

Research Article

Isolation and characterization of hainantoxin-IV, a novel antagonist of tetrodotoxin-sensitive sodium channels from the Chinese bird spider *Selenocosmia hainana*

Z. Liu, J. Dai, Z. Chen, W. Hu, Y. Xiao and S. Liang*

College of Life Sciences, Hunan Normal University, Changsha 410081 Hunan (China), Fax: + 86 7318861304, e-mail: liangsp@public.cs.hn.cn

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Abstract. A neurotoxin, named hainantoxin-IV, was purified from the venom of the spider *Selenocosmia hainana*. The amino acid sequence was determined by Edman degradation, revealing it to be a 35-residue polypeptide amidated at its C terminal and including three disulfide bridges: Cys2-Cys17, Cys9-Cys24, and Cys16-Cys31 assigned by partial reduction and sequence analysis. Hainantoxin-IV shares 80% sequence identity with huwentoxin-IV from the spider *S. huwena*, a potent antagonist that acts at site 1 on tetrodotoxin-sensitive

(TTX-S) sodium channels, suggesting that hainantoxin-IV adopts an inhibitor cystine knot structural motif like huwentoxin-IV. Under whole-cell voltage-clamp conditions, this toxin has no effect on tetrodotoxin-resistant voltage-gated sodium channels in adult rat dorsal root ganglion neurons, while it blocks TTX-S sodium channels in a manner similar to huwentoxin-IV, and the actions of both toxins on sodium currents are very similar to that of tetrodotoxin. Thus, they define a new family of spider toxins affecting sodium channels.

Key words. Spider toxin; HNTX-IV; HWTX-IV; disulfide bridges; sodium channels; inhibitor cystine knot (ICK) structural motif.

The inhibitor cystine knot (ICK) structural motif is known to be a common structural motif adopted by a number of ion channel toxins and related polypeptides from diverse sources, including spiders, coneshells, plants, and fungi [1]. This structural motif is characterized by triple-stranded, anti-parallel β sheets stabilized by a cystine knot containing three disulfide bridges (named the 2-5, 1-4, and 3-6 pattern) [2]. The compact and robust nature of this motif makes it an excellent scaffold for drug design [3]. Due to the diversity of spiders, they will certainly be a rich source of polypeptides based on this structural motif [1]. Spider venoms are known to contain several classes of neurotoxins, which display specific action

on ionic channel and receptor systems of the cell membrane. These toxins are widely employed as scalpels in electrophysiological and neurochemical studies [4–6]. The spider *Selenocosmia hainana* was recently identified as a new species of the genus *Selenocosmia*. It is distributed in the hilly areas of Hainan province in southern China. *S. hainana* is similar to the spider *S. huwana* in morphology, but they belong to different species by analysis of the components of their crude venom using biochemical methodology [7]. In our previous work, we found that the crude venom of *S. hainana* was toxic to mice, and it can block tetrodotoxin-sensitive (TTX-5) sodium currents in NG108-15 cells, while it has no effect on the activation of sodium channels [8].

In this paper, we describe the isolation, characterization, and biological activity of hainantoxin-IV (HNTX-IV)

* Corresponding author.

from the venom of the spider *S. hainana*. This toxin shares 80% sequence identity with huwentoxin-IV (HWTX-IV) [9] isolated from *S. huwena*. Both block TTX-S sodium channels in a similar manner to TTX. Thus, they can be reasonably classified as receptor site 1 toxins [10–12]. After comparison with other sodium channel toxins of spiders, we conclude that HNTX-IV and HWTX-IV define a new family of spider toxins affecting sodium channels.

Materials and methods

Materials and animals

Live spiders and Sprague-Dawley rats were maintained in the Laboratory of Protein Chemistry of Hunan Normal University. Cockroaches (*Periplaneta americana*) were from Peking University. The reagents and solvents for sequencing analysis were from Applied Biosystems. Tris(2-carboxyethyl)phosphine (TCEP), iodoacetamide and HEPES were purchased from Sigma. All other chemicals were of analytical grade.

