



Purification and characterization of Hainantoxin-V, a tetrodotoxin-sensitive sodium channel inhibitor from the venom of the spider *Selenocosmia hainana*

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Abstract

A neurotoxic peptide, named Hainantoxin-V (HNTX-V), was isolated from the venom of the Chinese bird spider *Selenocosmia hainana*. The complete amino acid sequence of HNTX-V has been determined by Edman degradation and found to contain 35 amino acid residues with three disulfide bonds. Under whole-cell patch-clamp mode, HNTX-V was proved to inhibit the tetrodotoxin-sensitive (TTX-S) sodium currents while it had no any effects on tetrodotoxin-resistant (TTX-R) sodium currents on adult rat dorsal root ganglion neurons. The inhibition of TTX-S sodium currents by HNTX-V was tested to be concentrate-dependent with the IC_{50} value of 42.3 nM. It did not affect the activation and inactivation kinetics of currents and did not have the effect on the active threshold of sodium channels and the voltage of peak inward currents. However, 100 nM HNTX-V caused a 7.7 mV hyperpolarizing shift in the voltage midpoint of steady-state sodium channel inactivation. The results indicated that HNTX-V inhibited mammalian voltage-gated sodium channels through a novel mechanism distinct from other spider toxins such as δ -ACTXs, μ -agatoxins I–VI which bind to receptor site three to slow the inactivation kinetics of sodium currents.

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Keywords: Spider; Sodium current; Whole-cell recording; Dorsal root ganglion

1. Introduction

Two species of Chinese bird spiders (*Selenocosmia huwena* and *Selenocosmia hainana*) have been found in the south area of China. Both of them can kill insects and some small vertebrates with their venoms. The crude venom of *S. huwena*, distributed mainly in the hilly areas of Yunnan and Guangxi in the south of China (Wang and Peng, 1993), contains a mixture of compounds with different types of biology activity (Liang et al., 1993a,b). In our previous work, three polypeptide toxins: Huwentoxin-I (HWTX-I) (Liang et al., 1993a,b, Huwentoxin-II (HWTX-II) (Shu and Liang, 1999) and *S. huwena* lectin-I (SHL-I) (Lü et al., 1999), which have 32–37 amino acid residues with three

disulfide bonds, have been isolated and characterized from the venom of the spider. The pattern of disulfide bridge is C1–C4, C2–C5, C3–C6 in both HWTX-I and SHL-I while that is C1–C5, C2–C3, C4–C6 in HWTX-II (Shu and Liang, 2001). HWTX-I and HWTX-II block the neuromuscular transmission in an isolated mouse phrenic nerve-diaphragm preparation, but only HWTX-II reversibly paralyze cockroaches (Shu and Liang, 1999). Further studies show that HWTX-I is a potent inhibitor of N-type calcium channel in the presynaptic membrane (Peng et al., 2001). The mechanism of activity for HWTX-II is unclear. SHL-I agglutinates human and mice erythrocytes at a minimum concentration of 125 and 31 μ g/ml, but it shows very low toxicity in both mammals and insects (Lü et al., 1999). The three-dimensional solution structures of these polypeptides have been determined using NMR spectroscopy. HWTX-I and SHL-I adopt an inhibitor cystine knot motif consisting of a small three-stranded antiparallel

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β -sheet and a knot of three disulfide bonds which have been adopted by several other small proteins such as ω -conotoxins, inhibitory polypeptides and gurmarin (Lü et al., 1999; Qu et al., 1997). However, for the special disulfide bridge linkage HWTX-II adopts a scaffold different from the cystine knot motif and forms two turns and a double-stranded antiparallel β -sheet (Shu and Liang, 2002).

Another Chinese bird spider, *S. hainana*, is distributed mainly in Tongshi county, Hainan province of South China (Liang et al., 1999) and its crude venom can inhibit tetrodotoxin-sensitive (TTX-S) sodium currents induced on neuroblastoma \times glioma hybrid (NG108-15) cells. The IC_{50} value of inhibition is 1.8 mg/l (Xiao et al., 2001). Hainantoxin-I (HNTX-I) is the most abundant component in the venom and has 33 amino acid residues with six cysteines (Liang et al., 1999), although HNTX-I does not show any significant toxicity in both vertebrate and insects. In this study, we report the isolation and characterization of a 3972 Da polypeptide toxin, named Hainantoxin-V (HNTX-V). We found that nanomolar concentration of HNTX-V evidently inhibited TTX-S sodium currents on adult rat dorsal root ganglion (DRG) neurons.

