Synthesis, Solution Structure, and Phylum Selectivity of a Spider δ-Toxin That Slows Inactivation of Specific Voltage-gated Sodium Channel Subtypes

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Magi 4, now renamed δ-hexatoxin-Mg1a, is a 43-residue neurotoxic peptide from the venom of the hexathelid Japanese funnel-web spider (Macrothele gigas) with homology to δ-hexatoxins from Australian funnel-web spiders. It binds with high affinity to receptor site 3 on insect voltage-gated sodium (Naᵥ) channels but, unlike δ-hexatoxins, does not compete for the related site 3 in rat brain despite being previously shown to be lethal by intracranial injection. To elucidate differences in Naᵥ channel selectivity, we have undertaken the first characterization of a peptide toxin on a broad range of mammalian and insect Naᵥ channel subtypes showing that δ-hexatoxin-Mg1a selectively slows channel inactivation of mammalian Naᵥ1.1, Naᵥ1.3, and Naᵥ1.6 but more importantly shows higher affinity for insect Naᵥ1.1 (para) channels. Consequently, δ-hexatoxin-Mg1a induces tonic repetitive firing of nerve impulses in insect neurons accompanied by plateau potentials. In addition, we have chemically synthesized and folded δ-hexatoxin-Mg1a, ascertainment of the bonding pattern of the four disulfides, and determined its three-dimensional solution structure using NMR spectroscopy. Despite modest sequence homology, we show that key residues important for the activity of scorpion α-toxins and δ-hexatoxins are distributed in a topologically similar manner in δ-hexatoxin-Mg1a. However, subtle differences in the toxin surfaces are important for the novel selectivity of δ-hexatoxin-Mg1a for certain mammalian and insect Naᵥ channel subtypes. As such, δ-hexatoxin-Mg1a provides us with a specific tool with which to study channel structure and function and determinants for phylum- and tissue-specific activity.

Voltage-gated sodium (Naᵥ) channels are responsible for the generation and propagation of electrical signals in excitable cells. At least nine different genes encoding distinct Naᵥ channels isoforms have been identified, and functionally expressed, in mammals (1). They are characterized by their sensitivity to TTX, with Naᵥ1.5, Naᵥ1.8, and Naᵥ1.9 being TTX-insensitive or TTX-resistant, and the remaining subtypes being sensitive to nanomolar concentrations of TTX. In addition, localization of the subtypes also varies, with Naᵥ1.1–1.3 mostly distributed in the central nervous system, Naᵥ1.6–1.9 principally located in the peripheral nervous system, and Naᵥ1.4 and Naᵥ1.5 found in skeletal and cardiac muscle, respectively. The structural diversity of Naᵥ channels also coincides with variations in physiological and pharmacological properties (2). In contrast, insects express only one gene (para) that undergoes extensive alternative splicing and RNA editing (3). The para-encoded Naᵥ channel is exceptionally well conserved across diverse orders of insects, with the level of identity ranging from 87 to 98% (3). This is one reason why insecticides that target insect Naᵥ channels have broad activity across many insect orders. In contrast, para-type Naᵥ channels have significantly lower levels of identity with the various types of mammalian Naᵥ channels with the level of identity typically around 50–60% (3). This explains why a high degree of phylogenetic specificity can be achieved with

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1The atomic coordinates and structure factors (code 2RDO) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4The abbreviations used are: Naᵥ channel, voltage-gated Na⁺ channel; HXTX, hexatoxin; Acm, acetamidomethyl; 4-AP, 4-aminopyridine; 3,4-DAP, 3,4-di-aminopropidine; Boc, tert-butoxycarbonyl; DRG, dorsal root ganglia; DUM, dorsal unpaired medial; DQF-COSY, double quantum-filtered correlation spectroscopy; ESI-Q-TOF MS, electrospray ionization/quadrupole time-of-flight mass spectrometry; GdnHCl, guanidine hydrochloride; ICK, inhibitory cystine knot; Kᵥ channel, voltage-gated K+ channel; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; RMP, resting membrane potential; RP-HPLC, reversed-phase high pressure liquid chromatography; TEA, tetraethylammonium; TOCSY, total correlation spectroscopy; TTX, tetrodotoxin.
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**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—δ-HXTX-Mg1a (43 residues) was chemically synthesized by native chemical ligation involving the coupling of a Cys(17)-(1–19)-thiopeptide (N-terminal 19 residues) and Cys-(20–43) peptide (C-terminal 24 residues). The N-terminal Cys residue of the former peptide was protected by the Acm group to prevent cyclization and oligomerization. The solid-phase synthesis of the Cys(17)-(1–19)-thioester and Cys-(20–43) peptides were performed using an ABI 433A peptide synthesizer using the Boc strategy on a Boc-Asn(xanthenyl)-S-CH2-CH2-CO-Leu-4-hydroxymethyl-phenylacatamidomethyl resin (0.5 mmol) and Boc-Cys(4-methylbenzyl)-Merrifield resin (0.3 mmol), respectively. The peptide chains were elongated using *in situ* neutralization protocols of coupling with Boc-amino acid/2-[1H-benzotriazole-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate/N-hydroxybenzotriazole/N,N-diisopropylethylamine (4:4:4:6 eq). After chain assembly was completed, the peptide resins obtained were treated with HF in the presence of p-cresol (to protect Trp residues) and 1,4-butanediol (as an oxygen scavenger) at −5 °C for 1 h to remove all of the protecting and anchoring groups except the Acm group on the Cys1 residue. The crude products were then purified by preparative RP-HPLC using a YMC-Pak ODS column (30 × 250 mm) to obtain 195 mg (16%) and 75 mg (8.4%) of purified peptides, respectively.

The N-terminal Cys(17)-(1–19)-thioester and C-terminal Cys-(20–43) peptides were then allowed to ligate by solubilizing them in freshly degassed 0.1 mM sodium phosphate buffer (10 ml), pH 8.4, containing 6 M guanidine hydrochloride (GdnHCl). Thiophenol (0.40 ml) was then added, and the whole mixture was stirred for 24 h at room temperature and then treated with diithiothreitol (1.6 mM). After 20 min, the pH was acidified to less than 2 using 1 mM HCl. The mixture was washed with ether, and Cys(17)–43 peptide was purified from the aqueous layers by preparative RP-HPLC using a linear gradient (20–40% CH3CN in 0.1% trifluoroacetic acid for 80 min) to obtain 51 mg (41%) of purified peptide.

To remove the Acm group, a 9.6 mM solution of Cys-(17)-(1–43) (50 mg) was made up in 5 ml of 95% trifluoroacetic acid containing anisole (50 μl), and 0.77 mmol of silver trifluoroacetate was added and stirred for 2 h at room temperature. The product was precipitated as the silver salt, by adding ether to the reaction mixture, and was dissolved in 0.1 M phosphate buffer, pH 8.4, containing 6 M GdnHCl. Dithiothreitol (3.8 mmol) was added to the solution and stirred for 0.5 h at room temperature. After the addition of 1 M HCl, the resulting AgCl was removed by filtration, and the filtrate was applied to preparative RP-HPLC using a linear gradient (20–40% CH3CN in 0.1% trifluoroacetic acid for 80 min) to obtain 23 mg (46% yield) of the reduced peptide (8 SH-peptide-(1–43)).

The eight free cysteines were allowed to oxidize in air for 4 days at 4 °C in 0.1 M aqueous ammonium acetate, pH 7.6, containing 2 M GdnHCl, 0.1 mM GSSG, and 1 mM GSH. The peptide/GSSG/GSH ratio was 1:10:100. The solution was then diluted 2-fold with cold water (4 °C) to a final peptide concentration of 5 μM. After 3 days at 4 °C, the mixture was acidified to pH 2 by adding trifluoroacetic acid, and the folded peptide was

Both NaV channel toxins and insecticides that target the NaV channel.