Isolation of HNTX-IV

The venom was obtained by electrical stimulation of female spiders, and the freeze-dried crude venom was stored at -20°C prior to analysis. Lyophilized venom, dissolved in double-distilled water, was applied onto a reverse-phase high-performance liquid chromatography (RP-HPLC) column Vydac C18 (300 Å, 4.6×250 mm) using a Waters Alliance system. Venom components were eluted using a linear acetonitrile gradient (5–40% acetonitrile/0.1% TFA in 50 min) at a flow rate of 1.0 ml/min. Elution of peptides was monitored at 215 nm.

Mass spectrometry

The molecular masses of peptides were determined using MALDI-TOF MS (Applied Biosystems, Voyager-DE STR Biospectrometry workstation). Ionization was achieved by irradiation with a nitrogen laser (337 nm), with a 20-kV acceleration voltage. α -Cyano-4-hydroxy-cinnamic acid (CCA) was used as matrix. Prior to each analysis in the reflection mode, the masses were calibrated internally using huwentoxin-I (HWTX-I) (MH^+ , 3750.45 Da).

Amino acid component and sequence analysis

Amino acid component analysis and carboxymethylation of native peptide were carried out as described previously [13, 14]. The carboxymethylated peptide was submitted to automatic N-terminal sequencing on an Applied Biosystems Model 491 gas-phase sequencer. Edman degradation was performed with a normal automatic cycle program.

Assignment of the disulfide bonds of HNTX-IV

The method of Gray was used [15, 16]. HNTX-IV (0.1 mg) was dissolved in 10 μl of 0.1 mol/l citrate buffer (pH 3) containing 6 mol/l guanidine-HCl. Partial reduction of HNTX-IV disulfide bonds was carried out by adding 10 μl of 0.1 mol/l TCEP at 40°C for 10 min at pH 3, and the intermediates were isolated by RP-HPLC (column: Vydac, C18, 300 Å, 4.6×250 mm) with linear gradient elution (18 ~ 40% acetonitrile/0.1% TFA in 40 min). The masses of the collected peptide isomers were determined by MALDI-TOF. Appropriate isomers containing free thiols were dried and then alkylated by adding 100 μl of 0.5 mol/l iodoacetamide (pH 8.3). The alkylated peptide was desalted by RP-HPLC and then submitted to an Applied Biosystem Model 491 gas-phase sequencer.

Biological assays

The toxicity of HNTX-IV was qualitatively assayed by intra-peritoneal injection into 18 to 20 g mice of both sexes and intra-abdominal injection into adult male cockroaches (*P. americana*) with body weights of 0.35 g using 20 μl solutions [in 0.9% (w/v) normal saline]. The LD_{50} was determined in six mice at each of four dose levels (from 0.16 $\mu\text{g/g}$ body weight to 0.32 $\mu\text{g/g}$ body weight with a 1.25-fold increment) and observed during a 24-h period following injection. The pharmacological study of HNTX-IV in mouse phrenic neuromuscular transmission preparations was carried out as described previously [17].

Electrophysiological studies

Whole-cell clamp recordings of voltage-gated sodium currents were made from rat dorsal root ganglion (DRG) neurons, which were acutely dissociated from 30-day-old Sprague-Dawley rats and maintained in short-term primary culture according to the methods described by Hu and Li [18]. DRG cells with a large diameter (around 50 pS in slow capacitance) and those with a relatively small diameter (around 20 pS in slow capacitance) were chosen for study of TTX-S and TTX-resistant (TTX-R) sodium currents, respectively. In addition, TTX (final concentration at 200 nmol/l) was used to separate the TTX-R sodium current from the TTX-S sodium current [19]. Patch-clamp experiments were performed at room temperature. Suction pipettes (2.0–3.0 M Ω) were made of borosilicate glass capillary tubes with a two-step pulling from a vertical micropipette puller. The pipette solution contained (in mM): CsCl 145, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 4, HEPES 10, EGTA 10, glucose 10, ATP 2, pH 7.2, the external solution contained (in mM): NaCl 145, KCl 2.5, CaCl_2 1.5, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.2, HEPES 10, glucose 10, pH 7.4). Experimental data were collected and analyzed using the program Pulse/Pulsefit (HEKA Electronics) 8.0. Macroscopic sodium currents were filtered at 10 kHz and digitized at 3 kHz with an EPC-9 patch-clamp amplifier

