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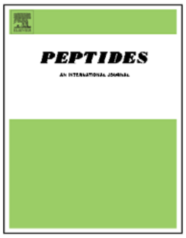

Molecular cloning and electrophysiological studies on the first K⁺ channel toxin (LmKTx8) derived from scorpion *Lychas mucronatus*

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ARTICLE INFO

Article history:
Received 2 September 2007
Received in revised form
5 October 2007
Accepted 5 October 2007
Published on line 22 October 2007

Keywords:
LmKTx8
Lychas mucronatus
α-KTx
Kv1.3
BK

ABSTRACT

LmKTx8, the first toxic gene isolated from the venom of scorpion *Lychas mucronatus* by constructing cDNA library method, was expressed and characterized physiologically. The mature peptide has 40 residues including six conserved cysteines, and is classified as one of α-KTx11 subfamily. Using patch-clamp recording, the recombinant LmKTx8 (rLmKTx8) was used to test the effect on voltage-gated K^+ channels (Kv1.3) stably expressed in COS7 cells and large conductance-Ca^{2+}-activated K^+ (BK) channels expressed in HEK293. The results of electrophysiological experiments showed that the rLmKTx8 was a potent inhibitor of Kv1.3 channels with an $IC_{50} = 26.40 \pm 1.62$ nM, but 100 nM rLmKTx8 did not block the BK currents. LmKTx8 or its analogs might serve as a potential candidate for the development of new drugs for autoimmune diseases.

1. Introduction

The roles of the K^+ channels can be elucidated in a wide variety of physiological processes, including cell excitability, the regulation of heart-beating, muscle contraction, neurotransmitter release, hormonal secretion, signal transduction and cell proliferation [5]. Scorpion toxins for K^+ channels (KTxs) can specifically interfere with the various existing K^+ channels, and thus they have been considered as rich sources for studying structural and functional features of K^+ channels [8,9,21]. KTxs are typically 23–64 amino acid residues in length and have a highly conserved secondary structure, composed of an α-helical segment connected to a double- or triple-stranded β-sheet and well-packed by three or four conserved disulfide bridges [13,21]. To date, over 120 K^+ channel toxins have been discovered [21,33,35]. According to similarities in their sequences and disulfide patterns of the peptides and specificity towards each corresponding subtype of K^+ channels, KTxs are grouped into four major subfamilies (α-, β-, γ- and κ-KTx). The α-KTxs, the largest subfamily, have proven to be powerful tools for testing

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doi:10.1016/j.peptides.2007.10.009

the pharmacological, physiological, biochemical, biophysical and even structural characteristics of K^+ channels and their associated ionic currents [7,20,25].

Since the first α-KTx NTX from scorpion venom was discovered nearly 25 years ago, these potassium channel blockers have greatly advanced our understanding of potassium channel structure and function. The first K^+ channel toxin from scorpion *Lychas mucronatus*, termed LmKTx8 (a trivial name abbreviated from the first letter of genus and species followed by 8, numbered in the cDNA library), is now the new member of the α-KTx toxin subfamily. In this study, the molecular cloning, recombination LmKTx8 (rLmKTx8) expression, purification, and electrophysiological effect of this toxin were performed. LmKTx8 is a short chain peptide with 40 amino acids including six cysteines. It blocks voltage-gated K^+ channel Kv1.3 at low nanomolar concentration, which can be used as a new material for further research towards the rational design of more specific drugs and the control of pathologies associated with K^+ channels.

2. Materials and methods

2.1. *cDNA library construction and screening*

L. mucronatus scorpions were collected in the Hainan Province of China. Their glands were collected 2 days after electrical extraction of their venom. Total RNA was prepared from the glands, using Trizol reagent (Invitrogen) method. Poly(A)-mRNA was purified by a Poly A Tract mRNA Isolation System (Promega), and cDNA library was constructed according to Superscript Plasmid System cDNA library construction kit (Gibco/BRL). cDNA was cloned into pSPORT1 plasmids and transformed into *Escherichia coli* DH5α cells. Randomly chosen cDNAs were sequenced to obtain a reliable representation of the toxin content in the venom gland [32,36].