2. Materials and methods

2.1. Venom and animals

The venom from the female adult *S. hainana* was collected as described in our laboratory earlier (Liang et al., 1999). Sprague-Dawley (SD) rats and Kunming albino mice were obtained from Hunan Medical University.

2.2. Toxin purification and sequencing

The venom was fractionated using ion-exchange high performance liquid chromatography (HPLC) and reverse-phase HPLC followed. Lyophilized venom (10 mg in 2 ml in distilled water) was applied to a Waters protein-Pak CM 8 H column (5 mm \times 50 mm) initially equilibrated with 0.2 M sodium phosphate buffer, pH 6.25 (buffer A). Then the column was eluted with a linear gradient of 0–90% of buffer B (2 M sodium chloride, 0.2 M sodium phosphate, pH 6.25) over 90 min at a flow rate of 3.0 ml/min. The aimed fraction, which was collected in ion-exchange HPLC, was then applied to a Vydac C18 analytical rpHPLC column (218TP54, 4.6 mm \times 250 mm) and eluted at a flow rate of 0.7 ml/min by a linear gradient of 0–40% of buffer B (acetonitrile containing 0.1% v/v trifluoroacetic acid (TFA)) over 50 min after an equilibrium period of 3 min with buffer A (distilled water containing 0.1% v/v TFA). The molecular mass was determined by matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry (MALDI-TOF MS) on a Bruker ProFlex-III mass spectrometer. Prior to amino acid sequencing, the toxin was reduced by DTT and then alkylated by iodoacetamide as described previously (Shu and Liang, 1999). The alkylated toxin was purified using reverse-phase HPLC as described earlier, then the entire amino acid sequence was obtained from a single sequencing run on an Applied Biosystem/Perkin Elmer Procise 491-A protein sequencer.

2.3. Cell isolation procedure

Rat DRG neurons were acutely dissociated and maintained in a short-term primary culture using the method described by Wang et al. (2001). Briefly, 30-day adult SD rats of either sex were killed by decapitation and the dorsal root ganglia were removed quickly from the spinal cord, and after having been cut into smaller tissues as possible, they were transferred into Dulbecco's modified eagle's medium (DMEM) 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. After transferred into 35 mm culture dishes (Corning, Sigma), the DRG cells were incubated in CO₂ incubator (5% CO₂, 95% air, 37 °C) for 1–4 h before patch-clamp experiment.

2.4. Electrophysiological studies

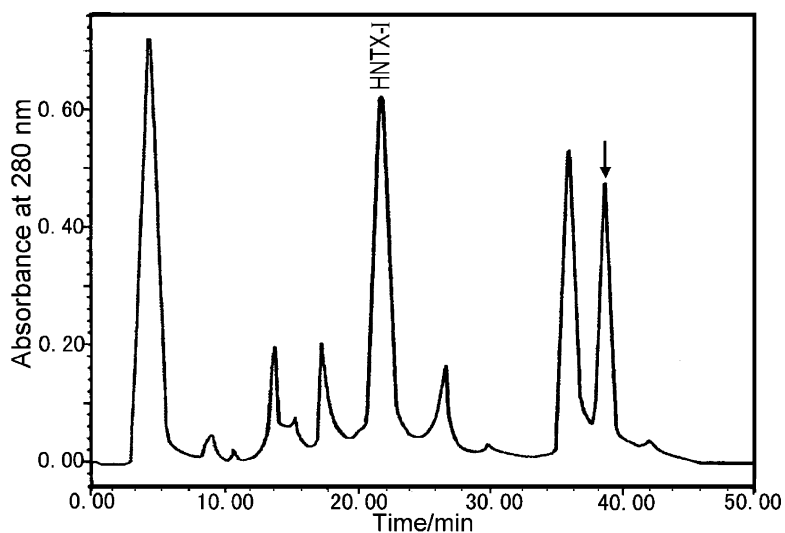
Sodium currents, which were filtered at 10 kHz and digitized at 3 kHz with a EPC-9 patch-clamp amplifier (HEKA Electronics, Germany), were recorded at room temperature (20–25 °C). Micropipettes (2–3 μ m diameter) were pulled from borosilicate glass capillary tubing by using a two-step vertical puller (PC-10, Narishige, Olypmus) and heat-polished with a microforge (MF-900, Narishige). The resistances of micropipettes were 1–2 M Ω after filled with internal solution contained (in mM): CsF 135, NaCl 10, HEPES 5, with the pH adjusted to 7.0 with 1 M CsOH. The external bathing solution contained (in mM): NaCl 30, CsCl 5, D-glucose 25, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, tetraethylammonium (TEA) chloride 20, tetramethylammonium chloride 70, with the pH adjusted to 7.40 with 1 M TEA hydroxide (Nicholson et al., 1998). The osmolarities of both internal solution and external solution were adjusted to 290–300 mOsm/l with sucrose. An Ag–AgCl pipette/3 M KCl-agar bridge was introduced between reference electrode and bathing solution to avoid disturbing the composition of the external solution. Capacitative and linear leak currents were digitally subtracted by P/4 procedures. Spider toxin was dissolved in external solution and about 10 μ l volume were applied by pressure injection with a microinjector (IM-5B, Narishige). All chemical reagents were purchased from Sigma.