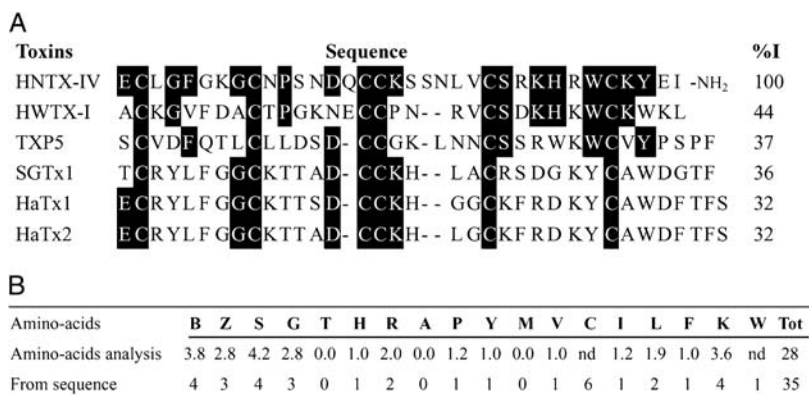


Figure 2. (A) Comparison of amino acid sequence of HNTX-IV with other spider toxins. Identical amino acid residues with HNTX-IV are shaded in black. Gaps were introduced to maximize the similarities. The percentage identity (%I) with HNTX-IV is shown to the right of the sequences. TXP5, from *Brachypelma smithii*; SGTx1 from *Scodra griseipes*; HaTx, from *Grammostola spatulata*. (B) Amino acid analysis of HNTX-IV (nd, not detected).

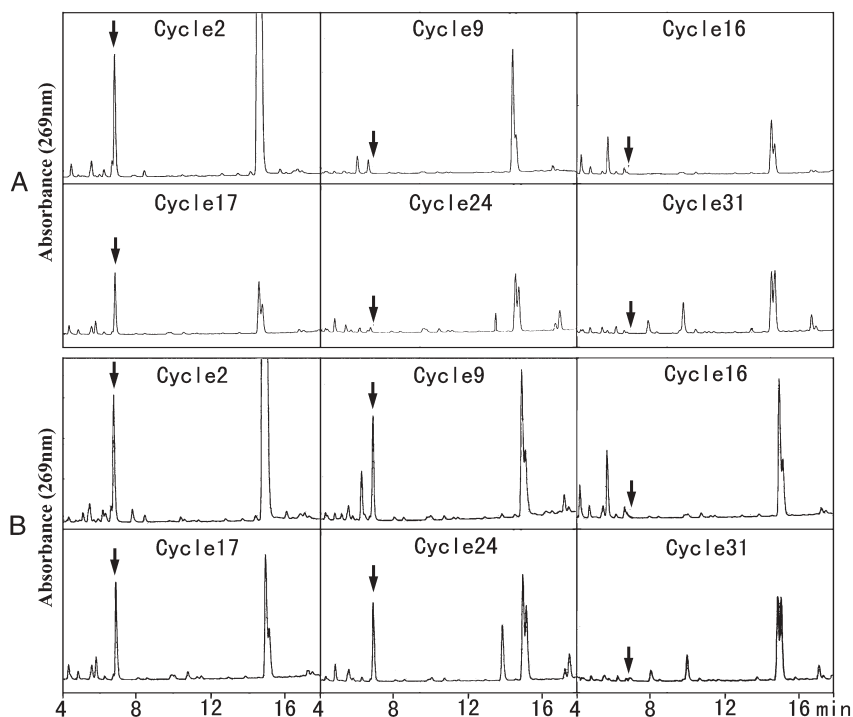


Figure 3. HPLC profiles of sequencing partial reduced intermediates after modification with iodoacetamide. Cysteine residues occurred at cycles 2, 9, 16, 17, 24, and 31. The elution position of Pth-CM-Cys is marked with arrows. (A) The cysteine residue cycles of alkylated peak II. (B) The cysteine residue cycles of alkylated peak Ia.

