Hainantoxin-IV (HNTX-IV) can specifically inhibit the neuronal tetrodotoxin-sensitive sodium channel and defines a new class of depressant spider toxin. The sequence of native HNTX-IV is ECLGFKGKCPNSDQC-CKSSNLVC8RKHRWC1YIE-NH₂. In the present study, to obtain further insight into the primary and tertiary structural requirements of neuronal sodium channel blockers, we determined the solution structure of HNTX-IV as a typical inhibitor cystine knot motif and synthesized four mutants designed based on the predicted sites followed by structural elucidation of two inactive mutants. Pharmacological studies indicated that the S12A and R26A mutants had activities near that of native HNTX-IV, while K27A and R29A demonstrated activities reduced by 2 orders of magnitude. 1H MR analysis showed the similar molecular conformations for native HNTX-IV and four synthetic mutants. Furthermore, in the determined structures of K27A and R29A, the side chains of residues 27 and 29 were located in the identical spatial position to those of native HNTX-IV. These results suggested that residues Ser12, Arg26, Lys27, identical spatial position to those of native HNTX-IV. The side chains of residues 27 and 29 were located in the more, in the determined structures of K27A and R29A, native HNTX-IV and four synthetic mutants. Further-activities reduced by 2 orders of magnitude.1H MR analyses, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

From the ‡College of Life Sciences, Peking University, Beijing 100871 and the College of Life Sciences, Hunan Normal University, Changsha 410081, People’s Republic of China

Dongling Li, Yucheng Xiao, Xia Xu, Xia Xiong, Shanyun Lu, Zhonghua Liu, Qi Zhu, Meichi Wang, Xiaocheng Gu, and Songping Liang‡

‘This work was supported by National Natural Science Foundation of China under Contract No. 30170193 39990 600. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The atomic coordinates and structure factors (code 1NIY (native HNTX-IV), 1RYV (K27A), and 1RYG (R29A)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

The 1H chemical shifts have been deposited in BioMagResBank (BMRB) with accession codes 5676, 6066, and 6067, respectively.

‡To whom correspondence should be addressed. Tel.: 86-731-8861304; Fax: 86-731-8861304; E-mail: liangsp@hunnu.edu.cn.

The abbreviations used are: VGSC, voltage-gated sodium channel; HNTX, hainantoxin; TTX, tetrodotoxin; TTX-S, TTX-sensitive; DRG, dorsal root ganglion; NOE, nuclear Overhauser effect; HPLC, high pressure liquid chromatography; DQF-COSY, double quantum-filtered correlation spectroscopy; TOCSY, total correlated spectroscopy; excitable cells. They are composed of a functional pore-forming α subunit (280 kDa) associated with up to four auxiliary β subunits (1–4). To date, at least six different receptor sites have been identified on VGSCs by different neurotoxins, but many [α] toxins targeting insect sodium channels have a common mode of action similar to that of scorpion α-toxins. They slow or block sodium channel inactivation (5, 6).

A relatively new family of spider toxins has been discovered that inhibit tetrodotoxin-sensitive (TTX-S) sodium currents, blocking most likely at site 1 of neuronal VGSCs (7–9). Hainantoxin-IV (HNTX-IV) is a 35-amino acid residue blocker of sodium channels that was isolated from the venom of the Chinese bird spider Ornithoctonus hainana Liang (Selenocosmia hainana Liang) (7). It has been shown to specifically inhibit the neuronal TTX-S VGSCs with an IC₅₀ value of 34.0 nM in adult rat dorsal root ganglion (DRG) neurons and seem to interact with neurotoxin receptor site 1 through a mechanism quite similar to that of TTX (10) without affecting the activation and inactivation kinetics. The toxin has a high proportion of basic residues, and it is cross-linked by three conserved intramolecular disulfide bonds. The linkage pattern of disulfide bridges in HNTX-IV is I-IV, II-V, and III-VI (Cys²-Cys¹⁷, Cys⁹-Cys²⁴, and Cys¹⁶-Cys²ⁱ) as assigned by partial reduction and sequence analysis. The synthetic HNTX-IV demonstrated the same disulfide pairings and biological activity as the native HNTX-IV, so the possibility that a very potent minor contaminant was present was ruled out (11).

