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A naturally-occurring carboxyl-terminally truncated α-scorpion toxin is a blocker of sodium channels

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ABSTRACT

α-Scorpion toxins constitute a multigene family of evolutionarily conserved venom peptides that inhibit sodium channel inactivation and increase its peak current. Here, we describe the characterization of a new α-scorpion toxin gene expressed in the venom gland of *Mesobuthus eupeus* that encodes a carboxyl-terminally truncated product of 38 residues (named MeuNaTxα(NT)-1). Synthetic MeuNaTxα(NT)-1 was oxidized to form two disulfide bridges in an alkaline environment and the refolded peptide exhibits different structure and function from the classical α-scorpion toxin. MeuNaTxα(NT)-1 blocks sodium channels on rat dorsal root ganglia (DRG) neurons without impact on the inactivation of the channels. This work provides a clue for evolution-guided design of channel blockers for therapeutic aims.

1. Introduction

Voltage-gated sodium channels (VGSCs) are crucial players of intrinsic neuronal, muscle and cardiac excitability. They are large, complex membrane proteins composed of an α-subunit of approximately 260 kDa associated with one or more accessory β-subunits. The α-subunit is composed of four homologous domains (DI–DIV) connected by intracellular and extracellular loops, each domain containing six helical transmembrane segments (S1–S6), and a hairpin-like P loop between S5 and S6 that forms the ion selectivity filter [1,2]. In the α-subunits, there are at least seven distinct receptor sites identified for various neurotoxins, where sites 3, 4 and 6 are targeted by several types of water-soluble polypeptide toxins from scorpions, spiders, sea anemone, and cone snails. Site 1 located at the extracellular pore opening of the α-subunits is shared by the peptide toxin μ-conotoxins from cone snails and tetrodotoxin (TTX) [3,4], a classical VGSC blocker isolated from diverse venomous species, which distinguish VGSCs into TTX-resistant (TTX-R) and TTX-sensitive (TTX-S) sodium channels [5]. It has long been known that VGSC blockers targeting site 1 are anaesthetics and analgesics and these blockers are also useful in treating cardiac heart arrhythmias and epilepsy [6–8].

Scorpion toxins affecting VGSCs constitute a family of evolutionarily conserved gating modulators composed of 61–76 residues [9]. They possess a common cysteine-stabilized α-helical and β-sheet (CSαβ) structural core with four disulfide bridges [10]. According to different pharmacological activities and binding features, these toxins can be classified into two distinct functional groups, α and β [11–14]. The α-toxins slow the sodium channel inactivation to induce prolongation of action potentials by binding to site 3, whereas β-toxins modify the activation process of channels by binding to site 4. In this work, we describe for the first time a naturally-occurring carboxyl-terminally truncated α-toxin that functions as a blocker of VGSCs rather than a gating modifier of these channels.

2. Materials and methods

2.1. Chemical synthesis and oxidative refolding

The reduced MeuNaTxα(NT)-1 was chemically synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). For oxidative refolding to form two disulfide bridges, the peptide was dissolved in 0.1 M Tris–HCl buffer (pH 8.0) to a final concentration of 1 mM and incubated at 25 °C for 48 h. The peptide was purified to homogeneity by reversed-phase high-pressure liquid chromatography (RP-HPLC). Purity and molecular mass of MeuNaTxα(NT)-1 was

Abbreviations: 3′-UTR, 3′-untranslational region; CD, circular dichroism; DRG, dorsal root ganglia; IC_{50}, 50% inhibitory concentration; VGPC, voltage-gated potassium channel; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NMD, nonsense-mediated mRNA decay; NMR, nuclear magnetic resonance; ORF, open reading frame; PSC, premature stop codon; RP-HPLC, reversed-phase high-pressure liquid chromatography; T_R, retention time; TTX, tetrodotoxin; VGSC, voltage-gated sodium channel.