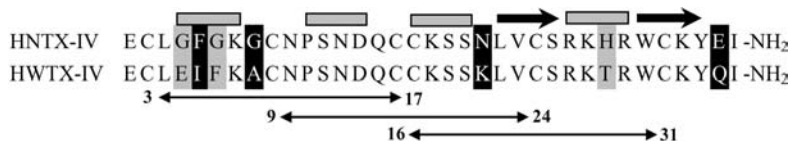


Figure 4. Sequence comparison of HNTX-IV and HWTX-IV. Conservatively substituted residues and non-conservatively substituted ones are shaded in black and gray, respectively. The disulfide bridge pattern (named 1-4, 2-5, and 3-6) of these two toxins was determined by partial reduction and sequence analysis. Secondary structure is shown at the top of the panel where shaded bars and black arrows represent β -turns and β -sheets, respectively.

(HEKA Electronics). Series resistance was kept near 5 M Ω and compensated 65–70%, linear capacitive and leakage currents were digitally subtracted using the P/4 protocol.

Results and discussion

Purification and characterization of HNTX-IV

Crude venom from the spider *S. hainana* was fractionated by RP-HPLC on a Vydac C18 column (fig. 1A). More than 20 peaks were observed on the chromatogram. The peak named HNTX-IV was eluted as a sharp peak at 27.6 min at a point in the gradient of about 24% acetonitrile/0.1% TFA and was found to be toxic to mice (intraperitoneal LD₅₀ in mice 0.2 \pm 0.07 mg/kg body weight). As assessed by mass spectrometry, the purified fraction contained one component, whose average molecular mass (M+H)⁺ was determined as 3,988.55 Da. The purity of HNTX-IV was over 98%, as judged by RP-HPLC (fig. 1) and N-terminal sequence analysis (data not shown). The complete amino acid sequence of HNTX-IV was identified by Edman degradation (fig. 2A), revealing it as a 35-amino-acid polypeptide containing six cysteine

residues, which is in agreement with the results of amino acid analysis (fig. 2B). The calculated monoisotopic molecular mass (M+H)⁺ of HNTX-IV was 3,985.82 Da for the HNTX-IV amide and 3,986.74 Da for HNTX-IV with a free carboxylic acid, assuming all cysteine residues are involved in disulfide bridges. The experimental monoisotopic molecular mass (M+H)⁺ of native HNTX-IV was 3,985.75 Da. Thus, we conclude that the native HNTX-IV is a C-terminal amidated peptide.

To determine the disulfide bridges, native peptide was partially reduced by TCEP under acidic conditions (fig. 1B). Five chromatographic peaks contained intact peptide and partially reduced isomers, as determined by MALDI-TOF analysis. Those with 0-Da, 2-Da, 4-Da, and 6-Da increases over the mass of native peptide corresponded to the three-disulfide (native, N), two-disulfide (II), one-disulfide (Ia and Ib), 0-disulfide (completely reduced, R) species, respectively.

Peaks (labeled II, Ia, and Ib) were collected and alkylated by adding iodoacetamide before further purification by analytical RP-HPLC. There was a 58-Da shift from the original molecular weight after the alkyl group was added to single free thiol upon alkylation. The masses of the three alkylated isomers determined by MALDI-TOF corresponded to the above mass results very well (data not shown). In figure 3A, Pth-CM-Cys signals were observed in the chromatograms at the 2nd and 17th cycles after Edman degradation of alkylated peak II, while no Pth-CM-Cys signals were shown at other cysteine cycles. The above result indicated that the only reduced disulfide bridge was Cys2-Cys17. When sequencing alkylated peak Ia, Pth-CM-Cys signals were observed only in the chromatograms at the 2nd, 9th, 17th, and 24th cycles in the HPLC profiles of cysteine cycles (fig. 3B), indicating that Cys16 was still linked to Cys31 by a disulfide bridge. By a process of elimination, the third disulfide bridge must be between Cys9 and Cys24, which is in agreement with the results of sequencing alkylated peak Ib (data not shown). All these results indicate that the disulfide linkage of HNTX-IV is Cys2-Cys17, Cys9-Cys24, and Cys16-Cys31 (named the 1-4, 2-5, 3-6 disulfide pattern) (fig. 4).