Among 28 spider peptides or homologues acting on VGSCs from the PubMed data base (www.pubmed.com), another four toxins from the venom of the Chinese bird spiders were found to not affect the activation and inactivation kinetics of Na⁺ channel. HNTX-I blocks rNa,1.2/β₁ and the insect Na⁺ channel para/tipE (12), while the other three toxins (HNTX-III and -V and HWTX-IV) inhibit TTX-S Na⁺ currents in adult rat DRG neurons similar to HNTX-IV (7–9), HNTX-IV is closely related in primary sequence to HNTX-III and -V and HWTX-IV (13) and they all have the same numbers and linkage modes of disulfide bonds (Fig. 1). However, these toxins show no significant sequence homology to any other known neurotoxins. So the four spider toxins (HNTX-III–V and HWTX-IV) define a new class of spider toxins affecting VGSCs. The three-dimensional solution structure of HWTX-IV has been determined using two-dimensional 1H NMR spectroscopy, and it was hypothesized that the positively charged residues of loop IV (residues 25–29), espe-
signal was multiplied by a sine bell or sine bell square window functions. However, this hypothesis has not been supported by the results of experiments with mutants. On the other hand, there is also no structural information available on HNTX-IV. Determination of the structure of HNTX-IV is therefore important to gain further information regarding the design of synthetic analogues for the structure-activity relationships of these toxins.

In the present study, we determined the solution structure of HNTX-IV by 1H NMR with distance geometry and simulated annealing. Based on the structural comparison and sequence assignment with other related toxins, we hypothesized that the positively charged residues Arg26, Lys27, and Arg29 in loop IV and valinyl polar residue Ser12 clustered on one face of HNTX-IV may be the potential interaction site. Mutants S12A, R26A, K27A, and R29A were synthesized by solid-phase Fmoc chemistry followed by oxidative refolding of purified peptides under the optimal conditions. 1H NMR analysis showed similar molecular conformations for native HNTX-IV and synthetic mutants. Pharmacological studies indicated that residues Ser12 and Arg26 were not important for the activities, while Lys27 and Arg29 were critical for the bioactivities. These results provide useful information for structure-activity relationships of toxins like HNTX-IV.

EXPERIMENTAL PROCEDURES

**NMR Spectroscopy of Native HNTX-IV**—An NMR sample was prepared by dissolving the native HNTX-IV in 500 μl of 20 mM sodium acetate buffer (H3OAc, pH 5.0) containing 0.002% NaN3 and 0.01 mM EDTA with a final concentration of 5.5 mM HNTX-IV and a pH of 4.0. Sodium 3-(trimethylsilyl)propionate-2,2,3,3-D4 was added to a final concentration of 200 μM as an internal chemical shift reference. For experiments in D2O, the sample used in H2O experiments was lyophilized and then redissolved in 500 μl of 99.996% D2O (Cambridge Isotope Laboratories).

All NMR spectra were observed on a 500-MHz Bruker DRX-500 spectrometer with a sample temperature of 295 K. Several sets of two-dimensional spectra were recorded in a phase-sensitive mode by standard pulse sequences and phase cycling. TOCSY spectra were obtained with a mixing time of 85 ms. NOESY spectra were recorded in D2O with a mixing time of 200 ms and in H2O with mixing times of 100, 200, and 400 ms. Solvent suppression was achieved by the presaturation method. All two-dimensional measurements were recorded with 1024 × 512 frequency data points and were zero-filled to yield 2048 × 1024 data matrices except for the high resolution DQF-COSY spectrum. The DQF-COSY spectrum was recorded with 2048 × 1024 data points in the 12 and 11 dimensions, respectively, and zero-filled to 4096 × 2048 points to measure the coupling constants. All spectra were processed and analyzed using Felix 98.0 (Biosem Technologies) software running on a Silicon Graphics O2 work station. Before Fourier transformation, the signal was multiplied by a sine bell or sines bell square window functions with a 2/2 phase shift.