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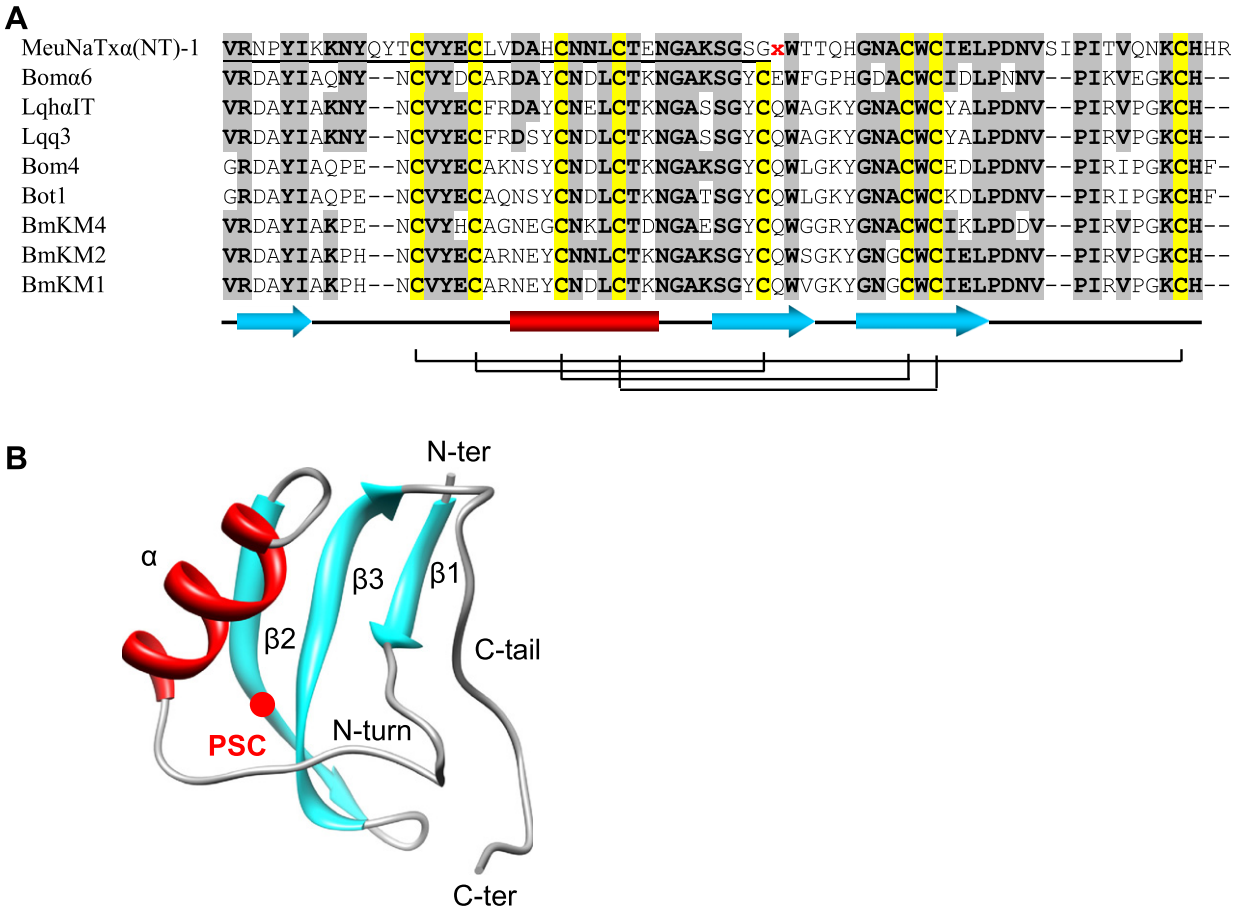

Fig. 1. MeuNaTxα(NT)-1 is a truncated analogue of α-scorpion toxins. "x" in red represents PSC. The mature MeuNaTxα(NT)-1 for chemical synthesis is underlined once. Cysteines for involving in the formation of disulfide bridges are shadowed in yellow and residues identical to those of MeuNaTxα(NT)-1 and -2 in gray. Secondary structure elements of BmKM1 [15,16] were extracted from its experimental structure (PDB code 1SN1) by STRIDE (http://webclu.bio.wzw.tum.de/stride/). Four conserved disulfide bridges are indicated by lines; (B) Ribbon structure of BmKM1 prepared by Chimera (http://www.cgl.ucsf.edu/chimera/), showing the location of the MeuNaTxα(NT)-1 PSC in a structural context. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) on a Kratos PC Axima CFR plus (Shimadzu Co., Ltd., Kyoto, Japan).