2.2. *Construction of expression vector pGEX-6p-1-LmKTx8*

The expression plasmid pGEX-6p-1-LmKTx8 was constructed on the basis of the full-length cDNA of LmKTx8. Primers were designed to match the mature region of LmKTx8. The forward primer was 5’-GCGGGATCCGATGACGATGACAAGGTACCAACAGGAGGATGC-3’ with a BamHI restriction enzyme site (underlined) and corresponding to five codons encoding an enterokinase cleavage site (underlined twice). The reverse primer was 5′-GCCCTCGAGTTACACGAGACATTTACA-3′ with restriction enzyme site XhoI (underlined). The PCR products were inserted into expression vector pGEX-6p-1 and sequenced with universal pGEX primers. *E. coli* Rosetta (DE3) cells were used for expression.

2.3. *Expression and purification of rLmKTx8*

E. coli Rosetta (DE3) cells containing pGEX-6p-1-LmKTx8 were proliferated at 37 °C in LB with 100 μg/ml ampicillin. Fusing protein synthesis was induced by the addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 28 °C for 4 h. Cells were harvested and resuspended in glutathione (GSH) wash buffer (pH 8.0, 50 mM Tris–HCl, 10 mM EDTA), digested by 1 mg/ml lysozyme for 30 min. After a brief sonication, the extract was clarified by a centrifugation at 10,000 × *g* for 15 min. The fusion protein was purified by GSH affinity chromatography [16] and enriched by centrifugal filter devices (Millipore).

High performance liquid chromatography (HPLC) analyses were used to purify the rLmKTx8. After cleavage of the fusion protein by enterokinase (Biowisdom, China) for 8 h at 37 °C, the mixture was filtered (Millex-HV, 0.45 μm, Millipore) and separated on a C18 column (EliteHPLC, China, 10 mm × 250 mm, 5 μ) using a linear gradient from 10% to 80% CH_3CN with 0.1% TFA in 60 min with a constant flow rate of 5 ml/min. Peaks were collected manually.

2.4. *Electrophysiological experiments*

2.4.1. *Expression of Kv1.3 and BK in cells*

Culture of COS7 and HEK293 (CCTCC, China) cells and construction of vectors containing Kv1.3 channels cDNA (kindly gifted from Dr. Stephan Grissmer) and BK channels cDNA (a gift of Dr. Christopher Lingle) were described previously [11,31]. With 40–50% confluence, the COS7 and HEK293 cells were transfected with 0.2 μg of recombinant plasmids using Sofast^{TM} transfection Reagent (Sunma, China).

2.4.2. *Electrophysiological measurements*

Currents from COS7 cells expressing Kv1.3 channels and HEK293 expressing BK channels were measured by using an EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by PULSE software (HEKA Elektronik). The solutions were prepared according to the previous reports [19,26]. The Kv1.3 channel currents were elicited by depolarizing voltage steps of 200 ms from the holding potential −80 mV to +50 mV. Inside-out patches were used in the measurement of BK currents, and channels were activated by a 50 ms voltage step to +100 mV, after a prepulse to −180 mV. For toxin application, the multi-channel microperfusion system MPS-2 (INBIO Inc., China) was used. All these experiments were performed at room temperature (22–25 °C). Currents were typically digitized at 100 kHz. Macroscopic records were filtered at 5 kHz. Patch-clamp recording data were analyzed with IGOR (Wavemetrics, Lake Oswego, OR) software.

3. Results

3.1. *Cloning and sequence analysis cDNA of LmKTx8*

After PCR random screening, several positive clones were got. One of these cDNAs, termed LmKTx8 (GenBank Accession No. EU118812) has the precursor nucleotide sequence of 251 bp, including three parts: 5′UTR, ORF and 3′UTR. The 5′ and 3′UTRs of LmKTx8 have 9 bp and 44 bp, respectively. At the 3′UTR end of the cDNA, a single AATAAA polyadenylation signal is found 12 nt upstream of the poly(A) tail. An ORF of 195 bp encodes a precursor of 65 amino acid residues (Fig. 1).

3.2. *Amino acid sequence analysis of LmKTx8*

The signal peptide has a common structure of a positively charged n-region followed by a hydrophobic h-region and a