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Identification and characterization of a novel O-superfamily conotoxin from *Conus litteratus*

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Abstract: A novel conotoxin named lt6c, an O-superfamily conotoxin, was identified from the cDNA library of venom duct of *Conus litteratus*. The full-length cDNA contains an open reading frame encoding a predicted 22-residue signal peptide, a 22-residue proregion and a mature peptide of 28 amino acids. The signal peptide sequence of lt6c is highly conserved in O-superfamily conotoxins and the mature peptide consists of six cysteines arranged in the pattern of C–C–CC–C–C that is defined the O-superfamily of conotoxins. The mature peptide fused with thioredoxin, 6-His tag, and a Factor Xa cleavage site was successfully expressed in *Escherichia coli*. About 12 mg lt6c was purified from 1L culture. Under whole-cell patch-clamp mode, lt6c inhibited sodium currents on adult rat dorsal root ganglion neurons. Therefore, lt6c is a novel O-superfamily conotoxin that is able to block sodium channels. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *Conus litteratus*; O-superfamily conotoxin; fusion expression; whole-cell patch-clamp; voltage-gated sodium channel

INTRODUCTION

Cone snails (genus *Conus*) are incredibly rich sources of bioactive small peptides. It is estimated that there are about 500–700 species of cone snails around the world. Each of the *Conus* species can express ~100 small, highly structured peptides in the venom with little interspecies overlap [1]. There are probably more than 50 000 different conotoxins present in the venoms of living species of Conus snails [2]. Conotoxins also surprise researchers with their abilities to discriminate different subtypes of their molecular targets or ion channels, which makes these peptides not only effective tools for neuroscience research but also promising therapeutic agents [2,3]. These venom peptides are generally classified into two groups: the non-disulfide-rich conopeptides and the disulfide-rich conotoxins. On the basis of the distinct signal peptide sequences of precursors and pattern of disulfide bonds, conotoxins can be categorized into several superfamilies. A-, L-, T-, O-, M-, P- and I-superfamilies are some of the identified conotoxin superfamilies [4]. Conotoxins from the same superfamily can be further divided into several families according to their pharmacological targets.

A great variety of O-superfamily conotoxins have been discovered from various cone snail species as described in previous reports [3,5–8]. Some identified conotoxins of O-superfamily can be roughly divided into four families: δ-, μO-, ω - and κ-conotoxins. Though they share the same Cys arrangement pattern (C–C–CC–C–C), they target different kinds of ion channels. δ- and μO-conotoxins inhibit inactivation and activation of sodium channels, respectively. ω-conotoxins inhibit calcium channels and κ-conotoxins block potassium channels [2]. Several conotoxins of O-superfamily are widely used as molecular probes in neuroscience and pharmacology research. For instance, ω-conotoxin GVIA is the most widely used conus toxin in neuroscience research, and ω-conotoxin MVIIA has been approved for the treatment of chronic pain by the United States Food and Drug Administration [9].

Here, we describe the identification of a novel O-superfamily conotoxin, lt6c, from the cDNA library of venom duct of *Conus litteratus*. In order to obtain enough mature peptide, the recombinant lt6c was produced in *Escherichia coli* by fusion with TRX and 6-His tag. The effect of recombinant mature lt6c on the sodium current of mature DRG was recorded under the whole-cell patch-clamp experiment.

MATERIALS AND METHODS

cDNA Library Construction

Cone snails *C. litteratus* were collected from Yalong Bay near Sanya (coastal city by South China Sea), Hainan Province. The venom duct of cone snails were dissected and immediately

Abbreviations: DRG, dorsal root ganglion; TRX, thioredoxin; HEPES, 4-(2-hydroxyethyl)-1-poperazineethanesulfonic acid; VGSC, voltage-gated sodium channel.

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preserved in liquid nitrogen. The following steps for total RNA isolation and cDNA library construction were performed as previously described [10]. Briefly, total RNA was isolated from the homogenized venom duct by using TRIZOL (Invitrogen, Carlsbad, USA). The cDNA was synthesized using SMART cDNA library construction kit (Clontech, Japan) and cloned into pcDNA3.0 according to the manufacturer's protocol.

