A disulfide tether stabilizes the block of sodium channels by the conotoxin μO§-GVIIJ

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A cone snail venom peptide, μO§-conotoxin GVIIJ from Conus geographus, has a unique posttranslational modification, 5-cysteinylation, of the channel, which makes possible formation of a covalent tetether to its target Na channels at a distal ligand-binding site. μO§-conotoxin GVIIJ is a 35-aa peptide, with 7 cysteine residues; six of the cysteines form 3 disulfide cross-links, and one (Cys24) is 5-cysteinylated. Due to limited availability of native GVIIJ, we primarily used a synthetic analog whose Cys24 was S-glutathionylated (abbreviated GVIIJSSG). The peptide-channel complex is stabilized by a disulfide tether between Cys24 of the peptide and Cys910 of rat (r) NaV1.2. A mutant channel of rNaV1.2 lacking a cysteine near the pore loop of domain II (C910L), was >103-fold less sensitive to GVIIJSSG than was wild-type NaV1.2. In contrast, although rNaV1.5 was >104-fold less sensitive to GVIIJSSG than NaV1.2, an rNaV1.5 mutant with a cysteine in the homologous location, rNaV1.5[L869C], was >103-fold more sensitive than wild-type rNaV1.5. The susceptibility of rNaV1.2 to GVIIJSSG was significantly altered by treating the channels with thiol-oxidizing or disulfide-reducing agents. Furthermore, coexpression of rNaV1.2 or rNaV1.4, but not that of rNaV1.1 or rNaV1.3, protected rNaV1.1 to -1.7 (excluding NaV1.1-5) against block by GVIIJSSG. Thus, GVIIJ-related peptides may serve as probes for both the redox state of voltage-gated sodium channels and for assessing which NaVβ-subunits are present in native neurons.

Significance

Voltage-gated sodium channels (VGSCs) are responsible for the uptake of action potentials in excitable tissues. Each VGSC is composed of a pore- and voltage sensor-bearing α-subunit and one or more auxiliary β-subunits. Mammals have nine α-subunit isoforms (NaV1.1 to -1.9) and four β-subunit isoforms (NaVβ1 to -β4) (1). An NaV1 has about 2,000-aa residues arranged in four homologous domains, where each domain has six transmembrane spanning segments with an extracellular “pore” loop between segments 5 and 6 (1, 2); furthermore, each NaV1 has about a dozen extracellular cysteine residues, all located in or near the pore loops. For the most part, not much is known about these cysteines (including whether they are disulfide bonded).

NaVβ-subunits can affect the function and cellular localization of NaV1s (1, 3–5). Each NaVβ-subunit has some 200-aa residues and consists of a single transmembrane segment with a large extracellular domain and a smaller intracellular domain (1). NaVβ2- and NaVβ4-subunits, unlike NaVβ1- and NaVβ3-subunits, are disulfide bonded to α-subunits (1, 6). A given neuron can have multiple isoforms of these subunits whose identities are challenging to appraise pharmacologically (7).

Toxins that target VGSCs have been invaluable for probing the structure and function of these channels. Venoms are a rich source of such toxins. For example, in Conus snails, four families of neuroactive peptides have been characterized that target VGSCs: μ-conotoxins and μO-conotoxins, which block VGSCs by plugging the ion-conductance pore and preventing channel activation, respectively; and γ-conotoxins and δ-conotoxins, which promote channel activation and block channel inactivation, respectively. Members within each conotoxin family have homologous structures (8–10). We have used μ-conotoxins to assess NaVβ1-isoforms (7, 11) and both μ- and μO-conotoxins to examine the pharmacological impact of NaVβ-subunit coexpression (12, 13).

Previously unidentified μO-conotoxin GVIIJ is the founding member of a fifth family of VGSC-targeting conotoxins. It is unusual among conopeptides in that it has an odd number of Cys residues in its primary amino acid sequence (Fig. 1). We present evidence that it potently blocks NaV1-channels and that its “extra” cysteine residue, Cys24, is disulfide bonded to an extracellular Cys residue in the peptide’s binding site on the channel, which we call “site 8” and which is distinct from previously identified neurotoxin-receptor sites 1 through 7 (14, 15).

Results

Discovery of μO§-GVIIJ, Its Sequence, and Its Synthesis and That of Its Analogos. We discovered μO§-GVIIJ by fractionating crude venom from Conus geographus by HPLC and assaying the eluate for activity against rNaV1.7 exogenously expressed in Xenopus laevis oocytes (Fig. 14). A partial sequence of the peptide was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained by conventional means, and the complete sequence was obtained.
by searching the transcriptome of \textit{C. geographus} (16) for sequences homologous to that of the peptide (Fig. 1B). Tandem mass spectrometry confirmed the sequence and identified three post-translationally modified residues: bromotryptophan, hydroxyproline, and S-cysteinylated cysteine (Fig. 1C and Figs. S2 and S3). Because of limited availability of the native peptide, most of the experiments were done with the two derivatives: GVII\textsubscript{SSG}[W2W; C24C(glutathione)] and GVII\textsubscript{SSG}[W2C24C(free thiol)], hereafter referred to as GVII\textsubscript{SSG} and GVII\textsubscript{SH}, respectively (Fig. 1D). Like the native peptide \( \mu \text{O}-\text{GVIIJ} \), the GVII\textsubscript{SSG} analog has Cys24 in a disulfide linkage whereas the GVII\textsubscript{SH} analog’s Cys24 is in the free thiol form; thus, GVII\textsubscript{SH} served as a reference to help investigate the role of the disulfide-bonded modification of Cys24.

GVII\textsubscript{SSG} and GVII\textsubscript{SH} Readily Block Six of Eight Na\textsubscript{v}1-Isosforms Tested. GVII\textsubscript{SSG} blocked the voltage-gated currents (\( I_{Na} \)) of all tetradotoxin-sensitive rNa\textsubscript{v}1-subtypes (Na\textsubscript{v}1.1 to -1.4, -1.6, and -1.7) with \( K_{IC50} \)s ranging from 5 to 360 nM whereas the IC\textsubscript{50} for rNa\textsubscript{v}1.5 was \( >200 \mu \text{M} \) (Table S3). No block of rNa\textsubscript{v}1.8 was observed at 100 \mu M, the highest concentration of the peptide tested. Similar results were obtained with human (h) Na\textsubscript{v}1-subtypes stably expressed in human embryonic kidney (HEK) 293 or Chinese hamster ovary (CHO) cell lines (Table S4). GVII\textsubscript{SSG} and GVII\textsubscript{SH} were extensively tested on oocytes expressing rNa\textsubscript{v}1.2. The recovery from block by GVII\textsubscript{SSG} was invariably only very slowly reversible following washout of peptide (Fig. 2A and Table S3) whereas the recovery from block by GVII\textsubscript{SH} usually had only two phases (Fig. 2B), a very rapid phase and a much slower phase (\( k_{off} = 2.0 \pm 0.2 \text{ s}^{-1} \) and 0.004 \pm 0.003 \text{ s}^{-1} (mean \pm SD, \( n = 6 \) oocytes), respectively); the latter value is statistically the same as the \( k_{off} \) of GVII\textsubscript{SH} (Table S3) (\( P = 0.57 \)). The native \( \mu \text{O}-\text{GVIIJ} \) behaved like GVII\textsubscript{SSG} insofar as its block of Na\textsubscript{v}1.7 reversed very slowly (Figs. 1A and 2A and Table S3).

**Treating Channels with Thiol Oxidizing and Reducing Agents Has Reciprocal Effects on GVII\textsubscript{SSG} Versus GVII\textsubscript{SH}** The structures and activities of GVII\textsubscript{SSG} and GVII\textsubscript{SH} suggested that they might interact, respectively, with a thiol and disulfide group on the channel. This possibility was explored by exposing oocytes expressing rNa\textsubscript{v}1.2 to two reagents that induce free thiols to form disulfide bonds [MTSET and Cu\textsuperscript{2+}-phenanthroline (CuPhen); see last paragraph in this section] and one reagent (DTT) that reduces disulfides to free thiols.

The methanethiosulfonate (MTS) derivative CH\textsubscript{3}SO\textsubscript{2}SC\textsubscript{2}H\textsubscript{2}NM\textsubscript{3}e\textsuperscript{+} (or MTSET) oxidizes thiols upon disulfide-bond formation with -SC\textsubscript{2}H\textsubscript{2}SC\text{Me\textsubscript{3}}\textsuperscript{+} (or ET) (17). Thus, MTSET treatment results in the attachment of an exogenous group, ET, to accessible free thiols on the channel via a disulfide bond. MTSET treatment completely protected the channel against block by GVII\textsubscript{SSG} (Fig. 2C). This protection was reversed by subsequent treatment of the oocytes with DTT (Fig. 2C). In contrast to the protection against GVII\textsubscript{SSG}, MTSET treatment of the oocytes converted the reversible block by GVII\textsubscript{SH} to one that was exclusively very slowly reversible (Fig. 2D), similar to that of GVII\textsubscript{SSG} on untreated oocytes (Fig. 2A).

At saturating concentrations, the level of block by GVII\textsubscript{SSG} was smaller and more variable than that by GVII\textsubscript{SH}; however, the block by GVII\textsubscript{SSG} was more less variable and accelerated to that by GVII\textsubscript{SH} by treatment of oocytes with DTT (Fig. 3B). The relative magnitude of the slow phase of recovery from block by GVII\textsubscript{SH} varied from oocyte to oocyte (e.g., Fig. 2A and D) and for a given oocyte it increased as the exposure time to a disulfide bond increased (Fig. S4).

When oocytes were exposed to CuPhen, which catalyzes air oxidation of thiols (18, 19), the magnitude of block by GVII\textsubscript{SSG} was decreased (Fig. 3A, b and B), and its off-rate accelerated (Fig. 3A, b). In stark contrast, the main effect of CuPhen treatment on the block by GVII\textsubscript{SH} was to make its off-rate very slow (Fig. 3A, b), similar to that following treatment with MTSET shown in Fig. 2D. (Note, unlike MTSET treatment, which adds an exogenous group to the free thiol of channel Cys residues, CuPhen-catalyzed air oxidation is expected to result in a disulfide linkage of two free thiol groups, presumably those of channel Cys residues that are in close proximity to each other.) Furthermore, the effects of CuPhen on the block by both peptides were largely reversed by subsequent treatment of oocytes with DDT (Fig. 3A, c and c’). The interactions between the peptides and the channel, modifiable by thiol-oxidizing and disulfide-reducing agents, are summarized in the hypothetical reaction scheme in Fig. 3C, which is described in detail in Discussion.