The hydrogen-deuterium exchange experiments were carried out by recording a series of one-dimensional spectra after the lyophilized sample was redissolved in D2O. A TOCSY spectrum was recorded after 2 h of exchange.

Structure Calculations of Native HNTX-IV—Distance constraints were obtained from the intensities of cross-peaks in NOESY spectra with a mixing time of 200 ms. All NOE data were classified into four distance ranges: 1.8–2.7, 1.8–3.5, 1.6–5.0, and 1.8–6.0 Å, correspondingly strong, medium, weak, and very weak NOE values, respectively. Pseudotautomers were applied to non-stereospecifically assigned methyl and methylene protons according to the method of Wuthrich (13).

Fourteen dihedral angle restraints derived from 1J,ν-Hα-Cα coupling constants were restrained to 120° ± 30 for 1J,ν-Hα-Cα ≥ 8.80 Hz and -65° ± 25 for 1J,ν-Hα-Cα ≤ 5.50 Hz. HNTX-IV contains 6 cysteine residues paired as Cys4-Cys7, Cys3-Cys4, and Cys4-Cys5 as assigned by partial reduction and sequence analysis. Three distance constraints for each disulfide bond were S(i)–S(i−1), S(i)–S(i+1), and S(i)–C(i) whose target values were set to 2.02 ± 0.02, 2.99 ± 0.5, and 2.99 ± 0.5 Å, respectively. Eight hydrogen bond constraints restrict the NH(i)–O(j) and N(i)–O(j) distance as 1.8–2.7 and 2.8–3.7 Å, respectively, according to slowly exchanging amide protons and NOEs patterns. Structural calculations were performed with 583 distance constraints and 14 dihedral angle constraints using the standard protocol of the X-PLOR 3.851 program (14).

Electrophysiological Experiments—The whole cell patch clamp experiments were made from rat DRG neurons as described previously (7). Rat DRG neurons were acutely dissociated and maintained in a short term primary culture using the method described by Hu and Li (16). Briefly 30-day-old adult Sprague-Dawley rats of either sex were killed by decapitation, and the dorsal root ganglia were isolated quickly from the spinal cord. Then they were transferred into Dulbecco’s modified Eagle’s medium containing trypsin (0.5 mg/ml, type III, Sigma), collagenase (1.0 mg/ml, type IA, Sigma), and DNase (0.1 mg/ml, type III, Sigma) to incubate at 34 °C for 30 min. Trypsin inhibitor (1.5 mg/ml, type II-S, Sigma) was used to terminate enzyme treatment. The DRG cells were transferred into 35-mm dishes (Corning, Sigma) with the culture medium and incubated in a CO2 incubator (5% CO2, 95% air at
Experimental constraints
Intraresidue NOE (i–j = 0) 190 171 156
Sequential NOE (|i–j| = 1) 139 90 94
Medium range NOE (|i–j| ≤ 5) 61 33 35
Long range NOE (|i–j| ≥ 5) 176 105 103
Dihedral angle (ϕ) 14 13 15
Average potential energies (kcal mol⁻¹)⁻¹
Eₑexcl⁻¹ 67.64 ± 5.470 55.24 ± 6.688 47.78 ± 6.583
Eₑend⁻¹ 5.518 ± 0.426 4.352 ± 0.400 5.574 ± 0.260
Eₑangle⁻¹ 42.24 ± 0.814 39.76 ± 0.860 42.12 ± 0.923
Eₑimproper⁻¹ 4.783 ± 0.183 4.371 ± 0.162 4.698 ± 0.117
Eₑdw⁻¹ 120.53 ± 5.445 105.81 ± 6.329 101.02 ± 6.346
EₑNOR⁻¹ 0.293 ± 0.161 0.613 ± 0.150 0.786 ± 0.264
EₑvdW⁻¹ 0.017 ± 0.031 0.012 ± 0.015 0.039 ± 0.041
r.m.s. deviation from experimental constraints
NOE distance (Å) 0.003 ± 0.001 0.005 ± 0.001 0.006 ± 0.001
Dihedral angle (°) 0.097 ± 0.08 0.097 ± 0.081 0.175 ± 0.117
r.m.s. deviations from idealized geometry
Bonds (Å) 0.003 ± 0.0001 0.003 ± 0.0001 0.003 ± 0.0001
Angles (°) 0.533 ± 0.005 0.523 ± 0.005 0.538 ± 0.006
Improper (°) 0.330 ± 0.006 0.331 ± 0.006 0.332 ± 0.004
Average r.m.s. differences versus mean structure (Å)
Backbone atoms (N, Cα, and C) 0.485 ± 0.064 0.620 ± 0.097 0.643 ± 0.111
Non-hydrogen heavy atoms 1.229 ± 0.131 1.313 ± 0.130 1.327 ± 0.129
Pairwise r.m.s. differences of 20 structures (Å)
Backbone atoms (N, Cα, and C) 0.689 ± 0.124 0.901 ± 0.159 0.925 ± 0.183
Non-hydrogen heavy atoms 1.746 ± 0.192 1.871 ± 0.215 1.874 ± 0.212