At least seven distinct toxin-binding sites have been identified by radioligand binding and electrophysiological studies on vertebrate and insect NaV channels (4, 5). Toxins interacting with these neurotoxin receptor sites have been instrumental in the study of NaV channel topology, function, and pharmacology (6). In particular, a wide range of scorpion α-toxins, sea anemone toxins, and spider δ-hexotoxins (formerly δ-atracotoxins (7)) compete for binding to receptor site-3 on the extracellular surface of NaV channels. These polypeptide toxins all inhibit the fast inactivation of NaV channels to prolong Na+ currents (I
\[\text{INa}\]), despite huge diversity in primary and tertiary structures (8, 9). Nevertheless, receptor site-3 has not yet been fully characterized but is believed to involve domains DI/S5-S6, DIV/S5-S6, as well as DIV/S3-S4 (9). Most importantly, however, toxin characterization is often limited to studies using whole-cell I\[\text{Na}\] or binding studies on neuronal membranes where there are mixed populations of NaV channel subtypes. For all of these toxins, the precise pattern of NaV channel subtype selectivity is either unknown or at best is incomplete.

Recently, it was found that receptor site-3 was also recognized by a 43-residue spider toxin, originally named Magi 4, from the hexathelid spider *Macrothele gigas* (Iriomote, Japan). It binds with high affinity to insect NaV channels but, similar to scorpion α-like toxins, does not compete for the related site-3 in rat brain synaptosomes, despite being lethal by intracranial injection (10). Magi 4 shares significant homology to four δ-hexotoxin (HXTX)-1 family peptides and δ-actinopoditoxin-Mb1a (formerly δ-missulenatoxin-Mb1a; Fig. 1) but no sequence homology to scorpion α-toxins. Neurochemical studies have shown that δ-HXTX-1 toxins compete at nanomolar concentrations with both anti-mammalian (e.g. Aah2 and Lqh2) and anti-insect (e.g. LqhαHIT) scorpion toxins for site-3 (11–13). The three-dimensional structures of δ-HXTX-Ar1a and δ-HXTX-Hv1a peptides have been determined (14, 15) and possess core β regions stabilized by four disulfide bonds, placing them in the inhibitory cystine knot (ICK) structural family (16).

The aim of this study was to first determine the solution structure of Magi 4 and second to investigate the ability of Magi 4 to discriminate between different NaV channel subtypes. Here we report the tertiary structure of Magi 4 by 1H NMR and show its disulfide bonding pattern and three-dimensional structure are homologous to δ-HXTX-1 toxins. We highlight the key residues in Magi 4 that appear to be topologically similar to those residues known to be part of the pharmacophore for site-3 scorpion α-toxins, despite Magi 4 having a different overall structure to scorpion α-toxins (11). In addition, we provide a detailed characterization of the selectivity and mode of action of Magi 4 on nine cloned mammalian and insect NaV channel subtypes, including a detailed characterization on insect neurotransmission. Given that the toxin potently slows the inactivation of NaV channels, it should be renamed δ-hexatoxin-Mg1a (δ-HXTX-Mg1a) in accordance with the rational nomenclature recently proposed for naming spider peptide toxins (7) (see ArachnoServer spider toxin database).
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desalted and purified by preparative RP-HPLC using a linear gradient (17–37% CH₃CN in 0.1% trifluoroacetic acid for 80 min) to obtain 5.3 mg from the previous 23 mg (23% yield).

ESI/Q-TOF Mass Spectrometry—All masses obtained during chemical synthesis were confirmed by electrospray ionization mass spectrometry using an ESI/Q-TOF mass spectrometer (Micromass, Manchester, UK). Peptides were mixed with 1% (v/v) formic acid, 70% (v/v) CH₃CN, and data were analyzed using MassLynx version 3.4 software (Micromass).

Capillary Electrophoresis—Capillary zone electrophoresis analyses were performed on a Jasco CE800 system (Jasco, Japan) equipped with a UV detector connected to a Shimadzu CR-4A recorder and a capillary (0.1 µm inner diameter, 70 cm length, 50 cm to detector). 20 µl sodium citrate buffer, pH 2.5, was used for the analyses. Samples dissolved in migration buffer were applied hydrodynamically to the capillary (height 20 cm, 15 s), and analyses were performed with a 20-kV constant voltage drop and monitored at 210 nm.

CD Measurements—CD spectra were obtained on a Jasco J-725 spectropolarimeter (Jasco, Japan). The spectra were measured from 260 to 180 nm in 60% trifluoroethanol, pH 7.1, at room temperature, with a 1-mm path length cell. Data were collected at 0.1 nm with a scan rate of 100 nm/min and a time constant of 1 s. The concentration of the toxins was 30 µM, as determined by amino acid analysis. Data from 10 separate recordings were averaged and analyzed by the method of Böhm et al. (18).

Iodination of Lqha1IT and Binding Assays—The toxin was radiiodinated with bovine milk lactoperoxidase (EC 1.11.1.7, Sigma) using 0.7 lactoperoxidase units, 1 nmol of toxin, and 0.5 mCi carrier-free Na¹²⁵I (Amersham Biosciences) (19). The disulfide bonds of the toxins were denaturated and desalted by preparative RP-HPLC using a linear gradient (5% acetic acid, 90% CH₃CN, data were analyzed using MassLynx version 3.4 software (Micromass).

The structural calculations were performed using the X-PLOR-NIH 2.9.1 program with 576 NOE-based distance restraints, which contain 207 intrarresidue, 181 sequential, 60 medium range, and 128 long range restraints. In the first stage, the starting extended strand structure was subjected to 10 ps and 1,000 steps of torsion angle molecular dynamics at 50,000 K. The structures were then subjected to 15 ps and 1,500 steps of a slow cooling torsion angle molecular dynamics stage in which the temperature was reduced from 50,000 to 298 K over 250 steps. Finally, the structures were subjected to 200 steps of conjugated gradient minimization. The initial runs for structure calculations were performed without hydrogen bond and disulfide bond restraints, and the obtained structure was examined.

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Stage V–VI oocytes were harvested from the ovarian lobes of anesthetized female X. laevis frogs as described previously (22). The oocytes were injected with up to 50 nl of cRNA at a concentration of 1 ng nl\(^{-1}\) using a Drummond microinjector (Ambion). The ND96 solution used for incubating the oocytes containing the following (in mM): NaCl 96, KCl 2, CaCl\(_2\) 1.8, MgCl\(_2\) 2, HEPES-acid 5, pH 7.4, supplemented with 50 mg liter\(^{-1}\) gentamycin sulfate. Whole-cell currents from oocytes were recorded 2–5 days after injection.

**Electrophysiological Studies**—Voltage or current clamp recordings from single DRG and DUM neurons were made using the whole-cell patch clamp technique of Hamill et al. (23). Recordings from X. laevis oocytes were performed using the two-electrode voltage clamp method as described by Liman et al. (22).

Experiments were performed at constant temperature 18–24 °C using either an Axopatch 200A patch clamp amplifier or GeneClamp 500 amplifier. Current and voltage pulse protocols were generated using the pClamp software system (Molecular Devices, Sunnyvale, CA). Data were digitized at 10–25 kHz, and low pass filtered at either 1 kHz (oocytes) or 5 kHz (DRG and DUM neurons) using a 4- or 5-pole Bessel filter (−3 dB). Leakage and capacitative currents were digitally subtracted with P-P/4 procedures and series resistance compensation set at >80% for all patch-clamped cells to minimize voltage errors. The extracellular Na\(^+\) concentration was reduced (see below) to minimize series resistance errors.

The voltage clamp data recorded in this study were rejected if there were large leak currents upon seal formation or currents showing signs of inadequate space clamping. At the commencement of each experiment, oocytes or DUM neurons exhibiting leakage currents at a holding potential of −90 mV of more than −200 and/or −600 pA, respectively, were discarded. Only cells exhibiting stable leakage currents throughout the whole experiment (with a maximal deviation of ±10% of initial value) were considered in the data analysis. To avoid overestimation of a potential toxin-induced shift in the current-voltage relationship as a result of inadequate voltage control when measuring large sodium currents in oocytes, only results from cells with currents lower than 1.5 μA were considered. Current clamp data were rejected if the initial resting membrane potential was more depolarized than −45 mV.

**Recording Solutions**—DRG neurons were perfused with an external solution containing the following (in mM): sodium acetate 30, MgCl\(_2\) 1, CaCl\(_2\) 1.8, cesium acetate 5, KCl 5, d-glucose 25, HEPES-acid 5, TEA-Br 100, CdCl\(_2\) 0.5, 4-AP 1 with the pH adjusted to 7.4 with 1 M TEA-OH. Recordings were performed using micropipettes filled with a solution containing the following (in mM): CsF 135, sodium acetate 8, HEPES-acid 5, with the pH adjusted to 7.0 with CsOH. The osmolarity of internal and external solutions was adjusted to 300–305 mosM liter\(^{-1}\) with sucrose, prior to use, to reduce osmotic stress. Pipettes with resistances of 0.8–2 megohms were used for recording \(I_{\text{Na}}\) from DRG neurons.