FIG. 1. Discovery of \( \mu \text{O}-\text{GVIIJ} \) from \textit{Conus geographus}. (A) Photograph of \textit{C. geographus} shell and RP-HPLC elution profile of \textit{C. geographus} venom. The star indicates the area from where the native peptide was isolated. The \textit{Inset} depicts the ability of the starred fraction to block sodium currents (\( I_{Na} \)) of an \( X. laevis \) oocyte expressing rNa\textsubscript{v}1.7 that was电压 snapshot (at \( 80 \text{ mV} \) and \( I_{Na} \) evoked by a voltage step to \( -10 \text{ mV} \) applied every 20 s) (Materials and Methods); the major plot shows the time course of block (the bar represents when oocyte was exposed to the starred fraction)—note the very slow recovery from block when the fraction was washed out; the minor plot illustrates traces of \( I_{Na} \) before and during block (large and attenuated responses, respectively). The amount of material applied to oocyte (in a 30-\textmuL bath) was an equivalent of 2 mg of starting crude venom. (B) The complete sequence of prepro GVIIJ from transcriptome. The arrow indicates the signal cleavage site predicted by Signal P; the underlined sequence was independently obtained from targeted PCR; the “LCD” sequence after the arrow is part of the propeptide; mature toxin is shown in bold; the asterisk indicates the stop codon. (C) Sequence and posttranslational modifications of native peptide determined by tandem mass spectrometry, where W is bromotryptophan (presumably \( 1-6\)-bromoTrp), a residue also found in other \textit{Conus} peptides (35, 36), O is hydroxyproline, \( ^{\wedge} \) indicates C-terminal free carboxyl group, and X\textsubscript{i} is cysteine (which is surmised to be disulfide bonded to the side-chain thiol of Cys24 based on the functional activities of synthetic analogs in D. (D) Synthetic analogs of GVIIJ with disulfide connectivities indicated by lines bridging Cys residues. X\textsubscript{i} represents glutathione (disulfide bonded to Cys24 in GVII\textsubscript{SSG} or hydrogen (of the free thiol of Cys24 in GVII\textsubscript{SH}) or cysteine (disulfide bonded to Cys24 in GVII\textsubscript{SH}). (E) RP-HPLC elution profiles of purified native GVIIJ (Left), synthetic GVIIJ (Center), and a mixture of both native and synthetic GVIIJ (Right) on an analytical C18 column with a gradient of 15−45% of solvent B (90% acetonitrile in 0.1% TFA) in 30 min at a flow rate of 1 mL/min. Methods and additional results are provided in SI Materials and Methods, Figs. S1−S3, Tables S1 and S2, and SI Appendix.)
Mutating VGSCs Pinpoint the Location of a Cys Residue in the Binding Site (Site 8). To help identify the cysteine(s) in the channel’s active site, the amino acid sequences of rNaV1.1 to 1.8 and those of hNaV1.1 to 1.7 were aligned, and the locations of extracellular Cys residues were compared. The sequences of all NaV1s that were highly sensitive to GVIIJSSG have Cys residues at 14 extracellular locations (which are at identical homologous positions, except for rNaV1.4, which has one fewer Cys residue). By comparison, NaV1.5, which was very poorly blocked by the peptides, lacks three Cys residues located in the N-terminal region near the pore loop of domain II in the other NaV1-isoforms. We constructed two complementary chimeras from hNaV1.1 and 1.5: chimera 7577 (composed of domain II of hNaV1.1, which is an otherwise hNaV1.7 background) and the reciprocal chimera 5775. The latter, like hNaV1.7, was sensitive to GVIIJSSG whereas chimera 7577, like hNaV1.5, was significantly less sensitive (Fig. S5), suggesting that one or more of these three cysteines may be involved in the peptide binding to the channel.

For rNaV1.2, the trio of Cys residues in domain II are Cys910, Cys912, and Cys918, and the mutant rNaV1.2[C910L] was made and tested. (Leu was chosen to replace Cys because Leu869 is near the homologous location of rNaV1.1.) rNaV1.2[C910L] was poorly blocked by both peptides (Fig. 2E and F and Table S3). Treatment of oocytes expressing rNaV1.2[C910L] with MTSET gave essentially no effect on the activity of either peptide (Fig. 2G and H), suggesting that Cys910 alone was largely responsible for the consequences of MTSET treatment of the wild-type channel seen in Fig. 2 C and D.

The rNaV1.5 mutant with a Cys replacing Leu869 near the pore loop of domain II, rNaV1.5[L869C], was expressed in oocytes and found to be significantly more sensitive than wild-type rNaV1.5 to both GVIIJSSG and GVIIJSH (Fig. 4). GVIIJSSG had a >100 higher affinity for the mutant over the wild-type channel (Table S3). Furthermore, in contrast to the rapidly reversible block of rNaV1.5 by both GVIIJSSG and GVIIJSH (Fig. 4A and B), the block of rNaV1.5[L869C] was slowly reversible, with GVIIJSSG being more slowly reversible than GVIIJSH (Fig. 4 C and D). These results further substantiate the importance of a cysteine near the pore loop of domain II in the active site, site 8, of the channel.

The Binding Site of GVIIJSSG (Site 8) on Rat NaV1.2 Does Not Overlap with Those of TTX and μ-KIIIA[K7A] (Site 1) or μ-O-MrVIB (Site 4). Identification of Cys910 of rNaV1.2 as a critical residue for both GVIIJSSG and GVIIJSH activity suggests that μ-O-conotoxins interact with a different fraction of members of the two other families of conotoxins that also block VGSCs: namely, μ-conotoxins and μ-O-MrVIB. As previously mentioned, Cys910 in rNaV1.2 is near the pore loop of domain II; in contrast, μ-conotoxins and TTX bind at site 1, closer to the channel’s ion-selectivity filter (14, 20, 21), and the binding site of μ-O-MrVIA/B is near the voltage sensor of domain II (22, 23) (for reviews of neurotoxin receptor binding site designations, see refs. 14 and 15). Thus, TTX and μ- and Ω-conotoxins might not be expected to compete with GVIIJSSG. Lack of competition was experimentally verified with oocytes expressing rNaV1.2 using TTX, μ-KIIIA[K7A], and μ-O-MrVIB (Fig. S6G). KIIIA[K7A] was used because, like TTX, it blocks by plugging the pore of the channel, but the plug is “leaky” (20, 24) and, after saturating concentrations of KIIIA[K7A], an ~25% residual current (rINa) persists with NaV1.2. This rINa was readily blocked by 3 μM GVIIJSSG (Fig. S6B), indicating that these two toxins occupy different sites on the channel. It should be noted that μ-KIIIA has 16 residues and is among the smallest μ-conotoxins; furthermore, KIIA or KIIA[K7A] can simultaneously occupy site 1 with TTX (20, 24). It is possible a larger μ-conotoxin, such as μ-GHIA (with 22 aa), might intrude into GVIIJSSG binding space, insofar as mutation of Cys910 of rNaV1.2 does affect the peptide binding to the channel. It should be noted that KIIIA[K7A] can simultaneously occupy site 1 with TTX (20, 24). It is possible a larger μ-conotoxin, such as μ-GHIA (with 22 aa), might intrude into GVIIJSSG binding space, insofar as mutation of Cys910 of rNaV1.2 does affect the peptide binding to the channel.

When coexpressed with rNaV1.4, rNaV1.1 through 1.6 were all rendered insensitive to 33 μM GVIIJSSG, except rNaV1.5, whose already-poor sensitivity was largely unaffected (Fig. 5F). Likewise, the poor sensitivity of rNaV1.2[C910L] toward GVIIJSSG was minimally affected by rNaV1.4 coexpression (Fig. 5F).

Functional Properties of GVIIJSSG and Synthetic μ-O-GVIII. Only a limited number of experiments were performed with GVIIJSSG (Fig. 1D with Cys for XJ) and synthetic μ-O-GVIII itself because the S-cysteinylation of a Cys residue and the presence of bromotrop were identified only after the electrophysiological experiments described above had been completed. Tests with rNaV1.2 and -1.7 showed that the koff values of both peptides were essentially the same (Table S3), which is expected; that is to say, for a given channel and peptide backbone, the identical peptide-channel complex is formed regardless of the SR-group disulfide bonded to Cys24 of the backbone because each peptide’s respective SR group leaves when displaced by the thiol group of the channel cysteine during the process of disulfide exchange at site 8 (Fig. 3C).

Discussion

Binding Site of μ-O-GVIII. Site 8 is distinct from those of members of the two other families of conotoxins that also block VGSCs, μ-conotoxins (14, 20) and μ-O-conotoxins (22); consistent with this conclusion, μ-KIIA and μ-O-MrVIB do not compete with GVIIJSSG, in blocking NaV1.2 (Fig. S6). Furthermore, site 8 can be modified by thiol-oxidizing and disulfide-reducing agents as summarized in Fig. 3C, which appears to be the most parsimonious scheme consistent with our results. The site exists in two basal states where, for one state (diagram a in Fig. 3C), the block by GVIIJSSG and GVIIJSH is slowly and rapidly reversible, respectively, whereas, for the other state (diagram b in Fig. 3C, where Cys910 is a channel cysteine), the converse is true. However, when SX in diagram b in Fig. 3C is ET, then GVIIJSSG cannot block at all. These two interconvertible states can be driven by treatment with DTT in one direction and MTSET or CuPhen-catalyzed acid oxidation in the other. Peptide binding results in three functionally blocked states; dissociation of the peptide occurs readily from two of the states (diagrams c and e in Fig. 3C) and slowly from the third.
disulfide-tethered state, which was achieved by disulfide exchange between peptide and channel (diagram d in Fig. 3C).

It might be noted that, although most of the GVIIJ-sensitive NaV1.1-isomers block with high potency (Kdis = 5–50 nM), a standout is NaV1.6 (Kdis = 360 nM), whose poor potency is largely due to its relatively large Koff (Table S3). Presumably, the disulfide tether between GVIIJ and NaV1.6 is more labile, and it would be interesting to examine this further.

Both MTSET Treatment and Coexpression of NaVβ2 or β4 Inhibit the Block by GVIIJSSG. Coexpression of NaVβ2- and NaVβ4-subunits, as well as MTSET treatment, produces the same effect; namely, the channels are protected against block by GVIIJSSG. Unlike NaVβ1 and NaVβ3, which are noncovalently attached to their NaV1-subunit, each of NaVβ2 and NaVβ4 is linked to its NaV1-subunit via a disulfide bond (1, 6). Thus, it is tempting to speculate that C910 of rNaV1.2 might be disulfide-linked to its counterpart in

Fig. 2. Block by GVIIJSSG and GVIIJSH of rNaV1.2 and rNaV1.2[C910L] and effects of thiol-oxidizing and disulfide-reducing agents. Oocytes expressing either native or mutant channels were voltage-clamped as described in Fig. 1A. (A–H) Representative plots of peak sodium currents (INa) as a function of time before, during (indicated by bar above each plot), and after exposure to toxin. (A) GVIIJSSG (10 μM) rapidly blocked INa, which very slowly recovered during toxin washout. (Inset) Averages of five INa traces obtained before, during, and after exposure to peptide (at t = 2, 4, and 15 min, respectively). (B) Block by GVIIJSH (1 μM) where recovery had fast and slow components. (C, D, G, and H) Vertical gray bars indicate when oocyte was exposed to 2 mM MTSET or 5 mM DTT, as indicated. Responses during washout of MTSET or DTT are not shown, accounting for blank area immediately following each vertical bar. (C) MTSET treatment prevented block by GVIIJSSG, whose block is restored by subsequent DTT treatment to produce a slowly reversible block like that in A. (D) Initial exposure to GVIIJSH produced a block that was essentially completely reversible, and MTSET treatment did not prevent block; on the contrary, the block was now only very slowly reversible, resembling that of GVIIJSSG in A. (E and F) Block of NaV1.2[C910L] mutant channel by GVIIJSSG (E) and GVIIJSH (F) was attenuated and transient. (G and H) MTSET treatment of mutant channel affected the activities of neither GVIIJSSG (G) nor GVIIJSH (H); note that both magnitude and time course of block remained essentially unaltered.

