Spider toxin inhibits gating pore currents underlying periodic paralysis

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Gating pore currents through the voltage-sensing domains (VSDs) of the skeletal muscle voltage-gated sodium channel Na\textsubscript{V}1.4 underlie hypokalemic periodic paralysis (HypoPP) type 2. Gating modifiers target ion channels by modifying the function of the VSDs. We tested the hypothesis that these toxins could function as blockers of the pathogenic gating pore currents. We report that a crab spider toxin Hm-3 from Heriaues melloteei can inhibit gating pore currents due to mutations affecting the second arginine residue in the S4 helix of VSD-I that we have found in patients with HypoPP and describe here. NMR studies show that Hm-3 partitions into micelles through a hydrophobic cluster formed by aromatic residues and reveal complex formation with VSD-I through electrostatic and hydrophobic interactions with the S3b helix and the S3–S4 extracellular loop. Our data identify VSD-I as a specific binding site for neurotoxins on sodium channels. Gating modifier toxins may constitute useful hits for the treatment of HypoPP.

Hypokalemic periodic paralysis (HypoPP) is characterized by episodes of muscle weakness or paralysis associated with reduced serum potassium levels (1). Attacks often begin in adolescence, range from mild and limited to severe full-body paralysis, and last from hours to days. Many patients with HypoPP develop permanent muscle weakness and require mobility aids later in life (2). Patients with HypoPP are counseled to avoid known triggers, while acetazolamide and other carbonic anhydrase inhibitors may help prevent episodes of periodic paralysis. However, these drugs have no effect or even worsen symptoms in some patients (3).

The resting membrane potential of muscle fibers from patients with HypoPP is excessively depolarized, leading to inactivation of voltage-gated sodium channels, inexcitability, and paralysis of the muscle (4). Mutations in the skeletal muscle voltage-gated sodium channel (Na\textsubscript{V}1.4) and calcium (Ca\textsubscript{V}1.1) channel genes, SCN1A and CACNA1S, are associated with HypoPP (5, 6). Na\textsubscript{V}1.4 and Ca\textsubscript{V}1.1 are responsible for excitability and excitation/contraction coupling in the muscle, respectively, and the molecular pathomechanism of HypoPP is similar for both channels despite their different role and selectivity (6). Mutations found in patients with HypoPP affect arginine residues in the S4 helices of the voltage-sensing domains (VSDs) of Ca\textsubscript{V}1.1 (HypoPP type 1) or Na\textsubscript{V}1.4 (HypoPP type 2).

Na\textsubscript{V} and Ca\textsubscript{V} channels are composed of four homologous repeats (Fig. L4), each closely related to a subunit of voltage-gated potassium channels (K\textsubscript{s}). Each repeat consists of six transmembrane a-helices (S1–S6) and contains a VSD formed by helices S1–S4. The central (main) pore is formed by helices S5–S6 from all four repeats (7) (Fig. 1B). Arginine residues in the S4 helices reposition relative to a hydrophobic charge transfer center when the transmembrane voltage changes (7, 8) (Fig. 1C). Upon depolarization, the S4 segment moves to the extracellular side of the membrane (“up” state), while hyperpolarization pulls it to the intracellular side (“down” state). Up and down conformations of the VSDs stabilize the open and closed states of the pore domain, respectively. The S4 helix moves within a structure called the gating pore that is formed by the VSD. When an S4 arginine is mutated, ions may leak through the gating pore (9, 10). This current is known as gating pore or \(\omega\)-current (\(I_{\omega}\)) that flows in addition to the main pore or \(\alpha\)-current (\(I_{\alpha}\)) and underlies the abnormal depolarization of HypoPP muscles (4). HypoPP-associated \(I_{\omega}\)-current have been described in Na\textsubscript{V}1.4 channels with mutations in VSD-I (11), VSD-II (12–14), and VSD-III (15–17), but not in VSD-IV (18, 19) (Fig. L4). \(I_{\omega}\)-current are voltage-dependent; for example, a mutation in one of the two outermost arginines (R1 and R2) of VSD-II results in \(I_{\omega}\) in the down state of the voltage sensor (12–14). In contrast, a mutation of the third arginine (R3) in VSD-II results in \(I_{\omega}\) when the voltage sensor is in the up state (19).

