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Huwentoxin-XVI, an analgesic, highly reversible mammalian N-type calcium channel antagonist from Chinese tarantula *Ornithoctonus huwena*

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ABSTRACT

N-type calcium channels play important roles in the control of neurotransmission release and transmission of pain signals to the central nervous system. Their selective inhibitors are believed to be potential drugs for treating chronic pain. In this study, a novel neurotoxin named Huwentoxin-XVI (HWTX-XVI) specific for N-type calcium channels was purified and characterized from the venom of Chinese tarantula *Ornithoctonus huwena*. HWTX-XVI is composed of 39 amino acid residues including six cysteines that constitute three disulfide bridges. HWTX-XVI could almost completely block the twitch response of rat vas deferens to low-frequency electrical stimulation. Electrophysiological assay indicated that HWTX-XVI specifically inhibited N-type calcium channels in rat dorsal root ganglion cells (IC_{50} ~60 nM). The inhibitory effect of HWTX-XVI on N-type calcium channel currents was dose-dependent and similar to that of CTx-GVIA and CTx-MVIIA. However, the three peptides exhibited markedly different degrees of reversibility after block. The toxin had no effect on voltage-gated T-type calcium channels, potassium channels or sodium channels. Intraperitoneal injection of the toxin HWTX-XVI to rats elicited significant analgesic responses to formalin-induced inflammation pain. Toxin treatment also changed withdrawal latency in hot plate tests. Intriguingly, we found that intramuscular injection of the toxin reduced mechanical allodynia induced by incisional injury in Von Frey test. Thus, our findings suggest that the analgesic potency of HWTX-XVI and its greater reversibility could contribute to the design of a novel potential analgesic agent with high potency and low side effects.

1. Introduction

Voltage-gated calcium (Cav) channels are found in presynaptic nerve terminals where they allow the entry of calcium into cells through activation of Cav channels directly affects membrane potential and contributes to electrical excitability, repetitive firing patterns, excitation-contraction coupling and gene expression. Native calcium channels are generally divided into low-threshold (T-type) and high threshold (L-, N-, P/Q- and R-types) by both their electrophysiological and pharmacological properties (Hofmann et al., 1994; Olivera et al., 1994; Snutch, 2005). N- and P/Q-type calcium channels seem to be critical for modulating neurotransmitter release (Yu et al., 2010; Zamponi et al., 2009, 2010). N-type calcium channel was the first dihydropyridine-insensitive isoforml identified, which was inhibited by ω-CTx-

Abbreviations: HWTX, Huwentoxin; ICK, inhibitor cystine knot; Cav channel, voltage-gated calcium channel; Nav channel, voltage-gated sodium channel; DRG, dorsal root ganglion; TTX, tetrodotoxin; TTX-R, TTX-resistant; TTX-S, TTX-sensitive; IC_{50}, median inhibitory concentration; Kv channel, voltage-gated potassium channel; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight.

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GVIA. The inhibition impairs neurotransmitter release in a series of mammalian preparations (Fox et al., 1987a, b; Olivera et al., 1994). N-type calcium channels are often highly expressed both in dorsal root ganglia cell bodies and in the synaptic terminals projecting to dorsal horn of the spinal cord (McCallum et al., 2011). These primary afferents are involved in the sensation of a number of noxious painful stimulation including mechanical, thermal and inflammatory. Moreover, block of N-type calcium currents inhibits the release of neuropeptides substance P and calcitonin gene-related peptide (CGRP) from sensory neurons (Baillie et al., 2012; Takasusuki and Yaksh, 2011; Wilson et al., 2012).