Fig. 3. Effects of CuPhen-catalyzed air oxidation of rNaV1.2 on the block by GVIIJSSG and proposed reaction scheme accounting for the behavior of GVIIJ and its analogs on channels treated with oxidizing and reducing agents. Oocytes expressing rNaV1.2 were voltage-clamped as described in Fig. 1A. (A) Representative time course of block by indicated peptide plotted as in Fig. 2. Treatment of oocytes with CuPhen (100 μM for 1 h) highly attenuated the block by GVIIJSSG and converted the slowly reversible block in control (a) to one that was mostly rapidly reversible (b). In contrast, CuPhen treatment converted the rapidly reversible block by GVIIJSH (a’ to one that was largely slowly reversible (b’). These effects of CuPhen were largely reversed by subsequent DTT treatment of oocytes with 2 mM DTT for 1 h (c and c’). Each plot is from a different oocyte. (B) Relative to controls (white bars), DTT-treatment of oocytes (2 mM for 1 h, black bars) increased the maximum block by GVIIJSSG (33 μM) but not that by GVIIJSH (10 μM); furthermore, DTT treatment reduced the variability of block by GVIIJSSG. In contrast, the Cu2+-phenanthroline (CuPhen) treatment (gray bars) decreased the maximum block by GVIIJSSG whereas that by GVIIJSH was more modestly decreased. Bars represent mean ± SD (n = 2 oocytes). * P < 0.0002; heights of the second, fourth, and fifth bars are not significantly different from each other (P > 0.5). (C) Hypothesized states of site 8, wherein P represents the peptide either as PSH (i.e., GVIIJSSG) or as PS-SR where Cys24 is disulfide bonded to R, which is either cysteine (GVIIJSSC and native peptide) or glutathione (GVIIJSSG). (Diagrams a and b) Channel with Cys whose side chain has a free thiol (diagram a) or is disulfided bonded to X (diagram b), where SX is either another Cys residue of the channel or ET (SC4-C2H2NMe3), resulting, respectively, from CuPhen-catalyzed air oxidation or MTSET treatment. (Diagram c) Peptide noncovalently and readily reversibly bound to site 8. (Diagram d) Peptide tethered via channel via a disulfide bond and therefore only very slowly dissociable. Not shown are other possible thiol or disulfide groups at site 8, including those that may, for example, facilitate the c-d transition. (Diagrams e and f) When SX is a channel cysteine, then PS-SR binding is readily reversible (diagram e); however, when SX is ET, then PS-SR is unable to bind the channel (diagram f), possibly due to steric hindrance by ET.

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Na\textsubscript{Vβ2} or Na\textsubscript{Vβ4}, a possibility also raised by Chen et al. for Na\textsubscript{Vβ2} based on other considerations [6]. Although Na\textsubscript{Vβ2} or Na\textsubscript{Vβ4}-subunit coexpression protected against the block by both GVII\textsubscript{SH} and GVII\textsubscript{SSG}, MTSET treatment actually enhanced the block by GVII\textsubscript{SH} (i.e., rendered it less reversible) (Fig. 2D), an explanation for which is depicted in Fig. 3C. The difference between the consequences of Na\textsubscript{Vβ2}- or Na\textsubscript{Vβ4}-subunit coexpression and MTSET treatment could be explained by steric hindrance, in view of the large disparity in size between a β-subunit versus ET and that between a proton in GVII\textsubscript{SH} versus glutathione in GVII\textsubscript{SSG} (Fig. 1D).

It should be noted that coexpression versus association of α- and β-subunits per se are not synonymous; thus, for example, Na\textsubscript{Vβ1} coexpression can affect the glycosylation of the α-subunit (27), and the glycosylation state of the α-subunit, rather than its physical association with a β-subunit, could be responsible for the altered pharmacology of the VGSC.

It remains to be shown whether the effects of Na\textsubscript{Vβ}-subunit coexpression in X. laevis oocytes is also manifested in mammalian expression systems and in neurons. Differences between VGSCs expressed in oocytes versus HEK cells with regard to Na\textsubscript{Vβ}-subunit coexpression have been observed; for example, in oocytes, the rate of fast inactivation of Na\textsubscript{V1.7} coexpressed with either Na\textsubscript{Vβ1} or Na\textsubscript{Vβ3} is faster than that of Na\textsubscript{V1.7} expressed alone (13, 28). This result was not observed when HEK 293 cells served as the expression system, where coexpression of any Na\textsubscript{Vβ} isoform (Na\textsubscript{Vβ1} to Na\textsubscript{Vβ4}) had no effect on the kinetics of Na\textsubscript{V1.7} currents (29).

**Other Unresolved Issues.** The incomplete block of sodium current, i.e., the residual current (rINa), that persists in the presence of saturating concentrations of GVII\textsubscript{SSG} or GVII\textsubscript{SH} is not understood. The maximum block produced by either peptide is about 90% (Fig. 3B). It could result from either (i) heterogeneity of the channel—i.e., ~10% of the channels are resistant to block, or (ii) incomplete, or partial, efficacy of block. Heterogeneity may play a role to some extent insofar as DDT treatment of oocytes can increase the efficacy of the block by GVII\textsubscript{SH} (although not that by GVII\textsubscript{SSG}). The rINa with GVII\textsubscript{SSG} is about 30% for Na\textsubscript{V1.2}, -1.3, -1.4, and -1.6, and -1.7 (Fig. 5E and F); however, that for Na\textsubscript{V1.1} is about twice as large (Fig. 5F). For all of these Na\textsubscript{V1}-isoforms, DTT treatment reduces the residual current to about 10%, like that shown for Na\textsubscript{V1.2} in Fig. 3B.

The mechanism by which binding of GVII\textsubscript{I} results in block of the channel remains to be determined. GVII\textsubscript{SSG} is not a classical pore blocker. GVII\textsubscript{SSG} is able to block Na\textsubscript{V1.2} with the IFM–QQQ mutation (in the linker between domains III and IV) that removes fast inactivation (30) (Fig. S7), but the peptide may stabilize the channel in some other inactivated state, such as a slow-inactivated state that may involve conformational coupling between the pore and voltage-sensor domains (31). Alternatively, the GVII\textsubscript{I} peptides may inhibit channel activation by interfering with the channels’ voltage sensors, similar to what gating modifiers such as μO-MrVIB, Protox II, and HwTx-IV do (22, 23, 32–34), but possibly allosterically. It might be noted that, at saturating toxin concentrations, the level (or efficacy) of block of Na\textsubscript{V1.7} by Protox II and of Na\textsubscript{V1.4} by HwTx-IV are 95% (32) and 41% (34), respectively, and they may represent precedents for the partial efficacy of block manifested by the GVII\textsubscript{I} peptides.

In conclusion, μO\textsubscript{S}-GVII\textsubscript{I} provides an exciting tool with which to explore the pharmacology, structure, and function of VGSCs.
Materials and Methods

The discovery of μO5-GVIIJ and its sequence determination are described in detail in SI Materials and Methods. The synthesis of μO5-GVIIJ and its analogs GVIIJsp, GVIIJsc, and GVIIJscd and disulfide-bond mapping of GVIIJsp are described in detail in SI Materials and Methods. Functional activities of peptides were assessed by two-electrode voltage clamp of X. laevis oocytes expressing rat channels and by patch clamp of HEK 293 or CHO cells expressing human channels as described in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Isolation of the Native μO§-GVIIJ and Characterization of Its Encoding cDNA. Isolation of μO§-GVIIJ from Conus geographus venom. Lyophilized venom from C. geographus (840 mg) was suspended in 25 mL of aqueous 35% (vol/vol) acetonitrile-0.1% (vol/vol) trifluoroacetic acid (TFA) and homogenized at room temperature with a glass-Teflon tissue grinder for six cycles at 1,500 rpm. The homogenate was centrifuged in a Beckmann F0650 rotor at 10 °C for 15 min at 20,000 rpm. The supernatant was divided into two portions, and each was diluted threefold with aqueous 0.1% (vol/vol) TFA (gradient buffer A) before application on a Vydac preparative C18 HPLC column at a flow rate of 7 mL/min. Elution was performed using a multistep gradient of 10-50% of buffer B [aqueous 90% (vol/vol) acetonitrile-0.1% (vol/vol) TFA] for 40 min followed by 50-60% of buffer B for 5 min and 60-100% of buffer B for 5 min. The HPLC profile at 280 nm is shown in Fig. 1A of the main text. Pooled aliquots of fractions were assayed for activity on NaV1.7 expressed in Xenopus laevis oocytes. Aliquots from each fraction in the active pool were subsequently assayed for activity, and the most active fraction is marked on the HPLC profile in Fig. 1A.

The most active preparative-HPLC fraction was subfractionated on a Vydac semipreparative C18 with a gradient of 0.2% per min change in the concentration of buffer B, at 45 °C; the active fraction from this semipreparative run was applied to a Vydac analytical monomeric C18 column. Elution from the monomeric C18 column was performed using buffers A′ and B′ [buffers A′ and B′ have 0.005% (vol/vol) TFA] over a gradient of 0.05% per min change in the concentration of buffer B′. The most active fraction was subjected to a final HPLC separation step using the monomeric C18 column and a gradient of buffers A′ and B′ [buffers A′ and B′ have 0.005% (vol/vol) TFA replaced with 0.2% formic acid] with a gradient of 0.05% of buffer B′ per min.

A sample of the purified native peptide, μO§-GVIIJ, was analyzed using matrix-assisted laser desorption ionization (MALDI) mass spectrometry at The Salk Institute for Biological Studies, and it had a mass of 3,934.49 Da [M+1].

