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Im10A, a short conopeptide isolated from *Conus imperialis* and possesses two highly concentrated disulfide bridges and analgesic activity

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ABSTRACT

In the present study, we isolated, synthesized and NMR structurally characterized a novel conopeptide Im10A consisting of 11 amino acids (NTICCEGCMCY-NH_2) from *Conus imperialis*. Unlike other conopeptides with four cysteine residues, Im10A had only two residues in loop 1 and one residue in loop 2 (CC-loop1-C-loop2-C), which formed a stable disulfide connectivity "I-IV, II-III" (framework X) with a type I β-turn. Interestingly, Im10A exhibited 50.7% analgesic activity on rat partial sciatic nerve ligation (PNL) at 2 h after Im10A administration. However, 10 μM Im10A exhibited no apparent effect on neuronal nicotinic acetylcholine receptor, and it did not target DRG voltage-dependent sodium, potassium and calcium ion channels and opioid receptor. To our knowledge, Im10A had the most concentrated disulfide bridges among conopeptides with four cysteine residues. This finding provided a new motif for the future development of biomimetic compounds.

1. Introduction

Conopeptides are small peptides consisting of 12–50 amino acids and two to five disulfide bridges, and they are derived from the venom salivary glands of cone snails. Conopeptides are grouped into several superfamilies [1,2], based on conserved signal peptide sequences and specific cysteine patterns within each superfamily. To date, more than 26 superfamilies have been identified [2–4]. Conopeptides selectively act on a wide variety of ion channels (Na^+, K^+, Ca^{2+}) and membrane receptors, such as nicotinic acetylcholine receptor (nAChR), 5-hydroxytryptamine (5-HT3R), N-methyl-D-aspartate receptors (NMDAR) and G-protein-coupled receptors [5–7], and they are highly valued as neuropharmacological probes and pharmaceutical development [8,9]. In addition, the tight disulfide bridges in conopeptides are good template for the design of structurally-restricted molecules [10]. For example, disulfide bridges have been engineered into enzyme inhibitors [11,12], antimicrobial peptides [13–15] and ion channel inhibitors [16] to improve the activity and stability of these molecules.

In the present study, we isolated a novel conopeptide Im10A with 11 amino acids (NTICCEGCMCY-NH_2) from *Conus imperialis* and obtained its complete sequence. The peptide was then synthesized and structurally characterized by NMR. Unlike other conopeptides, Im10A had only two and one residues between four cysteines (CC-C-C), which formed a stable disulfide connectivity "I-IV, II-III". Furthermore, Im10A exhibited 50.7% analgesic activity on rat partial sciatic nerve ligation (PNL) model at 2 h after intramuscular injection of 100 nmol/kg Im10A. To our best knowledge, Im10A possessed the most concentrated disulfide bridges among conopeptides with four cysteine residues. This finding provided a new motif for the design of functional compounds.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (SD, 220g, Beijing Animal Center, China) were group-housed (n = 8) and maintained under a 12 h light-/dark cycle (light cycle from 8AM–8PM) at a temperature of

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23 ± 2 °C and a relative humidity of 50%. Food pellets and water were available *ad libitum*. All experiments were conducted in accordance with the guidelines of Animal Research Advisory Committee in Beijing Institutes for Biological Sciences and conformed to the European Community directives for the care and use of laboratory animals.

2.2. Peptide isolation and sequencing

Specimens of *Conus imperialis* were collected in the Xisha islands of the South China Sea. The venom ducts were removed immediately, frozen and stored at −70 °C prior to further analysis. A total of 313 venom ducts were thawed and broken to pieces, and they were extracted by 5% acetic acid and homogenized in a glass vehicle. Subsequently, the homogenate was centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant was freeze dried to yield 8.2 g crude toxin powder. The crude venom extract was separated by Sephadex G-25 chromatography (3.2 × 100 cm) and eluted with 0.5% acetic acid at a flow rate of 0.3 mL/min. The peptide fraction was then isolated from QHP Sepharose (13mL, Amersham AKTA Prime) at a flow rate of 1 mL/min with a linear gradient under conditions as follows: 0–10 min, 0%B;10–20 min, 0–10%B;20–40 min, 10–50%B;40–70 min, 100%B; where A is 10 mM Tris·HCl (pH 7.5) and B is 10 mMTris·HCl + 1 M NaCl (pH 7.5). Finally, the lyophilized peptides were further separated on a Kromasil-C_{18} analytical column (5 μm, 250 × 4.6 mm, Beijing Analysis Apparatus Factory) at a flow rate of 1 mL/min with a linear gradient under conditions as follows: 0–2 min,0–5%B;2–25 min,5-50%B;25–35 min,50-80%B, 214 nm; where A is H_2O (0.1% TFA) and B is acetonitrile (0.1% TFA).

The amino acid sequences were analyzed by automated Edman degradation on an ABI 419 Sequencer as previously described [17].