The idealized geometry and energy values were defined by the CHARMm force field as implemented in the XPLOR program. All statistical values of energies, r.m.s. deviations, and r.m.s. differences are given as the mean ± S.D.

The statistics of experimental r.m.s. deviation of NOE and dihedral angle constraints were from the calculation with force constants of 50 kcal mol⁻¹ Å⁻² and 200 kcal mol⁻¹ radian⁻², respectively.

37 °C for 1–4 h before the patch clamp experiment.

Patch clamp experiments were performed at room temperature (20–25 °C) under the whole cell patch clamp configuration. Patch pipettes (2–3-μm diameter) were pulled from borosilicate glass capillary tubing by using a two-step vertical puller (PC-10, Narishige, Olypmus) and heat-polished with a microforge (MF-900, Narishige). The patch pipettes contained 135 mM CsF, 10 mM NaCl, 5 mM HEPES, 20 mM tetraethylammonium chloride, 0.75 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, with the pH adjusted to 7.0 with 1 M CsOH. The external bathing solution contained 30 mM NaCl, 5 mM CsCl, 25 mM D-glucose, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 20 mM tetraethylammonium chloride, 70 mM tetramethylammonium chloride with the pH adjusted to 7.40 with 1 M tetraethylammonium hydroxide. Peptide toxin was dissolved in external solution, and about a 10-pL volume was applied by pressure injection with a microinjector (IM-5B, Narishige). All chemical reagents were purchased from Sigma.

Conformational Analysis of Synthetic Peptides—The synthetic peptide samples used for ⁱH NMR spectrum analyses were dissolved in 500 μL of 20 mM deuterium sodium acetate buffer at pH 4.0. One-dimensional ¹H NMR spectra were obtained on S12A and R29A mutants at 295 K. One- and two-dimensional ¹H NMR spectra were acquired on K27A and R29A mutants at 295 K.

