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Two recombinant depressant scorpion neurotoxins differentially affecting mammalian sodium channels

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ABSTRACT

The scorpion depressant toxins are a group of evolutionarily conserved polypeptides targeting sodium channels, which show preferential ability to induce flaccid paralysis in insects, making them attractive candidates for the construction of transgenic plants or viral vectors to control pests. In this study, two new depressant toxin-like peptides (BmKITc and BmKITc2) differing only at position 52 (Lys for Thr) were produced in *Escherichia coli*. Circular dichroism analysis indicated that these two recombinant peptides display a typical structural feature similar to native scorpion toxins. They both cause a maintained current component at the last phase of inactivation of the insect sodium channel DmNav1/tipE expressed in *Xenopus oocytes* and interestingly, they do not produce a beta effect despite of their primary structure as beta-toxins. Furthermore, an inhibitory effect with BmKITc but not with BmKITc2 was observed on TTX-R sodium currents in rat DRG neurons. We hypothesize that such differential potency highlights a crucial role of lysine 52 in channel selectivity. Our results therefore indicate that, in spite of the general idea, not all scorpion depressant toxins interact with mammalian and/or insect sodium channels in the same manner.

1. Introduction

Voltage-gated sodium channels (VGSCs) are complex membrane proteins that regulate and control electrical excitability of insect and mammalian muscles and nerves (Catterall et al., 2007). Modification of the pharmacological activities of these channels by toxins from various venomous animals causes rapid immobilization of their preys. Scorpion venom-derived depressant toxins comprise a subfamily of bioactive polypeptides that contain 61–65 amino acids stabilized by four disulfide bridges (Possani et al., 1999; Rodriguez de la Vega and Possani, 2005; Gurevitz et al., 2007). These β-toxins bind to the receptor site 4 of the VGSCs and affect channel activation in a manner by shifting the voltage dependence of activation in the hyperpolarizing direction. In a recent work, Bosmans et al. have found that β-toxins can interact with multiple Na channel paddle motifs from domains II, III or IV in rNav1.2, but only with domain II paddle in rNav1.4 (Bosmans et al., 2008). Scorpion depressant toxins exhibit preferential ability in induction of a transient contraction paralysis of insect larvae followed by a progressive flaccid paralysis, making them attractive

Abbreviations: CD, circular dichroism; CSαβ, cysteine-stabilized α-helix/β-sheet; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglia; EK, enterokinase; GST, glutathione-S-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MBP, maltose-binding protein; RP-HPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid; VGSC, voltage-gated sodium channel; TTX-S, tetrodotoxin-sensitive; TTX-R, tetrodotoxin-resistant.

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candidates for construction of transgenic plants or viral vectors to control pests. In fact, by combination of viral promoters, LqhIT2 and Lqh-dprIT3 have shown strong insecticidal efficacy (Gurevitz et al., 2007).

So far, more than 24 depressant toxins have been functionally characterized (Fig. 1). Although most of them show high preference for insect VGSCs and modulate their activation (Bosmans et al., 2005; Karbat et al., 2007), some members are active on mammals, e.g. (1) BmKIM, a recombinant peptide toxic to insects and mammals, could inhibit Na^+ currents in rat DRG neurons and ventricular myocytes (Peng et al., 2002); (2) BmKdITAP3 was reported to have a dual bioactivity, a depressant toxicity on insects and an analgesic effect on mice (Guan et al., 2001); (3) BmKAEP had little toxicity on mice and insects but was found to have an anti-epilepsy effect in rats (Wang et al., 2001); (4) LqhIT2 was demonstrated to bind and affect rat skeletal muscle channels (Cohen et al., 2007).

In this study, we recombinantly produced two new depressant toxins (BmKITc and BmKITc2) in *E. coli* and characterized their structural and functional features. BmKITc and BmKITc2 differ by only one amino acid subsitution at position 52 (Thr52Lys) and therefore constitute an interesting starting point of investigation. As described below, we have found that this single-residue change resulted in functional diversification of these two toxins, making BmKITc rather than BmKITc2 a weak inhibitor of the TTX-R and TTX-S Na^+ channels in rat DRG neurons. This observation highlights the crucial role of lysine 52 in interacting with mammalian VGSCs. In addition, although lacking sequence conservation in the equivalent positions corresponding to the 'pharmacophore' of scorpion β-toxins (Karbat et al., 2007), these two peptides were able to produce a significant change on the slow phase of inactivation of the *Drosophila* DmNav1/tipE channels expressed in *Xenopus oocytes*.

