 (Fig. S4).

Given this structural similarity including identity of many bioactive residues (Fig. S3), the starting position for Lqh-dprIT3-c docking was set to match that of LqhIT2 in the MC-minimized complex with the channel. The starting structure of the toxin-channel complex was intensively MC-minimized yielding evidently a very similar model to the LqhIT2-BgNa1-1a model. Lqh-dprIT3-c established specific contacts with the same channel segments as LqhIT2 did. Of note is the similarity of H-bonds that K23 in LqhIT2 and N23 in Lqh-dprIT3-c form with Q867 in the IIS3-S4 loop of the channel (Fig. 7A,B and S5C,E). Other residues, whose substitution has affected the binding affinity of LqhIT2 [16], are also involved in H-bonds that Lqh-dprIT3-c forms with the same channel residues. The network of hydrophobic toxin-channel contacts is also conserved in the two toxin-channel complexes (cf. Figs. 7A,B and S5C,D). The main difference between the two complexes lies in the toxin contacts with the salt bridge between D802 and R1. W38 of LqhIT2 forms a π-cation contact with R1, but is distant from D802 (Fig. 7E), whereas W38 of Lqh-dprIT3-c is close to both R1 and D802, but does not form specific contacts with these residues (Fig. S5E,F).

All residues but one, whose substitutions have decreased LqhIT2 binding over 100-fold, are identical between the two toxins (Table 1). However, less functionally important residues in LqhIT2, glycines G17 and G20, are substituted in Lqh-dprIT3-c by N17 and F20, respectively (Table 1), and make specific contacts with the channel (Fig. 7B,C). This may explain why Lqh-dprIT3-c is 15 fold more active than LqhIT2 [12].

3.2.5. In-silico deactivation of II-VSM bound to Lqh-dprIT3-c. There is no structure available of a β-toxin bound to the II-VSM of a sodium channel at rest. Modeling of such a structure requires at least the coordinates of the II-VSM in its resting state. VSMs in crystal and cryo-EM structures of voltage-gated sodium channels, including NaPaS [20], are usually captured in their energetically preferable activated states, with the S4 voltage-sensing helices in their outward position. Among notable exceptions are the crystal structures of Na1.7-Ab where S4s remain in their resting state with engineered disulfide linkers [49], and the cryo-EM structure of the Na1.7-
Na,PaS chimeric channel where IVS4 cannot reach its outward position due to a bound scorpion α-toxin [22].

In the 3D-aligned crystal structures of Na,Ab with VSMs in the resting (PDB ID: 6P6W) and activated (PDB IDs: 6p6x, 6p6y) states, the z-coordinates of C α_R1 differ by ~10 and ~11 Å, respectively. In the 3D-aligned cryo-EM structures of the Na,1.7-Na,PaS channel with IV-VSM in the resting (PDB ID: 6nt4) and activated (PDB ID: 6nt3) states, the z-coordinates of C α_IVR1 differ by ~10.5 Å. However, employing the resting-VSM structures of Na,Ab or Na,1.7-Na,PaS as templates to model the resting II-VSM in BgNa,1-1a would be inappropriate due to two major reasons. Firstly, the mutual disposition of PD and VSMs in different P-loop channels varies. Secondly, such models would not suggest intermediary structures between the resting and activated II-VSM.

To overcome these problems, we in-silico deactivated II-VSM in the Lqh-dprIT3-c-BgNa,1-1a complex by stepwise shifting C α atoms of IIIS4 basic residues from their positions in the activated II-VSM in the intracellular direction with 0.5 Å steps and MC-minimizations at each step (see Methods). A total of 21 steps were computed resulting in overall displacements of the C α atoms by 10 Å along the z-axis. This is approximately equal to the displacement of C α atoms of S4 basic residues between the inward and outward positions of S4 helices in the crystal structures of the Na,Ab and Na,1.7-Na,PaS channels. No other constraints were imposed on the channel or toxin. Although this methodology has its limitations, it provided for the first time a glimpse of possible state-dependent changes in the channel-toxin complex upon movement of IIIS4.

The superposed 21 states are shown in Fig. 8A,B. The enforced displacement of IIIS4 caused IIIS4 basic residues to switch salt-bridges and H-bonds within II-VSM (Fig. S6A,B and Table S4). This is consistent with voltage-dependent formation of ion pairs between basic residues in S4 and acidic residues in S2 or S3 during activation of the voltage sensor of a bacterial sodium channel [42, 50, 51]. The displacement of IIIS4 downshifted the toxin by only 2–3 Å (Fig. 8 A-F), but caused considerable perturbations in linker IIIS3-S4 (Fig. 8C). These involve side-chains of Q867, L869 and S870 that in the activated II-VSM make strong contacts with the toxin (Fig. S5C). In particular, I16, the functionally most important residue in LqhIT2 (Table 1) and likely in Lqh-dprIT3-c, shifted noticeably due to maintaining hydrophobic contacts with the moving L869 and S870 (Fig. 8C,E), but did not follow them all the way because this would have destroyed other toxin-channel contacts. In contrast, the changes in linker IIS1-S2 were rather small (Fig. 8D). Displacements of some residues in II-VSM and toxin between the activated (green carbons) and resting (brown carbons) states of II-VSM are shown in Figure 8E,F. R1 shifted significantly, but D802 did not follow. Significant shifts of Q867 and L869 in linker IIIS3-S4 caused noticeable, yet smaller shifts of their contacts, N23 and I16, respectively.