Many toxins exert their effects by targeting ion channel function either by directly blocking ion permeation through the channel or by modifying the function of the voltage sensor (12).

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Fig. 1. Na₈ channel organization and Na₈,1,4 VSD-I sequence comparison with other voltage-gated channels. (A) Transmembrane topology of Na₈ channels. The S1–S4 helices are in blue, and the S5–S6 helices are in gray. Gating charges are marked by “+” signs, and those neutralized in HypoPP are marked by red diamonds. Colored frames indicate VSDs specifically targeted by gating modifier toxins, VSD-I studied by us is shown by magenta. (B) Spatial organization of Na₈ channels with one pore domain and four VSDs. (C) Alignment of Na₈,1,4 VSD-I with VSDs of other Na₈ and K₈ channels. Conserved aromatic/hydrophobic, charged, and polar residues are color-coded. Transmembrane segments are highlighted by gray background. The gating charge transfer center is marked by green asterisks. Conserved charged residues in the S4 helix are numbered. Mutation of R222 (red diamond) is associated with HypoPP. Residues of K₈,1,2.1 and Na₈,1,7-DI responsible for the interaction with hanatoxin (S4) and huwentoxin-IV (S3), respectively, are boxed. D, domain.

Results

Properties of I_{CP} in p.R222W Channels. The p.R222W mutation was found in patients with HypoPP (5), but its molecular pathomechanism was not described. We studied I_{CP} properties of the p.R222W channel in HEK293 cells (Fig. S1 A–H). The current density of p.R222W channels was reduced compared with wild-type channels (P < 0.001 at 0 mV), whereas the voltage of half-maximal activation and fast or slow inactivation (V₁/₂) were unaltered. The slope factors of activation and fast inactivation were less steep (P < 0.01) and steeper (P < 0.001), respectively, for p.R222W channels compared with wild-type channels. In addition, the time constant of open-state inactivation was increased in p.R222W channels [P < 0.01 for τ(0)]. Other parameters of p.R222W channels were unaltered.

Many HypoPP mutants show I_{CP} loss of function (21–23) analogous to reduced current density of p.R222W channels. This may result in hypoeexcitability and play a role in the clinical phenotype, but it cannot account for the depolarization observed in muscles from patients with HypoPP (4, 22, 24). The reduced rate of sodium channel inactivation is a gain-of-function property that has been associated with paramyotonia and hyperkalemic periodic paralysis, but not with HypoPP (1, 6). HypoPP is often defined as paralysis without myotonia, arguing that the slowed inactivation kinetics are not contributing to the symptoms experienced by mutation carriers.

Properties of I_{CP} in p.R222W Channels. We next asked if p.R222W mutant channels conduct I_{CP} using the Xenopus laevis oocyte expression system (Fig. 2 A–C). To isolate I_{CP}, I_{Na} was blocked with 1 μM tetrodotoxin. Leak-subtracted current-voltage data revealed hyperpolarization-activated inward currents for p.R222W channels. The current amplitude at −80 mV was 49 ± 11 nA (n = 21) for p.R222W channels and 25 ± 6 nA (n = 20) for wild-type channels. Guanidinium increases the amplitude of I_{CP} in channels where an S4 arginine has been substituted (10, 25) and can be used to confirm the presence of I_{CP} caused by such mutations. When half of the extracellular sodium was replaced by guanidinium, the current amplitude at −80 mV increased to −0.8 ± 0.1 μA in cells expressing p.R222W channels, whereas the current in cells expressing wild-type channels was 0 ± 15 nA. For uninjectected oocytes, these values were 28 ± 12 nA and 10 ± 3 nA in Na⁺ and Na⁺/Gn⁺, respectively (n = 4). These data indicate that p.R222W channels conduct hyperpolarization-activated I_{CP}, similar to channels containing the p.R222G mutation found in another patient with HypoPP and previously shown to conduct I_{CP} (11). The mean current amplitude of p.R222G channels at −80 mV was −138 ± 28 nA in a Na⁺ solution and −1.7 ± 0.2 μA in Na⁺/Gn⁺ solution (n = 42).