Sequence Analysis

cDNA clones were randomly sequenced using ABI Prism BigDye Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems, USA) by ABI3730 automatic sequencer (Applied Biosystems, USA). T7 and SP6 primers were used for the sequencing. The cDNA sequences and the putative proteins were used to BLAST, the protein database available from NCBI (National Center for Biotechnology Information). The signal peptide sequences and cleavage sites of the conotoxin precursors were predicted by SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP/).

Construction of Fusion Expression Vector pTRX-lt6c

The lt6c was fused to TRX in order to express it soluble in *E. coli*. The expression vector pTRX-lt6c was constructed as previously described [11]. Briefly, the artificial DNA sequence encoding lt6c was designed by replacing the rare or the low-usage codons with *E. coli* preferred codons. Four oligonucleotides were annealed to amplify lt6c peptide coding sequences (Table 1). Restriction endonuclease *KpnI* site and a region encoding protease Xa were included at the 5′ end, while *NotI* cleavage sites and a stop condon were contained at the 3′ end in the synthesized lt6c gene. The synthesized fragment was cloned into pTRX digested with *KpnI* and *NotI*. The recombinant plasmid pTRX-lt6c was confirmed by DNA sequencing. The 6-His tag was designed between TRX and lt6c to simplify the purification of fusion protein by using Ni^{2+} affinity chromatography (Figure 1). Protease Factor Xa cleavage site after 6-His tag facilitated removing of the fusion partner and purification of mature recombinant peptide.

Expression and Purification of lt6c

The pTRX-lt6c was transformed into *E. coli* BL21(DE3). A single colony was inoculated into 100 ml liquid Lysogeny Broth (LB) medium containing 100 μg/ml ampicillin and cultured at 37 °C for 15 h. The culture was expanded in a ratio of 1 : 50 into 2l rich liquid LB medium and shaken at 37 °C for another 2.5 h until OD600 reached 0.8. The expression of exogenous protein was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM at 21 °C for 8 h. Cells were harvested by centrifugation and 10 g of cell pellet was suspended in 100 ml sonication buffer (500 mM NaCl, 50 mM Tris-HCl, pH8.0) and sonicated. The cell debris was sedimented by centrifugation at 8000 g (Himac CR21G, Japan) for 40 min and the supernatant with soluble proteins was used for purification.

The supernatant was loaded onto Ni^{2+} chelating sepharose fast flow column (GE Healthcare, USA), which was charged with Ni^{2+} ions and pre-equilibrated using sonication buffer. The column was washed with the sonication buffer until the absorbance value at 280 nm reached the base line. The binding proteins were eluted with 50 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl and a gradual increase of concentration of imidazole. Each fraction was collected and analyzed by Tricine SDS-PAGE [12]. Fractions containing fusion proteins were applied to a Sephadex G-25 fine column to change buffer with cleavage buffer (50 mM Tris, 200 mM NaCl, 5 mM $CaCl_2$, pH 8.0). Fusion protein was cleaved with Protease Factor Xa (Novagen, Madison, USA) at 21 °C for 16 h. The cleavage product was loaded onto a Sephadex G50 Fine column (26 × 1000 mm) pre-equilibrated with 50 mM NH_4HCO_3. Each elution fraction was analyzed by Tricine SDS-PAGE. Fractions containing the target proteins were pooled and condensed by lyphilization (CHRIST BETA 1–8K, Germany). The lt6c was further purified through a C18 reverse-phase column (Inertsil ODS-3, 4.6 × 250 mm, 5 μm particle size, 300 Å pore size; GL Sciences Inc., Japan) on Waters 600E HPLC system (Waters, USA). The protein concentration was determined using the method of Lowry, and bovine serum albumin was used as the standard.