Sequence comparison of HNTX-IV with HWTX-IV [9] (fig. 4) revealed 80% sequence identity and only seven residues difference with three non-conserved substitutions: the residues Glu4, Ile5, Phe6, Ala8, Lys21, Thr28, and Gln34 in HWTX-IV are replaced by Gly4, Phe5, Gly6, Gly8, Gln21, His28, and Glu34 in HNTX-IV, respectively. The disulfide linkage in HWTX-IV was assigned experimentally to adopt the same disulfide bridge pattern as in HNTX-IV [9]. The three-dimensional structure of HWTX-IV was determined by 2D-NMR studies as an ICK structural motif containing two β sheets (Lue22-Ser25 and Trp30-Tyr33) and four β turns (Glu4-Lys7, Pro11-Asp14, Cys17-Ser20, and Arg26-Arg27)

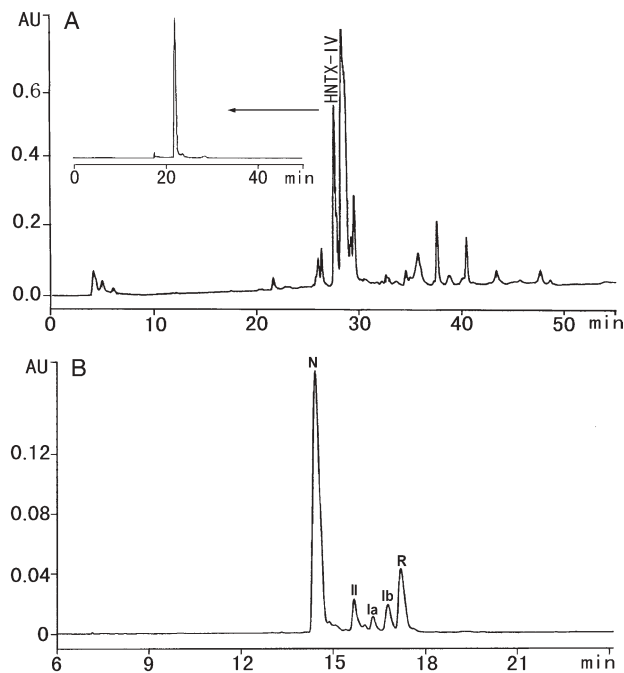


Figure 1. (A) HPLC chromatography of the *S. hainana* crude venom on a Vydac C18 column (300 Å, 4.6 \times 250 mm). The peptides were eluted using a linear gradient of 5–40% acetonitrile/0.1% TFA for 50 min at a flow rate of 1.0 ml/min. The inset shows that the further purification of HNTX-IV on analytical RP-HPLC yielded a single peak. (B) Analytical RP-HPLC profile of partial reduction of HNTX-IV by TCEP. Five chromatographic peaks represent intact HNTX-IV (N), the intermediate with two disulfide bridges (II), intermediates with one disulfide bridge (Ia and Ib), and completely reduced peptide (R), respectively.

(Protein Data Bank code: 1MB6). Due to the significant sequence identity, HNTX-IV should share a homologous three-dimensional structure with that of HWTX-IV.

The amino acid sequence of HNTX-IV was also used to search the protein database for possible homologues using an online BLAST search (www.ncbi.nlm.nih.gov/blast). Amino acid sequence alignment showed that HNTX-IV has considerable sequence identity with other spider peptide toxins (fig. 2A): HWTX-I [20] isolated from venom of the spider *S. huwena* (44% identity), TXP5 [21] from *Brachypelma smithii* (37% identity), SGTx1 [22] from *Scodra griseipes* (36% identity), and HaTX1 and HaTX2 [23] from *Grammostola spatulata* (32% identity). Moreover, these peptides share a similar disulfide bridge pattern, and their three-dimensional structures were reported to be an ICK motif [1, 24, 25]. In terms of amino acid sequence, they satisfied the well-known consensus sequence: $CX_{3-7}CX_{3-9}CX_{0-5}CX_{1-5}CX_{4-20}C$, where X can be any amino acid residue [2]. Relative to the sequence of HNTX-IV and HWTX-IV, since six amino acid residues were inserted between the disulfide bridge Cys17-Cys24, the fourth gap could be expanded to X_{1-6} . The longer sequence between Cys17-Cys24 just extends the loop between the two residues.