For voltage clamp experiments, DUM neurons were perfused with an external solution consisting of the following (in mM): NaCl 90, CsCl 5, CaCl\(_2\) 1.8, 4-AP 5, TEA-Cl 50, verapamil 0.01, NiCl\(_2\) 0.1, CdCl\(_2\) 1, HEPES-acid 10, pH adjusted to 7.4 with HCl. Micropipettes were filled with an internal solution consisting of the following (in mM): CsF 135, MgCl\(_2\) 1, NaCl 34, ATP-Na\(_2\) 3, EGTA 5, HEPES-acid 10, pH adjusted to 7.35 with CsOH. For current clamp experiments, DUM neurons were perfused with an external solution consisting of the following (in mM): NaCl 190, KCl 3.1, CaCl\(_2\) 5, MgCl\(_2\) 4, HEPES-acid 10, with the pH adjusted to 7.4 with NaOH. Micropipettes were filled with an internal solution consisting of the following (in mM): potassium gluconate 160, KF 10, ATP-Mg 1, CaCl\(_2\) 0.5, NaCl 15, MgCl\(_2\) 1, EGTA 10, HEPES-acid 10, with the pH adjusted to 7.4 with KOH. The osmolarity of all solutions was adjusted to 400 mosM with sucrose. Pipettes with resistances of 1.2–2 megohms were used for recording currents from DUM neurons.

Oocytes were perfused with ND96 solution. Voltage and current electrodes were filled with 3 m KCl and resistances were <1 megohms.

**Voltage and Current Clamp Recordings**—In DRG neurons, the predominant TTX sensitivity of the Na\(_{\text{v}}\) channels present in each cell was determined using a modified steady-state Na\(_{\text{v}}\) channel inactivation (\(h_{\text{inact}}\)) voltage clamp protocol (24, 25). This takes advantage of the separation of \(h_{\text{inact}}\) curves for TTX-sensitive and TTX-resistant Na\(_{\text{v}}\) channels (26). Only those found to have less than 10% TTX-resistant \(I_{\text{Na}}\) were used for TTX-sensitive experiments. Of the remaining cells, those expressing sufficiently large TTX-resistant \(I_{\text{Na}}\) were used for experiments by perfusing with external solution containing 200 nM TTX.

To determine the effect of the toxin on fast inactivation, currents were recorded in the absence and presence of a range of toxin concentrations. The action of the toxin on fast inactivation was assayed by measuring the late current remaining at 50 ms (\(I_{\text{50ms}}\)) for DRG and DUM neurons or 30 ms for oocytes, as a fraction of peak current (\(I_{\text{pk}}\)). Currents were elicited by test potentials to −10 mV in DUM and DRG neurons or at depolarizing test potentials corresponding to maximal activation in oocytes (ranging from −10 to +10 mV). Test potentials were applied at 10-s (DRG and DUM) or 5-s (oocytes) intervals. The normalized late current ratio (e.g. \(I_{\text{50ms}}/I_{\text{pk}}\)) gives the fractional probability for Na\(_{\text{v}}\) channels not to be inactivated at the end of the test pulse. The concentration dependence for removal of inactivation was measured by plotting the normalized late current as a function of toxin concentration according to the following Hill Equation 1,

\[
\frac{I_{\text{50ms}}}{I_{\text{pk}}} = \frac{100}{1 + \left(\frac{[\text{toxin}]}{E_{\text{C50}}}\right)^n},
\]

where \(E_{\text{C50}}\) is the concentration at half-maximal inhibition of fast inactivation, [toxin] is the toxin concentration, and \(n_H\) is the Hill coefficient.

The effect of 6-HHTX-MgI\(_2\) on the voltage dependence of Na\(_{\text{v}}\) channel activation was determined using depolarizing test pulses from −90 to +70 mV for 50 ms, in 5-mV (oocytes) or 10-mV steps (DRG and DUM). The values for sodium conductance (\(g_{\text{Na}}\)) were calculated according to Equation 2,

\[
g_{\text{Na}} = \frac{I_{\text{Na}}}{(V - V_{\text{rev}})}
\]
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where $I_{Na}$ is the absolute value of the sodium current at a given test potential ($V$), and $V_{rev}$ is the reversal potential. The values of $g_{Na}$ and $V - V_{rev}$ were then fitted to Boltzmann Equation 3,

$$\frac{g_{Na}}{g_{max}} = \frac{1}{1 + \exp(V/v/V_0/v/k_m)}$$  \hspace{1cm} (Eq. 3)

where $g_{max}$ is maximal $g_{Na}$; $V_0$ is the half-maximal conductance, and $k_m$ is the slope factor.

To determine the effect of δ-HXTX-Mg1a on the voltage dependence of steady-state Na, channel fast inactivation ($h_v$), a two-pulse protocol with a 0.5-ms interpulse interval was applied. This consisted of a 500-ms or 1-s conditioning prepulse ($V_{cond}$), in which the holding potential of −90 mV was stepped to potentials ranging from −130 up to +20 mV in 5-mV (oocytes) or 10-mV increments (DRG and DUM), followed by a 50-ms test pulse ($V_{test}$) to −10 mV in DRG or DUM neurons, or potentials corresponding to maximal activation in oocytes. Pulses were applied every 10 s. Data were normalized to the maximum peak control $I_{Na}$ and fitted using Boltzmann Equation 4,

$$h_v = \frac{1 - C}{1 + \exp(V - V_{1/2}/v/k_s)} + C$$  \hspace{1cm} (Eq. 4)

where $V_{1/2}$ is the voltage at half-maximal inactivation; $k_s$ is the slope factor; $V$ is the test voltage, and $C$ is a constant or non-inactivating fraction (usually zero in controls).

The effect of the toxin on the rate of recovery from Na, channel inactivation was examined by applying a standard two-pulse protocol with a variable interpulse interval ($\Delta T$). A 50-ms conditioning prepulse ($V_{cond}$) was applied from a holding potential of −90 to −10 mV, followed by a 50-ms test pulse ($V_{test}$), with an interpulse interval ranging between 0.5 ms and 4 s.

Mathematical curve fitting was accomplished using GraphPad Prism version 5.00 for Macintosh (GraphPad Software, San Diego). All curve-fitting routines were performed using nonlinear regression analysis employing a least squares method. Comparisons of two sample means were made using a paired Student’s t test. Multiple comparisons were assessed by repeated measures analysis of variance with a Bonferroni’s multiple comparison post hoc test. A test was considered to be significant with $p < 0.05$. All data are presented as means ± S.E. of n independent experiments.

RESULTS

Peptide Synthesis and Characterization—δ-HXTX-Mg1a was synthesized with its C terminus as the free carboxyl, as observed for the native toxin by mass spectrometry and from the cDNA-encoding gene (10). The structural identity between the synthetic and native toxins was verified by ESI/Q-TOF MS and capillary zone electrophoresis (supplemental Fig. S1 and supplemental Table S1). In a co-injection experiment, native δ-HXTX-Mg1a and synthetic δ-HXTX-Mg1a co-eluted in a single sharp peak (supplemental Fig. S1A). Moreover, the CD spectra of synthetic and native δ-HXTX-Mg1a superimposed indicating that their secondary structures were similar. Both native and synthetic δ-HXTX-Mg1a were able to displace the binding of 125I-LqhαIT from insect Na, channels in cockroach synaptosomes with similar IC_{50} values (supplemental Fig. S1, B and C).