I_{CP} Inhibition by Hm-3. Hm-3 is a gating modifier toxin from the crab spider Heriades melottei (26) and has been hypothesized to exert its effect by acting on the VSDs of Na₈ channels (27). We tested whether Hm-3 could inhibit guanidinium-enhanced I_{CP} of p.R222W and p.R222G channels (Fig. 2 D–F). Hm-3 inhibited I_{CP} for both mutant channels, with 10 μM Hm-3 suppressing 67 ± 5% of p.R222W currents (n = 4) and 64 ± 4% of p.R222G currents (n = 10) at −80 mV. The IC₅₀ for p.R222G channels measured from noncumulative data was 5.4 ± 0.8 μM at −80 mV. This is more than 10-fold higher than the IC₅₀ reported for the I_{Na} of Na₈,1,4 (27).

VSD-I Specific Effects of Hm-3. We tested the specificity of Hm-3 with a series of mutant channels that conduct I_{CP}: p.R672G (R2, VSD-II), p.R1132Q (R2, VSD-III), p.R219G (R1, VSD-I), and p.R225G (R3, VSD-I). Unlike p.R672G and p.R1132Q, p.R219G and p.R225G have not been identified thus far in patients with HypoPP. Guanidinium substantially increases the amplitude of nonlinear leak currents for all mutant channels (Fig. S1J), confirming the presence of I_{CP}. For p.R219G, p.R672G, and p.R1132Q channels, the currents were activated by hyperpolarization similar to p.R222W/G channels. In contrast, in p.R225G, I_{CP} was activated by depolarization, although the presence of guanidinium in the extracellular solution increased the current amplitude at all voltages. Hm-3 (10 μM) did not inhibit I_{CP} of mutant channels activated by hyperpolarization (n = 4 for p.R672G and p.R1132Q, n = 6 for p.R219G; Fig. 3A). Hm-3 (10 μM) inhibited the depolarization-activated I_{CP} of p.R225G channels (n = 9; Fig. 3A) at voltages negative to 0 mV; the I_{CP} was unaffected at positive voltages.
We also asked if the inhibition of the I<sub>Na</sub> by Hm-3 was affected by different VSD mutations (Fig. 3 B–D and Fig. S2 A and B). In response to voltage steps to −20 mV, inhibition by 1 μM Hm-3 was similar to wild-type for p.R672G and p.R1132Q channels. However, the reduction in current amplitude was significantly lower for p.R222G and p.R219G channels (for both mutants, <0.01 vs. wild-type channels). A nonsignificant trend toward a smaller effect on p.R222W and p.R225G was also seen. For p.R219G, p.R222G, and p.R225G, we also studied I<sub>Na</sub> inhibition with 10 μM Hm-3, with both p.R222G and p.R219G showing reduced inhibition compared with wild-type channels (P < 0.01; Fig. 3 D). Reduction in the inhibitory effect of Hm-3 on p.R222G channels is consistent with the higher IC<sub>50</sub> of I<sub>CM</sub> inhibition of p.R222G channels compared with the reported IC<sub>50</sub> of I<sub>Na</sub> inhibition of wild-type channels (27). A shift in the voltage dependence of activation by 10 μM Hm-3 was evident for p.R219G channels, although smaller than for wild-type channels. This indicates that the absence of inhibition of p.219G I<sub>CM</sub> by 10 μM Hm-3 is not due to a lack of Hm-3 binding to the channel. I<sub>Na</sub> and I<sub>CM</sub> data suggest that Hm-3 forms specific interactions with VSD-I. We proceeded to investigate this interaction using NMR spectroscopy.