Thus, the in silico deactivation of II-VSM weakened specific contacts of the toxin with linker IIIS3-S4, with little, if any, changes of the toxin contacts with PD-III. These results, along with previous studies [15, 28, 46] suggest that initially the toxin binds to PD-III and to II-VSM at rest. Upon II-VSM activation, the toxin contacts with IIIS3-S4 are strengthened, the toxin clamps the activated II-VSM to PD-III, and prevents II-VSM deactivation by maintaining strong contacts with the IIIS3-S4 linker. Substitutions in II-VSM may affect the toxin binding either at channel rest or at the activated state. The correlations between Lqh-dprIT3-c induced hyperpolarizing shifts of the conductance curves and increased fraction of the toxin-modified channel mutants (Sections 3.1.2 and 3.1.3) suggest that the toxin interacts with the activated II-VSM, while III-PD is in the open state.
3.2.6. Salt bridge D802-R1 opposes deep penetration of Lqh-dprIT-c into the II-VSM gorge.

In the model of Lqh-dprIT-c bound to WT BgNa1-1a, D802 and R1 form a salt-bridge (Fig. 9A). R2 forms a salt bridge with E860 in IIS3 and approaches R1. Due to the electrostatic repulsion between the positive charges of R1 and R2, the D802-R1 salt bridge is fortified. This prevents R1 from changing its conformation to form a salt-bridge with the nearby E864 in IIS3. The close proximity between R1 and R2 seems electrostatically unfavorable, yet analogous orientations of arginines R1 and R2 in activated II-VSMs appear in the cryo-EM structures of various Na channels, including Dc1a bound to Na3PaS [21] and the chimeric channel Na3Ab-Na1.7 with the bound spider toxin ProTx2 [52]. In our model, W38 of Lqh-dprIT-c is in close proximity to the salt-bridged D802 and R1 (Fig. 9A), but W38 is unable to penetrate deeper into the II-VSM gorge to form a π-stacking complex with R1. Nevertheless, W38 forms contacts with M806 in IIS1-S2 and K815 in IIS1. This may explain why substitution of W38 in the homologous depressant β-toxin LqhIT2 decreased 250 fold the binding affinity to an insect neuronal membrane preparation [16]. Furthermore, in our model I16 forms hydrophobic contacts with L869 of IIS3-S4 (Fig. 9A), in agreement with the fact that substitution of I16 for alanine decreased ~700 fold the binding affinity of LqhIT2 [16].

3.2.7. Lqh-dprIT-c interactions with channel mutants. Although the basal side of the toxin penetrates into the II-VSM gorge in both the activated and resting channel states, the contacts of Lqh-dprIT-c with linker IIS3-S4 seem to depend on the II-VSM state (Figure 8E). To shed more light on the toxin-channel interaction, we constructed models of the channel mutants with Lqh-dprIT-c bound to PD-III and II-VSM in the activated state.

In channel model D802A, R1 interacts with E864 and E860 and repels R2 that forms a salt-bridge with E864 (Fig. 9B). As R1 and R2 no longer limit W38 from entering deeper into the II-VSM gorge, this tryptophan now establishes a strong π-cation contact with K815 at IIS1, donates an H-bond to D811 at IIS1, and retains van der Waals contacts with M806 at IIS1-S2 (Fig. 9B). Other interactions of the toxin with the D802A channel mutant include an H-bond between D811 at IIS2 with W36 of Lqh-dprIT-c, as well as an H-bond between K815 and H18 of the toxin. Strikingly, despite the substantial alterations of contacts between the basal side of the toxin and II-VSM in channel mutant D802A, residues Q867 and L860 in the IIS3-S4 linker retain their contacts, respectively, with N23 and I16 of Lqh-dprIT-c. The deeper penetration of W38 into the II-VSM gorge and additional interactions between the toxin and channel mutant D802A may explain the increased sensitivity of this channel to Lqh-dprIT-c (Figure 2H).

In channel mutants D802A and D802K (Fig. 9 C,D), most of the toxin-channel interactions are similar, but an additional cation-π interaction is formed between K802 of channel mutant D802K and W38 of Lqh-dprIT-c. This interaction provides a unique cation-π-cation ‘sandwich’ involving W38 and two lysines of the channel (Fig. 9C).

In channel mutant R1E the glutamate substitution did not establish any salt bridge, yet it formed H-bonds with S870 at IIS3-S4 and Q1419 at IIS5 (Fig. 9D). The R1E substitution released D802 from its salt-bridge with R1 so that D802 formed an H-bond with W38 of Lqh-dprIT-c, which in turn forms a π-cation contact with K815. Still, W38 makes fewer contacts with II-VSM than in the model of Lqh-dprIT-c-D802K complex. This may explain the lower sensitivity of the R1E channel mutant to the toxin compared to channel mutant D802K (Fig. 4F).

In channel mutant R2E, the glutamate substitution does not restrict the conformational mobility of R1 as in the WT channel (Fig. 10A). The side-chain of R1 turns towards IIS3 to form salt bridges with E860 and E864 and π-stack with W38 of Lqh-dprIT-c. Such arginine-tryptophan
\(\pi\)-stacking pairs are seen in crystal structures of some proteins, where they contribute to the structure stabilization [53, 54]. Furthermore, W38 donates an H-bond to D802. The above interactions of W38 with R1 and D811 can explain why channel mutant R2E is much more sensitive to the toxin than the WT channel (Fig. 4F).