NMR Spectroscopy—Sequence-specific 1H resonance assignments for all residues were established using standard methods (27). NOE cross-peaks were converted to distance restraints for structural calculation. From NMR experiments, 582 distance constraints and 22 dihedral angle constraints were used for the structural calculations. The total of 582 distance restraints included 576 NOE constraints and 6 hydrogen bonds (Fig. 1B). In addition, disulfide bond constraints were also used (Cys1–Cys15, Cys8–Cys20, Cys14–Cys31, and Cys16–Cys43). Simulated annealing calculations were started from an extended structure, and 20 structures were selected with the lowest XPLOR energy, which had no $\geq 0.4$ Å NOE distance violations and no $\geq 5.0°$ angle violations from 100 structures obtained at the final calculation (28). Structural statistics for these 20 converged structures are summarized in Table 1. Fig. 2A shows a stereoview of the best fit superposition of the backbone atoms (N, C, Cα, and O) for 20 converged structures. The root-mean-square deviations with respect to the mean coordinate positions were 0.483 Å for backbone atoms and 1.178 Å for all heavy atoms of the disulfide-rich structured region (Gly2–Ala23 and Glu29–Glu22), excluding the nonstructured loop (Trp24–Gln27) and C-terminal regions (Arg33–Cys46). Analysis of the 20 structures using PROCHECK NMR and AQUA reveals that 95.1% of the backbone dihedral angles for the structured region lie in the most favored and additional allowed regions of the Ramachandran plot (29).

Six protons from amide groups were identified to form hydrogen bonds according to the amide proton temperature coefficients (supplemental Fig. S2). If the hydrogen bond was in agreement with amide temperature coefficients (more than −4.6 ppb/K), and a hydrogen bond acceptor and an oxygen atom of the backbone was within 2.6 Å of the amide–proton, the amide–proton was identified as a donor of the hydrogen bond. Hydrogen bond acceptors for all the protons were unambiguously determined from preliminary structure calculations. These restraints were then used in the following stage of structure calculations (1.8 $\leq d \leq 2.12$ Å for NH-O and 2.7 $\leq d \leq 3.2$ Å for N-O) (30).

Twenty two dihedral angles estimated from the $\delta_{Na-Ha}$ values were used as $\phi$ angle constraints within the range of −90° and −40° for $\delta_{Na-Ha} < 5.5$ Hz (Ala6, Trp7, Cys15, Trp24, Cys31, Arg23, Lys36, and Phe39) and between −160° and −80° for $\delta_{Na-Ha} > 8$ Hz (Ser3, Cys8, Lys9, Cys14, Tyr18, Asn19, Cys20, Ala23, Asn29, Glu28, Ser29, Glu32, Trp35, and Glu36) following standard parameterization (31) (Fig. 1B).

From the results of initial runs without using the disulfide bridge restraints, the disulfide patterns of Cys3–Cys15 and Cys16–Cys46 were unambiguously determined. Analyzing the distances between $\delta_T$ and $\delta_Y$ of the remaining four cysteines, three types of disulfide patterns were possible (pattern I, Cys8–Cys14 and Cys20–Cys31; pattern II, Cys8–Cys14 and Cys14–Cys20; and pattern III, Cys8–Cys20 and Cys14–Cys31). Three sets of structure calculations were performed with these possible restraints. The energy minimization was done in vacuo with the
Primary and secondary structure of δ-HXTX-Mg1a. A, comparison of the primary sequence of δ-HXTX-Mg1a and δ-HXTX-Mg1b (formerly Magi 14) with currently known members of the δ-HXTX-1 family and δ-AOTX-Mb1a (β-actinopodotoxin-Mb1a, formerly δ-missilenatoxin-Mb1a). Homologies are shown relative to δ-HXTX-Mg1a; identities are boxed in gray, and conservative substitutions are in gray italic text. Gaps (dashes) have been inserted to maximize alignment. The disulfide bonding pattern for the strictly conserved cysteine residues determined for δ-HXTX-Mg1a (this study), δ-HXTX-Ar1α (55), and δ-HXTX-Hv1a (15) is indicated above the sequences; it is assumed that δ-HXTX-Mb1a (36), δ-HXTX-Hs20.1a (8), and δ-HXTX-Hv1b (56) have the same disulfide bonding pattern. The percentage identity and homology with δ-HXTX-Mg1a is shown to the right of the sequences. B, summary of δ-HXTX-Mg1a NMR data. Sequential NOEs, classified as very weak, weak, medium, and strong, are represented by the thickness of bars. Filled diamonds indicate backbone amide protons that form hydrogen bonds. J_{HHHC} coupling constants are indicated by \( \uparrow \) (>8 Hz) and \( \downarrow \) (<5.5 Hz). Secondary structure is shown at the bottom of the figure where rectangles represent β-turns (the type of turn is indicated in the rectangle) and arrows represent β-sheets.

**TABLE 1**

| Structural statistics for the 20 lowest energy structures |
|----------------------------------------------------------|
| **Distance restraints**                                    |
| Intraresidue \((l - j) = 0\)                                |
| Residues in most favored regions                          |
| Residues in additional allowed regions                    |
| Residues in generously allowed regions                    |
| Residues in disallowed regions                            |
| Total                                                     |
| 207                                                       |
| 181                                                       |
| 60                                                        |
| 128                                                       |
| 576                                                       |
| **Ramachandran analysis (residues 2–23, 28–32)**          |
| Residues in most favored regions                          |
| Residues in additional allowed regions                    |
| Residues in generously allowed regions                    |
| Residues in disallowed regions                            |
| 70%                                                       |
| 25.1%                                                     |
| 4.2%                                                      |
| 0.7%                                                      |
| **Atomic r.m.s.d. differences between 20 conformers (Å)**   |
| Backbone atoms (residues 2–23, 28–32)                     |
| All heavy atoms (residues 2–23, 28–32)                     |
| 0.483 ± 0.005                                             |
| 1.178 ± 0.005                                             |
| **Deviations from idealized covalent geometry**            |
| Bonds                                                     |
| Improper                                                  |
| 0.0041 ± 0.00002Å                                         |
| 0.504 ± 0.002°                                            |
| 0.365 ± 0.002°                                            |
| **Mean Xplor energies (kcal/mol)**                        |
| Total                                                     |
| -50.30 ± 2.960                                            |

*PROCHECK-NMR was used to calculate these values.  
* r.m.s.d. means root-mean-square.  
* None of these structures exhibited distance violations \( \pm 0.4 \) Å and angle violations \( \pm 5.0° \). Root-mean-square differences are given as the means ± S.D.

GROMOS96 43B1 parameters set, without reaction field (32), to determine the lowest energy state and thus the most stable disulfide pattern of δ-HXTX-Mg1a. Energy computations were done with the GROMOS96 (32) implementation of Swiss-PdbViewer (33). Total energy calculations for patterns I, II, and III were \(-693.5, -892.7, \) and \(-1523.4 \) kcal/mol, respectively, therefore unambiguously determining the remaining disulfide bridge patterns as Cys\(^8\)–Cys\(^{20}\) and Cys\(^{14}\)–Cys\(^{31}\). These disulfide bridges, along with Cys\(^1\)–Cys\(^{15}\), form an ICK motif (Fig. 2D) and a pattern (I–IV, II–VI, III–VII, and V–VIII) identical to other δ-HXTX-1 family members (14, 15) (Fig. 1A).

**Description of the Three-dimensional Structure**—Fig. 2A shows the best fit superposition of the backbone atoms (N, C\(^\alpha\), and C) for the 20 converged structures of δ-HXTX-Mg1a. The three-dimensional structure of δ-HXTX-Mg1a includes several well-defined regions, consisting of an antiparallel β-sheet (strand 1, Asn\(^{273}\)–Ala\(^{327}\); strand 2, Glu\(^{28}\)–Glu\(^{33}\)) with some secondary structural elements (Fig. 1B and Fig. 2B). We identified turns by the standard definition that the distance between C\(_{\alpha}\) and C\(_{\alpha}+3\) is less than 7 Å (34, 35). The two turns involve Ser\(^{3}\) to Ala\(^{4}\) (turn 1) and Cys\(^{15}\) to Tyr\(^{18}\) (turn 2). The average dihedral angles for residues at positions \( i + 1 \) and \( i + 2 \) are follows: \( \phi_2 = -40\), \( \psi_2 = 122° \) for Lys\(^{4}\); \( \phi_3 = 57° \), \( \psi_3 = 84° \) for Arg\(^{5}\); \( \phi_5 = -45° \), \( \psi_5 = 128° \) for Cys\(^{16}\); and \( \phi_7 = 98° \), \( \psi_7 = -2° \) for Gly\(^{17}\). Turn 1 is classified as a miscellaneous type IV β-turn. There is no hydrogen bonding in this turn conformation. Arg\(^3\) in turn 1 shows a positive \( \varphi_3 \) value, because nonglycine residues at position \( i + 2 \) are rare in type II β-turns, and furthermore arginine shows low turn potential. Turn 2 is assigned as a typical type II β-turn (β\(_{al}\)). In this turn, the Cys\(^{15}\) oxygen atom forms hydrogen bonding with the Tyr\(^{18}\) amide-proton (Fig. 1B). The region Trp\(^{24}\) to Gln\(^{27}\) is not well defined by the NMR data.

