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DrTx(1–42), a C-terminally truncated analogue of drosotoxin, is a candidate of analgesic drugs

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ABSTRACT

Drosotoxin is an engineered tetrodotoxin-resistant (TTX-R) sodium channel-specific blocker with a non-toxic structural core (Zhu et al. Biochem Pharmacol 2010; 80:1296–302). Here, we report the discovery and functional characterization of a carboxyl-terminally truncated analogue of drosotoxin (named DrTx(1–42)) which selectively inhibited dorsal root ganglion (DRG) neuron TTX-R sodium current (I_{Na}) with an IC_{50} value of 1.74 ± 0.07 μM. Consistent with this effect, DrTx(1–42) significantly attenuates inflammatory hyperalgesia of mice in a formalin-induced pain model with stronger potency than indomethacin, a nonsteroidal anti-inflammatory and analgesic drug. Our mutational experiments indicate that the N-turn insertion is an essential functional determinant for the emergence of neurotoxicity from a non-toxic antifungal scaffold.

1. Introduction

Voltage-gated sodium channels (VGSCs) are integral membrane proteins composed of a pore-forming α-subunit associated with one or more β-subunits. They conduct Na^+ through plasma membrane of excitable cells and are vital determinants of neuronal excitability and signalling [1]. Mammal has nine VGSC α-subunits (Nav1.1–Nav1.9) with different tissue distributions [2]. For example, Nav1.3 is expressed in fetal neural tissue and its expression is down-regulated in adults. Nav1.2 is expressed in brain whereas Nav1.4 and Nav1.5 are respectively expressed in skeletal and cardiac muscle cells. As a major neuronal cell type, dorsal root ganglia (DRG) express all the other channel isoforms, including Nav1.1 and Nav1.6–Nav1.9 [3]. Pharmacologically, VGSCs can be divided into two groups based on their sensitivity to the neurotoxin tetrodotoxin (TTX), most of which can be blocked by nanomolar concentrations of TTX and are thus termed "TTX-sensitive (TTX-S)" sodium channels, such as Nav1.1–Nav1.4, Nav1.6, and Nav1.7. TTX-R sodium channels include Nav1.5, Nav1.8 and Nav1.9 [4]. Recent studies have highlighted key roles of the latter two TTX-R sodium channels as therapeutic targets in neurological disorders and pains [5–7].

Animal venoms represent a rich resource of peptide toxins that function as modulators or blockers of VGSCs to change the gated properties or Na^+ permeability of the channels [8,9]. The majority of these toxins were found to affect TTX-S sodium channels. From an evolutionary viewpoint, this is not unexpected in that targeting such channels will disrupt the functions of brain, skeletal and cardiac muscles, which undoubtedly is a benefit for prey and defence of venomous animals. At present, only one naturally occurring animal toxin was identified as a selective blocker of mammalian TTX-R sodium channels (μ-SIIIA from *Conus striatus*) [10]. Two additional conotoxins (μO-MrVIA and μ-CnIIIB) were found to have preferential ability in inhibiting mammalian TTX-R over TTX-S channels [11]. In addition, several conotoxins with selective blocking effect on amphibian TTX-R sodium channels have also been reported [12].

Very recently, an engineered neurotoxin – drosotoxin, was found to inhibit mammalian TTX-R sodium currents in rat DRG neuron with high potency and selectivity [13]. This peptide of 63 amino acids was designed by grafting the N-terminal turn and C-tail of a weak scorpion neurotoxin onto a non-toxic, antifungal scaffold (drosomycin) [14]. In the present work, we describe the molecular dissection of drosotoxin which clearly indicates the importance of the N-terminal turn insertion in emerging toxic function from a non-toxic scaffold. The C-terminally truncated peptide DrTx(1–42) exhibits high potency and selectivity on TTX-R sodium current (I_{Na}) and stronger analgesic effect than the clinical drug indomethacin, which make it an intriguing candidate for the development of therapeutic drugs for pain.

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2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were purchased from Sijiqing Biotech Co. Ltd. (Hangzhou, China) and Beijing Chemical Reagent Co. (Beijing, China), respectively; Enterokinase (EK) and dithiothreitol (DTT) were obtained from Sinobio Biotech Co. Ltd. (Shanghai, China) and SBS Genetech (Beijing, China), respectively; 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), tetrodotoxin (TTX), trypsin (type III), collagenase (IA), trypsin inhibitor (type II-S), Dulbecco's modified Eagle's medium (DMEM), tetraethylammonium chloride (TEA-Cl), D-glucose, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), CsF, CsOH, CsCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, and NaOH were purchased from Sigma–Aldrich (Shanghai, China). All these reagents are of analytical grade.

2.2. Recombinant production of DrTx(1–42) and DrTx(3DB)

The method for recombinant production of DrTx(1–42) and drosotoxin in *Escherichia coli* has been described previously [13]. However, in such conditions, the yield of DrTx(1–42) is rather low. To improve this, we modified the preparation procedure by direct digesting fusion proteins with enterokinase (EK) in the presence of a suitable amount of DTT at 4 °C for several days. The digested product was separated by reversed-phase high performance liquid chromatography (RP-HPLC).