Peptide sequencing. A small amount of the native μO§-GVIIJ was linearized by treatment with DTT. The peptide sample was dissolved in 100 μL of 50% (vol/vol) acetonitrile, and the pH of the sample was adjusted to 8.0 using 0.5 M Tris base. A 50 μM solution of DTT was added to a final concentration of 10 mM, and the sample was incubated at 65 °C for 15 min for complete reduction of disulfide bridges. Following reduction, cysteine thiols were modified by addition of 0.8 μL of 4-vinylpyridine (VP; Sigma). The sample was incubated, in the dark, for 30 min at room temperature. Sequencing by Edman degradation of the fully reduced and alkylated peptide was done by R. Shackmann from the DNA/Peptide Synthesis Facility at the University of Utah. Sequencing efforts yielded a nearly complete toxin sequence, GxCGDxGATCGKLRLYCCSOFGDCCYTtxxD, where x represents a cycle with an unreadable residue and residues marked in lowercase letters were not definitive. Due to ambiguities in the assignment of residues at the both N and C termini, the partial sequence was checked against the C. geographus transcriptome. A mature toxin sequence, with nearly 100% identity to the venom-derived peptide, was identified as described in Venom-duct cDNA cloning and sequencing.

Preparation of venom-duct RNA samples. Specimens of C. geographus were collected in the Philippines. Each specimen was dissected to isolate the venom duct, and the duct was immediately suspended in 1.0 mL of RNA later (Ambion) at ambient temperature, and then stored at −20 °C until used. Ducts were homogenized with a Tissue Tearor (Dremel model 985370), and total RNA isolated using a miRvana miRNA isolation kit (Ambion, Applied Biosystems) according to the manufacturer’s recommendations. Venom-duct cDNA cloning and sequencing. cDNA was prepared by reverse transcription of RNA isolated from the C. geographus venom duct. About 1 μg of total RNA was transcribed into cDNA using an In-Fusion SMARTer cDNA Library construction kit from Clontech. The resulting cDNA served as a template for PCR amplification. The 5′ and 3′ RACE experiments were carried out using a SMARTer RACE cDNA amplification kit (Clontech).

Based on the partial amino acid sequence of the peptide GVIIJ, we designed degenerate oligonucleotide primers, carried out 5′ and 3′ RACE experiments, and obtained a partial nucleotide sequence encoding GVIIJ. For 3′ RACE, the forward primer (5′ TAY TGY TGY TGY TGY TGY TGY TA 3′, where Y = A/G and N = A/T/G/C) was designed corresponding to the region YCCSGFCD in peptide. For 5′ ACE, the reverse primer (5′ TGTAACATGCAGAACCCCGC3′) corresponding to the region SGFCD CY in the peptide was used. The nucleotide sequences obtained from the 5′ and 3′ RACE experiments were assembled, and additional PCR was carried out using the specific primers (forward primer) 5′ GGA GGT ACG CAG AAT CAT CG 3′ and (reverse primer) 5′ ATG GAC TCG GGC AGA AAG GG 3′ to obtain the complete mature toxin sequence. The PCR amplification profile consisted of an initial denaturation for 2 min at 94 °C followed by 39 cycles of 94 °C for 30 s, 62 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 10 min. PCR products were analyzed by electrophoresis on 1% agarose gels. One prominent PCR product band around 250 bp was observed. The band was excised from the gel and purified with a QIAquick gel extraction kit (Qiagen). The purified PCR product was ligated to a pGEM-T-Easy vector (Promega) and used to transform Escherichia coli DH10B. Plasmids were isolated from transformed colonies containing the insert, and the sequence was determined at the DNA sequencing facility at the University of Utah. The conceptual translation of the nucleotide sequence contained the partial sequence of the propeptide, the mature toxin sequence, and the 3′ untranslated sequence. The complete sequence of μO§-GVIIJ was, however, identified in the transcriptome of the venom duct from C. geographus (1). The nucleotide sequence of the transcript and the conceptual translation product is shown in Fig. S1A.

The sequence of the peptide, established by Edman degradation and completed by targeted PCR and transcriptome information, was chemically synthesized and folded (GVIIJ_SH; see Chemical Synthesis of GVIIJ_SH GVIJIII SSG, GVIJIII_SSG, and μO§-GVIIJ). A difference in mass between the native peptide and GVIIJ_SH was observed, and further mass spectrometry studies were carried out to identify the posttranslational modifications as described immediately below.

Tandem Mass Spectrometry of Native GVIIJ. An aliquot of the purified toxin was loaded onto a New Objectives 360-μm OD × 75-μm ID column with an 8-μm integrated emitter and packed with 20 cm of HALO C18, 2.7 μm, 90 Å material using the autosampler of an EASY-nLC 1000 (Thermo Scientific). The toxin was eluted from the column directly into an Orbitrap Elite mass spectrometer (Thermo Scientific) using a 30-min gradient from 2% to 50% solvent B [solvent A was 2% (vol/vol) acetonitrile in 0.5% acetic acid; solvent B was 90% (vol/vol) acetonitrile in 0.1% formic acid].
0.5% acetic acid]. To obtain the accurate mass of the native toxin, high-resolution full-scan spectra were acquired with a resolution of 240,000 at 400 m/z, an AGC target of 5e5 with a maximum ion injection time of 500 ms, scan range of 400–1,400 m/z and polysiloxane 445 m/z as lock mass ion. For the sequence determination, an aliquot of the purified toxin was incubated for 1 h at room temperature with 25 mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and 1 μL of 2-mercaptoethanol in 500 mM triethylammonium bicarbonate (TEAB) buffer with 10% (vol/vol) acetonitrile. The mixture was acidified with TFA and the toxin solution was desalted by adding a slurry of 22 μM PMOS beads (Life Technologies) in 5% (vol/vol) formic acid and 0.2% TFA. The beads were loaded onto an equilibrated C18 ZipTip (Millipore) using a microcentrifuge for 30 s at 3,100 X g. The PMOS beads were rinsed three times with 0.1% TFA followed by further washes with 0.5% acetic acid. The toxin was eluted by the addition of 40% (vol/vol) acetonitrile in 0.5% acetic acid followed by the addition of 80% (vol/vol) acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator, and the sample was reconstituted in 0.5% acetic acid. An aliquot of the derivatized toxin was loaded on a New Objective HALO column as described at the beginning of this section, and spectra were acquired using the following instrument settings: The full scan was recorded with a resolution of 15,000 at 400 m/z, an AGC target value of 5e5 with a maximum ion injection time of 500 ms, and scan range of 300–1,500 m/z and polysiloxane 445 m/z as lock mass ion. Following each full scan, the +6, +7, and +8 charge states of the derivatized toxin were subsequently fragmented using electron-transfer dissociation (ETD), and the resulting MS/MS spectra were acquired using the following instrument parameters: a resolution of 15,000 at 400 m/z and an AGC target value of 1e5 with a maximum ion injection time of 800 ms and 4 scans. The resulting MS/MS spectra were interpreted manually and analyzed using Conovo and Byonic optimized for peptide toxins of ~3,000 Da (Figs. S3 and S4). The observed N-terminal ions show a characteristic bromine isotope pattern, and the observed mass is consistent with a bromination on tryptophan. The c6 ion is observed 15,994 Da higher, and the c5 ion is not observed, consistent with a hydroxypropylxime at the sixth position from the N terminus.

**Chemical Synthesis of GVIIaSS, GVIIbSS, GVIIcSS, and µO5-GVII. Solid-phase peptide synthesis of the linear peptide with and without bromoTrp.** The linear GVII with the sequence

\[ \text{GWCDDGATCGKLRLYCCSGFCDCYTKTCKD} \]

and the linear GVII[W2W] with the sequence

\[ \text{GWCDDGATCGKLRLYCCSGFCDCYTKTCKD} \]

(where W2W denotes 6-Br-Trp, 4-hydroxypropylxime, and free carboxyl terminus, respectively) were synthesized on an Apex 396 automated peptide synthesizer (AAPPtec) applying standard solid-phase Fmoc (9-fluorenylmethoxycarbonyl) protocols. The peptide was constructed on a preloaded Fmoc-t-Ala-Wang resin (substitution, 0.38 mmol-g⁻¹; Peptides International Inc.). All standard amino acids and pseudoproline dipeptide Fmoc-Tyr(tBu)-Thr(ωMe,Me,Me)-OH were from AAPPtec. N-α-Fmoc-O-t-butyl-l-trans-4-hydroxypropyl (HyP) was from EMD Millipore, and Fmoc-D,L-6-Br-Trp-OH was provided by Jean Rivier (The Salk Institute for Biological Studies, La Jolla, CA). Side-chain protection for the following amino acids was as follows: Arg, 2,2,4,6,7-pentamethylethylhydrobenzofuran-5-sulfonylethyl (Pbf); Asp and Glu, O-t-buty1 (Obu); Lys and Trp, tert- butyloxycarbonyl (Boc); Hyp, Ser, Thr, and Tyr, tert-butyl (tBu); and Asn and Cys, trityl (Trt). Peptides were synthesized on a 50-μmol scale. Coupling activation was achieved with 1 equivalent of 0.4 M benzotriazol-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) and 2 equivalents of 2 M N,N-diisopropylpropylthyl amine (DIEPA) using N-methyl-2-pyrrolidone (NMP) as the solvent. Ten-fold excess of standard amino acids was used except Hyp and 6-Br-Trp, which were used in fivefold excess. Double coupling, each in ten-fold excess, was used for every amino acid coupled after Cys and 6-Br-Trp. Standard amino acid coupling was conducted for 60 min except for Hyp and 6-Br-Trp, where the reaction was 90 min. Fmoc deprotection was carried out for 20 min with 20% (vol/vol) piperidine in dimethylformamide (DMF).

**Cleavage and purification of the linear peptides.** Peptides were cleaved from 50 to 100 mg of resin by a 3-h treatment with 1 mL of Reagent K (TFA/water/phenol/thioanisole/1,2-ethanediol 82.5/5.5/5/2.5 by volume) and subsequently filtered and precipitated with 12 mL of cold methyl-tert-butyl ether (MTBE). The crude peptides were then collected by centrifugation at 7,000 X g for 6 min and washed once with 10 mL of cold MTBE. The washed peptide pellets were dissolved in 10% acetonitrile in 0.1% TFA in water and purified by reverse-phase (RP) HPLC using a semipreparative C18 Vydac column (218TP510, 250 mm × 10 mm, 5-μm particle size) eluted with a linear gradient ranging from 15% to 45% of solvent B (GVII[W2W]) and 25–55% of solvent B (GVII [W2W]) in 30 min at a flow rate of 4 mL/min. The HPLC solvents were 0.1% (vol/vol) TFA in water (solvent A) and 0.1% TFA (vol/vol) in 90% aqueous acetonitrile (solvent B). The absorbance of the eluent was monitored at 220/280 nm. Purity of the linear peptide was assessed using an analytical C18 Vydac reversed-phase HPLC column (218TP54, 250 mm × 4.6 mm, 5-μm particle size) with a linear gradient ranging from 15% to 45% of solvent B (GVII [W2W]) and 25–55% of solvent B (GVII [W2W]) in 30 min with a flow rate of 1 mL/min. Peptides were quantified by absorbance at 280 nm using an extinction coefficient (ε) value of 8,480 M⁻¹ cm⁻¹. On average, 1,200 nmol (4.49 mg) of pure linear GVII[W2W] and 400 nmol (2.67 mg) of GVII was obtained from 100 mg of cleaved resin.