RESULTS
Structure Calculations and Evaluation of Native HNTX-IV—Sequence-specific resonance assignments were performed according to the standard procedures established by Wuthrich (13). All of the backbone protons and more than 95% of the side chain protons were identified. 583 intramolecular distance constraints and 14 dihedral constraints were used to calculate the structure of HNTX-IV by distance geometry and simulated annealing calculation with the program X-PLOR. A family of 20 accepted structures with lower energies and better Ramachandran plots were selected to represent the three-dimensional solution structure of HNTX-IV. A summary of the structural statistics for HNTX-IV is given in Table I. The structures have no distance violations greater than 0.2 Å and no dihedral violations greater than 2.0°. Furthermore they have favorable non-bonded contacts as evidenced by the low values of the mean Lennard-Jones potentials and good covalent geometry as indicated by the small deviations from ideal bond lengths and bond angles. Analysis of the structures in PROCHECK (17) shows that 85.3% of non-Pro, non-Gly residues lie in the most favored regions of the Ramachandran plot with a further 14.7% in additionally allowed regions.

Structure Description of Native HNTX-IV—Fig. 2A shows the best fit superposition of the backbone atoms (N, Cα, and C) for the 20 converged structures of native HNTX-IV. Analysis of the 20 converged structures indicated that the molecular structure of HNTX-IV contained a short triple-stranded antiparallel β-sheet formed by the strands Lys⁴–Cys⁵, Leu²²–Ser²³ and Trp²⁰–Tyr²¹, respectively (Fig. 2B). The information from strong sequential dₙ₋ₙ and weak dₙ₋ₙ, interstrand NOE connectivities, chemical shift index (18), large ³JHH-CαH coupling constants, and slowly exchanging amide protons also occurred in a β-sheet. The turns in HNTX-IV were also identified using a standard definition that states that the distance between C(i) and C(i + 3) should be less than 7 Å and that the characteristic NOE connectivities of backbone protons (13) for the corresponding turn segments are presented. These analyses led to the identification of four β-turns (Fig. 2B), which were classified according to Richardson (19). The four β-turns involve residues Gly⁴–Lys⁵ (Type II), Pro¹³–Asp¹⁴ (Type I), Lys¹⁸–Asn²¹ (Type I), and Arg²⁶–Arg²⁷ (Type I).

HNTX-IV adopts an inhibitor cystine knot motif commonly observed in toxic and inhibitory peptides (20). The cystine knot in HNTX-IV is formed by three disulfide bonds linked as Cys²⁵–Cys¹⁷, Cys²⁸–Cys²⁹, and Cys¹⁴–Cys¹⁵ in which the Cys²⁵–Cys¹⁷ disulfide bond passes through a 16-residue ring formed by the intervening polypeptide backbone and the Cys²⁸–Cys²⁹ disulfide bonds.
Peptide Synthesis and Characterization—Solid-phase synthesis of mutants S12A, R26A, K27A, and R29A, using Fmoc-protected amino acids and HOBt/TBTU coupling, yielded a major product as revealed by reverse-phase HPLC analysis and MALDI-TOF mass spectrometry. The purified reduced peptides were folded/oxidized in buffer 0.1 M Tris-HCl, 0.1 M NaCl, pH 7.4, containing 5 mM GSH, 0.5 mM GSSG for 24 h at room temperature. Purified products were homogeneous in analytical reverse-phase HPLC (Fig. 3), and their masses (3972.95 Da, 3903.45 Da, 3931.02 Da, and 3903.60 Da for S12A, R26A, K27A, and R29A, respectively) were in good accordance with the theoretical masses for all oxidized analogues.

Effects of Synthetic Analogues on Sodium Channel Currents—The biological activities of four analogues of HNTX-IV were studied by testing their capacity to inhibit the TTX-S sodium currents (Fig. 4). The dose-response curves shown in Fig. 5 illustrate the block of sodium channels at peptide concentrations ranging from 0.1 nM to 1.0 mM. The IC50 values of 58.3 and 99.6 nM were estimated for S12A and carboxyl termini, respectively. The backbones are shown in cyan, green, and gray, respectively. Positively charged side chains are shown in blue, and negatively charged side chains are shown in red. B, comparison of HNTX-IV to conotoxin GS (CTX-GS) and μ-conotoxin GIIIA (CTX-GIIIA) (conotoxin GS, Protein Data Bank code 1AG7; μ-conotoxin GIIIA Protein Data Bank code 1TCG). The β-sheet is shown in yellow, the turn is shown in blue, and the random coil structure is shown in green. Three disulfide bonds of each molecule are indicated. The letters N and C refer to the amino and carboxyl termini, respectively.