2.2. Circular dichroism (CD) spectroscopy

CD spectra of the refolded MeuNaTxα(NT)-1 and BmKM1, a typical α-scorpion toxin isolated from the venom of *Mesobuthus martensii* [10,15,16] were recorded on a JASCO J-720 spectropolarimeter (Tokyo, Japan) at a protein concentration of 0.3 mg/ml dissolved in water. Spectra were measured at 20 °C from 240 to 195 nm by using a quartz cell of 1.0 mm thickness and data were collected at 0.5 nm intervals with a scan rate of 50 nm/min. The measurement of CD spectra was performed by averaging three scans. Data are expressed as mean residue molar ellipticity ($[\theta]$).

2.3. Electrophysiological assays for DRG neurons

The method for isolating dorsal root ganglia (DRG) neurons from SD rats (100–150 g) has been described previously [17]. Sodium currents from these cells were recorded by the whole cell patch clamp recording technique. Resistances of micropipettes used here 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 TTX-R from TTX-S sodium currents.

2.4. Comparative modeling

To obtain templates with a similar cysteine pattern to MeuNaTxα(NT)-1 for comparative modeling, we searched the structural database (http://www.expasy.org/) by ScanProsite, in which the pattern C-x(3)-C-x(4,5)-C-x(3)-C derived from MeuNaTxα(NT)-1 was used as query. Once a reliable alignment between MeuNaTxα(NT)-1 and a template is available, comparative modeling is performed by Homer (http://protein.cribi.unipd.it/homer/).

3. Results

3.1. MeuNaTxα(NT)-1 is a carboxyl-terminally truncated α-scorpion toxin

In an attempt to isolate new α-scorpion toxin cDNA clones from the venom gland of *Mesobuthus eupeus*, we unexpectedly cloned an unusual cDNA of 271 bp that encodes a typical α-scorpion toxin with a premature stop codon (PSC) at site 39 which results in a C-terminally truncated peptide of 38 residues named MeuNaTxα(NT)-1 (accession number: HM 989915). When the PSC is

