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A new subfamily of conotoxins belonging to the A-superfamily

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Abstract

Two novel conotoxins from vermivorous cone snails *Conus pulicarius* and *Conus tessulatus*, designated as Pu14.1 and ts14a, were identified by cDNA cloning and peptide purification, respectively. The signal sequence of Pu14.1 is identical to that of α -conotoxins, while its predicted mature peptide, pu14a, shares high sequence similarity with ts14a, with only one residue different in their first intercysteine loop, which contains ten residues and is rich in proline. Both pu14a and ts14a contain four separate cysteines in framework 14 (C-C-C-C). Peptide pu14a was chemically synthesized, air oxidized, and the connectivity of its two disulfide bonds was determined to be C1–C3, C2–C4, which is the same as found in α -conotoxins. The synthetic pu14a induced a sleeping symptom in mice and was toxic to freshwater goldfish upon intramuscular injection. Using the *Xenopus* oocyte heterologous expression system, 1 μ M of pu14a demonstrated to inhibit the rat neuronal $\alpha 3\beta 2$ -containing as well as the mouse neuromuscular $\alpha 1\beta 1\gamma \delta$ subtypes of nicotinic acetylcholine receptors, and then rapidly dissociated from the receptors. However, this toxin had no inhibitory effect on potassium channels in mouse superior cervical ganglion neurons. According to the identical signal sequence to α -conotoxins, the unique cysteine framework and molecular target of pu14a, we propose that pu14a and ts14a may represent a novel subfamily in the A-superfamily, designated as $\alpha 1$ -conotoxins.

Keywords

Conotoxins; A-superfamily; cysteine framework 14; nicotinic acetylcholine receptors

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1. Introduction

Conotoxins are highly diversified peptides produced by marine cone snails for prey and defense. They target diverse ion channels, receptors, or neurotransmitter transporters in the muscular and nervous system. It is estimated that each of the 500–700 different species in the genus *Conus* can produce 50–200 different components in their venom. Thus, conotoxins represent an ideal natural peptide library to investigate toxin structural diversity, and to develop specific tools for neuropharmacological research or lead compounds for the treatment of nervous system diseases [25].

Most conotoxins are disulfide-rich peptides which are classified into different families based on their cysteine patterns and molecular targets. Families of conotoxins can be grouped into a certain superfamily if they share highly conserved signal peptides in their precursors. Thus far, A-, T-, M-, O-, P-, I-, L-, J-, V-, Y-, S-, and D-superfamily conotoxins have been identified [11]. Among these superfamilies, the A-superfamily of conotoxins is the best characterized one.

The A-superfamily of conotoxins contains three pharmacological families: α -conotoxins, α A-conotoxins, and κ A-conotoxins [25]. α -Conotoxins have four cysteines (CC-C-C) that form two disulfide bonds with the C1–C3 and C2–C4 connectivity, and they act on nicotinic acetylcholine receptors (nAChRs) with distinct selectivities depending on the residue number and sequence of their two intercysteine loops. α A-conotoxins also target nAChRs, but they have the CC-C-C-C-C cysteine framework. The first two long α A-conotoxins (α A_L-conotoxin), PIVA and EIVA, were found to adopt the C1–C5, C2–C3, and C4–C6 disulfide connectivity and share similar three dimensional structure [2, 6, 8, 10]. More recently, a new short α A-conotoxin subfamily (α A_S-conotoxins) was reported to have the distinct C1–C3, C2–C5, and C4–C6 disulfide linkages [24]. Similarly, κ A-conotoxins with the same cysteine pattern as α A-conotoxins also include two subfamilies, the long κ A- and short κ A-conotoxins (κ A_L- and κ A_S-conotoxins), but these toxins block K⁺ channels other than nAChRs [23]. In particular, α A_S-conotoxins and κ A_S-conotoxins have the same intercysteine spacing and disulfide linkage, but they clearly differ in physiological functions [23, 24].

Despite of the complicated classification of A-superfamily conotoxins, their structural diversity has not been fully explored. In this work, we describe the identification of two novel conotoxins with high sequence homology, Pu14.1 (GenBank nucleotide accession no. EU912017) and ts14a (UniProt: P86362), both of which possess the 14th cysteine framework (C-C-C-C) and have the same disulfide connectivity to that of α -conotoxins. The identical signal peptide of Pu14.1 to that of A-superfamily conotoxins, the molecular target of the predicted mature peptide of Pu14.1, pu14a, as well as the unique sequence of pu14a and ts14a that differ from all known α -conotoxins, suggest that these two toxins are defining members of a new subfamily belonging to the A superfamily, which we designated α 1-conotoxins.