Table 1 Four synthesized oligonucleotide fragments used to form lt6c gene with enzyme digestion sites and the corresponding coding sequences

Oligoes	DNA and the coding sequences of lt6c
Oligo I	5′ C ATT GAG GGC CGC TGG CCG TGC AAG GTT GCC GGT AGT CCT TGT GGT CTG 3′
Oligo II	3′ CATGG TAA CTC CCG GCG ACC GGC ACG TTC CAA CGG CCA TCA GGA ACA CCA GAC CAATCA 5′
	I E G R W P C K V A G S P C G L
Oligo III	5′ GTT AGT GAA TGC TGC GGA ACT TGC AAT GTT TTA CGC AAT CGT TGT GTG TGA GC 3′
Oligo IV	3′ CTT ACG ACG CCT TGA ACG TTA CAA AAT GCG TTA GCA ACA CAC ACT CGCCGG 5′
	V S E C C G T C N V L R N R C V *

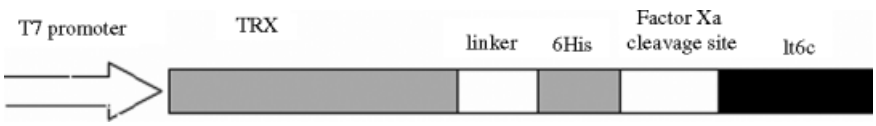

Figure 1 Schematic representation of the expression vector pTRX-lt6c.

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Electrophysiology

DRG neurons were acutely dissociated from 30-day-old Sprague-Dawley (SD) rats and maintained in short-term primary culture described previously [12]. Briefly, SD rats of either sex were killed by decapitation and the DRG were isolated quickly and cut into small pieces, and digested with collagenase followed by trypsin. Trypsin inhibitor (1.5 mg/ml, typeII-S) was added to inactivate enzyme and the DRG cells were suspended into Dulbecco's modified eagle's medium (DMEM) and incubated in CO_2 incubator at 37 °C for 3 h before patch-clamp experiment.

Micropipettes were pulled from borosilicate glass capillary tubing (1.0–2.0 mm diameter) using a P97 puller (Sutter Instrument Co, USA). The resistances of the micropipettes were 2–5 MΩ after filling with internal solution of the following composition (in mM): CsF 135, NaCl 10, N-2-HEPES 5, EGTA 5, MgATP 2, pH 7.0 adjusted with 1 M CsOH. The external solution contained the following components (in mM): NaCl 30, CsCl 5, D-glucose 25, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, TEA chloride 20, tetramethylammonium (TMA) chloride 70, LaCl 0.01, pH 7.4 adjusted with 1 M TEA hydroxide [13].

Whole cell recordings of sodium currents were performed using the method as described previously [14]. Briefly, whole cell recording was carried out at 25 °C with a patch/whole cell clamp Amplifier CEZ2400 amplifier (Nihon Kohden, Japan). Stimulation and recording were controlled by a pClamp data acquisition system (Axon Instruments). In all voltage-clamp experiments, the membrane potential was held at −80 mV.

RESULTS

Sequence Analysis of a Novel Conotoxin lt6c

A high quality cDNA library of the venom duct from *C. litteratus* was successfully constructed and 42 novel conotoxins were identified by expressed sequence tag (EST) sequencing and BLAST analysis [10]. One cDNA clone contains an open reading frame of 216 bp that encodes a 72-residue precursor. This precursor has 59% of amino acid identity to MVIIA and SVIB precursor, two representative O-superfamily conotoxins. The signal peptide cleavage site predicted by SignalP 3.0 Server located between Ser^{22} and Asp^{23}. The characteristic basic residue Arg^{44} before the first cysteine residue at the *N*-terminus of the mature peptide may act as the processing cleavage site. A mature peptide, designated as lt6c, with 28 amino acid residues was generated by the proteolytic cleavage of the precursor (Table 2). The signal sequence in the lt6c precursor exhibits high homology to the corresponding region of previously characterized O-superfamily conotoxins (Figure 2). The arrangement of cysteine residues in the primary sequence of the mature peptide of lt6c (C–C–CC–C–C) is similar to that of identified O-superfamily conotoxins indicating that lt6c could be categorized as O-superfamily of conotoxin. Similar to MrVIA, lt6c is a basic peptide with two net charges (Table 3).

Functional Expression and Purification of lt6c

The engineering recombinant *E. coli* strain BL21(DE3) harboring vector pTRX-lt6c was induced with IPTG for 8 h to express the fusion protein including TRX partner, 6-His tag, protease Factor Xa cleavage site and lt6c mature peptide. The resulting proteins were run on SDS-PAGE and a band with the size about 18 kD corresponding to the fusion protein appeared in the total cell lysate and supernatant of cell lysate (Figure 3). A weak band was seen in the cell pellet indicating that the fusion protein was expressed solubly in *E. coli*.