Large DRG cells with diameters of 20–40 μm were selected for experiments, for larger DRG cells from older animals tend to express fast TTX-S sodium currents while smaller ones (10–20 μm) tended to express slow TTX-resistant (TTX-R) sodium currents (Su et al., 1999). TTX (200 nM) added in external solution was used to separate TTX-R sodium currents from TTX-S sodium currents. After establishing the whole-cell recording configuration, experiments did not commence for a period of more than 10 min in order to allow adequate equilibration between the micropipette solution and the cell interior.

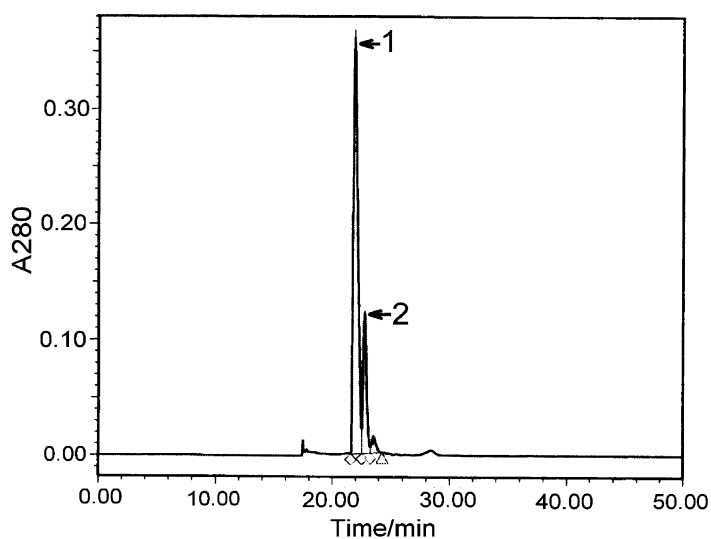
3. Results

3.1. Isolation and purification of HNTX-V

Fig. 1(A) shows a typical ion-exchange HPLC chromatogram of the venom from the Chinese bird spider *S. hainana*. There were eight evident absorbance peaks among which our interesting peak was the last one with retention time of about 42 min. By using C18 rpHPLC further purification of the peak yielded two different components (Fig. 1(B)). The molecular mass of peak 1 labeled in Fig. 1(B) was determined to be 3989 Da by MALDI-TOF MS while that of peak 2 was



A



B

Fig. 1. (A) Ion-exchange HPLC of crude *S. hainana* venom. The wanted peak was the last one labeled by arrow. (B) Purification of HNTX-V by reverse-phase HPLC. There were two absorbed peaks among which the first labeled by 1 was HNTX-IV while the next labeled by 2 was HNTX-V.