Biological assays

The intra-peritoneal LD_{50} in mice of HNTX-IV was 0.2 ± 0.07 mg/kg body weight ($n=4$). The symptoms were excitation, gasping, and spastic paralysis. However, no visible symptoms or behavioral changes were observed after injection of HNTX-IV into cockroaches (intra-abdominal dose 200 μ g/g body weight), which indicated that HNTX-IV was vertebrate selective. The twitch tension of phrenic preparations induced by electrical

stimulation of nerves could be blocked by 1.0 μ mol/l HNTX-IV within 18.1 ± 2.0 min ($n=6$) (data not shown). After block, the direct electrical stimulation of muscle was unaffected, which indicated that HNTX-IV did not affect the ion channels in skeletal muscle.

Electrophysiological studies

Electrophysiological studies showed that, under whole-cell voltage-clamp conditions, HWTX-IV can block TTX-S voltage-gated sodium channels in a similar manner to TTX with an IC_{50} of 30 nmol/l, while it had no effect on TTX-R sodium channels in DRG neurons [9]. Based on the high degree of sequence identity with HWTX-IV, we carried out patch clamp experiments to investigate the activity of HNTX-IV on the sodium channels in DRG neurons. Two types of sodium channels (TTX-S and TTX-R) exist on DRG neurons [26]. TTX-S sodium currents activate and inactivate more quickly than TTX-R sodium currents. TTX at a dose of around 200 nmol/l can eliminate TTX-S sodium currents, but TTX-R sodium currents remain almost unchanged at that dose. Larger DRG cells from older animals tended to express TTX-S currents while smaller cells tend to express slow TTX-R currents [19, 26].

Under whole-cell conditions, the inward sodium currents were elicited by a series of 20-ms depolarizations from a holding potential of -80 mV to 70 mV in 10-mV steps. Figure 5A shows that 100 nmol/l HNTX-IV blocked about 50% TTX-S sodium currents. The complete block of TTX-S sodium currents occurred at the HNTX-IV concentration of 10 μ mol/l (fig. 5A). At this concentration, this toxin had no detectable effects on the TTX-R sodium currents (fig. 5B). Effects of HNTX-IV on the current-voltage relationship are illustrated in figure 5C.

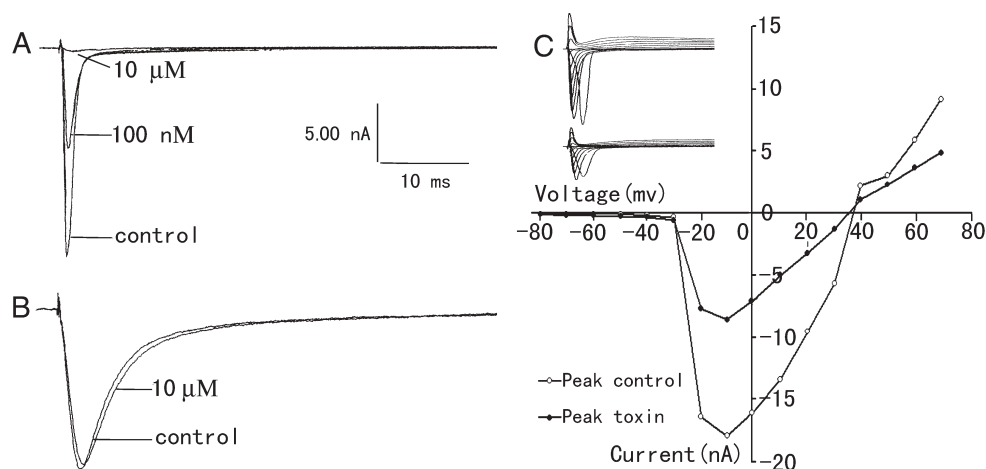


Figure 5. Different effects of HNTX-IV on TTX-R and TTX-S sodium currents in rat DRG neurons. Separation of TTX-R from TTX-S sodium currents was as described in Materials and Methods. (A) 100 nmol/l and 10 μ mol/l HNTX-IV blocked about 50% and complete TTX-S sodium currents, respectively. (B) 10 μ mol/l HNTX-IV has no effect on TTX-R sodium currents. (C) Partial reduction of TTX-S sodium currents by 100 nmol/l toxin showed that the block is not associated with the current-voltage relationships. The inset shows the control current trace and the partially blocked sodium currents.