Among the most paradoxical observations in the present study is the high sensitivity of channel mutant R3E to the toxin (Fig. 4F). The glutamate substitution is very far from the toxin, from D802 and from R1 (Fig. 10B). In terms of toxin-channel contacts, channel mutants R2E and R3E are rather similar (cf. Figs. 10A and 10B). It seems in the R2E channel model that the conformation of R1 is firmly restrained by the salt-bridges with E864 and E860 as well as by an electrostatic attraction to R2E, whereas in the R3E channel model arginine R2 electrostatically repels R1, likely increasing its conformational flexibility. This may facilitate the \(\pi\)-stacking interactions of R1 with W38 of Lqh-dprIT\(3\)-c.

Many toxin contacts in channel mutant D802K/R1E (Fig. 10C) are similar to those in channel mutant D802K. However, the cation-\(\pi\)-cation interactions involving W38 of Lqh-dprIT\(3\)-c in channel mutant D802K/R1E are weaker than in channel mutant D802K because K802 is attracted by R1E, whereas K815 is attracted by E864 and Y812. This may explain why double-mutant channel D802K/R1E is less sensitive to the toxin than the single-mutant channel D802K (Fig. 4F).

In channel mutant D802K/R2E, the side-chain of R1 turns in the cytoplasmic direction and towards IIIS3 to form salt bridges with R2E and E864 (Fig. 10D) and electrostatic interaction with E860. W38 of Lqh-dprIT\(3\)-c is involved in multiple interactions with the channel: (i) H-bond with D811, (ii) cation-\(\pi\)-cation interactions with K815 and K802, (iii) hydrophobic interactions with M806, L814 and methylene groups in K815 and K802, and (iv) edge-to-plane interactions with R1. These interactions may explain the higher sensitivity of channel mutant D802K/R2E to the toxin compared to channel mutant D802K/R1E (Fig. 4F).

4. Discussion

The interaction of scorpion \(\beta\)-toxins with their target voltage-gated Na-channels has been studied for more than two decades [10, 12, 15, 16, 28, 34, 42, 43, 46, 55-59] and yet, despite the elucidation of their pharmacological mode of action, bioactive surfaces and putative binding site at the channels, molecular details of the toxin-channel interactions have not been fully described. To this end we combined the data accumulated on toxin binding, electrophysiological effects, and structure with mutational analysis and structural modeling of an insect sodium channel with the objective to better understand at the atomic level the mechanism by which \(\beta\)-toxins obstruct the channel gating. We used the cryo-EM structure of Na-PaS [20] as template to first model the cockroach sodium channel BgNa\(1\)-1a (Song et al., 2004), and then the crystal structure of LqhIT2 depressant toxin (Karbat et al., 2007) to model BgNa\(1\)-1a in complex with LqhIT2 and with the super active depressant toxin Lqh-dprIT3-c [12]. Highly useful in the modeling process were the models of the mammalian brain channel Na\(1\)-1.2 with the bound scorpion \(\beta\)-toxin Css4 [42, 43] and the structure of Na-PaS with the spider toxin Dc1a [21].

**The critical role of D802 in channel gating.** An acidic residue at the extracellular end of IIIS1 is conserved in vertebrate and invertebrate voltage-dependent sodium channels, as well as in potassium and calcium channels. In the rNa\(1\)-1.2 channel, charge neutralization of E779, the homolog of D802 in BgNa\(1\)-1a, rendered a large depolarizing shift of \(\Delta V_{0.5}\) [46]. The Shaker potassium channel Kc2.1 bears E247 in the homologous position [60] and a 3D model based on
the crystal structure of the open channel [61] predicted a salt bridge between this glutamate and an arginine in IIS4. Substitution E247W that eliminated the salt bridge, destabilized the channel open state, shifting $V_{0.5}$ by +50 mV [62]. In the bacterial sodium channel NaChBac, neutralization of E43 at the extracellular end of S1 also resulted in a large (+47 mV) shift of $V_{0.5}$ [51]. Thus, our conclusion that the activated state of II-VSM of the BgNa$_{1.1a}$ channel is stabilized by the salt bridge between D802 and R1 (Fig. 6 C,D) is consistent with the experimental data available for a variety of voltage-gated ion channels.

When the voltage-sensing helix IIS4 is in the activated, outward position, D802 is the only acidic residue that forms a close contact with R1 (Fig. 9A). Therefore, contribution of the salt bridge to stability of the II-VSM activated conformation is particularly important. Such a salt bridge is likely formed also between R1 and D802E as seen in the cryo-EM structure of the brain channel Na$_{v}$.1.2 [45].

In lack of the salt bridge in channel mutants D802A/G/K, the activated conformation of VSM-II and hence the open conformation of PD are probably destabilized and so a stronger depolarization is required to activate the channel. This explains the depolarizing shift of $V_{0.5}$ (Fig. 2A) and the shift in voltage dependence of activation toward more depolarizing voltages measured in the channel mutants (K > A > G; Du et al., 2010). The largest depolarizing shift of $V_{0.5}$ in channel mutant D802K (Fig. 2A) is likely due to the electrostatic repulsion between K802 and R1, which further destabilized the activated state of II-VSM. Substitution D802E caused a shift in the hyperpolarizing direction because a flexible glutamate probably forms a more stable salt-bridge with R1, supporting the activation of II-VSM. The fact that the above substitutions do not affect channel inactivation (Table S2) suggests that the inactivation process starts when II-VSM is activated and IIS4 still has not returned to its resting position.