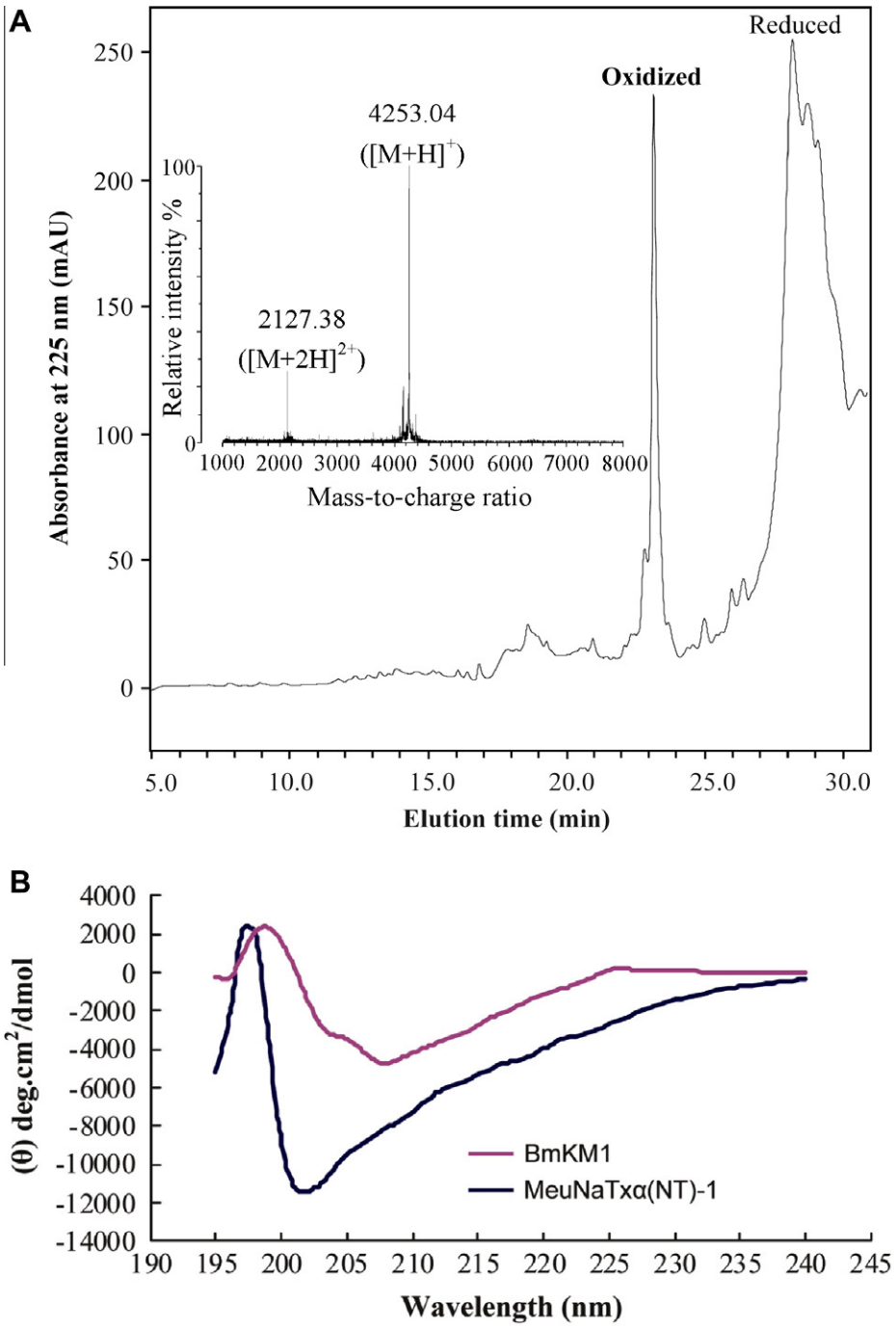

Fig. 2. Chemical and structural characterization of synthetic MeuNaTxα(NT)-1. (A) RP-HPLC profile of the synthetic MeuNaTxα(NT)-1 after oxidative refolding. Oxidized and reduced MeuNaTxα(NT)-1 products show different retention times. Peptides were eluted from the column with a linear gradient from 0% to 60% acetonitrile in 0.05% TFA within 30 min. Insert: MALDI-TOF of refolded MeuNaTxα(NT)-1,where there are two main peaks, corresponding to the singly and doubly protonated forms of the peptide; (B) Comparison of CD spectra of MeuNaTxα(NT)-1 and BmKM1.

neglected, we found that this encoded peptide is composed of 69 amino acids that share >50% sequence identity to some known α-scorpion toxins (e.g. Bomα6, LqhαIT, Lqq3, Bom4, BotI, BmKM4, BmKM2, and BmKM1) (Fig. 1A).

Because of the presence of a PSC, it is possible that this transcript is a target of nonsense-mediated mRNA decay (NMD), a process that degrades transcripts carrying PSCs in their open reading frames (ORFs) [18]. However, the corresponding genomic region of this truncated peptide contains no intron (accession number: HM 989915). Based on the generally accepted '50-nucleotide rule' (i.e. transcripts with PSCs located more than ~50 to 55-nucleotides upstream of an exon–exon junction are degraded), this transcript is predicted not to be a target of NMD [19]. In a structural context, the PSC is located within the second β-strand (Fig. 1B).