Electrophysiological Studies on Mammalian Na\(_\text{v}\) Channels—The effects of δ-HXTX-Mg1a were initially examined on neonatal rat DRG neurons as these were employed in the original characterization of δ-HXTX-1 and δ-AOTX-Mb1a toxins (36–38). Rat DRG neurons express two types of Na\(_\text{v}\) channel currents, TTX-sensitive (mainly Na\(_\text{v}1.1\), Na\(_\text{v}1.6\), and Na\(_\text{v}1.7\)) and TTX-resistant (Na\(_\text{v}1.8\) and Na\(_\text{v}1.9\)) (26, 39). Like δ-HXTX-Mg1a slowed inactivation of TTX-sensitive Na\(_\text{v}1.8\) and Na\(_\text{v}1.9\) (26, 39). Like δ-HXTX-Mg1a slowed inactivation of TTX-sensitive Na\(_\text{v}1.8\) and Na\(_\text{v}1.9\) (26, 39).
potentials to \(-10\) mV, in which \(I_{Na}\) values are fully inactivated in the absence of toxin. This is most likely the result of a marked slowing of transitions between open and open-inactivated states. The maximum degree of slowing of inactivation after 50 ms was \(26.3 \pm 4.4\%\) (\(n = 7\)) of control peak \(I_{Na}\) at 300 nM, with an \(EC_{50}\) of 46 nM (Fig. 3E). This steady-state current was completely blocked by 100 nM TTX indicating that it is mediated exclusively via NaV channels (data not shown). In parallel, the peak \(I_{Na}\) amplitude recorded at \(-10\) mV transiently increased, but after 5 min perfusion was slightly reduced by \(6.6 \pm 11.0\%\) (\(n = 7\)).

Similar to \(\delta\)-HXTX-1 toxins, however, the effect to slow \(Na_v\) channel inactivation in TTX-sensitive DRG neurons was almost completely reversible after washing with toxin-free solution with a \(\tau_{on}\) of 84.9 \pm 18.4 s and \(\tau_{off}\) of 81.5 \pm 9.2 s (\(n = 5\)).

Given the potent action of \(\delta\)-HXTX-Mg1a on DRG neurons, we then assayed the effect of the toxin on eight mammalian \(Na_v\) channel clones (\(Na_v1.1/\beta_1\) to \(Na_v1.8/\beta_1\)) by analyzing \(g_{Na}/V\) relationships and steady-state inactivation. Effects of the toxin on \(Na_v1.9\) channels were not investigated as this channel subtype currently fails to express in standard heterologous systems. Addition of \(\delta\)-HXTX-Mg1a (up to 5 \(\mu M\)) to the bath medium produced a marked slowing of \(I_{Na}\) inactivation with a rank order in magnitude of \(Na_v1.6/\beta_1 > Na_v1.1/\beta_1 > Na_v1.3/\beta_1\). However, with \(Na_v1.2/\beta_1\) and \(Na_v1.7/\beta_1\), there were only weak effects to slow \(I_{Na}\) inactivation (Fig. 4A, left-hand panels, and supplemental Table 2S). These effects were accompanied...
by ∼20% increase in peak $I_{\text{Na}}$ amplitude with NaV1.6/β1 and NaV1.1/β1 channels but less significant changes with the remaining channel clones (supplemental Table 2S).

The above effects were accompanied by only weak hyperpolarizing shifts in the voltage dependence of activation ($g_{\text{Na}}$) to a maximum $\Delta V_{1/2}$ of −8.9 mV for NaV1.3/β3 channels (Fig. 4A, right-hand panels). In contrast, the toxin failed to slow $I_{\text{Na}}$ inactivation of NaV1.4/β1, NaV1.5/β1, or NaV1.8/β1 channels at concentrations up to 5 μM (Fig. 4B). This lack of activity on NaV1.8/β1 channels is consistent with the lack of effects on TTX-resistant $I_{\text{Na}}$ in DRG neurons that express both NaV1.8 and NaV1.9.

In the mammalian NaV channel clones, steady-state inactivation ($h_{\text{inact}}$) in the absence of toxin was best described by a single Boltzmann function. δ-HXTX-Mg1a (5 μM) caused a strong −19.7 mV hyperpolarizing shift in the voltage at half-maximal inactivation ($V_{1/2}$) of NaV1.3/β3 but only weak nonsignificant shifts in $V_{1/2}$ in NaV1.1/β1, NaV1.2/β1, NaV1.6/β1, and NaV1.7/β1 channels of less than −4 mV (Fig. 4A, right-hand panels). Moreover, in the presence of δ-HXTX-Mg1a, steady-state inactivation became incomplete, causing the appearance of a noninactivating component at prepulse test potentials more depolarized than −40 mV, an effect previously noted with δ-HXTX-1 toxins (37, 38). This noninactivating component was up to 27 ± 10% ($n = 5$) of peak current in the case of the NaV1.1/β1 channel (determined from C in Equation 4) with a rank order in magnitude of NaV1.6/β1 > NaV1.1/β1 > NaV1.3/β3 > NaV1.2/β1 > NaV1.7/β1 (Fig. 4A, right-hand panels). This was sufficient to induce significant changes in the slope factor, $k_{\text{inact}}$ in all NaV channel clones except NaV1.2/β1. Although time-dependent shifts in $h_{\text{inact}}$ have been demonstrated in patch clamp configurations (41), these are unlikely to account for the observed changes given the magnitude of the shift in NaV1.3/β3 and the absence of a noninactivating component in controls.

Electrophysiological Studies on Insect NaV Channels—In contrast to the clear effects, but modest potency, of the toxin on mammalian NaV channel gating and kinetics, δ-HXTX-Mg1a showed high affinity for the Drosophila NaV channel clone DmA1/TipE. This was evident first by a concentration-dependent increase in control peak $I_{\text{Na}}$ amplitude up to a maximum of 290 ± 37% at 1 μM ($n = 5$; Fig. 5C). This was most likely the result of the marked concentration-dependent slowing of fast inactivation (Fig. 5, A and C) with an EC50 of 22.8 nM (Fig. 5H). Importantly δ-HXTX-Mg1a completely removed fast inactivation at concentrations of 1 μM (Fig. 5C). This was accompanied by a 21.8-mV hyperpolarizing shift in DmA1/TipE channel activation at 1 μM ($n = 3$; Fig. 5D). At a concentration of 15 nM, δ-HXTX-Mg1a also caused steady-state DmA1/TipE channel inactivation to become incomplete at prepulse test potentials more depolar-
Spider Toxin Structure That Targets Sodium Channel Subtypes

A

**Na\textsubscript{V} 1.1**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]

**Na\textsubscript{V} 1.2**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]

**Na\textsubscript{V} 1.3**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]

**Na\textsubscript{V} 1.6**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]

**Na\textsubscript{V} 1.7**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]

B

**Na\textsubscript{V} 1.4**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

**Na\textsubscript{V} 1.5**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

**Na\textsubscript{V} 1.8**

Control

5 μM \(\delta\)-HHTX-Mg1a

\[ \text{V}_m (\text{mV}) \]

\[ h_i (\bullet) \]

\[ \text{Norm. } g_{Na} (\square) \]
Spider Toxin Structure That Targets Sodium Channel Subtypes

FIGURE 4. Differential effects of δ-HTX1-Mg1a on mammalian Nav channels expressed in Xenopus oocytes. A, left-hand panels show superimposed current traces illustrating typical effects on mammalian Na1.1/Na1.3/β, and Na1.6/1.7/β, following a 5-min perfusion with 5 μM δ-HTX1-Mg1a. Currents were elicited every 5 s by 100-ms test pulses from −90 mV to the voltage of maximum activation of the Nav channel subtype under control conditions. Corresponding right-hand panels show normalized gNa/V (squares) and hNa/V (circles) relationships in the presence (open symbols) and absence (closed symbols) of 5 μM δ-HTX1-Mg1a (n = 3–6). The gNa/V curves were fitted using Equation 1 under “Experimental Procedures” although hNa/V curves were fitted according to Equation 4. B, superimposed current traces showing typical lack of effect on mammalian Na1.4/β, Na1.5/β, or Na1.8/β, channel currents following a 5-min perfusion with 5 μM δ-HTX-JβMg1a.