In the structural model of BgNa$_{1.1a}$-Lqh-dprIT$_{3c}$ complex (Fig. 9A) the D802:R1 salt-bridge does not contribute to the toxin binding energy. In channel mutant D802A, R1 is no longer engaged in the salt-bridge with the extracellular end of S2 and so it turns towards S3 to form salt-bridges with E860 and E864 (Fig. 9B). This new arrangement allows deeper penetration of W38 of Lqh-dprIT$_{3c}$ into the II-VSM gorge and formation of an H-bond with D811 as well as a π-cation contact with K815 (Fig. 9B). These two contacts, which are absent in the WT channel, may explain why mutant D802A is more sensitive to Lqh-dprIT$_{3c}$ than the WT channel. Similar contacts are likely formed between the toxin and channel mutant D802G. Moreover, channel mutant D802G is more sensitive to Lqh-dprIT$_{3c}$ than channel mutant D802A (Fig. 2H) probably because the flexible loop IIS1-S2 adjusts more easily to the toxin surface.

In channel mutant D802K, additional cation-π contacts are formed between K802 and W38 of the toxin resulting in a cation-π-cation ’sandwich’ (Fig. 9C). Analogous interactions of tryptophan with two basic residues (Arg-Trp-Arg) appear, for example, in a ubiquitin variant (PDB ID: 5TOG). The stronger attraction of the toxin W38 to II-VSM may explain why channel mutant D802K is even more sensitive to Lqh-dprIT$_{3c}$ than channel mutant D802A (Fig. 2H). Overall, the sensitivity of the channels to the toxin (WT = D802E < D802A < D802G < D802K) qualitatively correlates with the number of favorable toxin contacts with the activated II-VSM (Fig. 2H), and it also qualitatively correlates with the negative shifts of $V_{0.5}$ in toxin-bound vs. toxin-free channels (Fig. 2C-G). The structural models of the WT and D802A/K channel mutants (Fig. 9A-C) provide the basis of these interpretations, namely, the Lqh-dprIT$_{3c}$ contacts with the activated II-VSM in the mutants are fortified and increase the Lqh-dprIT$_{3c}$ hold over II-VSM. The stronger contacts likely shift the equilibrium between the resting and activated II-VSM towards the activated state and increase the probability of PD to remain open.
Do the basic residues in IIS4 affect the channel interaction with depressant toxins? Since the interaction between D802 and R1 in IIS4 stabilizes the activated state of the channel, and since the other basic residues are involved in the formation of intermediary states during the outward and backward movement (sliding helix) of IIS4 upon gating, we asked how substitution of these basic residues would affect the channel interaction with scorpion depressant toxins. Charge reversal of the basic residues at IIS4 has a smaller effect on the G-V curves than substitutions of D802. Charge reversal substitutions R1E and R2E slightly facilitated the activation, whereas substitutions R3E, K4E and K5E had an opposite effect (Fig. 3A). A possible reason is that the basic residues are attracted to the inner leaflet of the hyperpolarized membrane and thus facilitate the backward movement of IIS4 upon membrane hyperpolarization, while the negative charges of glutamate substitutes impede this movement, and so resist deactivation of II-VSM and the pore closure. Some analogy may be found in the study of the Shaker channel, where substitution of R4 by the electroneutral citrulline resulted in a substantial depolarizing shift of the G-V curve, suggesting that the positive charge of R4 was important for the S4 deactivation [63].

All double substitutions, except D802K/K4E, rendered negative shifts of $\Delta V_{0.5}$ in comparison to the background channel mutant D802K (Fig. 4A; Table S2). The shift increased with the distance of the glutamate substitutes from the membrane inner leaflet (D802K/R1E $\geq$ D802K/R2E $\geq$ D802K/R3E $> D802K/K4E$), likely a result of the electrostatic repulsion of the glutamate substitutes from the membrane inner leaflet. A large negative shift of $\Delta V_{0.5}$ in channel mutant D802K/R1E vs. the D802K background (-24.5 mV) (Table S2) can be explained by its structural model. The formation of a salt bridge between D802K and R1E in the D802K/R1E channel mutant stabilized the activated conformation of II-VSM (Fig. 10C), and so its deactivation required stronger hyperpolarization. The shift of $\Delta V_{0.5}$ in channel mutant D802K/R2E vs. the background channel D802K (-12.2 mV) was also larger than the shift of $\Delta V_{0.5}$ in channel R2E vs. the WT channel (-3.8 mV). This is consistent with the D802K/R2E channel model where contacts of R2E with two glutamates in IIS3 likely stabilize as well the activated conformation of II-VSM.

The increase in channel sensitivity to the toxin upon glutamate substitutions of basic residues (Fig. 4F) did not correlate with the shifts of $\Delta V_{0.5}$ (Fig. 3A). A possible reason is the state-dependency of electrostatic interactions of the ionized residues within II-VSM. The charge reversal might change the relative stability of the activated and resting states of II-VSM. Moreover, the glutamate substitutions may have decreased the electrostatic attraction of IIS4 to the cytoplasmic side of the hyperpolarized membrane.