**Formation of GVIIaSS by oxidative folding in the presence of oxidized and reduced glutathione.** One hundred nanomoles of a linear GVII [W2W] was resuspended in 0.5 mL of 0.01% TFA solution and added to a solution containing: 2.5 mL of 0.2 M Tris-HCl (pH 7.5) plus 0.2 mM EDTA, 0.25 mL of 20 mM reduced and 0.25 mL of 20 mM oxidized glutathione, and 1.5 mL of water. Final peptide concentration in the folding mixture was 20 μM. The reaction was conducted for 60 min except for Cys and 6-Br-Trp, where the reaction was 90 min. Fmoc deprotection was carried out for 20 min with 20% (vol/vol) piperidine in dimethylformamide (DMF). The resulting MS/MS spectra were acquired using the following instrument parameters: a resolution of 7, and a maximum ion injection time of 400 m/z, an AGC target value of 1e5 with a maximum ion injection time of 400 m/z and polysiloxane 445 m/z as lock mass ion. Following each full scan, the +6, +7, and +8 charge states of the derivatized toxin were subsequently fragmented using electron-transfer dissociation (ETD), and the resulting MS/MS spectra were acquired using the following instrument parameters: a resolution of 15,000 at 400 m/z and an AGC target value of 1e5 with a maximum ion injection time of 800 ms and 4 scans. The resulting MS/MS spectra were interpreted manually and analyzed using Conovo and Byonic optimized for peptide toxins of ~3,000 Da (Figs. S3 and S4). The observed N-terminal ions show a characteristic bromine isotope pattern, and the observed mass is consistent with a bromination on tryptophan. The c6 ion is observed 15,994 Da higher, and the c5 ion is not observed, consistent with a hydroxypropylxime at the sixth position from the N terminus.

**Formation of GVIIaSS by oxidative folding in the presence of oxidized and reduced glutathione.** The linear GVII [W2W] and 25–55% of solvent B (GVII [W2W]) in 30 min with a flow rate of 1 mL/min. Peptides were quantified by absorbance at 280 nm using an extinction coefficient (ε) value of 8,480 M⁻¹ cm⁻¹. On average, 1,200 nmol (4.49 mg) of pure linear GVII[W2W] and 400 nmol (2.67 mg) of GVII was obtained from 100 mg of cleaved resin.

**Formation of GVIIaSS by oxidative folding in the presence of oxidized and reduced glutathione.** The linear GVII [W2W] and 25–55% of solvent B (GVII [W2W]) in 30 min with a flow rate of 1 mL/min. Peptides were quantified by absorbance at 280 nm using an extinction coefficient (ε) value of 8,480 M⁻¹ cm⁻¹. On average, 1,200 nmol (4.49 mg) of pure linear GVII[W2W] and 400 nmol (2.67 mg) of GVII was obtained from 100 mg of cleaved resin.
Formation of GVIIJSC by oxidative folding in the presence of l-cystine. One hundred nanomoles of the linear GVIIJ[W2W] was suspended in 0.5 mL of 0.01% TFA solution and added to a solution containing 2.5 mL of 0.2 M Tris-HCl (pH 7.5) plus 0.2 mM EDTA, 2 mL of l-Cystine solution [3 mg of l-Cystine suspended in 10 mL of 5% (vol/vol) acetonitrile in 0.01% TFA and sonicated for 1 h and vortexed before adding to the reaction mixture]. The final peptide concentration in the folding mixture was 20 μM. The folding reaction was conducted for 22 h and quenched with formic acid to a final concentration of 8%. Peptides were purified as described for glutathione-assisted peptide folding. Pure GVIIJSSC was quantified by measuring absorbance at 280 nm as described for the linear peptide. On average, 22 nmol of GVIIJSSC was obtained from 100 nmol of linear peptide in the folding reaction. The molecular mass of GVIIJSSC was confirmed by MALDI MS (Table S1). The HPLC elution profile of the peptide is shown in Fig. S1B.

Formation of synthetic μO-S-GVIIJ by oxidative folding in the presence of l-cystine. The L-cystine assisted peptide folding protocol described for GVIIJSSC was used to form, purify, and quantify μO-S-GVIIJ from linear GVIIJ. On average, 8 nmol of μO-S-GVIIJ was obtained from 100 nmol of linear peptide in the folding reaction. The molecular mass of μO-S-GVIIJ was confirmed by MALDI MS (Table S1). The HPLC elution profile of the peptide is shown in Fig. S1B.

Determination of the Free Cys Residue in GVIIJSSC. Alkylation of free Cys residues of GVIIJSSC. Twelve nanomoles of GVIIJSSC was resuspended in 60 μL of 100 mM TEAB, pH 8.5, and 2.4 μL of VP was added [4% (vol/vol) solution]. The reaction was carried out for 30 min in the dark and quenched with 32 μL of 0.01% TFA. The reaction mixture was purified by RP-HPLC on an analytical C18 column eluted over a linear gradient of 35–65% solvent B in 30 min with a flow rate of 1 mL/min and dried down using speed vac.

Reduction and alkylation of the remaining Cys residues. For complete reduction, ~12 nmol of alkylated GVIIJSH was resuspended in 60 μL of 100 mM TEAB buffer, mixed with 60 μL of 10 mM TCEP, and incubated for 45 min at 45 °C. Next, 12 μL of 125 mM iodoacetamide (IAM) was added to the solution and incubated at room temperature in the dark for 1 h, followed by quenching with 12 μL of TCEP.

Endoproteinase Asp-N digestion. Without purification, the reduced and alkylated peptide was mixed with 8.75 μL of endoproteinase Asp-N (1 μg/100 μL of water) at a ratio of 20:1 (wt/wt), incubated at room temperature for 3 h, and purified by RP-HPLC equipped with a C18 analytical column, using gradient of solvent B as follows: 5% for 10 min and then 5–65% in 30 min with a flow rate 1 mL/min. The peaks were isolated and analyzed by MALDI-TOF. The mass of one of the peaks [retention time (RT) = 17.16 min, [M+H]+ = 1123.26] was found to be the same as the expected mass ([M+H]+ = 1123.93) for the peptide fragment DC(VP)YT(KTC)(IAM) of the digested GVIIJSH. The results identified Cys24 as the location of either free thiol of glutathione adduct in the synthetic GVIIJ peptide.

Disulfide-Bond Mapping of the Synthetic GVIIJSH. Alkylation of free Cys residues of the synthetic, folded GVIIJSH. The folded GVIIJSH (30 nmol) was resuspended in 150 μL of 100 mM TEAB buffer, pH 8.5, and then 6 μL of VP, 4% (vol/vol) solution was added, and the mixture was left at room temperature in the dark for 30 min. The peptide was purified by RP-HPLC using an analytical C18 column and a gradient of solvent B as follows: 5% for 10 min and then 5–65% in 30 min with a flow rate 1 mL/min.

Partial reduction and alkylation of the alkylated intermediate GVIIJSH. Partial reduction was initiated by dissolving ~30 nmol of GVIIJSH alkylated with VP in solvent B and adding 150 μL of 30 mM TCEP in 100 mM sodium citrate buffer, pH 3.0. The reaction was carried out at 37 °C for 45 min. Next, 30 μL of 250 mM N-ethylmaleimide (NEM) in citrate buffer, pH 3.0, was added, and the mixture was left at room temperature for 1 h, followed by fractionation on the C18 analytical column with a gradient of solvent B as described in Alkylation of free Cys24 residue. The masses of collected peaks (peak 1, peak 2, peak 3, peak 4, and peak 5) were analyzed by MALDI-TOF, and the results are shown in Table S2. The remaining material was dried and subjected to further reactions.

Reduction and alkylation of the remaining Cys residues in the intermediates. The above intermediates (labeled as peak 2, peak 3, and peak 4) were each resuspended in 50 μL of 1 M Tris-HCl, 10 mM EDTA, pH 7.5, and 30 μL of 40 mM TCEP in 1 mL Tris-HCl was added to each suspension. The mixtures were allowed to react for 1 h at 37 °C, and then 6 μL of 200 mM IAM was added to each mixture, which then was incubated at room temperature for 1 h in the dark. The samples then were purified by RP-HPLC as described in Alkylation of free Cys24 residue.

Trypsin digestion. Reduced, alkylated, and purified peaks 2, 3, and 4 were each mixed with modified trypsin (1 μg of trypsin was suspended in 100 μL of 100 mM TEAB) at a ratio of 20:1 (wt/wt) and incubated at 37 °C for 3 h. The reaction was quenched with formic acid (8% final concentration) and analyzed by ESI-MS/MS, and the results are shown in SI Appendix, Table S3.

Summary of the disulfide-mapping analysis of the synthetic GVIIJSH. In the step of the disulfide bond mapping, the synthetic GVIIJSH was alkylated with VP and then reduced with TCEP and realyzed, this time with NEM. The HPLC profile of the reaction revealed five different peaks. Peak 1 and peak 5 had zero and three reduced and NEM-alkylated disulfide bonds, respectively. MALDI-TOF analysis of the collected peaks indentified three intermediates: peak 2, peak 3, and peak 4. Peak 2 was found to have two disulfide bonds closed and one disulfide bond reduced and alkylated with NEM whereas both peak 3 and peak 4 were characterized as intermediates with one disulfide bond closed and two disulfide bridges reduced and alkylated with NEM (Table S2). In the next step, all three intermediates were reduced, and the remaining Cys residues were alkylated with IAM. Following HPLC purification, the intermediates were digested with trypsin and analyzed by ESI-MS/MS. For all three intermediates, MS analysis showed the presence of three peptide fragments (an identical set for each digested intermediate), in which Cys24 was modified with VP. Peak 2 (SI Appendix, Tables S1 and S2) was found to have Cys3 [m/z ion of 472.16 (b10), 1106.42 (y9)] and Cys18 [m/z ion of 1,413.53 (y10)] alkylated with NEM whereas four remaining Cys residues were modified with IAM. These findings implied the presence of the disulfide bond between Cys2 and Cys4 (Cys3–Cys18). Cys17 in peak 3 (SI Appendix, Tables S3 and S4) was found to be the only one modified with IAM [m/z ion of 437.18 (b10)], and the remaining Cys residues were alkylated with NEM. Cys29 was not identified by ESI-MS/MS analysis of the peptide fragments, which suggested the presence of a disulfide bridge between Cys3 and Cys7 (Cys17–Cys29). The last intermediate (peak 4) (SI Appendix, Tables S5 and S6) contained Cys10 [m/z ions of 1,146.40 (b10), 364.16 (y9)] and Cys22 [m/z ion of 894.35 (y10)] alkylated with IAM, and the rest of the Cys residues modified with NEM. The results suggested the presence of the disulfide bridge between Cys3 and Cys7 (Cys10–Cys22). The peptide fragments obtained from all intermediates show that the disulfide-bridging pattern of the synthetic GVIIJSH was Cys3–Cys4, Cys4–Cys7, Cys7–Cys17, and Cys17–Cys29.