**Hm-3 Binds to the S3–S4 Helix-Loop-Helix Motif in VSD-I.** VSD-I of human Na<sub>a</sub>1.4 (residues L114–S246) was produced by cell-free expression (Fig. S3). Mixed micelles of zwitterionic detergents [1:1 N-labeled VSD-I in DPC/LDAO] provided optimal conditions for NMR measurements (28). In this milieu, VSD-I has an expected α-helical content of ~60% (Figs. S3 D and S5 A), but its stability at the temperatures needed for NMR studies is limited (half-life of ~24 h at 45 °C). Nevertheless, the backbone resonance assignment was obtained for ~47% of residues belonging to the VSD sequence (Fig. 4 D and Fig. S4). Hm-3 has previously shown affinity to zwitterionic and anionic lipid vesicles (27). We studied the interaction between Hm-3 and DPC/LDAO micelles in the absence of VSD-I (Fig. S4 B and C). The equilibrium dissociation constant of the Hm-3/micelle complex (K<sub>d</sub>) of 3.6 ± 3.1 μM revealed that the toxin has a considerably high affinity to the micelle surface (Fig. S6 A). Hm-3 interacts with the micelle through a hydrophobic cluster formed by aromatic residues (W11, F12, W16, and Y25), while positively and negatively charged groups either contact with polar head groups of detergents (e.g., K32) or protrude into the aqueous environment (Fig. 4 C and Fig. S6 B and D). The spatial structure of the toxin does not change significantly upon micelle binding.

We then titrated 13<sup>N</sup>-labeled VSD-I in DPC/LDAO micelles with unlabeled Hm-3 and vice versa. Changes in the position and intensities of the backbone 1<sup>H</sup> N resonances (Fig. 4 A and B and Figs. S5 B and S6 C) indicated the formation of a toxin/channel complex and revealed the location of binding interfaces. Hm-3 interacts with the outer half of the S3 helix of Gly249 (S3b, residues S199–T207) and the C-terminal part of the S3–S4 extracellular loop (residues L212–L215) by the two-stranded, anti-parallel β-sheet (residues C23–K28 and L31–I33) and W11 and...
F12 belonging to the membrane-binding surface (Fig. 4 C and D). The S3–S4 loop accommodates two negatively charged groups (E208 and D211). Although the signals of these residues are not identified in the NMR spectra, significant changes are observed for the neighboring residues T207 and L212, implying the participation of their side chains in complex formation. In contrast, the absence of any responses from residues A217 and R225 (R3) in the S4 helix indicates that they do not participate in toxin binding; R219 and R222 (R1 and R2) remained unassigned. The absence of chemical shift perturbations indicates that the binding of Hm-3 does not introduce significant changes in the spatial structure of VSD-I outside the S3b/S3–S4 loop and the topology of Hm-3/micelle interaction is not significantly altered upon toxin binding to VSD-I. The equilibrium dissociation constant of the Hm-3/VSD-I complex (Kd) of 6.2 ± 0.6 μM was determined by the analysis of VSD-I chemical shift changes during Hm-3 titration (Fig. S7 C and D). A pull-down assay using VSD-I attached to an Ni⁺⁺ resin confirmed the formation of the Hm-3/VSD-I complex (Fig. S7F).

To confirm that Hm-3 interacts with the S3–S4 helix–loop–helix (“paddle”) motif of VSD-I, we tested the activity of Hm-3 on Kv1.2,1.8 channels where their paddle motif was substituted with the corresponding structures of NaV1.4 VSDs (29). The activity of the resulting hybrid channels was significantly reduced by 1.5 μM Hm-3 only when the paddle motif of VSD-I was introduced (Fig. 4E), but not that of any other VSDs (n = 3). In addition, we replaced NaV1.4 residues suggested by NMR to interact with Hm-3 (p. D211H and p.E208A). The inhibition of IKh by 1 μM Hm-3 was reduced significantly in p.D211H (n = 6; Fig. S2 A and B), while a small nonsignificant reduction was observed in p.E208A (n = 9). At 10 μM, Hm-3 inhibition of p.D211H (n = 4) and p.E208A (n = 3) channels was wild-type–like (Fig. S2 A and B).