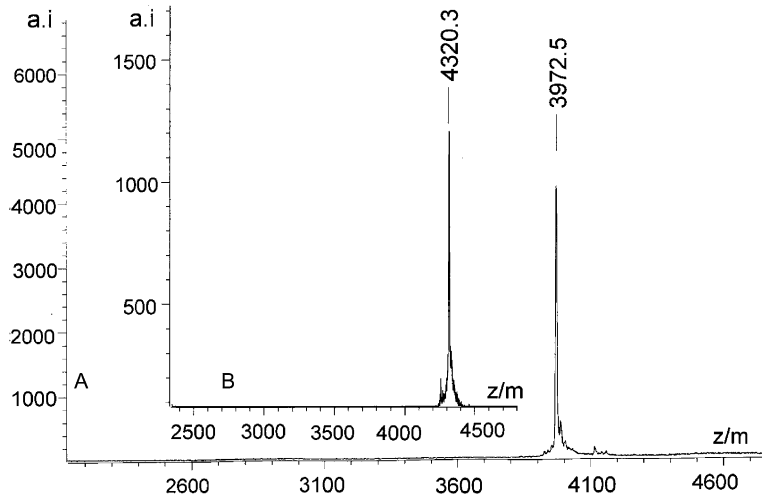


Fig. 2. MALDI-TOF mass spectra analysis of native HNTX-V (A) and alkylated HNTX-V by iodoacetamide (B).

determined to be 3972 Da (Fig. 2(A)). The former is Hainantoxin-IV (HNTX-IV) and its sequence is not determined. So the latter was named as Hainantoxin-V (HNTX-V).

3.2. Sequencing of HNTX-V

After having been reduced by DTT and alkylated by iodoacetamide, the molecular mass of the toxin became to be 4320 Da (Fig. 2(B)). This indicated that there were six cysteines and three disulfide bonds in HNTX-V. Determining amino acid sequence analysis of alkylated HNTX-V was performed in a single sequence run with 37 cycles. After cycle 35 (I) two blank cycles were produced, suggesting that HNTX-V contained 35 amino acid residues. The complete amino acid residues of HNTX-V was determined

to be $\text{NH}_2\text{-ECLGFGKGCNPSNDQCCKSANLVCSRKHRWCKYEI-COOH}$. The theoretical molecular mass of the sequence, in which three disulfide bonds were formed among the six cysteines, was consistent with the exactly mass of 3972 Da determined by MALDI-TOF MS.

3.3. Effects of HNTX-V on sodium currents

HNTX-V has high sequence identity with that of HWTX-IV which inhibited TTX-S sodium channels on adult rat DRG neurons (Fig. 3), suggesting that HNTX-V maybe affect sodium currents, too. The effects of HNTX-V on sodium currents were shown in Fig. 4: under voltage-clamp conditions, fast TTX-S sodium currents which were activated and inactivated completely within 2 ms were

Toxin	amino acid sequence	identity(%)	references
HNTX-V	1 ECLGFGKGCNPSNDQCCKSANLVCSRKHRWCKYEI 30	100	
HNTX-I	ECKGFGKSCVPGKNECCSGYA-CNSRDKWCEPIL	42	Liang et al., 1999
HWTX-I	ACKGVFDACPTPGKNECCP-NRVCSDKHKWCKWKL	36	Liang et al., 1993
HWTX-IV	ECL EIFKGCNPSNDQCCKSSKLVCSRKTRWCKYQI	77	Peng et al., 2002
SHL-I	GCLGD-KCDYNNG-CCSGY-VCSRTWKWCVLGPW	47	Lü et al., 1999

Disulfide bonding

Fig. 3. Comparison of the amino acid sequences of HNTX-V, HNTX-I, HWTX-I, HWTX-IV and SHL-I. The identical conserved residues in six toxins were shaded in black. HNTX-V and HNTX-I were isolated from the Chinese bird spider *S. hainana*. HWTX-I, HWTX-IV and SHL-I were isolated from another Chinese bird spider *S. huwena*. The disulfide bridge patterns of these peptides is C1–C4, C2–C5 and C3–C6. For six cysteines are conserved restrictedly at the same positions in the six peptides, it is assumed that HNTX-V has the same disulfide framework.