Although a number of central and peripheral splice variants of the N-type calcium channels have been reported so far (Lin et al., 1997), the roles of them in normal and disease states remain to be understood with the aid of high specific ligands. The venom of poisonous predators has been a great source of novel peptides inhibiting calcium and sodium channels with a remarkable potential for use in agriculture and in medicine (Adams et al., 2012; Estrada et al., 2007; Kauferstein et al., 2005). For example, ω-conotoxin CVID, a specific blocker of N-type calcium channels isolated from the venom of the marine snail *conus catus*, as an analgesic drug is currently in Phase II clinical trials in Australia. It is hoped that this peptide may overcome some of the side-effects associated with MVIIA use (Adams et al., 2003; Lewis et al., 2000; Penn and Paice, 2000; Smith et al., 2002). HWTX-I, is also a potent N-type calcium channels blocker isolated from the venom of spider *Ornithoctonus huwena* (Wang et al., 2007).

It is well-known that spiders are among the oldest animals on the earth. Spider venoms are known to contain several classes of toxins that are used as an efficient means to capture their prey or to defend themselves against predators. These toxins are of interest as tools for studying neurophysiology and as potential lead structures for pharmaceutics and insecticides (Escoubas and King, 2009; King et al., 2008; King and Hardy, 2013; Klint et al., 2012; Windley et al., 2012). The Chinese tarantula, *O. huwena*, is one of the most venomous spiders in China. The venom of this spider contains a mixture of peptide compounds with different types of biological activity. More than ten peptide toxins have been purified and characterized from the venom of this spider, with several peptide toxins exhibiting structural similarity but high functional diversity (Liang, 2004; Peng et al., 2002; Wang et al., 2007).

In this work, we reported the isolation, functional characterization and analgesic effect of Huwentoxin-XVI (HWTX-XVI), a novel neurotoxin from the venom of Chinese tarantula *O. huwena*. The toxin is a 39-residue polypeptide with three disulfide bonds and the C-terminal residue is not amidated. The toxin could block the twitch response of rat vas deferens to low-frequency electrical stimulation. We found that the toxin selectively inhibited N-type calcium channels in DRG neurons but had no effect on voltage-gated T-type calcium channels, potassium channels or sodium channels. Intramuscular or intraperitoneal injection of the toxin has potent analgesic effects when tested in two pain assays in rats.

2. Methods

2.1. Materials and animals

Female spiders, Sprague–Dawley rats and *Xenopus laevis* were maintained in the Laboratory of Protein Chemistry of Hunan Normal University. Adult Sprague–Dawley rats of either sex were used in almost all of rat experiments except vas Deferens assays. All sequencing reagents were purchased from Applied Biosystems (Foster City, CA, USA). Trypsin (type III) and collagenase (type IA) were from sigma. Trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid (CCA) were from Sigma. All other reagents are analytical grade.

2.2. Ethics statement

Our research was conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Central Southern University, P. R. China. All surgery was performed under sodium pentobarbital or metacaine anesthesia, and all efforts were made to minimize suffering.

2.3. Toxin purification and sequencing

The venom was obtained by electrical stimulation of female tarantula *O. huwena*, and the freeze-dried crude venom was stored at −20 °C prior to analysis. The venom was purified by ion-exchange chromatography and reversed-phase high performance liquid chromatography (RP-HPLC) as previous described (Peng et al., 2002). The molecular mass and purity of toxin were using a Voyager-DETM STR MALDI-TOF mass spectrometer of ABI Company. Ionization was achieved by irradiation with a nitrogen laser (337 nm), with a 20-kV acceleration voltage. α-Cyano-4-hydroxycinnamic acid (CCA) was used as matrix. Once purified to >98% homogeneity (assessed by reverse-phase HPLC and mass spectrometry), peptide was lyophilized and stored at −20 °C until further use. The entire amino acid sequence was obtained by automated Edman degradation using an Applied Biosystems 491 pulsed-liquid-phase sequencer from Applied Biosystems Inc.