2.3. Peptide synthesis

Im10A and its variants were synthesized using the previously described method [18]. Briefly, the peptides were synthesized and then cleaved from Rink resin with the cleavage solution (TFA, 8.8 mL; water, 0.5 mL; DTT, 0.5 g; Triisopropylsilane, 0.2 mL). The released peptides were oxidized in 0.1 M NH_4HCO_3 at room temperature, pH 8.0–8.2. The folded products were then purified using semi-preparative RP-HPLC and assessed using analytical reversed-phase HPLC.

2.4. Analysis of disulfide bridges

Because of the limited quantity of natural peptides, the disulfide arrangement of synthetic Im10A by one-step oxidative folding was determined through comparison of peptide folding products with known disulfide connectivity [19,20]. The linear peptides containing acetamidomethyl (Acm) protecting groups at positions C^2—C^3 or C^1—C^4 were folded in 0.1 M NH_4HCO_3 buffer (pH = 8.0) at room temperature for 24–48 h. The folded products were further oxidized with an iodine buffer containing 30% CH_3CN, 2% TFA, and 68% H_2O for 10 min to form different types of disulfide bonds such as "C^1—C^3, C^2—C^4" and "C^1—C^4, C^2—C^3". These secondary oxidization products were mixed with the one-step folding product of Im10A, and the connectivity disulfide bond was then determined.

2.5. NMR spectroscopy

Samples of Im10A were dissolved in 500 μL phosphate buffer (20 mM, pH 5.5) containing 10% D_2O. NMR experiments, including TOCSY, NOESY and DQF-COSY, were conducted on a Bruker Avance 800 MHz spectrometer using TXI cryoprobe. All spectra were collected at 298 K. TOCSY spectra were obtained with mixing times of 25 and 70 ms. NOESY spectra were obtained with mixing times of 400, 600, 800 ms. H/D exchange experiment was performed at 298 K by adding 500 μL D_2O to the lyophilized sample.

Spectra were processed using NMRpipe software and peak analyses were conducted with XEASY module in Cara (version 1.5.5). Spin systems were established based on DQF-COSY and TOCSY spectra. A total of 206 NOE peaks were identified. The NOE intensities in NOESY spectrum with an 800-ms mixing time were extracted by CARA 1.8.4.2 and converted into distance constraints. $^3J_{H\alpha-NH}$ coupling constants were determined from DQF-COSY. A total of 100 structures were calculated using CYANA (version 2.1) with 84 non-redundant NOE constraints and 10 dihedral-angle constraints [21]. The final 18 conformers with the lowest energies were refined using Amber11. Disulfide bond constraints were added in the structure calculation, with three upper limit distance constraints for each bond [22]. Moreover, 72.9% of the phi-psi torsion angles in the structure were in the most favored regions in the Ramachandran plot according to Procheck. The three-dimensional structure was created using MOLMOL graphics program. The data, including chemical shifts, were submitted to the BMRB database with access codes 21062 for Im10A.

2.6. Analgesic activity tests

The analgesic activity of Im10A was determined using the classic rat PNL model [23]. Briefly, the male rats (230–270 g) were anesthetized by intraperitoneal (i.p.) injection of entobabital sodium (55 mg/kg), and the PNL was then performed. At 7 days after surgery, the rats were screened for reduction of mechanical paw withdrawal threshold (PWT) for the ipsilateral hind paw by 30%–50%. They were then assigned into five groups as follows: sterile saline (negative control) group, Vc1.1 group, or Im10A group. Im10A was dissolved in 0.9% sterile saline to a volume of 200 μL and intramuscularly administered with (12, 100, 200 nmol/kg) to the injury site in the mid-thigh region. To assess mechanical hyperalgesia, the PWT was measured with Ugo Basile analgesimeter (Ugo Basile, Italy) to produce increasing pressure to the hind paw until paw withdrawal occurred. PWTs were measured at 2 and 4 h after intramuscular administration.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). All results were expressed as mean ± SEM, and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. The isolation and sequencing of Im10A

The venom ducts of *Conus imperialis* were broken to pieces, extracted by 5% acetic acid and homogenized in a glass vehicle. The soluble components from centrifugation were lyophilized, separated by Sephadex G-25 chromatography (Fig. 1A) and eluted with 0.5% acetic acid. The peptide fraction (dashed area) was then isolated from QHP Sepharose, and the eluent part 2 (20–40 min, Fig. 1B) was further lyophilized and purified with RP-HPLC, which gave several peaks (Fig. 1C). The mass spectral analysis showed that the molecular weight of the peptide with a retention time of 14 min was 1233.4 Da ($[M+H]^+$ = 1234.4). The initial sequencing of the pyridylethylated peptide combined with mass spectral analysis yielded an 11-amino-acid peptide (NTICCEGCMCY-NH_2), and it was designated as Im10A according to the rule of conopeptide nomenclature [24].