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Natural mutations change the affinity of μ-theraphotoxin-Hhn2a to voltage-gated sodium channels

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ABSTRACT

μ-Theraphotoxin-Hhn2a (HNTX-III) isolated from the venom of the spider *Ornithoctonus hainana* is a selective antagonist of neuronal tetrodotoxin-sensitive (TTX-S) voltage-gated sodium channels (VGSCs). Intriguingly, previous transcriptomic study revealed HNTX-III family consists of more than 15 precursors, in which the 20th and 24th residues of the mature sequences are variable. Try20 and Ser24 of HNTX-III are mutated to His20 and Asn24 of other members, respectively. In addition, the alkaline residue His26 of the potent VGSC inhibitor HNTX-III is substituted by acidic residue Asp of the weak VGSC inhibitor HNTX-I. Therefore, four mutants of HNTX-III, HNTX-III-Y20H, -S24N, -H26D and -Y20H/24N, were synthesized to examine the effects of these natural mutations on the inhibitory activity of HNTX-III. They were subjected to an electrophysiological screening on five VGSC subtypes (Nav1.3–1.5, Nav1.7 and Nav1.8) expressed on HEK293 cells by whole-cell patch clamp. Like HNTX-III, all mutants only displayed inhibitory activity on Nav1.3 and Nav1.7 among the five subtypes, but the inhibitory potency was much lower than that of HNTX-III. Regarding Nav1.7, the IC_{50} values of HNTX-III-Y20H, -S24N, -H26D and -Y20H/S24N were increased by approximately 62-, 8.4-, 49- and 19.5-folds compared with that of HNTX-III, respectively. Similar data were obtained for Nav1.3. Our results provide new insights into the activity-related residues of HNTX-III at genic level. Furthermore, the reduced potency of the four mutants probably reflects natural selection might favor and reserve the most potent bioactivity of HNTX-III which is one of the most abundant fractions of the venom.

1. Introduction

Venoms from venomous animals, such as snake, scorpion, cone snail, spider, sea anemone et al., are complex cocktails composed of a variety of chemical compounds, including salts, small organic molecules, peptides and proteins, but the major components are small and disulfide-rich peptides and small proteins that have evolved for prey capture and/or defense (Saez et al., 2010; Dutertre and Lewis, 2010). These venom peptides target a diverse range of ion channels, cell receptors and enzymes in a wide range of vertebrate and invertebrate species (Dutertre and Lewis, 2010; Liang, 2008), which provides researchers highly potent and selective molecular probes and drug leads that have been proved invaluable in unraveling ion channel structure and function and clinical importance (Lewis and Garcia, 2003). Nevertheless, the mining of animal venoms is only the tip of the iceberg. For instance, more than 44,906 extant species of spiders have been described so far (Platnick, 2014), and a single venom may contain as many as hundreds of peptides, so that it has been conservatively estimated that >10 million bioactive peptides are likely to be present in the venoms of spiders (Escoubas et al., 2006). Actually, more than 32,000 ESTs of spider venom peptides derived from the spider cDNA libraries are deposited in GenBank database, but to date, only 916 peptide toxins from 85 spider species have been described in the database of ArachnoServer 2.0 (Herzig et al., 2011), with only 0.01% of this diversity having been characterized. Therefore, the investigation of venom peptides that exist naturally or at the level of genes will contribute to probe ion channels and develop peptide therapeutics.

The tarantula *Ornithoctonus hainana* is a very venomous spider found in the hilly areas of Hainan province in southern China (Tang et al., 2010). Its venom contains a variety of toxic

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components with different pharmacological properties. A total of 192 mature sequences were identified by a venomic strategy through a combination of transcriptomic, peptidomic and genomic techniques, which provides a dataset for detailed venom peptide investigations. Of the 192 mature sequences, HNTX-III family contains more than 15 precursors, in which the 20th and 24th residues of the mature sequences are much variable (Fig. 1A), namely Try20 and Ser24 of HNTX-III are mutated to His20 and Asn24 of other members, respectively. HNTX-III is one of main fractions in the venom and a highly potent and selective antagonist of neuronal TTX-S VGSCs (Xiao and Laing, 2003; Liu et al., 2013), while those natural mutants were not detected at peptide level. Studies have indicated that natural mutations are often observed in many venom peptide families from venomous animals, such as scorpion, snake, cone snail, centipede, sea anemones and so on (Ma et al., 2012; Fry et al., 2008; Jacobsen et al., 2000; Yang et al., 2012; Peigneur et al., 2012). However, for most of these mutations, the pharmacological activities and biological significance have been rarely elucidated. On the other side, our previous studies showed that the residues located on the C-terminal are important for the inhibitory activity of HNTX-III on TTX-S VGSCs. HNTX-I, another high abundant toxin in the venom, also belongs to HNTX-III family, although both HNTX-I and HNTX-III have 55% sequence identity. One of the different residues of the two toxins is the 26th residue, acidic Asp26 for HNTX-I and basic His for HNTX-III. Compared with HNTX-III, HNTX-I is a very weak antagonist of TTX-S VGSCs (Li et al., 2003). Therefore, we also want to know if this residue substitution may contribute to the inhibitory potency difference.