2.4. HWTX-XVI cDNA cloning and characterization

The full-length HWTX-XVI cDNA was obtained from a directional full-length cDNA library of the venomous glands of the spider *O. huwena*. The total RNA was extracted with Trizol Reagent (Invitrogen). The cDNA library was constructed using a standard protocol. Approximately 1000 clones with PCR fragments ≥400 bp in length were submitted for sequencing on an ABI 3730 automatic DNA sequencer (completed by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd). The cDNA sequences were derived by removal of the vector, primer sequences and poly (A) tails. The signal peptide was predicted with the SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/), and the pro-peptide cleavage site was ascertained from the known start site of previously characterized mature toxins.

2.5. Biological assays

The toxicity of HWTX-XVI was qualitatively assayed by intraperitoneal injection into 18–20 g mice of both sexes and intra-abdominal injection into adult male cockroaches (*P. americana*) with body weights of 0.3–0.5 g using 20 μl solutions [in 0.9% (w/v) normal saline]. Vas Deferens assays were performed according to the method of Liang et al. (2000). Briefly, adult male sprage–dawley rats were killed by CO_2 anesthesia followed by decapitation. Vasa deferentia were mounted in 5-ml organ baths, with the top of each tissue attached to an isometric force transducer and the bottom attached to a movable support and straddled with platinum stimulating electrodes. The vasa were stretched by a passive force about 10 mN in 5-ml organ baths containing Krebs solution containing the following (mM): 119 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 25.0 $NaHCO_3$, 5.5 glucose, 2.5 $CaCl_2$, 0.026 EDTA, at 37 °C and gassed with 95% O_2/5% CO_2. After an equilibration period of 30 min with frequent changes of medium, the vasa were stimulated by single electrical field pulses (30 V, 0.1 ms duration) at frequency of 10 Hz which were delivered every 10 s. After the addition of the highest concentration of the peptides, the toxins were typically washed out with Krebs solution and the twitch responses monitored. The resulting twitch responses mediated by sympathetic nerves were recorded on a chart recorder (RM6240B).

2.6. Whole-cell patch clamp experiments

According to the procedures adapted from Deng et al. (2009), adult rat DRG neurons were acutely dissociated and maintained in a short-term primary culture 4 weeks old adult Sprague–Dawley rats of either sex, were killed by deep ethyl ether anaesthesia followed by decapitation, and the dorsal root ganglia were removed quickly from the spinal cord, and then transferred into Dulbecco's modified Eagle's medium containing trypsin (0.5 g/l, type III) and collagenase (1.0 g/l, type IA) to incubate at 34 °C for 30 min. Trypsin inhibitor (1.5 g/l, type II-S) was used to terminate enzyme treatment. The DRG cells were transferred into 35-mm culture dishes (Corning, Sigma) containing 90% Dulbecco's modified Eagle's medium, 10% newborn calf serum, $NaHCO_3$ (3.7 g/l) and then incubated in the CO_2 incubator (5% CO_2, 95% air, 37 °C) for 1–4 h before the patch clamp experiment.

Calcium currents were recorded from experimental cells using whole-cell patch clamp technique at room temperature (22–25 °C). The patch pipettes were fabricated from borosilicate glass tubing using a two-stage vertical microelectrode puller (PC-10, Narishige, Japan). After establishing the whole-cell recording configuration, the resting potential was held at −80 mV for at least 5 min to allow adequate equilibration between the micropipette solution and the cell interior. Calcium channel currents were measured using Ba^{2+} as the charge carrier (I_{Ba}^{2+}). The external solution contained (in mM): 160 TEA-Cl, 10 HEPES, 2 $BaCl_2$, 10 glucoses, adjusted to pH 7.4 with TEA-OH. The internal solution contained (in mM): 120 CsCl, 5 Mg-ATP, 0.4 Na_2-GTP, 10 EGTA, 20 HEPES-CsOH, and adjusted to pH 7.2 with CsOH (Lewis et al., 2000). I_{Ba}^{2+} was evoked at −50 or 0 mV from a holding potential of −90 mV or −40 mV. To minimize rundown, cells which did not have stable baseline Ba current amplitude or did not respond to drugs or toxin with a rapid, monophasic change in Ba current amplitude were not considered for analysis. Ionic currents were