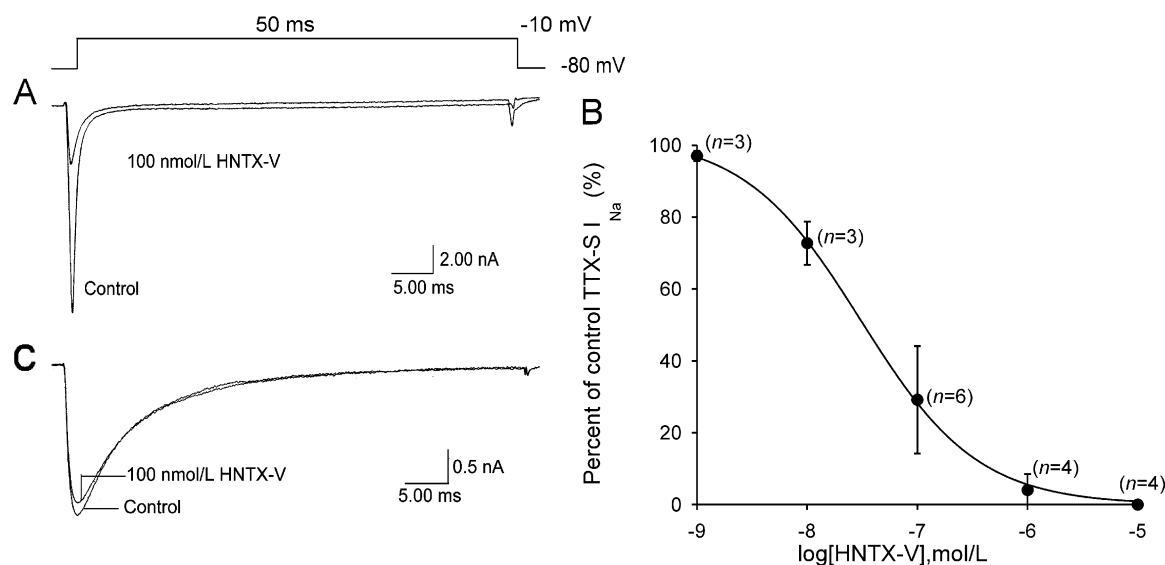


Fig. 4. Effects of HNTX-V on the TTX-S (A) and TTX-R sodium currents (C) and the concentration-dependent block of TTX-S sodium currents (B). Currents were induced by a 50 ms depolarizing potential of -10 mV from a holding potential of -80 mV. In (B) Data points (mean \pm SD) show current relative to control.

induced on adult rat DRG neurons by a 50 ms depolarization of -10 mV from a holding potential of -80 mV. After added 100 nM HNTX-V, the amplitude of TTX-S sodium currents were immediately depressed and a maximal depression was achieved within 4 min or so. Hundred nano molar of HNTX-V reduced the amplitude of TTX-S sodium currents by $63.5 \pm 13.2\%$ (mean \pm SD, $n = 6$) (Fig. 4(A)). The reduction was concentration-dependent with the IC_{50} value of 42.3 nM (Fig. 4(B)). After reduced by the neurotoxin, the shape of currents was similar to that of control, and the same results were found at other concentrations such as 10 nM and 1 μ M, indicating that HNTX-V did not affect the activation and inactivation kinetics of TTX-S sodium channels. In contrary, after 100 nM HNTX-V treatment for 10 min, the reduction of the amplitude and the change of the shape of TTX-R sodium currents were not observed ($n = 5$, Fig. 4(C)). The result indicated that HNTX-V have no effect on the inactivation and inactivation kinetics of TTX-R sodium channels.

3.4. Effects of HNTX-V on the I–V relationship of sodium currents

When the membrane potential of DRG cells were held at -80 mV, TTX-S sodium currents were initial elicited by a depolarization of -50 mV and reached a maximal amplitude by a depolarization of around -20 mV, while TTX-R ones were initial activated by a depolarization of -40 mV and reached a maximal amplitude by a depolarization of around -10 mV. After the depression of TTX-S sodium current amplitude by 100 nM HNTX-IV, there were no changes of both the threshold of activation and the active voltage of peak inward currents, and there was either no

shift of the membrane reverse potential ($n = 4$, Fig. 5(C)). On the other hand, 100 nM HNTX-V failed to affect the I–V relationship of TTX-R sodium currents ($n = 5$, Fig. 5(F)).