Conformational Analysis of Synthetic Analogues—One- and two-dimensional 1H NMR spectroscopy were used to analyze rigorously the structures of the synthetic analogues to determine the extent of isomer purity and to assess conformational integrity. The fact that one-dimensional 1H NMR spectra of S12A and R26A (data not shown) were similar to that of native HNTX-IV (34.0 nM). In contrast, K27A and R29A caused reductions in potency of 2 orders of magnitude in K27A and R29A, mainly R29A, the changes were found to be small, typically less than 0.1 ppm, with only two greater than 0.13 ppm. The deviations of Cα chemical shifts were typically less than half the size of the NH deviations, and so no large changes in backbone conformation were expected for K27A and R29A, a fact in accord with the calculated structures of K27A and R29A.

A quantitative characterization of the structure determinations is given in Table I. The solution structures of K27A and R29A, as shown in Fig. 2A, belong to the inhibitor cystine knot structural family. The atomic root mean square (r.m.s.) difference of K27A and R29A from the native HNTX-IV was 0.742 and 0.732 Å, respectively, for the backbone atoms (N, Cα, and

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**Fig. 2.** Structural comparison of HNTX-IV and correlated toxin molecules. A, ensembles of 20 energy-refined conformers representing the solution structures of native HNTX-IV, K27A, and R29A. The backbones are shown in cyan, green, and gray, respectively. Positively charged side chains are shown in blue, and negatively charged side chains are shown in red. B, comparison of HNTX-IV to conotoxin GS (CTX-GS) and μ-conotoxin GIIIA (CTX-GIIIA) (conotoxin GS, Protein Data Bank code 1AG7; μ-conotoxin GIIIA, Protein Data Bank code 1TCG). The β-sheet is shown in yellow, the turn is shown in blue, and the random coil structure is shown in green.

**Fig. 3.** Analytical reverse-phase HPLC profiles of the oxidation products of S12A, R26A, K27A, and R29A at different times. Oxidation was performed in 5 mM GSH, 0.5 mM GSSG, 0.1 M NaCl, 0.1 M Tris-HCl buffer, pH 7.4. N and R indicate isomers with three native and fully reduced disulfides, respectively.

**Fig. 4.** Effects of native HNTX-IV and four analogues at a concentration of 1.0 μM on TTX-S sodium channels in rat DRG neurons. Current traces were evoked by depolarizations ranging from a holding potential of −80 to −20 mV. A, native HNTX-IV; B, S12A; C, R26A; D, K27A; E, R29A.
from five to eight cells, shows current relative to control. Apparent IC50
R26A, K27A, and R29A.

Hydrogen bonds. HNTX-IV contains seven positively charged
activities (31, 32). Hence it seems clear that inhibitors bind to the
acidic residues Glu758, Asp762, Glu765, and Asp1241 at site 1 of
VGSCs (24–27). One of the most important factors for its binding to TTX-S VGSCs, but Lys77 and Arg29 were critical for the bioac-
tivities. The detailed NMR investigation of the inactive ana-
logue structures showed that the decrease in the blocking ac-
tivity was primarily the result of the loss of positively charged
side chain interactions with the binding site of sodium channel
molecules rather than the conformational change associated with
the substitutions.