Substitutions R1E, R2E and R3E dramatically increased the channel sensitivity to Lqh-dprIT3-c (Fig. 4F). The structural models of channel mutants R1E, R2E and R3E with bound Lqh-dprIT3-c (Fig. 9D and 10A,B) predict stronger toxin-channel interactions due to elimination of the D802:R1 salt bridge, leading to increased number of specific toxin-channel contacts. The sensitivity of channel mutant K4E to the toxin is comparable with that of the WT channel (Fig. 4F) likely because K4E is far from salt bridge D802:R1 and from R2, which stabilizes the salt bridge (Fig. 9A). Channel mutants R2E, R3E and K4E are as sensitive to the toxin as the background channel mutant D802K (Fig. 4F). The reason is that in the D802K channel mutant, W38 of the toxin is engaged in several specific contacts with II-VSM (Fig. 9C), and so charge reversal of the basic residues in IIS4 has a small impact on these contacts as seen in the structural model of the D802K/R2E channel mutant (Fig. 10C).
General resemblance in the interaction of scorpion gating modifier toxins with their target Na-channels. Neutralization or charge reversal of the two outermost arginines in IIS4 enhanced the action of the scorpion β-toxin Css4 on the mammalian channel Na1.2, supposedly by facilitating the toxin-mediated trapping of S4 in its outward position [46, 50]. Our findings that charge reversals of R1, R2 or R3 facilitated as well the action of Lqh-dprIT3-c on the insect sodium channel (Fig. 3B-D) may be explained similarly by the structural models of channel-toxin complexes where each glutamate substitution causes more favorable toxin-channel interactions. The LqhIT2-BgNa1.1a model shares a few commonalities with the model of Css4-Na1.2 complex: (i) Aromatic residues in sequentially matching positions of Css4, LqhIT2 and Lqh-dprIT3-c (Fig. 1A,C) are located in the loop between two beta-strands that penetrate deeply into the gorge of II-VSM (Fig. 4E). F48 between two beta-strands in Dc1a also penetrates deeply into the II-VSM gorge of Na1PaS; (ii) Most of the ionizable residues in Css4, LqhIT2 and Dc1a are exposed extracellularly and do not interact with the channels; (iii) External residues in the helical segment of LqhIT2, Lqh-dprIT3-c and Css4 interact with loop IIS3-S4; (iv) The beta-strands in LqhIT2, Lqh-dprIT3-c and Css4 do not form contacts with the channel; (v) The C-termini of Css4 and LqhIT2, and Lqh-dprIT3-c interact with the extracellular loop PD-III.

Interestingly, the cryo-EM structures of the chimeric channel bearing the pore domain from Na1PaS and IV-VSM from Na1.7 in complex with the scorpion α-toxin AaH2 [22] exhibits as well various analogies with the LqhIT2-BgNa1.1a structural model. The general orientation of the α-toxin AaH2 over IV-VSM is similar to that of β-toxins over II-VSM in several aspects: (i) The helical segment of both α- and β-toxins is distant from the PD and is closer to loop S3-S4 than to loop S1-S2; (ii) The C-termini of both α- and β-toxins approach loop S5-P1 in the PDs; (iii) In both α- and β-toxins an aromatic residue at the apex of the loop between the β-strands penetrates most deeply into the VSM gorge; (iv) Comparison of cryo-EM structures of the Na1PaS/Na1.7 chimeric channel in the apo- and AaH2-bound states [22] shows that major changes upon toxin binding occur in loop IVS3-S4, which forms multiple stabilizing contacts with the toxin. A dramatic toxin-induced shift of linker IVS3-S4 is also seen in the superimposed cryo-EM structures of rNa1.5 and its complex with the deathstalker scorpion toxin [23]. In agreement with the experimental structures, our BgNa1.1a-Lqh-dprIT3-c models with the activated and in-silico deactivated VSD-II also show large shift of IIS3-S4, which affect its contacts with the toxin (Fig. 8).

Like in the case of Css4 interaction with Na1.2, the effect of Lqh-dprIT3-c on \( αV_{0.5} \) can be detected only after a strong depolarizing prepulse to fully activate BgNa1.1a. However, while a short (5 ms) prepulse is sufficient for Css4 to trap Na1.2 in the activated state, such a prepulse was insufficient to detect action of Lqh-dprIT3-c on BgNa1.1a [34]. Complete channel activation and putative IIS4 trapping by Lqh-dprIT3-c could be observed only after applying a 20 Hz train of fifty conditional depolarizing prepsules of 5 ms to 50 mV. The requirement of multiple depolarizing prepsules to observe the full action of Lqh-dprIT3-c suggests that formation of multiple toxin-channel contacts requires intensive shuttle-like movements of II-VSM which facilitate mutual adjustment of the toxin and channel residues. This effect is analogous to use-dependent action of various ligands that target the inner pore of Na1s, where multiple depolarizations are necessary to achieve the maximal effects.

In a recent study, experimental, phylogenetic and molecular modeling approaches were used to propose models of LqhIT2-Na1PaS with activated and resting II-VSM [26]. The models were generated using web servers ZDOCK and SWISS-MODEL. Noteworthy, any protein-protein docking method yields structures, which are critically dependent on the respective starting...
approximations. The toxin orientation in our LqhIT2-BgNa1-1a model is different from that in the LqhIT2-Na,PaS model [26]. In particular, Q604 in loop IIS3-S4 of the LqhIT2-Na,PaS model interacts with the toxin cavity formed by T3, K11 and W53. In our LqhIT2-BgNa1-1a model, Q867 (homolog of Q604 in Na,PaS) accepts an H-bond from K23, whereas K11 and W53 form specific contacts, respectively, with D1443 and N1445 in loop IIS5-P1 (Fig. 7D). Furthermore, in the LqhIT2-Na,PaS model, N58 interacts with G602 of the channel [26], whereas in our model N58 donates an H-bond to D1443 (Fig. 7D).