**NMR-Based Model of Hm-3/VSD-I Complex.** The Hm-3 complex with the up state of VSD-I was modeled using protein-to-protein docking with specific restraints imposed by NMR (Fig. S8). A resulting solution (Fig. S4) shows the toxin peripherally attached to the S3b–S4 region of VSD-I. The complex is stabilized by two salt bridges (K24–E208 and K28–D211) and by hydrophobic/stacking interactions: W11 side chain is sandwiched between F198, I201, M202, and Y205; Y25 makes contacts with M202, M203, and L206; and I27 is in contact with L206 and I215. In addition, F209 of VSD-I may participate in hydrophobic interactions with I33 and V35 residues in the C-terminal β-strand of the toxin.

**Discussion**

**VSD-I as a Binding Site for Gating Modifier Toxins.** We present several lines of evidence that the crab spider toxin Hm-3 affects channel gating by interacting with VSD-I. Hm-3 inhibits IKh evoked by mutations in VSD-I (Fig. 2 D–F), but not in VSD-II or VSD-III (Fig. 3A), and Hm-3 inhibition of IKh is reduced by mutations of R1 and R2 in VSD-I, while unaffected by mutations of R2 in VSD-II or VSD-III (Fig. 3C and D). In addition, Hm-3 alters NMR signals in the paddle motif of VSD-I (Fig. 4D), and when this region is inserted in K2.1 channels, it conveys Hm-3 sensitivity to these channels (Fig. 4E). Finally, a mutation in the VSD-I S3–S4 loop predicted to break a salt bridge reduces the inhibitory effect of Hm-3 (Fig. S2A and B). VSD-I has been proposed as one of the binding sites for ProTx-II from the tarantula *Thrixopelma puncri*, but it is not the principal site (30, 31). We therefore identify VSD-I as a specific binding site for gating modifier toxins (Fig. 5B).

Hm-3 inhibits NaV1.4 at submicromolar concentrations but does not affect all NaV isoforms (27). Alignment of the proposed binding site shows that the S3–S4 loop is one of the most variable regions of VSD-I (Fig. S9), and some of the nonsensitive isoforms lack the aspartate corresponding to D211 (NaV1.2 and NaV1.3) or a hydrophobic residue corresponding to M203 (NaV1.1). These residues form contacts with Hm-3 in our model (Fig. S4). Another Hm-3–resistant channel, NaV1.8, contains an additional positively charged residue in the S3–S4 loop. In agreement with our mutagenesis data, E208 is missing in the insect DmNaV1 channel, which is sensitive to Hm-3.

NMR data suggest that Hm-3 can anchor onto the membrane surface in a position compatible with binding the paddle motif (Figs. 4C and 5A and B). A similar mechanism was proposed for some other gating modifier toxins from spider venom acting on Kv1.4 and NaV channels (e.g., Vstx1, ProTx-II, huwentoxin-IV, SgfTx1, and Vstx1–ProTx-II constructs containing the S3–S4 helix–loop–helix motif of one of the four NaV1.4 VSDs before (black) and following (colored) addition of 1.5 μM Hm-3 (n = 3 each, error bars show SEM). D: domain; G: is the conductance measured at the voltage indicated in x axis, Gmax is the normalized maximal conductance measured in control condition.
Hm-3 Mode of Action. Several lines of evidence suggest that Hm-3 stabilizes the down state of VSD-I: (i) the toxin inhibits \( \text{Na}_{\text{AT1.4}} \) by shifting channel activation to more positive voltages (27), (ii) Hm-3 inhibits the hyperpolarization-activated \( I_{\text{GP}} \) of p.R222G/W channels (Fig. 2D–F), (iii) Hm-3 shifts the voltage dependence of p.R222G \( I_{\text{GP}} \) to more depolarized voltages (Fig. S2C), and (iv) Hm-3 inhibits the depolarization-activated p.R222G \( I_{\text{GP}} \) similar to \( \text{Na}_{\text{AT1.4}} \). Conduction increases at more positive voltages (Fig. 3A). However, Hm-3 does not alter the voltage dependence of p.R219G \( I_{\text{GP}} \) in contrast to \( \text{Na}_{\text{AT1.4}} \). This may be due to the reduced shift of p.R219G \( I_{\text{GP}} \) by Hm-3 and the fact that its \( I_{\text{GP}} \) is activated at more hyperpolarized voltages than p.R222G \( I_{\text{GP}} \) (Fig. 2C vs. 3A).