3.2. MeuNaTxα(NT)-1 structurally differs from α-scorpion toxins

To structurally and functionally characterize this truncated α-scorpion toxin, we chemically synthesized its reduced form firstly and then carried out air oxidation in an alkaline environment [20].

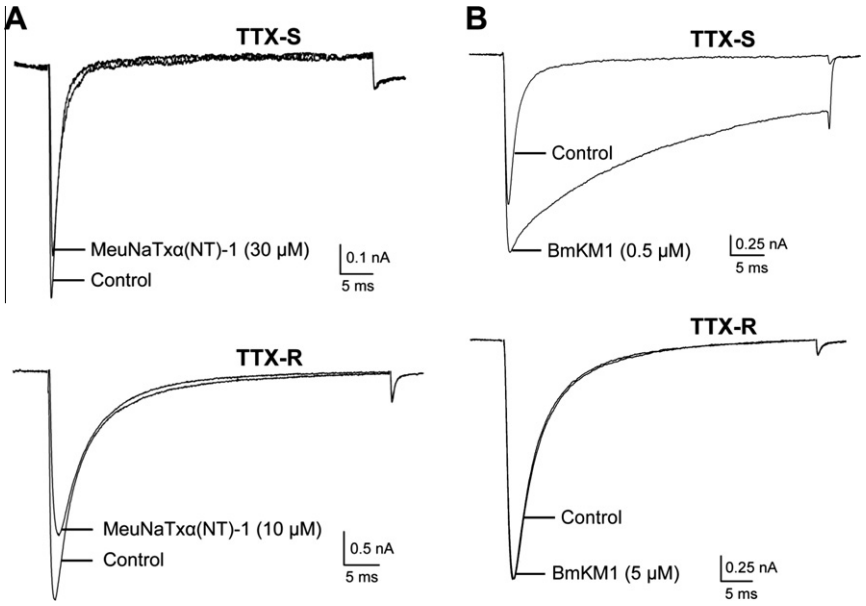

Fig. 3. Effects of MeuNaTxα(NT)-1 and BmKM1 on TTX-R and TTX-S currents in rat DRG neurons. Under voltage-clamp conditions, the inward Na^+ currents were elicited by 50-ms depolarization from a holding potential of −80 mV to −10 mV ($n \geqslant 3$). (A) MeuNaTxα(NT)-1; (B) BmKM1.