Oocyte Electrophysiology. rNa1.1 and rNa1.2 clones. Clones for rNa1.1 (NM_030875), rNa1.2 (NM_012647), rNa1.3 (NM_013119), rNa1.4 (NM_013178), rNa1.5 (NM_013125), and rNa1.6 (NM_017288) and rNa1.2 (NM_012877.1) were obtained from Alan Goldin (University of California, Irvine). Clones for...
rNa_{1.7} (NM_133289) and rNa_{1.8} (NM_017247) were obtained from Gail Mandel (Vollum Institute) and John Wood (University College London), respectively. The clone for rNa_{1.6} (NM_019266.2) was prepared as previously described (2). Clones for rNa_{β3} (NM_139907.3) and rNa_{β4} (NM_001008880.1) were obtained from Lori Isom (University of Michigan). rNa_{β1} and rNa_{β2} DNA were linearized with NotI and transcribed with T7. rNa_{β3} DNA was linearized with XbaI and transcribed with T7. rNa_{β4} DNA was linearized with BamHI and transcribed with T7.

**rNa_{1.2} and rNav1.5 mutant construction.** The rNa_{1.2} [C910L] and rNav1.5 [L869C] mutants were prepared by PCR. Forward and reverse primers were designed with 18 base pairs flanking the desired mutation. Using the nonstrand displacing action of Pfu Turbo DNA polymerase, the mutagenic primers were extended 40-120 bp. The rNav1.5[C910L] mutants were prepared by PCR. Forward and reverse primers were designed to PCR amplify the desired area of one subunit, followed by an overhang belonging to the other subunit. In a subsequent PCR, the two DNA pieces were allowed to hybridize first at the overhangs, and then amplified using primers at the 5′ and 3′ ends, used to introduce restriction sites NotI and XhoI respectively. The PCR product was gel-extracted and purified using Qiaquick PCR purification kit (Qiagen Sciences). The chimeras were subcloned into the pSGEM oocyte expression vector (which contains the 5′ and 3′ Xenopus globin regions) using the NotI and XhoI restriction sites, transformed into DH10B competent cells, and grown in ampicillin-containing LB. DNA was isolated using a Qiagen Spin mini prep kit (Qiagen Sciences) and linearized. Sense RNA was transcribed using T7 polymerase (mMessage mMachine RNA transcription kit; Ambion).

**rNa_{β} chimera construction.** All chimeras were made by PCR as previously described (2). Briefly, primers were designed to PCR amplify the desired area of one subunit, followed by an overhang belonging to the other subunit. In a subsequent PCR, the two DNA pieces were allowed to hybridize first at the overhangs, and then amplified using primers at the 5′ and 3′ ends, used to introduce restriction sites NotI and XhoI respectively. The PCR product was gel-extracted and purified using Qiaquick PCR purification kit (Qiagen Sciences). The chimeras were subcloned into the pSGEM oocyte expression vector (which contains the 5′ and 3′ Xenopus globin regions) using the NotI and XhoI restriction sites, transformed into DH10B competent cells, and grown in ampicillin-containing LB. DNA was isolated using a Qiagen Spin mini prep kit (Qiagen Sciences) and linearized. Sense RNA was transcribed using T7 polymerase (mMessage mMachine RNA transcription kit; Ambion).

**Oocytes.** Oocytes were harvested and prepared essentially as previously described (3). Briefly, freshly excised oocytes were treated with 2.5 mg/mL collagenase A (Roche Diagnostics) in OR-2 (82.5 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl₂, and 5 mM Heps, pH 7.3) for 1-2 h on a rotary shaker at room temperature. Half-way through the treatment, the solution was exchanged with fresh collagenase solution. The oocytes were then rinsed with OR-2 and incubated until used at 16 °C in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, and 5 mM Heps, pH 7.3) supplemented with the antibiotics penicillin (100 U/mL), streptomycin (0.1 mg/mL), Septra (0.2 mg/mL), and Amikacin (0.1 mg/mL). Use of X. laevis frogs, which provided oocytes for this study, followed protocols approved by the University of Utah Institutional Animal Care and Use Committee that conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Injection of cRNA into oocytes.** Injection of cRNA from rat Na_{1.1} to -1.8 and rNa_{β1} to -β4 clones into oocytes was done as previously described (2, 4). Briefly, a given oocyte was injected with 30–50 nL of cRNA in distilled water for one of the followings sodium channel isoforms: rNa_{1.1}, rNa_{1.2}, rNa_{1.3}, rNa_{1.4}, rNa_{1.5}, rNa_{1.6}, rNa_{β1}, or rNa_{1.8} (3, 1.5, 0.2, 0.6, 3, 30, 15, or 35 ng, respectively) without or with an equal weight of a rNa_{β} cRNA, except for coinjections of Na_{1.3} and Na_{β4}, in which case 0.2 ng of Na_{1.3} and 0.6 ng of Na_{β4} cRNAs were used. Oocytes were incubated at 16 °C for 1-6 d in ND96 supplemented with antibiotics, as described in the preceding paragraph.

**Two-electrode voltage clamping of oocytes.** Oocytes were voltage clamped with a Warner OC-725C amplifier (Warner Instruments) using 3 M KCl-filled microelectrodes (<0.5 MW resistance) essentially as previously described (2). A holding potential of −80 mV was used, and voltage-gated sodium currents (I_{Na}) were induced every 20 s with a 50-ms depolarizing step to −20 mV (Na_{1.5}), −10 mV (Na_{1.1}, -1.2, -1.4, -1.6, and -1.7), 0 mV (Na_{1.3}), or +20 mV (Na_{1.8}). Current signals were filtered at 2 kHz, digitized at a sampling frequency of 10 kHz and leak-subtracted by a P/S protocol using in-house software written in LabVIEW (National Instruments). The recording chamber was a 4-mm-diameter well (30 μL of total volume) sunk in the silicone elastomer, Sylgard (Dow Corning). Conopeptides were dissolved in ND96, and oocytes were exposed to toxin by applying 3 μL of peptide solution (at ten times the final concentration) to a static bath with a pipette and manually stirring the bath for a few seconds by gently aspirating and expelling a few μL of bath fluid several times with the pipette. A static bath was used to conserve peptide. Peptides were washed out by continuous perfusion with ND96, initially at a rate of 1.5 mL/min for 20 s, then at a steady rate of 0.5 mL/min. Exposures of oocytes while in the recording chamber to DTT (Sigma-Aldrich) or MTSET (Toronto Research Chemicals) were performed in a static bath, just as for toxin exposures. Exposures to DTT or copper phenanthroline (CuPhe) before introducing the oocytes into the recording chamber were done in 35-mm-diameter plastic culture dishes followed by rinsing with ND96. CuPhe was prepared from CuSO₄ and 1–10 phenanthroline (Sigma-Aldrich) as described by Bénitah, et al. (5).

All experiments were done at room temperature. **Analysis of oocyte data.** Percentage block of peak I_{Na} by peptide was determined by obtaining the average peak of ≥3 control traces and the average peak of ≥3 traces acquired at steady state in the presence of peptide, then dividing the latter by the former and multiplying by 100. Fitting of time-course data to a single-exponential function was done with homemade software written with LabVIEW. The interaction of toxin with channel was assumed to be that of a simple bimolecular reaction whose kinetics are described by the equation, $k_{obs} = k_{on}[\text{toxin}] + k_{off}$ where [toxin] is toxin concentration. The time course of peak I_{Na} was plotted before, during, and after exposure to toxin. The on-rate constant was determined as follows: The onset of block at a given peptide concentration was fit to a single-exponential function to yield the observed rate constant, $k_{obs}$, following which $k_{on}$ was obtained from the linear-regression slope of a $k_{obs}$ versus peptide concentration plot for at least three different peptide concentrations (where each concentration was tested on ≥3 oocytes), as previously described (2, 6). The off-rate constant was determined by fitting the toxin-washout curve to a single-exponential function; however, when recovery from block was very slow (less than 50% recovery after 20 min; i.e., $k_{off} < 0.035\text{min}^{-1}$), $k_{off}$ was estimated from the level of recovery observed after 20 min of washing and assuming recovery followed a single-exponential time course (2, 4). Times longer than 20 min were not used to avoid error due to possible baseline drift. Each $k_{off}$ Value was the average of ≥9 oocytes.

All oocyte data are presented as mean ± SD. Statistical comparisons were performed by two-tailed unpaired t tests.

**Mammalian Cell-Line Electrophysiology. Culture of cell lines expressing hNav1.5s.** Human embryonic kidney (HEK) 293 cells stably expressing human Na_{1.2} (supplied by H. A. Hartmann, University of Maryland Biotechnology Institute), Na_{1.4}, Na_{1.5}, or Na_{1.7} (Millipore) were cultured in DMEM/F-12 media (1:1), supplemented with 10% (vol/vol) FBS, 1% penicillin/streptomycin, 400 μg/mL G418 (Geneticin), and 100 μM Non Essential Amino
Acids (NEAAs) (all reagents from Invitrogen). Chinese hamster ovary (CHO) cells stably expressing human Na$_{\text{a}1.3}$ were cultured in IMDM (Iscove’s media with t-Glutamine), supplemented with 10% (vol/vol) dialyzed FBS, 1% HT supplement (a mixture of 10 mM hypoxanthine and 1.6 mM thymidine), 1% NEAAs, and 400 µg/mL G418. Tetracycline-inducible CHO cells stably expressing human Na$_{\text{a}1.1}$ or Na$_{\text{a}1.6}$ were cultured in Ham’s F-12 media (Mediatech), supplemented with 10% (vol/vol) FBS, 100 units/mL penicillin/streptomycin, 10 µg/mL blasticidin, and 400 µg/mL zeocin (Invitrogen) and induced with 1 µg/mL tetracycline at least 24 h before use. Cells were maintained at 37 °C and in 5% CO$_2$ and harvested for QPatch experiments upon reaching ~50-90% confluency.

**Preparation of cells for, and their use with, QPatch.** Cell preparation for assay with QPatch HT (Sophion) was performed essentially as previously described (7). Briefly, cells were dissociated using 0.05% trypsin (5 min at 37 °C), centrifuged, resuspended in CHO-S-SFM media (Life Technologies), and gently triturated to break up cell clumps. Cell density was adjusted to 1–2 × 10$^6$/mL with the same media, and cells were transferred to a cell “hotel” in QPatch and used in experiments for several hours. For gigahm seal formation and whole-cell patch clamp recording, the extracellular solution contained 137 mM NaCl (except for Na$_{\text{a}1.5}$), 5.4 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, and 10 mM Hepes pH 7.4, and an osmolality of 315 mOsM. For Na$_{\text{a}1.5}$, the 137 mM NaCl was replaced by 27.4 mM NaCl and 109.6 mM choline chloride. The intracellular solution contained 135 mM CsF, 10 mM CsCl, 5 mM EGTA, 5 mM NaCl, and 10 mM Hepes, pH 7.3, and an osmolality of 290 mOsM.