In addition, to study the impact of the fourth disulfide bridge to the function of drosotoxin, we constructed a mutant of drosotoxin whose two cysteines forming the fourth disulfide bridge were substituted by two serines (called DrTx(3DB)). Methods for the expression, purification and identification of DrTx(3DB) are similar to those previously described for recombinant drosotoxin [13] except DTT omitted.

2.3. Chemical synthesis and oxidative refold of peptides

Reduced peptides (DroTx(N-turn)(R) and Bm(N-turn)(R)) with >95% purity were synthesized by Xi'an Huachen Bio-Technology Co., Ltd. (Xian, China). To form an intra-molecular disulfide bridge, we oxidized these two peptides in 0.1 M Tris–HCl (pH 8.0) by air. Refolded peptide was purified to homogeneity by RP-HPLC.

2.4. MALDI-TOF mass spectra and Edman degradation

Molecular weights (MWs) of recombinant or synthetic peptides were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on a Kratos PC Axima CFR plus (Shimadzu Co. Ltd., Kyoto). The automated Edman degradation by ABI Procise 492cLC protein sequencer was used for sequencing the N-terminus of DrTx(1–42) (Shanghai GeneCore BioTechnologies Co., Ltd., Shanghai, China).

2.5. Electrophysiological assays for DRG neurons

Sprague–Dawley (SD) rats (100–150 g) were used for isolating DRG neurons in this work [13,15]. A rat was killed by decapitation without anesthetization and its dorsal root ganglia were rapidly removed and placed into Dulbecco's modified Eagle's medium (DMEM). After cut to pieces, they were digested for 15–25 min at 34 °C in DMEM containing 1.56 mg collagenase I and 0.66 mg trypsin (1:250). And then 1.0 mg trypsin inhibitor II-s was added to terminate the reaction. DRG neurons were distributed on coverslip in a 35-mm dish and incubated at 37 °C (5% CO_2 and 95% air) for 30–60 min, and then 2 ml DMEM containing 10% fetal calf serum and 100 U/ml penicillin–streptomycin were added into every dish. Cells were cultured in an incubator for 2–3 h before patch clamp experiments

Sodium channels from DRG cells were recorded by the whole cell patch clamp recording technique. Micropipettes were pulled by P-97 (Sutter Instrument Co.) and the resistances of micropipettes were 3.0–6.0 MΩ after filled with the internal solution containing (in mM): CsF 135, NaCl 10, HEPES 5, adjusted to pH 7.0 with 1 M CsOH. The external bathing solution contains (in mM): NaCl 30, KCl 5, CsCl 5, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, TEA-Cl 90, D-glucose 25, adjusted to pH 7.4 with NaOH. Membrane currents recordings were made by an AxoClamp 700A amplifier (Axon Inc.) and DIGIDATA 1322A (Axon Inc.). Pulse stimulation and data acquisition were controlled by Clampex 9.0 software (Axon Inc.). 200 nM TTX was used to separate the TTX-R from the TTX-S sodium currents.

2.6. Formalin test

Healthy male ICR (imprinting control region) mice (25–30 g) were housed in a temperature controlled room (22–25 °C) with free access to food and water. The formalin test was performed according to the standard method [16]. All animal handling procedures were in accordance with the China Animal Protection Law and officially approved by the Ethics Committee of Institute of Zoology, Chinese Academy of Sciences. Animals were treated with sterile saline (0.1 ml, i.v.), indomethacin (7.5 mg/kg, i.v.), or DrTx(1–42) (0.1, 1 mg/kg, i.v.), 30 min prior to formalin injection. 20 μl of 2.5% formalin (v/v in distilled water) was injected into the ventral surface of each mouse's right hind paw. Immediately after formalin injection, animals were individually placed in a suitable chamber for observation. The summation of time (in seconds) that animals spent licking the injected paws was recorded from 0 to 5 min (neurogenic phase, corresponding to a direct chemical stimulation of nociceptors) and from 15 to 30 min (inflammatory phase, involving release of inflammatory mediators) after injection of formalin.

2.7. MTT assay

The human GC-2 cell line was used in the MTT assay [17] for the evaluation of the cytotoxic effect of DrTx(1–42). GC-2 cells were cultured at 37 °C in a 5% CO_2: 95% air incubator in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS). Cells distributed in 96-well plates at a density of 3×10^4 cells/well were incubated overnight. After removing the overnight medium, the cells were incubated with fresh medium alone (control) or various concentrations of peptides for 24 h at 37 °C. To determine cell viability, the cells were treated for 4 h with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). Then the medium was removed and 200 μl dimethyl sulfoxide (DMSO) was added. Cell viability, as indicated by the absorbance at 570 nm of the purple formazan product formed by reduction of the MTT dye by live cells, was measured by the VersaMax reader (Molecular Devices).

3. Results and discussion

3.1. Purification and identification of recombinant DrTx(1–42)

In our previous studies, we isolated and identified a recombinant fragment of drosotoxin, which is a C-terminally truncated analogue composed of the N-terminal first 42 amino acids (called drosotoxin(1–42), hereafter abbreviated as DrTx(1–42)) [13] (Fig. 1). This truncated product was derived from non-specific cleavage between ^{42}K and ^{43}C of drosotoxin during recombinant