The fusion protein was purified by the Ni^{2+} chelating sepharose fast flow and eluted from the column with 180 mM imidazole in 50 mM Tris-HCl and 500 mM NaCl

Table 2 The full-length cDNA and putative amino acid sequence of lt6c

ggagcctga cttcaccttt cttcgccgcc tcctttggca tcacccagac catcatcaga

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1    ATG AAA CTG ACG AGT GTG GTG ATC GTC GCT GTG TTG TTC CTG GCG GCC TGT CAA CTC ACT   60
1     M   K   L   T   S   V   V   I   V   A   V   L   F   L   A   A   C   Q   L   T   20

61   ACA TCT GAT GGC TCC AGA GGT ACG TGG AAG GAT CGT GCT GTG AGG TCG ATC ACC AAA GTC  120
21    T   S   D   G   S   R   G   T   W   K   D   R   A   V   R   S   I   T   K   V   40

121  TCC ATG TTG CGA TGG CCC TGC AAG GTT GCC GGT AGT CCT TGT GGT CTT GTT AGT GAA TGC  180
41    S   M   L   R   W   P   C   K   V   A   G   S   P   C   G   L   V   S   E   C   60

181  TGT GGA ACT TGC AAT GTT TTA CGC AAT AGA TGT GTG TGA GTG GCT GAT CCG GCG TCT GGT  240
61    C   G   T   C   N   V   L   R   N   R   C   V   *

241  ctttccgcct  tctgtgctct  atccttgttc  gcctgcgtcc  tccatagctg tgagtggtca  tgggccactc  aacacctact
321  cctctggagg  cttcagagga  actacattca  aataaagccg  cattgcaatg aaaaaaaaaa  aaaaa
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The signal peptide sequence is shaded and the mature toxin region is underlined. The coding sequence is in uppercase while untranslated region in lowercase letters. The polyadenylation signal 'aataaa' is double underlined.

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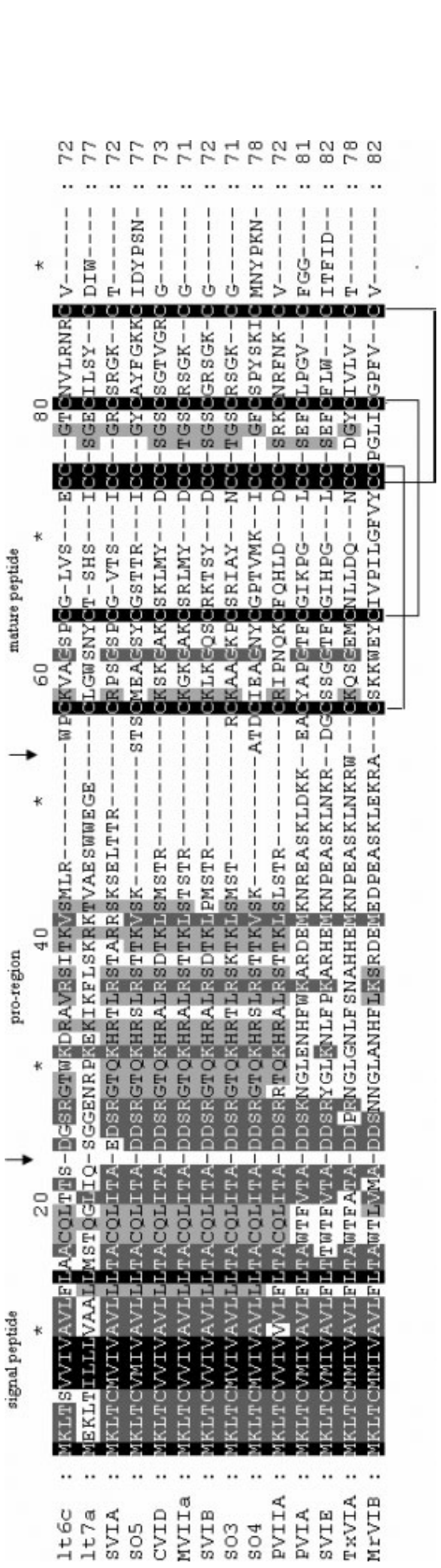

