Peptides 34 (2012) 19–25

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

The effects of huwentoxin-I on the voltage-gated sodium channels of rat hippocampal and cockroach dorsal unpaired median neurons

Meichi Wang, Mingqiang Rong, Yucheng Xiao, Songping Liang*

Key Laboratory of Protein Chemistry and Developmental Biology of the Ministry of Education, The College of Life Science, Hunan Normal University, Changsha 410081, PR China

ARTICLE INFO

Article history:
Received 20 May 2011
Received in revised form 28 October 2011
Accepted 28 October 2011
Available online 7 November 2011

Keywords:
Tetrodotoxin-sensitive voltage-gated sodium channel
Dorsal root ganglion
Dorsal unpaired median
Hippocampal neuron

ABSTRACT

Huwentoxin-I (HWTX-I) is a 33-residue peptide isolated from the venom of *Ornithoctonus huwena* and could inhibit TTX-sensitive voltage-gated sodium channels and N-type calcium channels in mammalian dorsal root ganglion (DRG) neurons. However, the effects of HWTX-I on mammalian central neuronal and insect sodium channel subtypes remain unknown. In this study, we found that HWTX-I potently inhibited sodium channels in rat hippocampal and cockroach dorsal unpaired median (DUM) neurons with the IC_{50} values of 66.1 ± 5.2 and 4.80 ± 0.58 nM, respectively. Taken together with our previous work on DRG neurons ($IC_{50} \approx 55$ nM), the order of sodium channel sensitivity to HWTX-I inhibition was insect central DUM $\gg$ mammalian peripheral > mammalian central neurons. HWTX-I exhibited no effect on the steady-state activation and inactivation of sodium channels in rat hippocampal and cockroach DUM neurons.

1. Introduction

Voltage-gated sodium channels (VGSCs) are major contributors to the initiation and propagation of action potentials in excitable cells. They are composed of a functional pore-forming α-subunit (260 kDa) and up to two auxiliary β-subunits (β1–β4). Nine distinct VGSC α-subunit subtypes (Nav1.1–1.9) with over 75% amino acid sequence identity to each other have been cloned from mammals [21]. The subtypes Nav1.1–1.3 are principally found in central neurons, the subtypes Nav1.6–1.9 are predominantly distributed in peripheral neurons, and the subtypes Nav1.4–1.5 selectively express in skeletal and cardiac muscles, respectively [8].

Besides mammalian VGSCs, several insect VGSCs, e.g. Para/TipE and BSC1, have been cloned from Drosophila *melanogaster* [18] and German cockroach *Blattella germanica* [6]. Unsurprisingly, a number of plant and animal toxins have evolved to target VGSCs as a means to combat predators or to subdue or capture food sources [15]. Some animal toxins such as Tx4 (6-1) and δ-PaluIT are found to specifically target insect VGSCs without affecting vertebrate VGSCs [3–5,8–10]. Some toxins such as Hainantoxin-I (HNTX-I), Jingzhaotoxin-I (JZTX-I) and δ-atracotoxins (δ-ACTXs) target both vertebrate and invertebrate VGSCs [7,9,15,23].

Chinese bird spider *Ornithoctonus huwena* is distributed in the hilly area of Guangxi province in Southern China. Our previous work has determined the lethality of Huwentoxin-I (HWTX-I), a major component of *O. huwena* venom, through peripheral or direct injections to central nervous system [12]. HWTX-I could block N-type calcium channels and TTX-sensitive (TTX-S) VGSCs, but had no effect on TTX-resistant (TTX-R) VGSCs in mammalian peripheral neurons [19]. Recently, HWTX-I was found to selectively inhibit neuronal TTX-S VGSC subtypes by binding to domain II S3–S4 linker and trapping the domain II voltage sensor in the closed state [21]. However, the effects of HWTX-I on mammalian and insect central VGSC subtypes remain unknown. In this study, we investigated the action of HWTX-I on the VGSC subtypes from rat hippocampal and cockroach dorsal unpaired median (DUM) neurons.

2. Experimental procedures

2.1. Materials and animals

Sprague-Dawley rats were purchased from Xiangya School of Medicine, Central South University. All procedures conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of the Hunan Normal University College of Medicine. Cockroaches were from our laboratory stock colonies. All synthesis reagents were purchased from Chemassist Corp. Trifluoroacetic and α-cyano-4-hydroxycinnamic acids were from Sigma.

* Corresponding author. Tel.: +86 731 88872556; fax: +86 731 88861304.
E-mail address: liangsp@hunnu.edu.cn (S. Liang).

0196-9781/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2011.10.029

All other reagents were of analytical grade. Natural Huwentoxin-I was purified from freeze-dried crude *O. huwena* venom [12].

2.2. Synthesis, refolding, and purification of HWTX-I

HWTX-I was synthesized using an Fmoc (*N*-(9-fluorenylmethoxycarbonyl)/*tert*-butyl strategy and HOBt/TBTU/NMM [14]. We purified the primary synthetic product using semi-preparative Elite C18 column (10 mm × 250 mm). The linear gradient of 10–50% buffer B (analytic purity of ACN) over 40 min at a flow rate of 3 ml/min. The crude linear peptide was diluted to a final concentration of 30 μM by 0.1 M Tris–HCl solution (pH 8.0) containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione. The solution was stirred slowly at room temperature for 24 h and the folding reaction was monitored by reverse phase high performance liquid chromatography (4.6 mm × 250 mm Vydac C18colum) and analytic matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The natural and synthetic toxins were quantified by HPLC.