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To further probe the mode of action and insect selectivity of δ-HTX1-Mg1a, we assayed the toxin on cockroach DUM neurons, previously employed in the characterization of δ-HTX1-Hv1a (42). Like δ-HTX1-Hv1a, δ-HTX1-Mg1a produced a potent time- and concentration-dependent slowing of INa inactivation (Fig. 5, E and H). Maximum slowing after 50 ms was 46.1 ± 4.5% (n = 10) of control peak INa at 300 nm δ-HTX1-Mg1a (Fig. 5E). The EC50 for the slowing of inactivation in DUM neurons was 823 pM, ~56-fold lower than in DRG neurons (Fig. 5H). Hence, although this toxin is not insect-specific, it shows a marked selectivity for insect Nav channels. The toxin also caused 14.3 and 13.5 mV hyperpolarizing shifts in the Vg of Nav channel activation and steady-state inactivation, respectively (Fig. 5F).

In DUM neurons, the slowing of inactivation described above was accompanied initially by a slight increase in the peak INa. This effect would reach a maximum after ~70 s followed immediately by a decrease in peak and late INa amplitude (Fig. 5E). If depolarizing test pulses were abolished for 2 min after reaching steady-state toxin effects (e.g. after 4–5 min perfusion), peak and late INa amplitude increased markedly (Fig. 5G, trace marked 120 s). If depolarizing test pulses (Δt = 10 s) were then reapplied, peak and late INa amplitude would decline back to steady-state levels within ~1 min (Fig. 5G, trace marked 180 s). This effect may result from a slowed rate of recovery from fast inactivation or a voltage-dependent dissociation of the toxin from the channel. To test these possibilities, we first assessed the effect of δ-HTX1-Mg1a (30 nm) on the rate of recovery from inactivation. Although the toxin markedly increased repriming kinetics at interpulse intervals less than 3 ms, presumably reflecting a more rapid transition from open to closed states, the major effect of the toxin was to prolong the rate of recovery from fast inactivation at interpulse intervals greater than 3 ms, reflecting a slowed transition between closed-inactivated and closed states (Fig. 6A). This action resulted in a use-dependent decrease in peak INa amplitude during repetitive stimulation at 30 Hz (Fig. 6B).

To determine whether the effect of the toxin was dependent on the holding potential, the amplitude of the toxin-modified INa measured at the end of the test pulse was compared with peak INa at hyperpolarized holding potentials. In comparison with currents recorded at −90 mV, the fraction of the sustained INa measured at the end of the 50-ms depolarizing test pulse (I50ms), compared with the peak INa amplitude (Ip), was not significantly increased except at −150 mV (p < 0.05, n = 7; Fig. 6C). In addition, to test if depolarizing pulses could cause dissociation of the toxin, post-pulses up to +200 mV were applied immediately following a test pulse to −10 mV from −90 mV every 2 s. At this stimulation frequency there was some initial rundown in peak and late INa amplitude, but the depolarizing post-pulses failed to cause any significant decrease in fractional I50ms/Ipk compared with data recorded in the absence of the post-pulse (p > 0.05, n = 5; Fig. 6D).

To investigate the effects of the toxin on membrane excitability, DUM neurons were held under current clamp conditions, and action potentials were evoked by depolarizing current pulses. δ-HTX1-Mg1a (100 nm) initially produced a prolongation of the repolarizing phase of the action potential (Fig. 7A). The falling phase of the action potential developed a broad shoulder in the last two-thirds of the repolarization phase (Fig. 7A). This caused a suppression of the after hyperpolarization and an increase in spike duration resulting in “plateau” action potentials (Fig. 7, A–C). Although a small depolarization of 3.1 ± 0.6 mV (n = 5) was observed, neither resting membrane potential (RMP) nor spike amplitude was significantly altered. Applying artificial hyperpolarization to a level 20–40 mV more negative than the RMP facilitated the appearance of these plateau potentials. In addition, the duration of plateau potentials were heavily influenced by the stimulation frequency such that the duration increased from ~70 ms at a RMP of ~60 and stimulus interval of 10 s (Fig. 7A) to ~300 ms at stimulus intervals of 1 min at an RMP held at ~90 mV (Fig. 7C). Interestingly, 100 nm δ-HTX1-Mg1a co-applied with 500 μM 3–4 diaminopyridine (3,4-DAP), a Kv channel blocker, markedly prolonged the action potential duration to ~150 ms at ~60 mV (Fig. 7D) and ~800 ms at ~90 mV (Fig. 7E). In this case, the action potential duration was more than 80-fold longer than with 3,4-DAP applied alone.

As described previously, DUM neurons are spontaneously active (43). At resting membrane potentials, most DUM neurons were capable of generating repetitive action potentials with firing frequencies of 31 ± 4.6 Hz (n = 4; Fig. 7F). In the presence of 100 nm δ-HTX1-Mg1a, repetitive plateau action potentials of ~70 ms duration would also occur spontaneously, albeit with a much reduced firing frequency of 3.2 ± 0.6 Hz (n = 4; Fig. 7G). However, when the membrane potential was hyperpolarized to ~80 mV, spontaneous plateau action potential duration increased up to ~500 ms, but firing frequency was still low at 0.7 ± 0.2 Hz (n = 4; Fig. 7H). Using a ramp current from ~80 mV at 0.2–0.4 nA/s, there was also a ~26 ± 2.0 mV shift in the threshold of spontaneous firing from ~44.6 ± 4.0 mV in controls to ~70.6 ± 3.5 mV (n = 7) in the presence of 100 nm δ-HTX1-Mg1a.

DISCUSSION

Actions on Nav Channel Gating—In all cases, δ-HTX1-Mg1a modulated channel gating and kinetics in a similar fashion to other δ-HTX1 toxins by causing the following: (i) a slowing/removal of Nav channel inactivation typically associ-
ated with an increase in peak $I_{Na}$ channel currents; (ii) a hyperpolarizing shift in the voltage dependence of channel activation; and (iii) a hyperpolarizing shift in the voltage dependence of steady-state inactivation. In insect neurons, further characterization revealed that the toxin (iv) slowed $Na_{\text{V}}$ channel repriming kinetics and (v) caused a use-dependent reduction in $I_{Na}$ amplitude.

The toxin-induced increase in peak $I_{Na}$ seen with DmNaV1 and to a lesser extent in DUM neurons, rNaV1.1, rNaV1.3, and NaV1.6, was in general well correlated with the magnitude of the sustained current at the end of the depolarizing test pulse. This increase in peak $I_{Na}$ can be explained by a delay in $Na_{\text{V}}$ channel fast inactivation from open to open-inactivated states. In contrast, $\delta$-HXTX-Mg1a did not slow $I_{Na}$ inactivation as markedly in NaV1.2 or NaV1.7 and therefore did not appreciably increase peak $I_{Na}$ amplitude. This slowing of the transition from open to open-inactivated states also generated a noninactivating component in the steady-state inactivation curve. This occurred at prepulse test potentials more depolarized than $-40 \text{ mV}$, an effect previously noted with $\delta$-HXTX-1 toxins (37, 38). This is the result of a more rapid rate of recovery from fast inactivation when test pulses are separated by less than 3 ms. This reflects a higher proportion of transitions between open and closed states than open-inactivated to closed states via a closed-inactivated intermediate.

This study demonstrates that $\delta$-HXTX-Mg1a displays differential NaV channel subtype specificity for NaV1.1/$\beta_1$, NaV1.3/$\beta_1$, and NaV1.6/$\beta_1$ channels. This is consistent with its action on TTX-sensitive $I_{Na}$ in rat DRG neurons that express mainly NaV1.1, NaV1.3, and NaV1.7 channels (39). Importantly, however, $\delta$-HXTX-Mg1a showed considerably higher affinity for invertebrate Drosohila DmNaV1 and cockroach NaV channels. Differences in the $EC_{50}$ of $\delta$-HXTX-Mg1a for dipterans (DmNaV1) versus orthopterans (DUM neurons) have been previously noted for Lqh3 and $\delta$-HXTX-Hv1a with cockroach versus locust NaV channels (11, 44).