Figure 2 Alignment of amino acid sequences of lt6c precursor with several known O-superfamily conotoxins. The identical and similar amino acids are shaded in dark and gray background, respectively. Gaps are inserted to maximize similarity. The arrows indicate the cleavage sites during the proteolytic process of prepeptide and proregion. The disulfide connectivity was indicated by the straight lines. The sequences of other O-superfamily conotoxin precursors were obtained from Genbank (The accession no is : lt7a, ABC74981; SVIA, AF146361.1; SO5, Q9XZK4; CVID, P58920; MVIIA, P05484; SVIB, P28881; SO3, Q9XZK2; SO4, Q9XZK3; PVIIA, P56633; PVIA, P58913; SVIE, Q9XZK5; TxVIA, P18511; MrVIB, q26443).

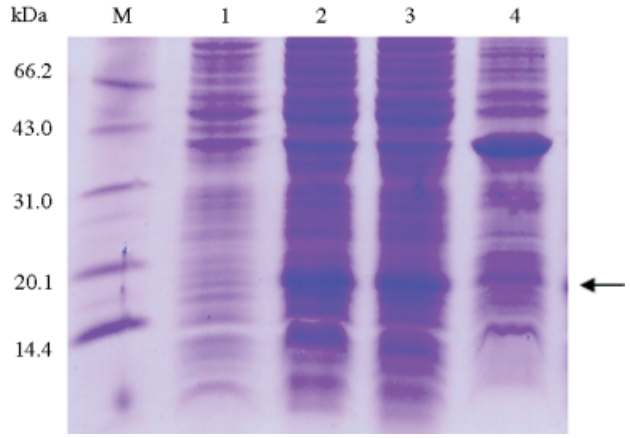

Figure 3 SDS-PAGE analysis of recombinant TRX-lt6c expressed in BL21(DE3). M, the standard protein marker; Lane 1, total proteins of preinduced *E. coli*; Lane 2, total proteins of *E. coli* with plasmid pTRX-lt6c induced; Lane 3, supernatant from induced *E. coli* with plasmid pTRX-lt6c. Lane 4, pellet from induced *E. coli* with plasmid pTRX-lt6c.

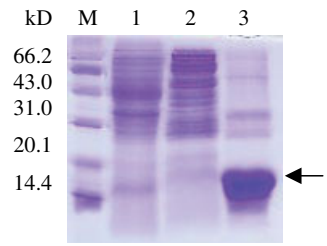

Figure 4 Purification of TRX-lt6c fusion protein. The standard protein marker M; Lane 1, the flow-through fraction of TRX-lt6c from Ni^{2+} Chelation Sepharose Fast Flow Chromatograph; Lane 2, elution fraction of TRX-lt6c with 75 mM imidazole; Lane 3, elution fraction of TRX-lt6c with 180 mM imidazole.

(Figure 4). After the buffer was changed to the cleavage buffer through G25 sephadex chromatography, the purified fusion protein was cleaved with protease Factor Xa at 21 °C for 16 h. The recombinant lt6c was purified by Sephadex G-50 column and the protein was lyophilized for further verification.

Verification of Recombinant lt6c

To analyze the characterization of recombinant lt6c, the lyophilized protein was resolved in *dd*H_2O and analyzed using reverse-phase HPLC, and a single peak was eluted at about 13.5–14 min. This peak was further analyzed using MALDI-TOF-MS. A peak with molecular weight of 2948.50 was detected, which is similar to the molecular weight (2949.46) of recombinant lt6c calculated from the amino acid composition. Twelve milligrams of recombinant lt6c was obtained from 1 liter culture.

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Table 3 Characterization of several known O-superfamily conotoxins