Meanwhile, some experimental data of Zhu and co-authors [26] which became available as we were finalizing our model, can be rationalized using our model. First, substitution F609N near IIR1 of Na,PaS resulted in channel resistance to 100 μM LqhIT2 [26]. In our model, L872 (homolog of F609 in Na,PaS) is far from the toxin in both the resting and activated states of II-VSM. Instead, L872 forms a strong hydrophobic contact with L856 in the resting (Fig. S7B), but not in the activated state of II-VSM (Fig. S7A). Analogos of the two leucine residues in BgNa1-1a are phenylalanine residues, F609 and F593, in Na,PaS (Fig. 1D), which likely form a stacking contact in the resting II-VSM. Substitution F609N in Na,PaS would eliminate the stacking contact and destabilize the II-VSM resting state, which is targeted by the toxin. Furthermore, substitution L40A decreased only slightly the LqhIT2 toxicity in two insect species, whereas substitutions L40N and L40R decreased significantly the toxicity [26]. In our models, L40 is located above loop IIS1-S2, approaches L1493 in loop IIIP2-IIIP6, and M810 in loop IIS1-S2, but does not form tight contacts with these residues (Fig. S7C), consistent with the little decrease of LqhIT2 toxicity upon substitution L40A [26]. However, L40R or L40N would render repulsion from the hydrophobic residues L1493 and M810 (and their homologs in Na,PaS), thus weakening the toxin-channel interactions, in agreement with the experimental data [26]. Also, substitution Q604E in IIS3-S4 (Na,PaS number) of DmNa1 abolished the channel sensitivity to LqhIT2 [26]. In our model, Q867, the analog of Q604 in Na,PaS, is H-bonded to E19 and K23 (Fig. 7A). Its substitution for glutamate (Q867E) would electrostatically repel E19 and so reduce the binding of the toxin. Thus, despite the fact that our LqhIT2-BgNa1-1a model is essentially different from the LqhIT2-Na,PaS model [26], it is quite consistent with the experimental data.

Finally, it should be noted that voltage dependence of channel activation and variations in sensitivity to the toxin of the channel mutants depend as well on various factors, such as membrane potential, possible transitions between alpha-helical and 3_10 helical conformations in some portions of the transmembrane helices upon channel gating, and impact of lipids, ions and the aqueous environment on toxin-channel interactions. Despite these limitations, the structural models constructed in this study were useful in explaining most of the experimental data obtained in this and some previous studies.

5. Conclusion

The strong activity of the scorpion β-toxin Lqh-dprIT3-c on the cockroach splice variant BgNa2-1 bearing G802 at the extracellular side of IIS1 (all other BgNa variants bear D802) led us to examine substitutions D802G/A/K in the most common channel variant BgNa1-1a (referred here as the wild-type channel). These substitutions increased prominently the channel sensitivity to the toxin. Furthermore, substitutions R1E, R2E and R3E, as well as double substitutions R1E/D802K, R2E/D802K and R3E/D802K, also increased the channel sensitivity to the toxin. Therefore, this study focused on the structural entity surrounding residue 802, its putative interactions with the positively-charged IIS4 residues and the channel interactions with Lqh-
Structural models of the channel in complex with the depressant scorpion β-toxin LqhIT2 or its super-active homolog Lqh-dprIT3-c were generated using the crystal structure of LqhIT2 and the cryo-EM structure of Na₃PaS as template. The reliability of these models is supported by the results from previous binding studies using toxin mutants [16] as well as electrophysiological assays using BgNa₁-1a mutants (present study). In our model, all functionally important LqhIT2 residues [16] make specific contacts with channel residues in the II-VSM extracellular loops IIS₁-S₂ and IIS₃-S₄, as well as with the III-PD extracellular loops IIS₅-P₁ and IIIP₂-S₆. The models suggested that the low sensitivity of BgNa₁-1a to the toxin is due to the salt-bridge between D802 and R1. All substitutions that eliminated the salt bridge enabled formation of additional toxin-channel contacts that increased the channel sensitivity to Lqh-dprIT3-c. BgNa₁-1a residues, which interact with the toxins, are homologous to residues in the mammalian brain channel rNa₁.2, that are involved in binding of the β-toxin Css₄ [42, 43] and also Na₃PaS residues that interact with Dc₁a [21]. In-silico deactivation of II-VSM in the Lqh-dprIT₃-c-BgNa₁-1a complex rendered a limited shift of the toxin, but weakened its contacts with linker IIS₃-S₄, in agreement with the data indicating that the toxin stabilized the activated state of II-VSM. Overall, these models describe how scorpion β-toxins interact with their target insect sodium channels, and enable rationalization of previous results obtained in mutagenic dissection of depressant toxins as well as in mutagenesis of the channel. Our models integrated results from a large body of previously published mutational and binding data as well as new mutational and electrophysiological experiments from our study and also another recent study (Zhu et al. 2020). Future studies involving mutational and electrophysiological analyses of multiple toxin-channel contacts, or cryo-EM structural analysis of toxin-channel complexes are needed to further validate our models.

Supplemental data
The Supplemental data include seven Figures and four Tables.