3.5. Effects of HNTX-V on channel inactivation

Using a standard two-pulse protocol as detailed in Fig. 6, we quantified the changes of the voltage dependence of steady-state sodium channel inactivation produced by HNTX-V. After 100 nM HNTX-V treatment for 4 min, when TTX-S sodium current amplitude were elicited by a 50 ms depolarization potential of -10 mV from different holding potentials: -130 , -90 , -70 and -50 mV, the reductions were enhanced ($n = 4$, Fig. 6(A)). It suggested HNTX-V changed the steady-state sodium channels inactivation. In order to quantify the changes, peak sodium currents recorded during the test pulse were normalized to the maximal value and plotted against the conditioning prepulse potential. The results showed 100 nM HNTX-V caused the half-maximal inactivation potential of sodium channels to shift approximate 7.7 mV in hyperpolarizing direction from -67.6 ± 0.8 to -75.3 ± 1.4 mV (mean \pm SD, $n = 5$) and the slop factor (k) was increased by 2.1 from 8.3 ± 0.6 mV of control to 10.4 ± 1.2 mV (Fig. 6(B)).

4. Discussion

Our previous works demonstrated that the venom of *S. hainana* inhibited voltage-gated sodium currents with the IC_{50} value of 1.8 mg/l on NG108-15 cells (Xiao et al., 2001), suggesting that its venom contain components altering