Name	Family	Cysteine motif	Net charge	Species	Molecular target	Reference
lt6c	μO-	$CX_6CX_5CCX_2CX_6C$	+2	*C. litteratus*	Rat DRG neuron Na channel	This work
lt7a	μO-	$CX_6CX_5CCX_3CX_4C$	−2	*C. litteratus*	Rat DRG neuron Na channel	1
SVIA	ω -	$CX_6CX_5CCX_2CX_4C$	+4	*C. striatus*	Ca channel	15
CVID	ω -	$CX_6CX_6CCX_3CX_6C$	+4	*C. catus*	$Ca_v2.2$	16,17
MVIIa	ω -	$CX_6CX_6CCX_3CX_4C$	+5	*C. magus*	$Ca_v2.2$	2
SVIB	ω -	$CX_6CX_6CCX_3CX_5C$	+5	*C. striatus*	Ca channel	15
SO3	ω -	$CX_6CX_6CCX_3CX_4C$	+6	*C. striatus*	Ca channel	25
PVIIA	κ-	$CX_6CX_6CCX_3CX_5C$	+3	*C. purpurascens*	*Shaker*-Δ6–46 K Channel	2
PVIA	δ -	$CX_6CX_6CCX_3CX_4C$	0	*C. purpurascens*	Amphibian Na_v, $rNa_v1.2$, 1.4, 1.7	18
SVIE	δ -	$CX_6CX_6CCX_3CX_4C$	−3	*C. striatus*	Amphibian Na_v, $rNa_v1.4$	18
TxVIA	δ -	$CX_6CX_6CCX_3CX_4C$	−2	*C. textile*	Molluscan Na_v	18
MrVIB	μO-	$CX_6CX_9CCX_4CX_4C$	+1	*C. marmoreus*	TTX-R($hNa_v1.8$) > TTX-S	18
MrVIA	μO-	$CX_6CX_9CCX_4CX_4C$	+2	*C. marmoreus*	TTX-R($hNa_v1.8$) > TTX-S	18

Effect of Recombinant lt6c on Sodium Currents

The effects of lt6c on sodium currents were shown under voltage clamp conditions (Figure 5). Both TTX-S and TTX-R currents were induced on adult rat DRG neurons by a 50 ms depolarization of −10 mV from a holding potential of −80 mV. The amplitude of sodium currents was reduced by 800 nM lt6c (Figure 5). Although the sodium current was inhibited by conotoxin lt6c, the shape of currents was similar to that of control, indicating that lt6c did not affect the activation and inactivation kinetics of sodium channels.

When the membrane potential of DRG cells was held at −80 mV, sodium currents were initially elicited by a depolarization of −40 mV and reached maximal amplitude by a depolarization of around −10 mV. After the reduction of the sodium current amplitude by 800 nM lt6c, there was no change in both the threshold of activation and the active voltage of peak inward currents (Figure 5(C)).

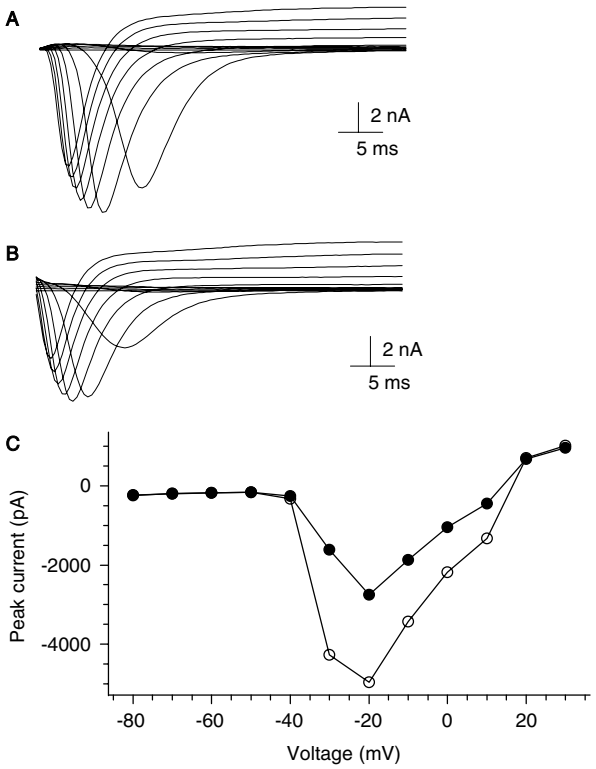

Figure 5 Effect of recombinant lt6c on the transient sodium channels of rat DRG neurons. Both TTX-S and TTX-R currents were induced by 50 ms depolarizing steps to various potentials from a holding potential of −80 mv. Test pulses were from −80 to +30 mV in 10 mV steps. Addition of 800 nM lt6c leads to an apparent reduction in currents (B) compared to control (A). The I–V curves showed the relationships of sodium current under control conditions (open circles) and after 20-min exposure to 800 nM lt6c (filled circles) (C).