Previously it was mentioned that the mainly acidic residues in Na+
channel molecules interacted with basic residues and
vicinal polar amino acids in toxins via electrostatic interactions or
hydrogen bonds. Residues Lys77 and Arg29 in HNTX-IV are
positively charged residues with long side chains, hence it is
plausible that the positive charges of two residues are an im-
portant factor for its binding to TTX-S VGSCs, similar to the
function of basic residues in µ-conotoxin GIHIIA. As a result,
the electrostatic potentials seem to play an important role in con-
ducting the block of the TTX-S Na+ channels by HNTX-IV.

Many studies have revealed that the binding interface of the
toxin molecule is rather wide, and multiple amino acid residues
of toxins are involved in the interaction with Na+ channels (5,
6, 25). The NMR structure of HNTX-IV displayed that Ser12, Arg26,
Lys77, and Arg29 were clustered on one side of the
molecule with their side chains being more surface-exposed

Structure-Activity Relationships of Hainantoxin-IV

C of all residues (Fig. 7B). The r.m.s. difference of two mutants
was 0.440 Å for the backbone atoms (N, Cα, and C). The convergence
of structures for the two synthetic analogues and
native HNTX-IV did not differ significantly, suggesting that
either the chemical properties or the conformations of their
constituent amino acid side chains account for their different
activities.

Discussion

HNTX-IV and previously characterized HNTX-III, HNTX-V,
and HWTX-IV have been shown to specifically inhibit the
mammal neuronal TTX-S sodium channels. They seem to
interact with neurotoxin receptor site 1 through a mechanism
quite similar to that of TTX without affecting the activation
and inactivation kinetics (7–9). Although we have not con-
ducted an isotope-labeled toxin binding assay to test whether
they share the same binding site with TTX or saxitoxin (23), we
have demonstrated that among all reported spider toxins, the
four toxins (HNTX-III–V and HWTX-IV) are a new class of
depressant spider toxins affecting VGSCs. They could prove to
be a class of novel, useful ligands to investigate the multiple
molecular forms of VGSCs in vertebrates.

Previous studies on sodium channel blockers have revealed that
1) three-dimensional structures were essential for the activity,
2) one important characteristic required for blockage of
sodium current was the presence of a positively charged
region on the toxin, and 3) the guanidine group and hydroxyl
group were important for the high inhibitory activity (24–27).
The most well known example is µ-conotoxin GIHIIA among
neurotoxins acting at site 1 affecting VGSCs (24–30). Studies of
site-directed mutagenesis in VGSCs and µ-conotoxin
GIHIIA/B have also been carried out to identify that the main
acidic residues Glu756, Asp762, Glu765, and Asp1241 at site 1 of
VGCS and residues Arg15, Gln14, Lys16, Hyp17, and Arg19 of
µ-conotoxin GIHIIA are involved in the binding and blocking
activity (31, 32). Hence it seems clear that inhibitors bind to the
sodium channels at least in part by electrostatic interactions or
hydrogen bonds. HNTX-IV contains seven positively charged
residues (Lys7, Lys18, Arg26, Lys27, His28, Arg29, and Lys29) and
three negatively charged residues (Glu1, Asp14, and Glu24),
producing a net positive charge (theoretical pI = 8.66) on this
molecule (Fig. 2A). Evidently the positively charged side chains
are located on the surface of the peptide. In particular, the
surface of loop IV (Arg26, Lys27, His28, and Arg29) bears the
majority of the positively charged residues with Arg26, Lys27,
His28, and Arg29 being the most solvent-exposed. In addition,
respectively. The fit was done using the common secondary structure elements. The letters N backbon superposition of native HNTX-IV (blue), K27A (green), and R29A (red). 27 and 29 indicate the positions of substituted amino acid residues. The fit was done using the common secondary structure elements. The letters N and C refer to the amino and carboxyl termini, respectively.

Fig. 6. Chemical shift differences of K27A and R29A from native HNTX-IV, respectively.

