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A single-point mutation enhances dual functionality of a scorpion toxin

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ABSTRACT

Scorpion venom represents a tremendous, hitherto partially explored peptide library that has been proven to be useful not only for understanding ion channels but also for drug design. MeuTXKα3 is a functionally unknown scorpion toxin-like peptide. Here we describe new transcripts of this gene arising from alternative polyadenylation and its biological function as well as a mutant with a single-point substitution at site 30. Native-like MeuTXKα3 and its mutant were produced in *Escherichia coli* and their toxic function against *Drosophila Shaker* K^+ channel and its mammalian counterparts ($rK_v1.1$–$rK_v1.3$) were assayed by two-electrode voltage clamp technique. The results show that MeuTXKα3 is a weak toxin with a wide-spectrum of activity on both *Drosophila* and mammalian K^+ channels. The substitution of a proline at site 30 by an asparagine, an evolutionarily conserved functional residue in the scorpion α-KTx family, led to an increased activity on $rK_v1.2$ and $rK_v1.3$ but a decreased activity on the *Shaker* channel without changing the potency on $rK_v1.1$, suggesting a key role of this site in species selectivity of scorpion toxins. MeuTXKα3 was also active on a variety of bacteria with lethal concentrations ranging from 4.66 to 52.01 μM and the mutant even had stronger activity on some of these bacterial species. To the best of our knowledge, this is the first report on a bi-functional short-chain peptide in the lesser Asian scorpion venom. Further extensive mutations of MeuTXKα3 at site 30 could help improve its K^+ channel-blocking and antibacterial functions.

1. Introduction

Scorpion venom is a combinational library of ion channel-targeted neurotoxins, cytolytic peptides, proteinases and inhibitors, antimicrobial peptides, and other toxic components (Diaz et al., 2009; Ma et al., 2012; Abdel-Rahman et al., 2013; Cao et al., 2013; He et al., 2013; Mille et al., 2014), and its essential biological function is involved in capturing prey (e.g. insects) and defending against predators (e.g. birds, lizards, and mammals) (Polis, 1990; Inceoglu et al., 2003). Peptide neurotoxins are a major component of scorpion venom, most of which impair functions of Na^+ and K^+ channels. Scorpion toxins affecting K^+ channels (abbreviated as KTxs) typically contain 23–64 amino acids with three or four disulfide bridges. They fold into a typical cysteine-stabilized α/β scaffold (CSαβ) shared with insect and fungal defensins (Quintero-Hernández et al., 2013; Zhu et al., 2014). According to sequence similarity and disulfide pattern, these molecules can be grouped into four large families: α-, β-, ϒ-, and κ-KTxs (Quintero-Hernández et al., 2013). The α-KTx family, usually containing 23–42 amino acids with 3 or 4 disulfide bridges (Tytgat et al., 1999; Quintero-Hernández et al., 2013), is the most diverse scorpion toxin group among those affecting voltage-gated K^+ channels (K_v), which in turn reflects their targets′ diversity. These molecules have been proven to be useful tools for studying pharmacological, physiological, and structural characteristics of different subtypes of K^+ channels (Wickenden, 2002; López-González et al., 2003; Rodriguez de la Vega et al., 2003).

MeuTXKα3 is a functionally unknown scorpion toxin-like peptide of 38 residues, belonging to the α-KTx family. It was identified by screening a venom gland cDNA library from *Mesobuthus eupeus* (Zhu et al., 2011). Despite low sequence similarity to other known KTxs, this peptide contains typical structural residues (six cysteines and one glycine in the GKC motif) involved in the formation of a CSαβ folding and a functional dyad (Lys27 and Phe36) for K^+ channel blockade. In this work, we describe for the first time the bi-functional feature of MeuTXKα3 as a K^+ channel toxin with antibacterial activity, and guided by prior knowledge, we designed a mutant to improve its dual functions.

2. Materials and methods

2.1. cDNA cloning

Reverse-transcriptional PCR was used to isolate new transcripts encoding MeuTXKα3 via two rounds of amplifications with nested PCR primers (MeuTxKα3-F0 and MeuTxKα3-F1) (Table 1). Nucleotide sequences reported here have been deposited in GenBank (http://www.

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Table 1
Primers used in this study.

Name	Sequence	Usage
MeuTxKα3-F0	5′-ATAATTATAACGAGATATAGACA-3′	Gene isolation
MeuTxKα3-F1	5′-CGCTTTTCCATTGTTCTACAATTA-3′	Gene isolation
MeuTXKα3-FP	5′-GGATCCGATGACGATGACAAGGTA GATTTTCCTAATAAA-3′	Construction of expression vector
MeuTXKα3-RP	5′-GTCGACTTATCCTGGAAAACATCT GCA-3′	Construction of expression vector
MeuTXKα3-30FP	5′-**AA**TAATTATTGCAGATGTTTTCCA GGA-3′	Mutation
MeuTXKα3-30RP	5′-AAAGCATTTTCCTCTGTAATTAAG-3′	Mutation

Note: Mutated nucleotides are boldfaced. All primers listed here were synthesized by SBS Genetech (Bejing, China).

ncbi.nlm.nih.gov/) under accession numbers of EF442052, EF442053, KR493338–KR493340.

2.2. Sequence and structure snalysis

Sequences used in this study were retrieved from GenBank and were aligned by CLUSTAL (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The model structure of P30N was built according to the previously described method for MeuTXKα3 (Zhu et al., 2011). The NMR structure of ChTX (PDB entry 2CRD), a well-characterized scorpion α-KTx, was used as template for comparative modeling on SWISS-MODEL, a fully automated protein structure homology-modeling server (http://swissmodel.expasy.org) and models were evaluated by the Verify 3D (Eisenberg et al., 1997).