Data availability
The 3D models of the WT channel BgNa₁-1a and channel mutants in complex with Lqh-dprIT₃-c have been submitted to BioModels (https://www.ebi.ac.uk/biomodels/). Submission identifiers: MODEL2103300001, MODEL2103310001

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Table 1. Mutagenic dissection of LqhIT2 affects the binding to *Periplaneta Americana* neuronal membrane preparation [16] and the contacts a of LqhIT2 and Lqh-dprIT3-c with side-chains in the Na\(_{\text{v}}\)PaS-based model of BgNa1.1a

| Mutant | Ki Mutant/Ki WT | Contact | Fig. 3 | Lqh-dprIT3-c | Fig. 4 | Matching Css4-sensing residues in Na\(_{\text{v}}\)1.2 | Matching Dc1a-binding residues in Na\(_{\text{v}}\)PaS | LqhIT2 Mutant | Contact | Residue b | Contact | Residue | Matching Contact | Matching Contact |
|--------|-----------------|---------|--------|--------------|--------|---------------------------------|---------------------------------|--------------|---------|-----------|---------|-----------|----------------|-----------------|
| I16A   | 700             | L869    | B      | 780          | C      | L846A                           | L869                            | L869         | S870    | B         | S870    | C         | E844N/R        | [42]            |
| K23A   | 600             | Q867    | B.D    | 780          | C      |                                 |                                 | E844N/R      | N23     | Q867      | C,E     |           |     | [42]            |
| W53V   | 650             | N1445   | C,E    | 780          | C      |                                 |                                 | E779Q/C      | D1443   | D         |  |          |     | M783C         | [28]            |
| N58D/G/A | 50/590/110     | D1443   | E      | 780          | C      |                                 |                                 | E779Q/C      | D1443   | D         |  |          |     | M783C         | [28]            |
| W36A   | 250             | K808    | F      | 780          | C      |                                 |                                 | I830         |  |          |     |           |     |               |                 |
| W38A   | 250             | R1      | F      | R1           | E,F    |                                 |                                 | R1           |  |          |     |           |     |               |                 |
| K11A/T/R | 240/122/25     | D1443   | E      | D1443        | D      |                                 |                                 | D802         |  |          |     |           |     |               |                 |
| E24A/R | 119/460        | K1495   | B      | K1495        | C      |                                 |                                 | L1439A/E     |  |          |     |           |     |               |                 |
| A13E/S/W | 61/2.7/0.8   | H1494   | C      | S13          | H1494  |                                 |                                 | E1348A       |  |          |     |           |     |               |                 |
| G20A   | 61              | L869    | F20    | L869         | C      |                                 |                                 | L846A        |  |          |     |           |     |               |                 |
| G9N    | 55              | -       | -      |              | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| N18A   | 35              | K815    | D      | H18          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| G17A   | 33              | G868    | N17    | E865         | E      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| L15A   | 24              | L1493   | B      | V15          | L1493  | C                                | V1493            | -            |  |          |     |           |     |               |                 |
| Y3F    | 21              | -       | -      |              | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| Y28A/W | 8/27            | -       | -      | A28          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| R6A    | 4.3             | G,H     | -      | G6           | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| D49A   | 2.4             | -       | "     | A49          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| D50A   | 2.4             | -       | "     | E50          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| R3D    | 2               | -       | "     | G7           | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| Y34A   | 1.9             | -       | "     | -            | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| E19A   | 1.2             | -       | "     | V19          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| K5A    | 1.1             | -       | "     | R5           | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| K51G   | 1               | -       | "     | R51          | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| E58G   | 1               | -       | "     | -            | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |
| D22A   | 0.9             | -       | "     | -            | -      |                                 |                                 | -            |  |          |     |           |     |               |                 |

a Shown are specific contacts (H-bonds, salt bridges, hydrophobic, cation-π) of the toxin with the channel side-chains within 4 Å from the toxin residue. b Lqh-dprIT3-c residues identical to sequentially matching residues of LqhIT2 (Fig. 1A) are not shown. c PDB ID: 6A90. d BgNa1.1a numbers. e D802 and W38 are 6 Å apart. f K11 and N1445 are less than 4 Å apart, but do not form an H-bond.
**Figure Legends**

**Figure 1.** Substitution D802G increases the sensitivity of BgNa,2-1 to Lqh-dprIT3-c. A, Membrane topology of the sodium channel protein. B, The protocol used to measure the effect of Lqh-dprIT3-c. C, I-V curves. D, Conductance curves of channel variant BgNa,2-1 in the absence or presence of Lqh-dprIT3. E, Modification of BgNa,2-1G802D activation by Lqh-dprIT3-c. F, Dose-response curves (the percentage of modified channels) of BgNa,2-1 and BgNa,2-1G802D in the presence of Lqh-dprIT3-c. G, Hyperpolarizing shift in the voltage dependence of activation of BgNa,2-1 following substitution G802D.

**Figure 2.** Effects of D802 substitutions on BgNa,1-1a gating properties and sensitivity to Lqh-dprIT3-c. A, Voltage-dependent activation of toxin-free channels. B, Voltage-dependent inactivation of toxin-free channels. C-G, Voltage-dependent activation of channel mutants in the absence (filled circles) and presence (open circles) of Lqh-dprIT3-c. H, Sensitivity (the percentage of modified channel) of the WT and mutant channels to Lqh-dprIT3-c. (*) indicates significant difference from the WT channel using one-way ANOVA with Scheffe's post hoc analysis (p < 0.05).

**Figure 3.** Effects of charge reversal of five basic residues in IIS4 on the voltage-dependent activation of toxin-free and toxin-bound BgNa,1-1a. A, Voltage-dependent activation of toxin-free WT and mutant channels. B-F, Effects of charge reversals on the voltage-dependent activation of toxin free (filled circles) and toxin-bound (open circles) channels.