In cockroach DUM neurons, the slowing of NaV channel inactivation by $\delta$-HXTX-Mg1a induced the development of pro-
longed plateau action potentials. This was accompanied by spontaneous repetitive firing as a result of a hyperpolarizing shift in the threshold of action potential generation because of a negative shift in the voltage dependence of \( \text{Na} \) channel activation. The reduction in firing frequency observed in tonically active neurons probably reflects the reduced rate of recovery from fast inactivation at interpulse intervals greater than 3 ms, presumably resulting from a slowing of the frequency of transition between the closed-inactivated and closed states. This is supported by the use-dependent decrease in \( I_{\text{Na}} \) amplitude during repetitive pulses. Interestingly, in DUM neurons, \( \delta \)-HXTX-Mg1a produced greater inhibition of \( I_{\text{Na}} \) binding to site-3 in rat brain synaptosomes, \( \delta \)-HXTX-Mg1a showed only weak effects on \( \text{Na}_\text{v}_1.2/\beta_1 \) at 5 \( \mu \)M. In addition, the toxin had even weaker activity on \( \text{Na}_\text{v}_1.7/\beta_1 \) and no activity on \( \text{Na}_\text{v}_1.4/\beta_1, \text{Na}_\text{v}_1.5/\beta_1, \) and \( \text{Na}_\text{v}_1.8/\beta_1 \) channels (Fig. 4A).

Although there is currently no reliable expression system for \( \text{Na}_\text{v}_1.9 \) channels, the lack of effect of \( \delta \)-HXTX-Mg1a on \( I_{\text{Na}} \) in DRG neurons would indicate that the toxin partially dissociates from the channel at more depolarized potentials. Voltage-dependent dissociation of bound toxins from vertebrate \( \text{Na}_\text{v}_1.9 \) channels has already been demonstrated with scorpion and sea anemone toxins (9, 11, 45, 46) and has also been observed with \( \delta \)-HXTX-Hvl1 on insect DUM neurons (42). However, there was no indication of any depolarization-dependent dissociation of toxin from DUM neurons using hyperpolarized holding potentials or depolarizing post-pulse protocols.

Comparison with Orthologous \( \delta \)-HXTX-1 Toxins—These actions on TTX-sensitive \( \text{Na}_\text{v} \) channel subtypes in rat DRG and insect DUM neurons resemble the effects of other spider \( \delta \)-HXTX-1 toxins, with which \( \delta \)-HXTX-Mg1a shares some sequence homology (37, 38, 42). In contrast to \( \delta \)-HXTX-1 toxins, however, \( \delta \)-HXTX-Mg1a is more efficacious on insect rather than mammalian \( \text{Na}_\text{v} \) channel subtypes, including markedly different actions on repriming kinetics and use-dependent activity (this study and see Ref. 10). \( \delta \)-HXTX-Mg1a therefore shows greater similarity in its phylum-selective actions with scorpion \( \alpha \)-like toxins. Like \( \delta \)-HXTX-Mg1a, \( \alpha \)-like toxins are toxic by direct injection into rat brain but fail to compete for site-3 on rat brain synaptosomes (9). Rat brain synaptosomal membranes are rich in mainly \( \text{Na}_\text{v}_1.2/1.2a \) (78%) and \( \text{Na}_\text{v}_1.1 \) (15%) (47). Consistent with a lack on inhibition of \( ^{125} \text{I}-\text{Lqh}2 \) binding to site-3 in rat brain synaptosomes, \( \delta \)-HXTX-Mg1a showed only weak effects on \( \text{Na}_\text{v}_1.1/\beta_1, \text{Na}_\text{v}_1.5/\beta_1, \) and \( \text{Na}_\text{v}_1.8/\beta_1 \) channels (Fig. 4A).

Although there is currently no reliable expression system for \( \text{Na}_\text{v}_1.9 \) channels, the lack of effect of \( \delta \)-HXTX-Mg1a on TTX-resistant \( I_{\text{Na}} \) in DRG neurons would indicate that \( \delta \)-HXTX-Mg1a does not have any appreciable affinity for this channel subtype.

Scorpion \( \alpha \)-like toxins, similar to \( \delta \)-HXTX-Mg1a, specifically target \( \text{Na}_\text{v}_1.1, \text{Na}_\text{v}_1.3, \) and \( \text{Na}_\text{v}_1.6 \) channels (48). Indeed

**FIGURE 6.** Typical effects of \( \delta \)-HXTX-Mg1a on DUM neuron \( \text{Na}_\text{v} \) channel gating. A, effects of 30 nM \( \delta \)-HXTX-Mg1a on \( \text{Na}_\text{v} \) channel repriming kinetics in cockroach DUM neurons. \( \text{Na}_\text{v} \) channel repriming rate was determined by normalizing peak \( I_{\text{Na}} \) elicited during a 50-ms test pulse from \(-90 \text{ mV} \) to \(-10 \text{ mV} \) against peak \( I_{\text{Na}} \) recorded during a 50-ms conditioning pulse from \(-90 \text{ mV} \) to \(-10 \text{ mV} \) and plotted as a function of the interpulse interval. With interpulse intervals greater than 2 ms, 30 nM \( \delta \)-HXTX-Mg1a (open circles, \( n = 6 \)) slowed the rate of recovery from inactivation in comparison with controls (closed circles, \( n = 6 \)). B, use-dependent actions of \( \delta \)-HXTX-Mg1a on \( \text{Na}_\text{v} \) channels in cockroach DUM neurons. Effects of 30 nM \( \delta \)-HXTX-Mg1a on use-dependent decline in \( I_{\text{Na}} \) during 20 depolarizing test pulses from \(-90 \text{ to } -10 \text{ mV} \) at 30 Hz are shown. Currents were normalized to the peak \( I_{\text{Na}} \) amplitude of the first pulse in the train in the absence (filled symbols, \( n = 6 \)) and presence (open symbols, \( n = 4 \)) of 30 nM \( \delta \)-HXTX-Mg1a (30 nM) at different hyperpolarized holding potentials. Late current \( (I_{\text{Na}, \text{late}}) \) amplitude only increased at holding potentials more negative than \(-140 \text{ mV} \), resulting in a modest but significant increase in the normalized late current \( (I_{\text{Na}, \text{late}}/I_{\text{Na}, \text{peak}}) \) \( (n = 3, *, p < 0.05, \text{one-way repeated measures analysis of variance}) \). C, effects of \( \delta \)-HXTX-Mg1a (30 nM) on the \( \text{Na}_\text{v} \) currents \( (I_{\text{Na}}) \) at different hyperpolarized holding potentials. Late current \( (I_{\text{Na}, \text{late}}) \) amplitude only increased at holding potentials more negative than \(-140 \text{ mV} \), resulting in a modest but significant increase in the normalized late current \( (I_{\text{Na}, \text{late}}/I_{\text{Na}, \text{peak}}) \) \( (n = 3, *, p < 0.05, \text{one-way repeated measures analysis of variance}) \). D, effects of depolarizing post-pulses on dissociation of 30 nM \( \delta \)-HXTX-Mg1a were assessed using 10-ms post-pulses to \(+140 \text{ or } +200 \text{ mV} \) applied immediately following a test pulse to \(-10 \text{ from } -90 \text{ mV} \) every 2 s (\( n = 5 \)). NS, not significant at \( p < 0.05 \).
Spider Toxin Structure That Targets Sodium Channel Subtypes

Inhibitory Cystine Knot Structural Motif—δ-HXTX-Mg1a shows sequence homology with δ-HXTX-1 family peptides and δ-missulelenatoxin-Mb1a (Fig. 1A). Indeed, the disulfide bonding arrangement of δ-HXTX-Mg1a was determined from the NMR data and found to be Cys(I–IV), Cys(II–VI), Cys(III–VII), and Cys(V–VIII), the same as δ-HXTX-Hv1a and δ-HXTX-Ar1a (14, 15). Like δ-HXTX-1 family peptides, δ-HXTX-Mg1a also conforms to an ICK motif (8). The ICK motif consists of double- or triple-stranded antiparallel β-sheets connected by three disulfide bonds, which forms a small stable globular domain and has been observed in a variety of scorpion, spider, cone snail, and snake toxins (16). Specifically, δ-HXTX-Mg1a includes a double-stranded antiparallel β-sheet and a core region (Gly2–Ala23 and Gln28–Glu32) (Fig. 2B). The unstructured C terminus (Arg33–Cys43) is most likely the result of a lack of medium and long range NOE constraints (Fig. 2A).