Fig. 7. Solution structure characterization of HNTX-IV. A, surface profile of HNTX-IV. a, surface profile of predicted active sites. B, surface profile of putative active sites. Blue, mauve, and cyan regions represent positively charged, polar, and hydrophobic residues, respectively. B, backbone superposition of native HNTX-IV (blue), K27A (green), and R29A (red). 27 and 29 indicate the positions of substituted amino acid residues. The fit was done using the common secondary structure elements. The letters N and C refer to the amino and carboxyl termini, respectively.

(Fig. 7A, a). Unexpectedly our present results showed that mutations of only Lys27 and Arg29 to Ala had large effects on HNTX-IV binding to TTX-S Na+ channels. Therefore, we hypothesize that HNTX-IV binds to the sodium channels with the surface encompassing mainly Lys27 and Arg29, as well as other uncharacterized residues, but not the surface formed by Ser12, Arg26, Lys27, and Arg29. After comparison of these structures and activities with the correlated toxins, we proposed that residues Lys27 and Arg29 and vicinal residues His28, Lys32, and activities with the correlated toxins, we proposed that HNTX-IV binds to the sodium channels with the surface encompassing mainly Lys27 and Arg29, as well as other uncharacterized residues, but not the surface formed by Ser12, Arg26, Lys27, and Arg29. After comparison of these structures and activities with the correlated toxins, we proposed that residues Lys27 and Arg29 and vicinal residues His28, Lys32, and Arg29 were responsible for binding to TTX-S VGSCs in rat DRG neurons.

Structural Comparison of HNTX-IV with μ-Conotoxins—μ-Conotoxins are receptor site 1 sodium channel blockers purified from the venom of marine cone snails that act selectively to occlude the pore of VGSCs by competing with TTX and saxitoxin. Among the μ-conotoxins, GIIIA/B and GS specifically inhibit rat skeletal muscle sodium channels (rNa(ν)1.4), whereas PIIIA inhibits neuronal as well as muscle TTX-S sodium channels (33–39). The three-dimensional structure of HNTX-IV has little resemblance to the three-loop μ-conotoxins (GIIIA/B and PIIIA) in agreement with the low sequence identity between them and their different cystine frameworks (27, 40, 41). Unlike the three-loop μ-conotoxins, both conotoxin GS and HNTX-IV belong to the inhibitor cystine knot structural family with the same disulfide bond pattern despite little sequence identity (42). Fig. 2B shows the secondary structures of HNTX-IV, conotoxin GS, and μ-conotoxin GIIIA. The main structure of the three-loop μ-conotoxins is composed of a small β-hairpin, a distorted 310 helix comprising residues 13–22, and several turns, whereas HNTX-IV and conotoxin GS consist mainly of β-strands and turns. Further structural comparison of HNTX-IV with conotoxin GS reveals that although the two molecules contain a Type II β-turn in loop I, several local differences in backbone conformation are observed. First, in HNTX-IV, the first strand constitutes a complete β-sheet at Lys7–Cys9, whereas in conotoxin GS it consists solely of a β-bridge at Ser7. Second, the size of loops II and IV is strikingly different between the two molecules. In particular, loop II of conotoxin GS lacks three residues relative to HNTX-IV and has a markedly different conformation. Moreover another distinct difference is observed in the relative orientation of loops II and IV in these two structures. Such differences in local structures and in the amino acid sequences between HNTX-IV and μ-conotoxins may play a vital role in discriminating the specific target subtype of sodium channels.

Conclusion—In conclusion, the solution structure of HNTX-IV determined by 1H NMR proved that the molecule adopted a typical inhibitor cystine knot motif. Based on the predicted active sites, we synthesize four analogues of HNTX-IV followed by structural elucidation of two inactive mutants. The solution structure of native HNTX-IV and its inactive analogues reported here should provide a basis for the further understanding of the structure-activity relationships of these toxins like HNTX-IV and insight into the structural difference of different subtypes of VGSCs.

Acknowledgment—We are grateful to Guangzhong Tu of Beijing Institute of Microchemistry for collecting the 1H NMR spectra.

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