2.3. Construction of recombinant expression vectors

To construct pGEX-4 T-1-MeuTXKα3 expression vector, we amplified the MeuTXKα3 cDNA using primers MeuTXKα3-FP and MeuTXKα3-RP (Table 1) by standard PCR, as described previously (Yuan et al., 2007). We introduced a *Bam* HI site and codons of the enterokinase (EK) cleavage site (DDDDK) at the 5′ end of the FP and a *Sal* I site and a stop codon at the 5′ end of the RP. PCR product was firstly cloned into pGM-T and sequenced by T7 primer. Recombinant plasmid confirmed was digested by *Bam* HI and *Sal* I and then ligated into pGEX-4 T-1.

2.4. Site-directed mutagenesis

Inverse PCR, as previously described (Zhu et al., 2008; Wang et al., 2015), was used to generate the mutant P30N. Phosphorylation of the 5′-end of primers (Table 1) was performed with polynucleotide kinase and ATP. PCR products were circularized by T4 DNA ligase and transformed into *E. coil* DH5α competent cells. Positive clones were confirmed by DNA sequencing.

2.5. Expression, purification, and characterization of recombinant peptides

Expression of glutathione-S-transferase-MeuTXKα3 and P30N in *E. coli* Rosetta (DE3) was induced by 0.5 mM IPTG. Fusion proteins were obtained from the supernatant after sonication, followed by affinity chromatography with glutathione-Sepharose 4B beads from GE Healthcare (Shanghai, China). The fusion proteins in 1× PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, and 1.8 mM KH_2PO_4, pH7.3) were then digested with EK (Sinobio Biotech Co. Ltd., Shanghai, China) at 25 °C overnight. RP-HPLC was applied to separate MeuTXKα3 and P30N from the enzymatic product. Molecular masses of recombinant peptides were determined by MALDI-TOF mass spectra.

2.6. Circular dichroism spectroscopy

CD spectra of MeuTXKα3 and P30N were recorded on Chirascan™-plus circular dichroism spectrometer (Applied Photophysics Ltd, United Kingdom), which were measured at room temperature from 190 to 260 nm with a quartz cell of 1.0 mm thickness. Data were collected at 1 nm intervals with a scan rate of 60 nm/min. Percentages of peptide secondary structure elements were calculated with the DICHROWEB software, an online server for protein secondary structure analysis from CD data (http://dichroweb.cryst.bbk.ac.uk).

2.7. Expression of K^+ channels in Xenopus oocytes

For the expression of K_v channels ($rK_v1.1$–$rK_v1.3$ and *Shaker* IR) in *Xenopus* oocytes, linearized plasmids were transcribed with the T7 mMESSAGE-mMACHINE transcription kit (Ambion, USA) (Gao et al., 2010). Oocytes were obtained from anesthetized female *Xenopus laevis*, as described previously (Liman et al., 1992). The oocytes were digested for 1–2 h by treatment with 0.5 mg/ml collagenase I at room temperature in Ca^{2+} free ND96 solution, and then washed 3 times with Ca^{2+}-free ND96 and another 3 times with ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH7.4 with NaOH). Oocytes of stage V–VI were selected and cultured in ND96 containing 50 mg/l gentamycin sulfate at 18 °C. After 2–3 h incubation, oocytes were injected with cRNA by micro-injector (NANOLITER 2000, WPI) and then incubated in ND96 solution with 50 mg/l gentamycin sulfate at 18 °C for 1–5 days.

2.8. Electrophysiological recording

Two-electrode voltage-clamp recordings were performed at room temperature with an Oocyte Clamp Amplifier (OC-725C, Harvard Apparatus Company) controlled by a data acquisition system (Digidata 1440A, Axon CNS) dominated by pCLAMP10.2 software (Axon Inc., USA). Whole-cell currents from oocytes were recorded 2–5 days after injection. Bath solution was ND96 solution. Voltage and current electrodes were pulled by P-97 Flaming/Brown Micropipette Puller (Sutter Instrument Co., USA) with resistance of 0.1–1.0 MΩ when filled with 3 M KCl. The elicited currents were filtered at 1 kHz and sampled at 2 kHz by a four-pole low pass Bessel filter. Leak subtraction was performed with a P/4 protocol. Currents were evoked by 250 ms depolarizations to 0 mV followed by a 250 ms pulse to −50 mV, from a holding potential of −90 mV. Data were analyzed by pClamp Clampfit 10.0 (Molecular Devices) and SigmaPlot 11.0 (Systat Software, CA, USA).

2.9. Antibacterial assays

Antibacterial assays were carried out according to the literature (Hultmark, 1998). *Bacteria* were incubated at 37 °C in Broth medium until the OD_{600} reached 0.6. 10 μl of bacterial culture was mixed in 6 ml of Broth medium containing 0.8% agar and poured into Petri dishes of 9.0 cm diameter. Wells with a diameter of 2 mm were punched into the medium, filled with 2 μl of sample each well. Bacteria were incubated at 37 °C for 12 h and then zone of inhibition was measured. Lethal concentration (C_L) was calculated according to the Hultmark method

Table 2
Comparison of lethal concentration (C_L) of MeuTXKα3 and P30N on different bacterial species.

Bacteria	MeuTxKα3	P30N	Fold
Bacillus megaterium CGMCC 1.0459	14.26	4.95	2.9
Bacillus subtilis CGMCC 1.2428	10.28	6.11	1.7
Micrococcus luteus CGMCC 1.0290	4.66	5.50	0.9
Streptococcus mutans	33.80	24.06	1.4
Xanthomonas oryzae	52.01	17.68	2.9

Note: Concentrations are given in μM and fold is calculated as C_L of the wild peptide/C_L of the mutant.