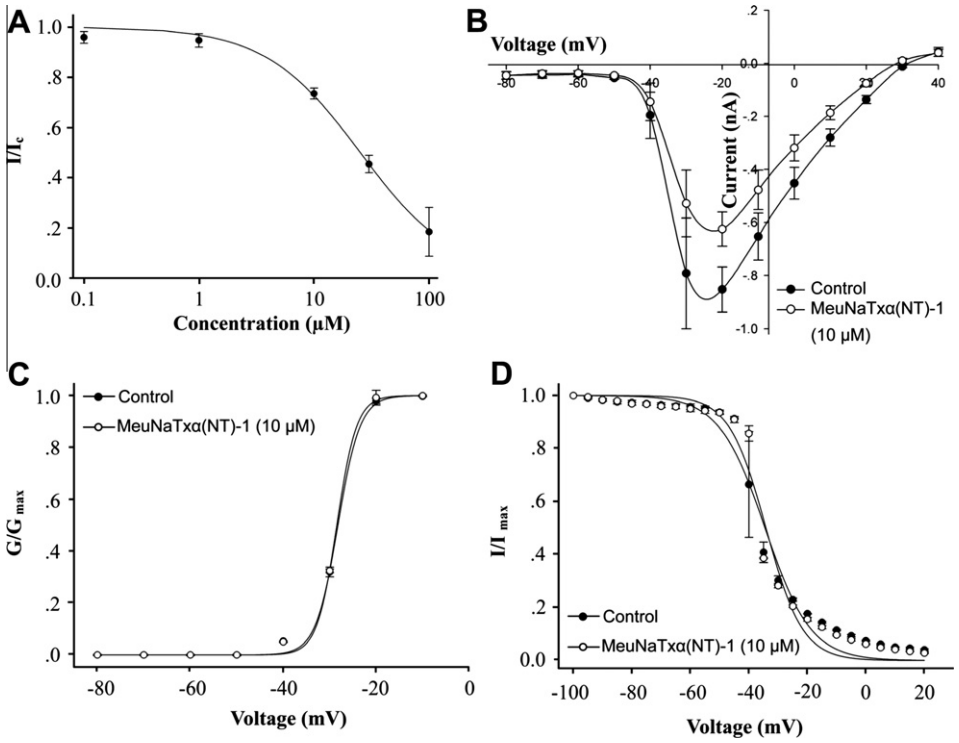

Fig. 4. Functional features of MeuNaTxα(NT)-1 on TTX-R currents. (A) Dose–response relationship of this peptide against TTX-R Na + channels. Each point represents the mean ± S.E. ($n \geqslant 3$). All data points were fitted according to the Hill equation; (B) The current–voltage (I–V) relationships of TTX-R currents before (●) and after (○) the treatment of 10 μM MeuNaTxα(NT)-1. Cells were held at −80 mV, and families of sodium currents were elicited by 60-ms depolarizing steps to various potentials ranging from −80 to 40 mV at increments of +10 mV. Points represent the mean ± S.E. from 3 to 4 different experiments; MeuNaTxα(NT)-1 has no effect on steady-state activation (C) and inactivation (D) of TTX-R channels. The current of steady-state activation were elicited by 60-ms depolarizing steps to various potentials ranging from −80 to −10 mV at increments of +10 mV. The current of steady-state inactivation was measured by 60-ms depolarizing steps to various potentials ranging from −100 to +20 mV at increments of 5 mV. The steady-state activation and inactivation were obtained after fitting with Boltzmann function. The normalized activation curves for the peptide: the midpoint ($V_{1/2}$) = −28.23 ± 0.32 mV, slop factor (s) = 2.39 ± 0.36 mV; Control: $V_{1/2}$ = −28.45 ± 0.37 mV, s = 2.14 ± 0.47 mV. The normalized inactivation curves for the peptide: $V_{1/2}$ = −34.17 ± 0.84 mV, s = 6.28 ± 0.74 mV; Control: $V_{1/2}$ = −34.47 ± 0.82 mV, s = 7.85 ± 0.72 mV.

The oxidized product was purified by RP-HPLC, in which the reduced and oxidized MeuNaTxα(NT)-1 were respectively eluted at 28 and 23 min (Fig. 2A). The shift of retention time (T_R) indicates an increase of polarity and a decrease of hydrophobicity of the oxidized MeuNaTxα(NT)-1 in solution. This is an indicative that more hydrophobic residues are buried in a structured molecule probably stabilized by two disulfide bridges. To verify this, we firstly analyzed samples using MALDI-TOF MS. The detected

molecular mass of the oxidized peptide is 4253.04 Da (Fig. 2A, about 4 Da less than the calculated value (4256.77 Da) from its primary sequence, indicating that four hydrogen atoms in the cysteines of the reduced peptide have been removed to form two disulfide bridges. Subsequently, we studied the structural feature of MeuNaTxα(NT)-1 by circular dichroism (CD) analysis. MeuNaTxα(NT)-1 displayed a characteristic minimum at 201–202 nm and a positive ellipticity at 197–198 nm. In contrast, BmKM1 (as a control) displayed a minimum at 208 nm and a maximum at 198–200 nm (Fig. 2B), indicating that they have significantly different structures.

Since the three-dimensional structure of MeuNaTxα(NT)-1 could not be experimentally determined due to poor refolding efficiency that led to difficulty in obtaining plenty of peptides for nuclear magnetic resonance (NMR) studies, a model for the core region of this molecule (residues 11–29) has been built instead. This model was generated from the NMR structure of polyphemusin-1 by comparative modeling. Polyphemusin-1 is an antimicrobial peptide of 18 residues isolated from horseshoe crab hemocytes [21] with a similar cysteine pattern to MeuNaTxα(NT)-1 (Fig. S1A, provided as electronic supplementary materials). The model supports that the core region of MeuNaTxα(NT)-1 can fold into a two-strands of β-sheet with two identical disulfide parings (C1–C4, C2–C3) to polyphemusin-1 (Fig. S1B). The β-sheet content calculated from this model is 26.3%, highly consistent with that estimated from the CD data (29%).