**Voltage protocol.** From a holding potential that corresponds to ~V$_{1/2}$ value for steady-state inactivation for each Na$_{\text{a}}$ and harvested for QPatch experiments upon reaching ~50-90% confluency.

**Production and patch-clamp recording of HEK cells expressing chimeras of humang Na$_{\text{a}1.5}$ and -1.7.** cDNAs encoding human Na$_{\text{a}1.5}$ (NM_198056.1) and human Na$_{\text{a}1.7}$ (NM_002977.1) were obtained from Origene. To create the chimera 7577 (composed of domain II S1–S6 of hNa$_{\text{a}1.5}$ in an otherwise hNa$_{\text{a}1.7}$ background), the amino acid sequence FIVMDPFVDL......LVVLNLFLALLL of human Na$_{\text{a}1.7}$ (corresponding to amino acids 739–968 of Q15858-1) was replaced with the amino acid sequence LVMDPFTD......LVVLNLFLALLL of human Na$_{\text{a}1.5}$ (corresponding to amino acids 712–939 of Q14524-1). The reciprocal chimera, 7575 (composed of domain II S1–S6 of hNa$_{\text{a}1.5}$ in an otherwise hNa$_{\text{a}1.7}$ background), was constructed by replacing the amino acid sequence LVMDPFTD......LVVLNLFLALLL of human Na$_{\text{a}1.5}$ (corresponding to amino acids 712–939 of Q14524-1) with the amino acid sequence FIVMDPFVDL......LVVLNLFLALLL of human Na$_{\text{a}1.7}$ (corresponding to amino acids 739–968 of Q15858-1). The DNA sequences encoding the donor and recipient channel fragments plus the mammalian expression vector pcDNA4/TO (Invitrogen/Life Technologies) were amplified by PCR using Prime Star GXL polymerase (Takara/Clontech). The donor and recipient cDNA fragments (plus the vector) were cloned together using the In-Fusion technology (Clontech). HEK293 cells were transiently transfected with cDNA encoding either chimera channel using FuGENE HD DNA-Charge Reversal Protocol and cultured with CD4 using manual patch-clamp 3–5 d posttransfection. Before conducting patch-clamp experiments, cells were incubated with paramagnetic CD4 beads (Dynabeads; Invitrogen; 11331D) to aid in detection of transfected cells.

Whole-cell patch-clamp recordings were carried out at room temperature using an AxoPatch 200B amplifier (Molecular Devices). Electrodes were made from thin-wall capillary glass (Cat. no. TW150F-4; WPI) using a P-97 puller from Sutter. The intra- and extracellular solutions were those used for QPatch. The resistances of the filled pipettes were ~2 MΩ. Voltage errors were minimized using 80% series resistance compensation. Membrane currents were filtered at 5 kHz and sampled at 25 kHz. Cells were held at the holding potential when the above voltage protocol was not being executed.

**Aflag l. The test compound concentrations were chosen to determine the blocking efficacy of the test compound at each concentration. All experiments were performed at room temperature (~22 °C).**


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**Fig. S1.** Nucleotide and conceptual translation product obtained from analysis of *Conus geographus* transcriptome, and HPLC elution profiles of GVIIJ<sub>SH</sub>, GVIIJ<sub>SSG</sub>, GVIIJ<sub>SSC</sub>, and synthetic μOS-GVIIJ. (A) Underlined are propeptide and the mature toxin obtained from the targeted PCR; mature toxin is shown in bold. The signal sequence and the remaining segment of the propeptide were inferred from the transcriptome sequence. The partial sequences of the 5′ and 3′ untranslated regions are also shown. (B) An analytical C<sub>18</sub> column was used with a flow rate of 1 mL/min and a gradient ranging from 15% to 45% of solvent B in 30 min. Absorbance was monitored at 220 nm.
**Fig. S2.** MS/MS spectrum of native μO§-GVIIJ. MS/MS ETD spectrum of the (M + 5H)+5 ion of GWCDOGATCGLRLYCCSGFCDCTKCTKDKSSA after reduction and alkylation with 2-methylaziridine acquired on the Orbitrap Elite with 15,000 resolution (at 400 m/z). N-terminal fragment ions (c) are indicated by ⌈, and C-terminal fragment ions (z) are indicated with ¨. Doubly charged ions are indicated with +2, and z ions resulting from cleavage at cysteine and loss of the cysteine side chain are indicated with # (1). Due to space limitations, different charge states of already labeled peptide bond cleavages are not all labeled. W is a brominated tryptophan and O is a hydroxylated proline. Eleven scans were averaged for this spectrum. The mass accuracy for all fragment ions is better than 15 ppm.


**Fig. S3.** Detection of cysteinylation in native μO§-GVIIJ. Shown are the extracted ion chromatograms for the +5 ion of the native toxin (Upper) and the +7 ion of the reduced and 2-methylaziridine alkylated toxin (Lower). The Insets show the isotopic distribution of each charge state. The observed shift in mass from the native to the reduced and alkylated toxin is consistent with a cysteinylated toxin. Reduction results in breakage of all disulfide bonds and leading to the addition of one proton (H+ = 1.008 Da) per cysteine that took part in a disulfide bond. Subsequent alkylation with 2-methylaziridine leads to a mass increase of 54.0578 Da per cysteine residue. The measured mass of the native toxin was 3,934.4215 [M+H]+1, which is within 0.25 ppm of the theoretical mass of 3,934.4214 [M+H]+1. The measured mass of the reduced and alkylated toxin was 4,220.8978 [M+H]+2, which is within 6.6 ppm of the theoretical mass of 4,220.8699 [M+H]+2. Note that cysteinylation of Cys at position 24 (in the amino acid sequence above the plots) is assumed based on the structure and activity of the synthetic GVIIJSH (see “Determination of the free Cys residue in GVIIJSH” in SI Materials and Methods and Table S3).
Fig. S4. The magnitude of the slow component of the biphasic recovery of Na\textsubscript{V}1.2 following washout of GVIIJ\textsubscript{SH} increased as toxin-exposure time was increased. The first exposure to 10 \(\mu\)M GVIIJ\textsubscript{SH} was for 1.3 min (first horizontal bar) whereas the second was for 13 min (second horizontal bar). The level of block with slow off-rate was larger with longer time-of-exposure to peptide.

Fig. S5. GVIIJ\textsubscript{SH} blocks human Na\textsubscript{V}1.5:Na\textsubscript{V}1.7 chimera 5755 but not chimera 7577. (Upper) Shown are diagrams depicting the channel’s four domains, illustrating that chimera 5755 (A) consisted of domain II of Na\textsubscript{V}1.7 (gray) with the remaining three domains those of Na\textsubscript{V}1.5 (white), whereas chimera 7577 (B) had the reciprocal composition of domains. (A and B, Lower) Representative \(I_{\text{Na}}\) traces of patch-clamped HEK 293 cells (SI Materials and Methods) expressing each chimera before and after exposure to 1 \(\mu\)M GVIIJ\textsubscript{SH}. The peptide largely blocked \(I_{\text{Na}}\) of chimera 5755 (A) but not that of chimera 7577 (B). Chimera 5755 was inhibited by 81.1 \(\pm\) 3.5\% (mean \(\pm\) SD, \(n=5\)) whereas chimera 7577 was inhibited by only 12.6 \(\pm\) 4.5\% (\(n=5\)).
Fig. S6. GVIIJSSG competes with neither TTX, μ-KIIIA[K7A], nor μ-O-MrVIB for their sites on rNa1.2. (A–D) Oocytes expressing rat Na1.2 were voltage clamped as in Fig. 1A. (A) Oocyte was exposed first to a saturating TTX concentration (10 μM) (white bar) and then additionally to a saturating concentration of 10 μM GVIIJSSG (black bar), following which both toxins were washed out. Inset shows the readily reversible block by 1 μM TTX alone (white bar). TTX did not reduce the level of slowly reversible block produced by GVIIJSSG that persisted after both toxins were washed out (compare levels of block following toxin washout of both toxins here with that during washout following exposure to GVIIJSSG alone in Inset of B). (B) Saturating concentration (30 μM) of μ-KIIIA[K7A] was added first (at the time and duration shown by the white bar) leaving a residual current of ∼25%. This exposure was followed by 3 μM GVIIJSSG (black bar), which blocked a large fraction of μ-KIIIA[K7A]'s residual current. Finally, both toxins were washed out at the same time. This result shows that GVIIJSSG's block was not impeded by the presence μ-KIIIA[K7A], as evident by comparison with the block produced by 3 μM GVIIJSSG alone (Inset). (C) Saturating concentration (33 μM) of GVIIJSSG was added first (black bar) followed by 10 μM μ-O-MrVIB (white bar), and then both peptides were washed out; after several minutes, conditioning-depolarizing pulses (300-ms rectangular steps to 120 mV that preceded each test pulse by 3.3 s) were applied (gray bar), which accelerated the washout of μ-O-MrVIB to a steady-state level expected of that if GVIIJSSG had been added alone. The Inset shows that exposure to 10 μM μ-O-MrVIB (white bar) largely blocks I Naughty which slowly recovers following washout, but rapidly recovers during depolarizing-conditioning pulses (gray bar) essentially completely. (D) μ-O-MrVIB (10 μM) was added first (white bar) followed by 33 μM GVIIJSSG (black bar), and then both toxins were washed out; after several minutes, conditioning-depolarizing pulses were applied (gray bar), which accelerated the washout of μ-O-MrVIB to a steady-state level similar to that arrived at in C. A more detailed description of these results is as follows. TTX blocked in a readily reversible manner (A, Inset). A saturating concentration of TTX present before and during exposure to GVIIJSSG did not prevent the peptide from producing its slowly reversible block (A), and we conclude that the two toxins don't compete for the same site on the channel. Like TTX, μ-KIIIA[K7A] also blocks by plugging the pore of the channel, but the plug is leaky (1, 2); thus, at saturating concentrations of μ-KIIIA[K7A], a ∼25% residual current (I Na ) persists with Na1.2. When μ-KIIIA[K7A] was added first, this I Na was readily evident (8), and when 3 μM GVIIJSSG was added to the bath already containing μ-KIIIA[K7A], most of the I Na was readily blocked (8), which indicates that the presence of μ-KIIIA[K7A] did not impede the block by GVIIJSSG; therefore, these two toxins occupy mutually exclusive sites on the channel. A protocol similar to the preceding was used to see whether GVIIJSSG and μ-O-MrVIB competed for a common site on rat Na1.2. The off-rate of μ-O-MrVIB can be accelerated with strong depolarizing-conditioning pulses; i.e., 300-ms rectangular steps to 120 mV that preceded each test pulse by 3.3 s (C, Inset). When 30 μM GVIIJSSG was added first, its residual current was readily and largely blocked when the bath was supplemented with 10 μM μ-O-MrVIB (Fig. S6C). When both toxins were washed out, recovery from block was slow but could be accelerated by strong depolarizing pulses to a steady-state level of block that was essentially the same as the level of block initially produced by GVIIJSSG alone, before μ-O-MrVIB was added. Because at least part of GVIIJSSG's residual current is likely due to heterogeneity of channels (Discussion), this result was not unexpected. To more rigorously test for competition, the order of addition of the toxins was reversed: that is, 10 μM μ-O-MrVIB was added first, which blocked most of the current; then, the bath was supplemented with 30 μM GVIIJSSG and then both toxins were washed out, following which slow recovery from block ensued that could be accelerated by strong depolarizing pulses to a steady-state level of block (D). Because the final level of steady-state block was similar to the level of block produced by GVIIJSSG alone (e.g., see C), it appears that prior exposure to μ-O-MrVIB did not protect the channel against occupation by GVIIJSSG, which indicates that the two toxins do not share a common binding site.