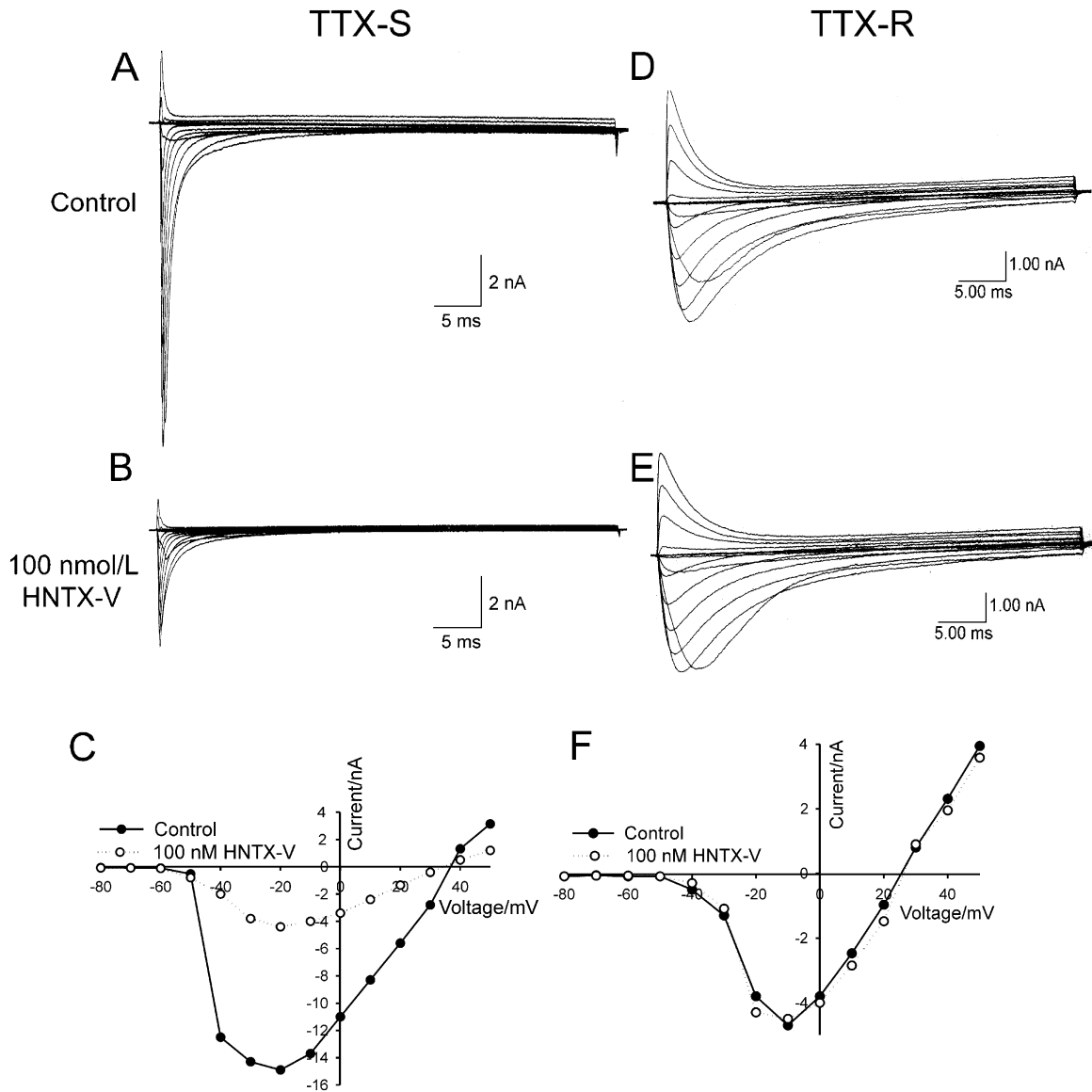


Fig. 5. Effects of HNTX-V on the current-voltage relationships of two kinds of sodium channels. TTX-S and TTX-R sodium currents were induced by 50 ms depolarizing steps to various potentials from a holding potential of -80 mV. Test potentials ranged from -80 to $+50$ mV. (C) was the relationship of TTX-S sodium currents before and after 100 nM HNTX-V treatment for 4 min (A, B) while (F) was that of TTX-R ones before and after 100 nM HNTX-V treatment for at least 10 min (D, E).

sodium channels. HNTX-I is the most abundant component in the venom and has 33 amino acid residues with six cysteines, but it does not show any significant toxicity in both vertebrate and insects (Liang et al., 1999). Hainantoxin-V (HNTX-V) is another component with the molecular mass of 3972 Da from the venom. Since the venom of *S. hainana* has no effect on outward delayed-rectifier potassium currents (Xiao et al., 2001), it is possible that HNTX-V fails to alter outward delayed-rectifier potassium channels. Further studies showed that the peptide toxin was a potent inhibitor of TTX-S sodium currents instead of TTX-R ones on

mammalian sensory neurons. When the membrane potential was held at -80 mV, low nanomolar concentration of HNTX-V reduced peak TTX-S sodium current amplitude. The reduction was in a concentration-dependent fashion with the IC_{50} value of 42.3 nM.

At present, there are almost 20 spider toxins such as δ -ACTXs (Szeto et al., 2000), μ -agatoxins I–VI (Skinner et al., 1989), PhTx2 (Araujo et al., 1993) found to affect sodium channels. These toxins are peptides consisted of 40–80 amino acid residues and four disulfide bonds. All of them slow the inactivation kinetics of currents in a similar