As can be seen from the current-voltage curve, the block was not associated with a change in the current-voltage relationship. The experiments with six HNTX-IV concentrations (from 0.1 nmol/l to 10 μ mol/l with a tenfold increment) showed that the block of HNTX-IV on TTX-S sodium currents was concentration dependent. The IC_{50} calculated from data collected from 4–11 DRG neurons was about 34 nmol/l (fig. 6). All these results suggested the HNTX-IV has a similar activity to HWTX-IV. We concluded that the seven non-conserved residues between HWTX-IV and HNTX-IV are not critical for targeting of the two toxins to sodium channels.

Voltage-gated sodium channels play important roles in initiating action potentials and nervous influx conduction in sensory nerves. They are the molecular targets for several types of neurotoxins that act at six or more distinct receptor sites [11]. Several spider toxins have been confirmed to interact with sodium channels. The μ -agatoxins (μ -Aga-I to VI) were isolated from venom of the spider *Agelenopsis aperta*, and induced a shift of the curve of insect sodium channel activation to more negative potentials [27, 28]. The δ -atracotoxins (Hv1a, Hv1b, and Ar1a) from the venom of Australian funnel-web spiders are peptide toxins that slow sodium current inactivation by interacting with neurotoxin receptor site 3 of voltage-gated sodium channels [19, 29, 30]; and PhTx2 (Tx2–5 and 2–6), isolated from the venom of *Phoneutria nigriventer*, can modify sodium current inactivation and activation in frog skeletal muscle [31, 32]. Unlike these toxins, HNTX-IV and HWTX-IV had no effect on the activation and inactivation kinetics of TTX-S sodium channels, and

their actions on TTX-S currents are very similar to that of TTX [11]. We presume that the two toxins are pore-blocking toxins, which act at receptor site 1 on TTX-S voltage-gated sodium channels, in the same manner as TTX [10–12]. Thus, HNTX-IV and HWTX-IV define a new family of spider toxins affecting sodium channels, which should prove to be novel ligands useful in the investigation of the multiple molecular forms of voltage-gated sodium channels

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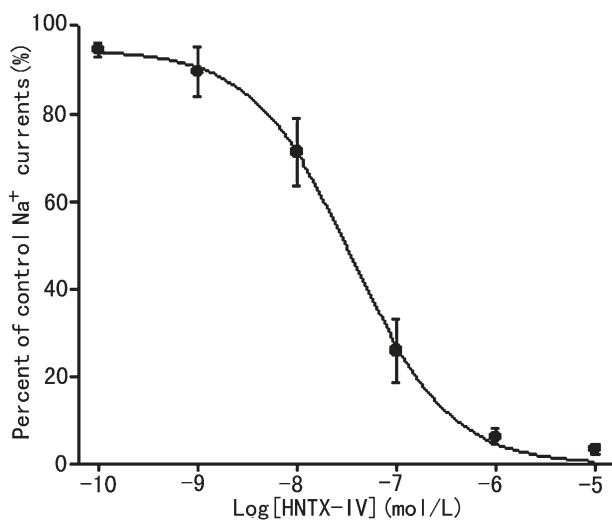


Figure 6. Concentration-dependent inhibition of HNTX-IV on TTX-S sodium current. HNTX-IV at doses from 0.1 nmol/l to 10 μ mol/l with a tenfold increment was applied. Cells with a run-down of sodium current above 5% in 3 min were excluded from further statistics. Block was determined after toxin had been applied for more than 1 min. Data represent the mean \pm SE of 4–11 cells per point.

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