2. Materials and methods

2.1. Materials

Male Sprague-Dawley rats (180–200 g) were purchased from the Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). All primers used in this study were synthesized by SBS Genetech (Beijing, China): Bm-IT-F (5′-ATGGATCC*GATGACGATGACAAG*GATGGATATATAAGAGGAAGT-3′); Bm-IT-R (5′-ATGTCGACTTAGCTACCGCATGTATTACTTTC-3′); Bm-IT-RRn (5′-TTTCATTATCAGGAAGGCCTTCACACCA-3′) and Bm-IT-FRn (5′-AATGGAAATATGAAAGTAATACATGCGGT-3′), in which *Bam* HI and *Sal* I sites are underlined once and codons for enterokinase (EK) is italized. Reagent sources: Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech Inc.); Enterokinase (Sinobio Biotech Co. Ltd., Shanghai, China); Amylose affinity resin (New England Biolabs, Ipswich, USA); Trypsin (Ameresco); Collagenase and trypsin inhibitor (Sigma).

2.2. Construction of pGEX-6P-1-BmKITc2 expression vector and its expression in E. coli

Isolation of the BmKITc2 clone has been described previously (Tian et al., 2008) and its ligation into pGEX-6P-1 was performed according to the published method (Yuan et al., 2007). In order to remove the carrier glutathione-S-transferase (GST) to exactly obtain the peptide, we introduced an EK cleavage site at the *Bam* HI downstream (Fig. S1, provided as supplementary data). The recombinant plasmid was transformed into *E. coli* DH5α and positive clones were confirmed by DNA sequencing.

Expression of the fusion protein GST-BmKITc2 in *E. coli* BL21(DE3) was induced by 0.5 mM IPTG at 25 °C when an OD_{600} reached 0.7. The fusion protein obtained from a sonication supernatant by affinity chromatography with glutathione-Sepharose 4B beads was digested with EK at 22 °C for 5 hr. The released BmKITc2 was separated from GST using reverse-phase HPLC on C8 column (Agilent Zorbax, Eclipse XDB-C8, 4.6 mm × 150 mm, 5 μm). Elution was carried out using a linear gradient of 10–70% acetonitrile in 0.1% trifluoroacetic acid (TFA) in watere (v/v) within 40 min with a flow rate of 1 ml/min.

2.3. Construction of pMAL-p2X-BmKITc expression vector and its expression in E.coli

For constructing pGEX-6P-1-BmKITc, we mutated site 52 by inverse PCR to amplify the template pGEX-6P-1-BmKITc2 by two phosphated primers (Bm-IT-FRn and Bm-IT-RRn) (Fig. S1). The PCR condition is as follows: 35 cycles of 1 min at 94 °C, 45 sec at 62 °C, and 8 min at 72 °C. Self-circularization of the PCR product was carried out by DNA ligase and then the circularized product was transformed into *E. coli* DH5α. Positive clones were confirmed by DNA sequencing. Subsequently, we cut the coding region of BmKITc by *Bam* HI and *Sal* I and ligated it into the downstream *mal*E gene, which encodes maltose-binding protein (MBP), of the pMAL-p2X expression vector (Fig. S1). The constructed vector was transformed into *E. coli* DH5α for protein expression.

Expression of fusion proteins was induced with 0.2 mM IPTG at 37 °C until an OD_{600} reached 0.5. Cells were harvested 5 hr after addition of IPTG by centrifugation and the pellet was resuspended in the column buffer (20 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA) for sonication. The supernatant was then loaded to an amylose affinity resin for 2 h, followed by washing unbounded proteins using the column buffer containing 10 mM β-mercaptoethanol. Fusion proteins bound on amylose resin were eluted using the column buffer containing 10 mM maltose and then were digested with EK at 22 °C for 24 h until about 80% BmKITc was released. The recombinant product was purified using RP-HPLC on C18 column (Agilent Zorbax 300SB-C18, 4.6 mm × 150 mm, 5 μm) as described previously.

2.4. Characterization of recombinant protein

CD spectra of recombinant toxins were recorded on a JASCO J-715 spectropolarimeter (Tokyo) at a protein concentration of 0.2 mg/ml dissolved in 10 mM sodium phosphate buffer, pH 6.8. Spectra were measured at 25 °C from 250 nm to 190 nm using a quartz cell of 1.0 mm length. Data were collected at 0.2 nm intervals with a scan rate of 200 nm/min. CD spectra measurement was performed by averaging three scans. Secondary structure