Structure-Function Relationships—Given that δ-HXTX-Mg1a has a similar solution structure to δ-HXTX-1 toxins (Fig. 2C) and comparable actions on Na\textsubscript{v} channel gating and kinetics, we posited that δ-HXTX-Mg1a might also share key residues important for binding to their Na\textsubscript{v} channel target. Fig. 8 (A–C) shows a comparison of residues that are conserved in spider δ-toxins targeting site-3 (14, 15). A number of residues of these spider toxins are oriented in a similar fashion because of the sequence and structural homology between δ-HXTX-Mg1a and δ-HXTX-1 toxins (Fig. 1A and Fig. 2C). A cluster of positively charged residues of δ-HXTX-Mg1a (Lys\textsubscript{3}, Arg\textsubscript{5}, and Arg\textsubscript{33}) appears in a similar position on the surface of δ-HXTX-Hv1a and δ-HXTX-Ar1a (Lys\textsubscript{3}, Lys\textsubscript{4}, and Arg\textsubscript{5}). The positively charged Lys\textsubscript{3} of δ-HXTX-Mg1a also seems to be oriented similarly to Lys\textsuperscript{10} of δ-HXTX-Hv1a and δ-HXTX-Ar1a, whereas aromatic residues Trp\textsubscript{7} and Tyr\textsubscript{22} are conserved in all these spider toxins. Finally, δ-HXTX-Mg1a possesses Ser\textsuperscript{6} in the same position as the nonpolar Asn\textsubscript{6} of the δ-HXTX-1 toxins. However, other variations in the sequence of δ-HXTX-Mg1a are most likely responsible for the dramatic loss in affinity for the lethal actions of δ-HXTX-Mg1a, when injected intracranially, most likely results from its action on these channels and not Na\textsubscript{v,1.2}. However, in this study δ-HXTX-Mg1a was not active on Na\textsubscript{v,1.4}/β\textsubscript{1}, which α-like, α-insect, and anti-mammalian α-toxins specifically target (49). Moreover, it was only weakly active on Na\textsubscript{v,1.7}/β\textsubscript{1}, which the novel scorpion toxin OD1 specifically targets (50). δ-HXTX-Mg1a is therefore unique from scorpion α-toxins in that it does not target Na\textsubscript{v,1.2}, Na\textsubscript{v,1.4}, or Na\textsubscript{v,1.7} channels with high affinity.
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rNa\textsubscript{1.2} observed in \textsubscript{125}I-Lqh2 binding experiments (10) and the present voltage clamp experiments (Fig. 4A). In particular, the addition/removal of charged side chains at positions 3, 10–12, 19, 34, 36, and 40–43 are dramatically different to the corresponding residues in \(\delta\)-HXTX-1 toxins. Site-3 toxins are proposed to slow the fast inactivation of Na\textsubscript{V} channels by preventing the outward movement of the S4 segment (6). \(\delta\)-HXTX-Mg1a has been previously shown to compete with the scorpion \(\alpha\)-insect toxin Lqh\textsubscript{IT} at subpmol concentrations for binding to insect site-3 (10). Recently, extensive mutagenesis studies have identified the pharmacophore of Lqh\textsubscript{IT} (51). Fig. 8E shows the critical residues in Lqh\textsubscript{IT} form a functional surface composed of two domains, the NC domain and core domain. The positively charged residues of Lys\textsubscript{5}, Arg\textsubscript{6}, and Arg\textsubscript{32} in \(\delta\)-HXTX-Mg1a, are oriented similarly to Lys\textsubscript{5}, Arg\textsubscript{36}, and Lys\textsubscript{32} in the NC domain of Lqh\textsubscript{IT}. The aromatic Trp\textsubscript{7} and Tyr\textsubscript{22} and basic Lys\textsubscript{9} residues of \(\delta\)-HXTX-Mg1a occupy similar positions to Phe\textsubscript{17}, Trp\textsubscript{38}, and Arg\textsubscript{18} in the core domain of Lqh\textsubscript{IT}. These aromatic residues in \(\delta\)-HXTX-Mg1a are also present in \(\delta\)-HXTX-1 toxins. Despite these topologically similar residues, \(\delta\)-HXTX-Mg1a and \(\delta\)-HXTX-1 toxins are smaller in bulk than scorpion \(\alpha\)-toxins and lack aliphatic residues corresponding to the bioactive Ile\textsubscript{57} and Val\textsubscript{59} residues of Lqh\textsubscript{IT}. Indeed, \(\delta\)-HXTX-Mg1a shows the greatest similarity in phylum selectivity to scorpion \(\alpha\)-toxins, e.g. Lqh3. Results from a mutagenesis study suggest that the core domain of Lqh3 plays an important role in interaction with the receptor site and its toxin selectivity (52). Aromatic (Phe\textsubscript{17} and Phe\textsubscript{38}), positively charged (His\textsubscript{13}), and aliphatic residues (Pro\textsubscript{18} and Leu\textsubscript{45}) were assigned to the bioactive surface in Lqh3. Importantly, the aliphatic Val\textsubscript{21} and Ala\textsubscript{23} of \(\delta\)-HXTX-Mg1a are oriented similarly to those of Lqh3. The core domain of \(\delta\)-HXTX-Mg1a therefore seems to be involved in hydrophobic-aromatic interactions and play a role in its phylum selectivity, although this awaits experimental confirmation.

In conclusion, although peptide neurotoxins binding with site 3 on Na\textsubscript{V} channels are known to possess phylum selectivity, our present study highlights a novel feature of \(\delta\)-HXTX-Mg1a, namely target selectivity for distinct Na\textsubscript{V} channel subtypes. This discriminating activity of the spider toxin is somewhat unexpected given that, like homologous \(\delta\)-HXTX-1 toxins, it interacts with both mammalian and insect Na\textsubscript{V} channels. The effects of \(\delta\)-HXTX-Mg1a on DmNa\textsubscript{1.1}/TipE, Na\textsubscript{V,1.1}/\(\beta\)\textsubscript{1}, Na\textsubscript{V,1.3}/\(\beta\)\textsubscript{1}, and Na\textsubscript{V,1.6}/\(\beta\)\textsubscript{1} channels as opposed to the limited or lack of activity on Na\textsubscript{V,1.2}/\(\beta\)\textsubscript{1}, Na\textsubscript{V,1.4}/\(\beta\)\textsubscript{1}, Na\textsubscript{V,1.5}/\(\beta\)\textsubscript{1}, Na\textsubscript{V,1.7}/\(\beta\)\textsubscript{1}, Na\textsubscript{V,1.8}/\(\beta\)\textsubscript{1}, and Na\textsubscript{V,1.9}/\(\beta\)\textsubscript{1} channels are remarkable because they represent the first exhaustive characterization of a selective interaction of any peptide neurotoxin across the complete range of Na\textsubscript{V} channel subtypes. Our findings indicate that specific insect and mammalian Na\textsubscript{V} channel subtypes can be pharmacologically discriminated by their sensitivity to \(\delta\)-HXTX-Mg1a as has only been partially described for scorpion \(\alpha\)-toxins, sea anemone, and other spider toxins (53, 54). This should provide new tools to study the functional role and distribution of various Na\textsubscript{V} channel subtypes. Despite very low sequence homology with all three scorpion \(\alpha\)-toxin groups (e.g. Lqh3, Lqh\textsubscript{IT}, and Aah2), the three-dimensional structure of \(\delta\)-HXTX-Mg1a reveals an apparently similar functional surface with a number of these site-3 toxins and spider \(\delta\)-toxins. Several positively charged and aromatic residues of \(\delta\)-HXTX-Mg1a are arranged in a topologically similar manner to those of site-3 toxins. Thus, the structure of \(\delta\)-HXTX-Mg1a provides an important lead for understanding phylum and subtype specificity and awaits the determination of the pharmacophore of the toxin.

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