2.3. Acute isolation of dorsal root ganglion neurons

Dorsal root ganglion neurons were isolated from 180 to 200 g adult Sprague-Dawley rats of both sexes [23]. Dorsal roots and spinal nerves were removed from the spinal cord and transferred immediately into ice-cold Dulbecco's Modified Eagle's Medium (DMEM). Dorsal root ganglions were stripped of their connective tissue capsules, transferred into another ice-cold DMEM aliquot and minced with scissors. They were then transferred into 5 ml DMEM containing 1.5 mg trypsin and 3.5 mg collagenase and incubated at 34 °C for half an hour. 7.5 mg trypsin inhibitor was used to terminate enzyme treatment. The neurons were resuspended in 90% DMEM/10% newborn calf serum. Cultured neurons were kept at 37 °C in a CO_2 incubator containing 5% CO_2 and were used between 4 and 24 h after initiation of the culture.

2.4. Acute isolation of dorsal unpaired median neurons

Experiments were carried out on single dorsal unpaired median neurons acutely isolated from the adult cockroach *Periplaneta americana* [23]. Adult cockroaches (*P. americana*) were desheathed, and their abdominal ganglia were removed and then incubated in insect physiological solution (in mM): 90 NaCl, 6 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 HEPES, 140 glucose, pH 6.6 containing 1 mg/ml trypsin for 5 min. Following this, the ganglia were removed and stored in physiologic solution for 1 h to restore. The large dorsal unpaired median cells situated in the dorsal midline of the ganglia were separated using thin silver needles. The cell viability was assessed by microscopic observation: only those cells which are bright under phase contrast were used.

2.5. Culture of hippocampal neurons

Hippocampal neurons were obtained from embryonic rats (E18) [2]. Hippocampal tissues were dissected and treated with 0.25% trypsin in Ca^{2+}–Mg^{2+}-free Hank's Buffered Salt Solution at 37 °C for 15 min. Then dissociated by trituration with a glass Pasteur pipette and plated in 35 mm culture dishes. Approximately 35,000 cells in DMEM containing 10% fetal bovine serum were plated in the glass area of each dish (35 mm diameter). On the second day after plating, the culture medium was replaced by serum-free Neurobasal medium containing 2% B27 supplement. Neurons were maintained in a CO_2 incubator at 37 °C, and one-half volume of the culture medium was replaced with fresh Neurobasal medium containing 2% B27 supplement every other day. The neurons were used for electrophysiological studies after culture for 10 days.

2.6. Electrophysiological studies

Sodium currents were recorded using an EPC-9 patch clamp amplifier (HEKA Electronics, Germany). The micropipette solution for dorsal root ganglion neurons contained (in mM): 105 CsF, 35 NaCl, 10 HEPES, 10 EGTA (pH 7.4). The external solution contained (in mM): 50 NaCl, 100 TEA.Cl, 2 KCl, 1.5 $CaCl_2$, 10 HEPES, 1 $MgCl_2$, and 5 glucose (pH 7.4). The external solution for hippocampal neurons contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4). The micropipette solution for the hippocampal neurons contained (in mM): CsCl 35, CsF 105, EGTA 10, Hepes 10, $CaCl_2$ 1, $MgCl_2$ (pH 7.2). The external solution for insect dorsal unpaired median neurons contained (in mM): 80 NaCl, 30 TEA.Cl, 2 $CaCl_2$, 4 KCl, 10 HEPES, 10 glucose, 50 choline-Cl, and 1 4-AP (pH 6.8), and the micropipette internal solution for these neurons contained (in mM): 140 CsF, 2 $MgCl_2$, 10 EGTA, and 10 HEPES (pH 6.8). All experiments were conducted at room temperature (22–25 °C). Experimental data were acquired and analyzed using the program Pulse/Pulsefit 8.0. Leakage and capacitive currents were digitally subtracted with the P/4 protocol and series resistance was kept at 3–5 MΩ.

Channel conductance was calculated by the equation:

$$G(\mathrm{Na}) = \frac{I}{V - V_r}$$

where V is test potential and V_r is reversal potential. Both steady-state activation and inactivation were fitted with a Boltzmann function: $y = 1/(1 + \exp(V - V_{0.5})/k))$, where $V_{0.5}$ is the voltage for 50% activation or inactivation, and k is slope factor. Statistical data are presented as means ± S.E. All comparisons between means were tested for significance using Student's unpaired test.

3. Results

3.1. Synthesis, refolding, purification and identification of HWTX-I

In order to rule out the possibility that inhibition of sodium channels by HWTX-I results from the contamination, we chemically synthesized HWTX-I using solid-phase peptide synthesis method. As shown in Fig. 1A, the synthetic product was found to be a single major peak eluted at 18.8 min by reverse-phase high performance liquid chromatography (RP-HPLC). The molecular mass of purified peptide was 3756.511 Da, which is the same as the theoretical mass of linear (reduced) HWTX-I peptide (Fig. 1B). The oxidative refolding product of interest was eluted at the retention time of 29 min by RP-HPLC (Fig. 1C). MALDI-TOF mass analysis demonstrated that the mass was 6 Da less than that of linear peptide (Fig. 1D), indicating that three disulfide bonds were formed. When the mixture of natural and synthetic HWTX-I (1:1) was co-eluted on an analytical C18 column, only one single elution peak was detected in Fig. 2A, inferring that synthetic peptide has the same three-dimensional structure as natural HWTX-I. Electrophysiological experiments also showed that synthetic peptide, like natural HWTX-I, significantly inhibited TTX-S sodium channels in rat DRG neurons (Fig. 2B, inset). The IC_{50} of synthetic peptide was 35.5 ± 1.54 nM, which is close to the value of natural HWTX-I (40.7 ± 4.3 nM, Fig. 2B) [15]. Thus, our data indicated that inhibition of TTX-S sodium channels by HWTX-I results from its nature rather than from contamination.