In this study, we synthesized the four natural mutants of HNTX-III (named HNTX-III-Y20H, HNTX-III-S24N, HNTX-III-H26D and HNTX-III-Y20H/S24N) (Fig. 1B) and compared their potency on five VGSC subtypes (Nav1.3–1.5, Nav1.7 and Nav1.8) with that of HNTX-III. Our results indicate that, compared with HNTX-III, the four mutants all demonstrated greatly reduced inhibitory activity on Nav1.3 and Nav1.7, and were also ineffective on the other three subtypes, which indicates that the three residues, Try20, Ser24 and His26, might be critical for the inhibitory potency of HNTX-III. Meanwhile, we hypothesized natural selection might drive the spider to retain the most potent HNTX-III that is one of the most abundant fractions of the venom, which is applied by the spider in prey capture and/or defense.

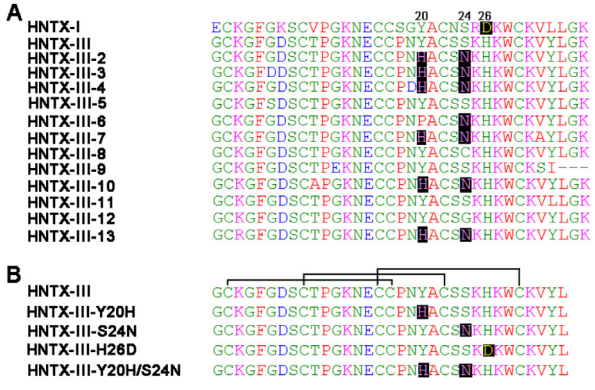

Fig. 1. The amino acid sequences of HNTX-III family and the four mutants. A. The amino acid sequences of HNTX-III family. The mutated residues, Try20, Ser24, His26, are shaded in black. B. The amino acid sequences of HNTX-III and the four mutants. The disulfide bonds are indicated above the sequences.

2. Materials and methods

2.1. Peptides synthesis, folding and purification

The five peptides were synthesized through an Fmoc/tert-butyl strategy and HOBt/TBTU/NMM coupling method by an automatic peptide synthesizer (PS3, PTI). Peptide synthesis was accomplished on a 0.1 mmol scale. The Fmoc-amino acids with side chain protection were purchased from GL Biochem Ltd (Shanghai, China). The terminal Fmoc group was removed by treatment with 25% piperidine/*N*, *N*-dimethylformamide (v/v). After the completion of synthesis, the peptide was cleaved from the resin with simultaneous removal of side chain protecting groups by treatment with reagent K (82.5% trifluoroacetic acid, 5% double distilled H_2O, 5% phenol, 5% thioanisole and 2.5% ethanedithiol) for 2 h at room temperature. The five peptides were precipitated in cold ether at 4 °C. Then, after centrifugation and washing once with cold ether, the peptides were dissolved in 20% acetic acid and lyophilized.

The reduced synthetic peptides were purified by semi-preparative RP-HPLC using a linear gradient of 10–60% eluant B (0.1% trifluoroacetic acid in acetonitrile) in eluant A (0.1% trifluoroacetic acid in double distilled H_2O) on a C18 column (Luna, 10 mm × 250 mm). The linear peptides were refolded and oxidized with glutathione, and purified according to the protocol described by Zhu et al. (Zhu et al., 2002). Briefly, the purified reduced peptides were refolded/oxidized in a buffer consisting of 0.1 M Tris–HCl and 0.1 M NaCl, pH 7.4, and containing 5 mM GSH and 0.5 mM GSSG for 24 h at room temperature and then fractionated by RP-HPLC.

2.2. MALDI-TOF MS analysis

The fractions from RP-HPLC separation were collected and their molecular weights were determined using MALDI-TOF-MS (Ultraflex I, Bruker Daltonics). A 1 μL of each peptide fraction was applied to the well overlaid with 1 μL CCA solution (oversaturated α-cyano-4-hydroxycinnamicacid dissolved in 50% acetonitrile and 0.1% TFA). The mixture was allowed to dry at room temperature. Ionization was achieved by irradiation with nitrogen laser (337 nm), with a 20 kV acceleration voltage.

2.3. Circular dichroism (CD) determination

CD spectra were obtained for the five peptides on a Jasco J-700 spectropolarimeter (Jasco, Osaka, Japan). The spectra were measured from 260 to 190 nm in 0.01 M sodium phosphate solution (pH 7.0) at room temperature, with a 1 mm path-length cell. Data were collected at 0.5 nm with a scan rate of 100 nm/min and a time constant of 1 s. Concentration of the toxins as determined by amino acid sequencing was 30 μM. The spectra were expressed as mean residue ellipticity (θ) in degrees per square centimeter per decimole.

2.4. Transient transfection

The Nav1.3–1.5, Nav1.7, or Nav1.8 channel plasmid and a plasmid for green fluorescent protein were transiently co-transfected into human embryonic kidney 293 (HEK293) cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. HEK293 cells were grown under standard tissue culture conditions (5% CO_2, 37 °C) in DMEM (10% FBS). A mixture of Lipofectamine, a channel plasmid and a green fluorescent protein reporter plasmid were added to a culture dish and were replaced by fresh medium after 4–6 h. Cells with green fluorescence were selected for whole-cell patch clamp recording at 24–36 h after transfection. For Na^+ current recording, the pipette