DISCUSSION

The venoms of *Conus* snails yield a complex library of about 50 000 bioactive peptides in nature [19]. However, the precursor organization is constant: a highly conserved signal sequence at the *N*-terminals, an intervening proregion and the hypervaried biologically active mature conotoxin in the *C*-terminals [2,19]. Here, we report a novel conotoxin isolated from the cDNA library of venom duct of *C. litteratus*. Its highly conserved signal peptide sequence of O-superfamily conotoxin and characterized Cys arrangement pattern suggested that this novel toxin belonged to O-superfamily (Figure 2). Except for the location of Cys residues, the sequence of lt6c mature peptide is greatly different from other O-superfamily conotoxins including lt7a, an O-superfamily conotoxin from the venom of the same cone snail species (*C. litteratus*) [10,11]. It is revealed that the cone snail toxins are extremely

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complex even in the same superfamily or the same cone snail species [6,8].

VGSCs are large transmembrane proteins that mediate the rising phase of the action potential in excitable cells. VGSCs are the molecular targets for a diverse range of neurotoxins, such as tetrodotoxin, saxitoxin, scorpion, sea anemone toxins, which strongly alter channel function by binding to specific receptor sites [20]. VGSCs-targeted conotoxins has been well reviewed previously [18]. μO-conotoxins inhibit Na channel conductance like μ-conotoxins, but most likely through a different mechanism [2]. δ-conotoxins increase sodium currents by inhibiting the inactivation process [21,22]. Because most of the O-superfamily conotoxins have effect on VGSCs (except for ω- and κ-conotoxins), we tested the effect of lt6c on the sodium currents of DRG neurons. Sodium currents recorded from DRG neurons were inhibited by recombinant lt6c, and the activation and inactivation kinetics of sodium channels were not affected by lt6c. It is suggested that the electrophysiological activity of lt6c is similar to that of μO-conotoxins and O-superfamily conotoxin lt7a, although the subtype-selectivity and the mechanism of interaction with sodium channels remain to be further studied. Therefore, lt6c together with lt7a, described previously by us [11,] might be classified into μO-conotoxin family and would be the new molecular probes for the structure–function research of sodium channels.

There are three ways to obtain sufficient conotoxins for further study of biological activity: biochemical purification from the natural cone snail resources, chemical synthesis and recombinant expression in different expression systems. Conventional biochemical purification could not meet the need for further functional research because of the difficulty in collecting enough samples. Chemical synthesis is the first choice for the short peptides. But chemical synthesis is not an economic strategy for the production of long peptides. Recently, the reports about recombinant expression of conotoxin were increasing and the widely used recombinant strategy of conotoxin is fusion toxin with a partner. The first recombinant conotoxin precursor pro-ω-MVIIA-Gly was expressed as a fusion protein with TrpLe protein in *E. coli*, but the aimed protein appeared insoluble [23]. A recombinant ω-MVIIA was recently produced in *E. coli* by fusion with TRX and a His-tag [24]. In our previous study, lt7a, an O-superfamily conotoxin, was successfully expressed as a fusion protein with TRX in *E. coli*. In this work, lt6c, a novel O-superfamily conotoxin, was successfully expressed and purified from *E. coli*. The expression level of lt6c and lt7a fusion protein are similar, but the production of the mature peptide lt7a (cleavage of fusion partner and the subsequent purification of mature peptide) is more difficult than that of lt6c (data was not shown). The yield of lt6c (about 12 mg/l) is higher than that of lt7a (about 6 mg/l). It was proposed that the primary amino acid sequences and/or the spacing of Cys residues (lt6a, $CX_6CX_5CCX_2CX_6C$; lt7a, $CX_6CX_5CCX_3CX_4C$) might affect the proper refolding of recombinant conotoxins [23]. However, recombinant expression is becoming an alternative strategy for producing disulfide-rich and long conotoxins [23].

In conclusion, a novel O-superfamily conotoxin was identified and the mature peptide was obtained by fusion expression in *E. coli*. The whole cell-patch clamp recording revealed that the novel conotoxin blocked VSGS in rat dorsal ganglion neurons. This study will lay a foundation for further application of this conotoxin.

Acknowledgements

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