**Figure 4.** Effects of double substitutes on BgNa,1-1a gating and sensitivity to Lqh-dprIT3-c. A, Effect of double substitutions (charge swapping between D802 and each of the five basic residues in IIS4) on the voltage-dependent activation of BgNa,1-1a. B-E, Effect of 100 nM Lqh-dprIT3-c on the voltage-dependent activation of the single and double mutants. Conductance curves in the absence and presence of the toxin are shown by filled and open circles, respectively. F, Sensitivity (the percentage of modified channels) of the WT and channel mutants to Lqh-dprIT3-c. (*) indicates significant difference from the WT channel using one-way ANOVA with Scheffe's post hoc analysis (p < 0.05).

**Figure 5.** Four views on LqhIT2 crystal structure oriented against II-VSM and III-PD. Residues whose substitution reduced toxin binding to the Periplaneta americana channel over 100-fold (Table 1) are colored. Names of the channel segments proximal to the toxin are shown. A, The basal side of the toxin is exposed to the II-VSM gorge. B, Solvent-exposed side of the toxin opposite to the basal side. C, The lower part of the toxin side is exposed to linker IIS1-S2. D, The lower part of the toxin side, which is exposed to linker IIS3-S4, bears several functionally important residues (Table 1).

**Figure 6.** Na,Pas-based model of BgNa,1-1 with bound LqhIT2. Repeats I, II, III and IV are in pink, yellow, green and gray, respectively. The toxin is in blue. A and B, Extracellular and side views with space-filled toxin. C and D, Detailed views. Channel repeats are colored as in panels B and the toxin is in blue.

**Figure 7.** Contacts between LqhIT2 and BgNa,1-1a. See text (section 3.2.3) for details. Residues involved in hydrophobic toxin-channel contacts (A) and knob-into-hole contacts (B) are space-filled. Salt-bridges and H-bonds in toxin-channel contacts are indicated by dashed
lines in A-D. E, The functionally-important W36 (Table 1) is engaged in \( \pi \)-cation contacts with K808 in IIS2. D802 is salt-bridged with R1, which forms a weak cation-\( \pi \) contact with W38. F and G, Side and extracellular views of ionizable residues whose substitutions had only a minute impact on binding of LqhIT2 to the Periplaneta Americana membrane preparations [16]. The residues exposed to the extracellular space, do not interact with the toxin, but enable its hydration. Note that these residues are most different between toxins LqhIT2 and Lqh-dprIT3-c (Table 1 and Fig. S3).

**Figure 8. In-silico deactivation of II-VSM with bound Lqh-dprIT3-c.** A and B, Overview. Although toxin-channel constraints were not imposed, the toxin swung to the cytoplasmic direction. For example, the \( z \)-coordinate of atom C\( ^\beta \) in Ile16 changed by 2.7 Å. The deeper binding of the large bulky toxin to rather a narrow gorge of II-VSM was opposed by sterical repulsion with IIS1-S2 and IIS3-S4 and attraction to PD-III. C, Residues Q867, L869 and S870 in linker IIS3-S4 moved substantially upon the II-VSM deactivation, changing contacts with the toxin. D, Upon II-VSM deactivation, the conformational changes in linker IIS1-S2 are rather small. E and F, Superposition of the activated (green) and deactivated (brown) states of II-VSM. Shifts of some residues in the channel and toxin are indicated by arrows.

**Figure 9. Models of the WT channel, BgNa1-1a, and channel mutants D802A, D802K and R1E with bound Lqh-dprIT3-c.** II-VSM is in the activated state (IIS4 is in the outward position). A, WT channel. B, Channel mutant D802A. C, Channel mutant D802K. D, Channel mutant R1E. In all models of the channel mutants, the general orientation of the toxin and its interactions with the P-loop of domain III are similar to those observed in the WT channel. See sections 3.2.4 and 3.2.5 for more details.

**Figure 10. Models of channel mutants D802K/R1E, R2E, D802K/R3E, and R3E with bound Lqh-dprIT3.** A, Double channel mutant D802K/R1E. B, Channel mutant R2E. C, Double channel mutant D802K/R2E. D, Channel mutant R3E. See section 3.2.5 for more details.
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**Figure 2**

(A) Plot showing the relationship between G/Gmax and Voltage (mV) for WT, D802E, D802A, D802G, and D802K Na channels.

(B) Plot showing the relationship between I/Imax and Conditioning Voltage (mV) for WT, D802E, D802A, D802G, and D802K Na channels.

(C) Plot showing the relationship between G/Gmax and Voltage (mV) for BgNa_v1-1a.

(D) Plot showing the relationship between G/Gmax and Voltage (mV) for D802E.

(E) Plot showing the relationship between G/Gmax and Voltage (mV) for D802A.

(F) Plot showing the relationship between G/Gmax and Voltage (mV) for D802G.

(G) Plot showing the relationship between G/Gmax and Voltage (mV) for D802K.

(H) Bar graph showing the modification (%) of BgNa_v1-1a, D802E, D802A, D802G, and D802K Na channels.

* Denotes significant difference.
Figure 4

A

B

C

D

E

F

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Figure 5

A. View from IIS4

B. Extracellular view

C. View from IIS1-S2

D. View from IIS3-S4
Figure 6

VSD-I
VSD-III
VSD-IV
LqhIT2
Extracellular view

PD-III
II-D-I
II-S-A
Side view

IIIS5-P1
IIIP2-S6
IIS3
IIS4
D802
IIIP1
IIIP2
Q867
K23
IIS5
IIS6
IIIS5
IIIS6
IIIS3
IIIS4
R1
D802
Q867
K23
R1
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Figure 8