Our NMR data likely describe the up state of VSD-I as the recordings were made in the absence of voltage. This suggests that similar to other gating modifier toxins from spider venom (38), Hm-3 interacts with both up and down states of VSD-I but by \( \text{Na}_{\text{AT1.4}} \) has a higher affinity to the down state. Indeed, Hm-3 inhibition of \( \text{Na}_{\text{AT1.4}} \) (27) and \( I_{\text{GP}} \) (Fig. S2D) can be reversed by applying prolonged depolarization voltage pulses. At a constant tail voltage of −100 mV, inhibition by Hm-3 is at its maximum following hyperpolarizing prepulses. However, after depolarizing prepulses the tail current amplitude increases, suggesting that the inhibition by the toxin is relieved when the voltage sensor moves to the up state.

Binding of Hm-3 to the up state is likely to be membrane-mediated. A major free energy contribution to the stability of the Hm-3/VSD-I complex comes from the partition of the toxin into the micelle (free energy of −6.5 kcal/M; Fig. S7E), while binding to VSD-I within the micelle adds only −1.1 kcal/M. However, this comparatively low free energy gain does not necessarily indicate the weakness of the toxin/domain complex. It rather suggests that Hm-3 forms hydrophobic and electrostatic contacts similar to those already present between VSD-I and lipids.

Currently, high-resolution structural data of ion channel VSDs are available only for the up state. Consequently, the binding of Hm-3 to the down state of VSDs cannot be modeled accurately. It is evident, however, that the relative orientation of Hm-3 binding elements, the S3b helix and S3–S4 loop, changes from two spatially separated regions in the up-state models to a continuous surface in the down-state models of VSDs (39) (Fig. 5C). This compaction of the binding interface likely underlies the increased affinity of the toxin to the resting state.

Our data suggest that stabilization of the voltage sensor by Hm-3 in the down state does not, per se, result in the inhibition of p.R222G/W \( I_{\text{GP}} \). Rather, by preventing the up movement of S4 in VSD-I, Hm-3 stabilizes the active state of \( I_{\text{GP}} \) (Fig. S2C). Our data also suggest that Hm-3 forms specific and state-dependent interactions to inhibit \( I_{\text{GP}} \). We propose that Hm-3 stabilizes VSD-I in the down state where p.R222G \( I_{\text{GP}} \) is active. Hm-3 either induces a local conformational change sufficient to constrict the gating pore or directly occludes it. The reduced \( \text{Na}_{\text{AT1.4}} \) inhibition of R1 and R2 mutant channels by Hm-3 suggests that it may directly interact with these residues in the down state and, in the absence of R2, prevent the flow of ions through the gating pore.