3.3. MeuNaTxα(NT)-1 inhibits DRG neuron sodium channels

By using whole-cell patch clamp recording techniques, we assessed the activity of MeuNaTxα(NT)-1 against TTX-S and TTX-R sodium channels on rat DRG neurons. We found that MeuNaTxα(NT)-1 inhibited both TTX-R and TTX-S Na^+ currents at micromolar concentrations (Fig. 3A). On the contrary, BmKM1 displayed a typical α-effect on TTX-S rather than TTX-R channel at low micromolar concentrations, as identified by prolonging the channel inactivation and increasing the peak currents (Fig. 3B). The dose-dependent effects of MeuNaTxα(NT)-1 on TTX-R currents was studied by using five different peptide concentrations (from 0.1 to 100 μM). The yielded 50% inhibitory concentration (IC_{50}) from these data is 25.14 ± 1.78 μM (Fig. 4A). The effect of 10 μM MeuNaTxα(NT)-1 on the current–voltage (*I–V*) curves of TTX-R channels is illustrated in Fig. 4B. From the curves, it can be seen that the inhibition of currents was observed at all tested potentials after the depression of current amplitude by 10 μM MeuNaTxα(NT)-1, and the current inhibition was not associated with a change of the shape of the *I–V* relationship. MeuNaTxα(NT)-1 also displayed no effect on steady-state activation and inactivation of DRG TTX-R Na^+ channels (Fig. 4C and D).

4. Discussion

The resistance of the garter snakes *Thamnophis sirtalis* to its toxic prey, the newts *Taricha granulose* [22], is associated to crucial amino acid point mutations in its VGSCs, which render this channel insensitive to TTX [23]. On the other hand, as part of arms-race between predator and prey, venomous animals need to continuously evolve their arsenals to adapt the changes of their competitors. Scorpion is one of the oldest arthropods whose evolutionary history dates back to the middle Silurian, about 425–450 million years ago. Along the long evolutionary history, scorpion has developed several efficient strategies to evolve its toxins with high affinity for various VGSC subtypes and different phylogenetic preference, in which amino acid substitutions combined with indels appears to be two major evolutionary events in reshaping of new bioactive surfaces of these toxins via structural reconfiguration of the C-tail [24–26].

In addition to these two modifications mentioned above, C-terminal truncation may represent a new mechanism to diversify the scorpion venom reservoir. Several C-terminally truncated scorpion sodium channel toxins have been identified so far [27–29], in which one peptide termed KAaH1 has been found to switch a VGSC modifier activity to a voltage-gated potassium channel (VGPC) blocker (Fig. S2). Despite C-tail truncation together with functional diversification, all these natural variants retain a typical CSαβ structure core stabilized by three conserved disulfide bridges. Intriguingly, MeuNaTxα(NT)-1 represents the first C-terminally truncated α-toxin that significantly changes its structure and function. It is particularly remarkable that MeuNaTxα(NT)-1 acts as a blocker of VGSCs rather than a gating modulator. Although the precise molecular mechanism of MeuNaTxα(NT)-1 for channel blockade remains to be demonstrated, our work is of value in understanding of functional innovation of the α-scorpion toxin multigene family. Furthermore, given potentials of VGSC blockers as drug leads for some human diseases, our observations provide a clue for C-terminal truncation-based molecular design of novel VGSC blockers from the α-scorpion toxin resource for therapeutic aims.

Author contribution

Shunyi Zhu designed the research. Bin Gao and Limei Zhu performed the experiments. The three authors contributed to data analysis and manuscript writing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.178.

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