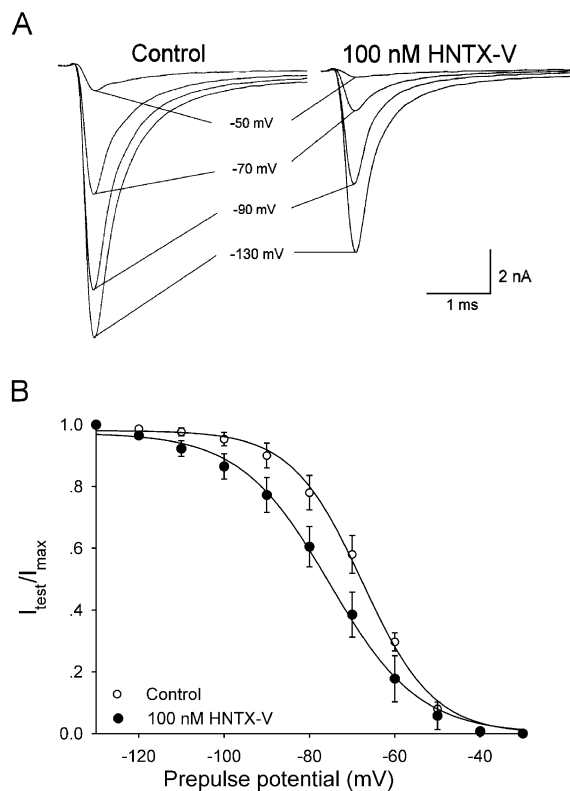


Fig. 6. Effects of HNTX-V on the steady-state inactivation of sodium channels on DRG neurons ($n = 5$). Sodium currents were induced by a 50 ms depolarizing potential of -10 mV from various prepulse potentials for 1 s which ranged from -130 to -30 mV at $+10$ mV increment. (A), Typical current traces were obtained following a 50 ms depolarization to -10 mV from prepulse potential of -130 , -90 , -70 and -50 mV. (B), The dots were fitted according to the Boltzmann equation: $I/I_{\text{max}} = 1/(1 + \exp((V - V_{1/2})/k))$ where V is holding voltage, $V_{1/2}$ is the voltage at which I is $0.5 I_{\text{max}}$, and k is the slope factor.

manner to α -scorpions (Wang and Strichartz, 1985) by binding to site three on sodium channels, so that they cause spontaneous contractions of isolated rat vas deferens smooth muscle (Szeto et al., 2000). Compared with those known spider peptide toxins, HNTX-V is a smaller one containing 35 amino acid residues and three disulfide bonds. The effects of the new spider toxin on TTX-S sodium channels from this study includes: (1) reducing the amplitude of currents; (2) no changes of the activation and inactivation kinetics of currents; (3) no shifts of the voltage dependence of activation. Therefore, we believe that HNTX-V inhibited mammalian voltage-gated sodium channels through a novel mechanism distinct from other spider toxins known so far such as δ -ACTXs. According to the categories that there are six neural receptor sites (1–6) on sodium channels (Cestele et al., 1995), we assume that HNTX-V maybe interact with neural receptor site one similar to TTX and μ -conotoxins (Shon et al., 1998). HNTX-V did not shift the normal

conductance of sodium currents on DRG neurons, implying that the toxin did not change the ion selectivity of channels. Like δ -ACTXs (Araujo et al., 1993; Nicholson et al., 1998), HNTX-V caused a shift of the steady-state inactivation curve for TTX-S sodium channels, but when the membrane potential was even held at -130 mV, the amplitude of currents did not recover completely to the control size after 100 nM HNTX-V treatment, suggesting that HNTX-V made profound alterations in the voltage dependence of steady-state inactivation. In addition, similar to any other spider toxins, HNTX-V did not have any modification of TTX-R sodium channels in mammalian sensory neurons.

The neural receptor site one, localizing in the extracellular end of the transmembrane pore of the channels, contains abundant negatively charged residues (Li et al., 2000). The positively charged guanidinium in STX, TTX and μ -conotoxins is critical for them to dock at the pore outside so that sodium currents do not pass the channels. HNTX-V has limited sequence identity with them and other spider toxins targeting sodium channels whilst it has high sequence identity with that of HNTX-I, isolated from the venom of *S. huwena*, HWTX-I, HWTX-IV and SHL-I isolated from the venom of *S. huwena* (Fig. 3). They are the natural mutants of HNTX-V and through the comparison of their sequences we speculate on the critical residues in HNTX-V. There are seven positively charged residues (four Lys, two Arg and one His) in HNTX-V and there are Lys-18 and Arg-29 conserved only in HWTX-IV, another sodium channel inhibitor (Peng et al., 2002). Although Lys-27 in HNTX-V is also conserved in HWTX-I at the corresponding position and HWTX-I lack the ability of affecting sodium channels, there is an acidic residue Asp-24 which neutralizes the alkalinescence of Lys-25 to counteract the residue's function in HWTX-I. In addition, four of the seven positively charged residues locate in Arg26–Arg29 where β -turn is formed in HWTX-I at the corresponding positions and Qu et al. (1997) suggested that the main functional residues in HWTX-I located in the β -turn (Asp24–Lys27). So we assume that Lys-27 and Arg-29 may be more important than other positively charged residues for inhibiting sodium channels in HNTX-V. The putative conclusion, however, need to be confirmed by the experiments of the alanine (Ala) substituted mutants of this toxin. Further investigations of the three dimensional structure and the function-structure relationship of HNTX-V are in progress in this laboratory.

Acknowledgements

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