Toxins as Hits for Development of HypoPP Therapies. The main pathomechanism of HypoPP is presumed to consist of gating pore leak currents through VSDs of \( \text{Na}_{\text{AT1.4}} \) or \( \text{Ca}_{\text{AT1.1}} \) that depolarize and paralyze the muscle. Thus, compounds blocking the leak currents may prevent the depolarization and paralysis. We identify the gating modifier toxin Hm-3 as an inhibitor of \( I_{\text{GP}} \) of HypoPP p.R222G/W channels. Hm-3 also suppresses \( I_{\text{NSA}} \) of wild-type and mutant channels, limiting its clinical usefulness. However, our study proposes several ways forward to develop agents with improved selectivity toward \( I_{\text{GP}} \) and with minimum activity on the wild-type channel. First, gating modifier toxins are a useful source for identifying novel \( I_{\text{GP}} \) inhibitors. Second, application of guanidinium will increase the throughput of \( I_{\text{GP}} \) pharmacological studies, allowing characterization of a large number of toxins on a set of mutant VSDs. Finally, NMR studies can identify key toxin-channel interactions that may help direct the development of hit compounds, clarify the pharmacophores, and eventually improve the therapeutic options of HypoPP.

Materials and Methods

Hm-3 Production. Hm-3 was produced recombinantly following a published procedure (27) as part of a fusion protein with thioredoxin, which was cleaved at methionine residues by cyanogen bromide (40). For NMR studies, \(^{15}N\)-labeled Hm-3 was produced. In this case, transformed Escherichia coli cells were first cultured in LB medium. Having reached the mid-log phase, the cells were pelleted and resuspended in the minimal growth medium M9 containing 1 mM MgSO\(_4\), 0.1 mM CaCl\(_2\), 0.2% glucose, 0.1% \(^{15}\text{NH}_4\)Cl, 0.6% NaH\(_2\)PO\(_4\), 0.3% K\(_2\)HPO\(_4\), 0.05% NaCl, and 1 mM thiamine (pH 7.0). All other steps were carried out as with cold toxin.

Molecular Biology and Electrophysiology. Materials and methods for mutagenesis, the \( \text{Na}_{\text{AT1.4}} \) channel patch clamp in HEK293 cells, and the two-electrode voltage clamp of \( X. \) laevis are described by Zaharieva et al. (16) and in SI Materials and Methods. Chimeric rat \( \text{Na}_{\text{AT1.4K-2.2}} \) constructs were generated as described by Bosmans et al. (29).

Oocytes for \( \text{Na}_{\text{AT1.4}} \) expression work were isolated from adult female \( X. \) laevis in accordance with the UK Animal (Scientific Procedures) Act 1986 or the Animal Care and Use Committee of Johns Hopkins University.

VSD-I Sample Preparation and NMR Spectroscopy. Samples of the unlabeled and \(^{15}N\) and \(^{13}C\),\(^{15}N\)-labeled VSD-I were produced using a cell-free expression system in the insoluble form as described elsewhere (28, 41). The precipitate of the reaction mixture was solubilized in 500 \( \mu \)L of 20 mM Tris-HCl, 300 mM NaCl, and 10% DPC (pH 8.0), and purified by Ni\(^{2+}\) chromatography in 0.5% DPC. LDAO was added to the purified protein (SI Materials and Methods).
NMR spectra were recorded on Bruker Avance-III 600 and 800 spectrometers equipped with cryoprobes at pH 5.5 and 45 °C. 1H, 13C-N TROSY spectra were used to monitor binding of Hm-3 to 13C-labeled VSD-I and vice versa. Detergent concentration in the samples was kept constant during titrations. Equilibrium dissociation constants of Hm-3/micelle and Hm-3/VSD-I complexes (Kd and Kp, respectively) were determined from the chemical shift titration data assuming fast (on the NMR time scale) exchange of Hm-3 molecules between three different states (Fig. 57e).

**Computer Modeling.** Homology models of VSD-I in the up and down states were constructed using as a template the VSD-I from the cryo-EM structure of the Na+,PAS channel (42) and the structures from the molecular dynamics (MD) trajectory of the Kc1.2/2.1 chimeric channel (39), respectively. The recently published cryo-EM structure of the NaV1.4 channel from the electric eel was not used for the modeling because VSD-I is poorly resolved in this structure (43). The structural model of the Hm-3/VSD-I complex was generated with the HADDOCK2.2 web server (44).

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