Fig. S7. GVIIJ$_{SSG}$ is able to block an Na$_V$1.2 channel that is deficient in fast inactivation. Oocytes were used that expressed the rNa$_V$1.2 mutant where the IFM sequence of residues in the intracellular link between domains III and IV were replaced by QQQ to remove fast inactivation (1). Oocytes were voltage clamped as described in Fig. 1A. Both traces, before (control) and after 2-min exposure to 33 $\mu$M GVIIJ$_{SSG}$, showed impairment of fast inactivation (compare time course of these traces with those shown in Fig. 2A). The attenuated trace obtained in the presence of GVIIJ$_{SSG}$ illustrates that the mutant channel can be blocked by the peptide.

Table S1. HPLC retention times, purity, and MS data for GVIIJ<sub>SH</sub> and GVIIJ<sub>SSG</sub>

<table>
<thead>
<tr>
<th>Peptide</th>
<th>HPLC RT&lt;sup&gt;+&lt;/sup&gt;, min</th>
<th>Purity, %</th>
<th>[M] + (Da, calc.)</th>
<th>[M+H] + (Da, found)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVIIJ&lt;sub&gt;SH&lt;/sub&gt;</td>
<td>19.13</td>
<td>99</td>
<td>3,736.54</td>
<td>3,737.58</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>17.50</td>
<td>99</td>
<td>4,041.61</td>
<td>4,042.44</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSC&lt;/sub&gt;</td>
<td>18.31</td>
<td>99</td>
<td>3,855.70</td>
<td>3,856.90</td>
</tr>
<tr>
<td>μOs-GVIIJ</td>
<td>20.43</td>
<td>99</td>
<td>3,933.46</td>
<td>3,934.40</td>
</tr>
</tbody>
</table>

*Retention time (RT) was assessed by RP-HPLC using an analytical C<sub>18</sub> column and an elution gradient ranging from 15% to 45% of solvent B in 30 min.

Table S2. Fragments identified by MS after partial reduction and alkylation of GVIIJ<sub>SH</sub>

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mass [M+H]&lt;sup&gt;+&lt;/sup&gt; found</th>
<th>Disulfide bonds closed</th>
<th>NEM-alkylated disulfide bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,842.37</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4,093.56</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>4,345.69</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>4,345.66</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>4,598.60</td>
<td>0</td>
<td>3</td>
</tr>
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</table>

Table S3. Kinetics of block by GVIIJ<sub>SSG</sub> of rNav<sub>1.1</sub> to -1.8 (top 10 rows), of rNav<sub>1.7</sub> + β1 or β3 (rows 11 and 12), and of rNav<sub>1.2</sub> and -1.7 by GVIIJ<sub>SSC</sub> or μOs-GVIIJ (bottom four rows)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Na&lt;sub&gt;V&lt;/sub&gt;</th>
<th>k&lt;sub&gt;on&lt;/sub&gt;&lt;sup&gt;‡‡&lt;/sup&gt;, μM&lt;sup&gt;−1&lt;/sup&gt;min&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>k&lt;sub&gt;off&lt;/sub&gt;&lt;sup&gt;‡‡&lt;/sup&gt;, min&lt;sup&gt;−1&lt;/sup&gt;</th>
<th>K&lt;sub&gt;d&lt;/sub&gt;&lt;sup&gt;‡‡&lt;/sup&gt; or IC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;‡‡&lt;/sup&gt;, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.1</td>
<td>0.41 ± 0.05</td>
<td>0.0047 ± 0.0006</td>
<td>0.011 ± 0.002²</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.2</td>
<td>0.48 ± 0.04</td>
<td>0.0051 ± 0.0026</td>
<td>0.011 ± 0.006²</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.2[C910L]</td>
<td>NA</td>
<td>3.1 ± 1.3</td>
<td>88 ± 2 (160 ± 2)⁵</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.3</td>
<td>0.32 ± 0.02</td>
<td>0.0047 ± 0.0002</td>
<td>0.015 ± 0.006³</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.4</td>
<td>0.34 ± 0.02</td>
<td>0.0016 ± 0.0008</td>
<td>0.0047 ± 0.0024⁴</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.5</td>
<td>NA</td>
<td>3.7 ± 2.2</td>
<td>207 ± 12 (579 ± 8)³</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.5[L869C]</td>
<td>0.035 ± 0.004</td>
<td>0.0046 ± 0.0032</td>
<td>0.13 ± 0.09⁴</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.6</td>
<td>0.33 ± 0.02</td>
<td>0.12 ± 0.016</td>
<td>0.36 ± 0.05⁵</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.7</td>
<td>0.12 ± 0.02</td>
<td>0.0049 ± 0.0032</td>
<td>0.041 ± 0.027⁴</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.8</td>
<td>NA</td>
<td>NA</td>
<td>&gt;1,000⁸</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.7 + β1</td>
<td>0.1 ± 0.02</td>
<td>0.0045 ± 0.0037</td>
<td>0.045 ± 0.038⁸</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSG&lt;/sub&gt;</td>
<td>1.7 + β3</td>
<td>0.11 ± 0.012</td>
<td>0.0005 ± 0.0025</td>
<td>0.05 ± 0.023⁸</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSC&lt;/sub&gt;*</td>
<td>1.2</td>
<td>1.66 ± 0.05</td>
<td>0.0057 ± 0.0022</td>
<td>0.0034 ± 0.0013⁷</td>
</tr>
<tr>
<td>μOs-GVIIJ&lt;sub&gt;SSC&lt;/sub&gt;*</td>
<td>1.2</td>
<td>2.60 ± 0.12</td>
<td>0.0075 ± 0.0010</td>
<td>0.0029 ± 0.0004⁴</td>
</tr>
<tr>
<td>GVIIJ&lt;sub&gt;SSC&lt;/sub&gt;*</td>
<td>1.7</td>
<td>0.42 ± 0.06</td>
<td>0.0055 ± 0.0025</td>
<td>0.013 ± 0.006⁶</td>
</tr>
<tr>
<td>μOs-GVIIJ&lt;sub&gt;SSC&lt;/sub&gt;*</td>
<td>1.7</td>
<td>2.00 ± 0.22</td>
<td>0.0064 ± 0.0026</td>
<td>0.0032 ± 0.0013⁴</td>
</tr>
</tbody>
</table>

Values represent mean ± SD (n ≥ 3 oocytes for each of ≥3 different concentrations of peptide).

*On-rate constants were determined from onsets of block at different [peptide] as described in SI Materials and Methods.

1<sup>Off</sup>-rate constants were determined from toxin-washout curves as described in SI Materials and Methods.

2<sup>K<sub>on</sub></sup> was determined from the ratio of <i>k<sub>off</sub>/K<sub>corr</sub></i>. When rates were too fast to measure, steady-state IC<sub>50</sub> was obtained as a measure of affinity instead of K<sub>d</sub>.

3Steady-state IC<sub>50</sub> were determined by fitting % block versus peptide concentration ([peptide]) curves to the equation (using GraphPad Prism or KaleidaGraph software): % block = % maximum block/(1 + IC<sub>50</sub>.[peptide]) for ≥3 different [peptide]; % maximum predicted block was 66 ± 0.2% and 42 ± 2% for Na<sub>1.2</sub>[C910L] and Na<sub>1.5</sub>[L869C], respectively. The value in parentheses indicates the IC<sub>50</sub> obtained when % maximum block was fixed at 100%.

4Less than 5% block of Na<sub>1.8</sub> was observed with 100 μM peptide, the highest concentration tested.

5<sup>k<sub>off</sub></sup> and <sup>K<sub>d</sub></sup> values with β1- or β3-subunit coexpression not significantly different from those of Na<sub>1.7</sub> without β-subunit coexpression (P > 0.6).

6GVIIJ<sub>SSC</sub> is synthetic peptide of Fig. 1D where X<sub>2</sub> is cysteine, and μOs-GVIIJ is synthetic native peptide (i.e., GVIIJ<sub>SSC</sub> with brominated Trp2).

7**GVIIJ<sub>SSG</sub> is synthetic peptide of Fig. 1D where X<sub>2</sub> is cysteine, and μOs-GVIIJ is synthetic native peptide (i.e., GVIIJ<sub>SSC</sub> with brominated Trp2).

8Value not different from <sup>k<sub>off</sub></sup> of GVIIJ<sub>SSG</sub> with Na<sub>1.2</sub> (P = 0.63).

9Value not different from <sup>k<sub>off</sub></sup> of GVIIJ<sub>SSG</sub> with Na<sub>1.7</sub> (P = 0.70). Two-tailed unpaired <i>t</i> tests were used for these statistical comparisons.
Table S4. Isochronal IC$_{50}$s for the block by GVII(SSG) and GVII(SH) of human Na$_V$1.1 to -1.7 expressed in HEK 293 or CHO cells

<table>
<thead>
<tr>
<th>hNa$_V$</th>
<th>GVII(SSG)</th>
<th>GVII(SH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ nM</td>
<td>SD</td>
</tr>
<tr>
<td>1.1*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1.2†</td>
<td>1,110</td>
<td>372</td>
</tr>
<tr>
<td>1.3*</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>1.4†</td>
<td>812</td>
<td>515</td>
</tr>
<tr>
<td>1.5†</td>
<td>19 ± 2% at 10 μM</td>
<td>7</td>
</tr>
<tr>
<td>1.6*</td>
<td>756</td>
<td>62</td>
</tr>
<tr>
<td>1.7†</td>
<td>783</td>
<td>110</td>
</tr>
</tbody>
</table>

Values were determined as described in SI Materials and Methods. NA, not available.

*Expressed in CHO cells.
†Expressed in HEK 293 cells.
‡Percentage block of hNa$_V$1.5 by 10 μM GVII(SSG) was 19 ± 2%.
§Percentage block of hNa$_V$1.5 by 10 μM GVII(SH) was 0.3 ± 6.5%.

Other Supporting Information Files

SI Appendix (DOC)