2. Materials and methods

2.1. Materials

The TRIzol reagent and Rapid Amplification of cDNA 3'-Ends Kit were purchased from Invitrogen (Carlsbad, CA, USA), *Taq* DNA polymerase from TaKaRa (Dalian, China), all Fmoc amino acids from GL Biochem Ltd. (Shanghai, China), trifluoroacetic acid (TFA) and acetonitrile (ACN) for HPLC from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Preparation of total RNA and cDNA cloning

The specimens of *Conus pulicarius* were collected from the South China Sea, Hainan, China. The total RNA of venom duct was extracted and purified from 6 specimens of *C. pulicarius* using the TRIzol reagent according to the manufacturer's protocol.

The venom duct cDNAs of *C. pulicarius* were prepared from approximately 5 µg total RNA using the Rapid Amplification of cDNA 3'-Ends Kit following the supplier's method. The resulting cDNAs served as the template for PCR. The forward primer (5'-ATG GGC ATG CCG ATG ATG TT-3') paired with the reverse primer (5'-GTC GTG GTT CAG AGG GTC-3') were used to amplify the ORFs encoding α -conotoxins, based on the conserved sequences of signal peptide and 3'-untranslated region of the α -conotoxin precursors, respectively. PCR amplification was performed with a touch-down cycling protocol as described previously [28]. All PCR products were analyzed on 2% agarose gel and ligated into the pGEM-T Easy vector (Promega) for sequencing.

2.3. Peptide synthesis and in vitro oxidative folding

The linear peptide of pu14a (DCPPHPVPGMHKCVCLKTC) was synthesized on an ABI 433A peptide synthesizer (ABI, Foster City, CA, USA) using the standard Fmoc chemistry. All cysteine residues were protected with trityl groups. The peptide was cleaved from the resin with TFA/H₂O/ethanedithiol/phenol/thioanisole. Side chains of non-Cys residues were de-protected during the cleavage. The crude peptide was then purified by reverse-phase HPLC (RP-HPLC) on a ZORBAX 300SB C18 semi-preparative column (9.4 × 250 mm, 7 µm particles, Agilent Technologies, Santa Clara, CA, USA) with a flow rate of 1.5 ml/min using the following gradient: 0–5 min, buffer A (0.1% TFA in H₂O); 5–7 min, 0–20% buffer B (0.1% TFA in ACN); 7–17 min, 20–30% buffer B. The linear peptide was characterized by electro-spray mass spectrometry (ESI-MS) on a Q-trap mass spectrometer.

The *in vitro* folding of pu14a was carried out by air oxidation. The purified linear pu14a (50 µM) was dissolved in 50 mM NH₄HCO₃, pH 7.8, and stirred at 4 °C for 72 h. The folded product was separated on the ZORBAX 300SB C18 semi-preparative column, lyophilized, and analyzed by ESI-MS.

2.4. Purification and sequence determination of ts14a

A single specimen of *C. tessulatus* was collected from the South China Sea near Hainan, China, and its venom was extracted as previously described [18]. The lyophilized crude venom was resuspended in buffer A. After centrifugation, the supernatant was loaded onto the ZORBAX 300SB C18 semi-preparative column, and eluted at a flow rate of 1.5 ml/min with a gradient of 0–50% buffer B over 80 min. All major peaks were collected and freeze-dried. Further purification of ts14a was carried out using a PepMapTM C18 analytical column (4.6 × 250 mm, 5 µm particles, LC Packings, San Francisco, CA, USA) with a flow rate of 0.5 ml/min and a gradient of 0–15% buffer B for 3 min and 15–30% buffer B for 30 min.

The purified ts14a was reduced by using 50 mM DTT in a solution of 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, and incubating at 37 °C for 1 h, immediately alkylated with 2 µl of 4-vinylpiperidine in dark for 1 h at room temperature, and then purified by HPLC. The alkylated derivative of ts14a was subjected to N-terminal amino acid sequence analysis by Edman degradation on an ABI Model 491A Procise Protein Sequencing System (Applied Biosystems, Foster city, CA, USA).

2.5. Disulfide connectivity analysis

The disulfide bond connectivity of pu14a was determined using the partial reduction and alkylation procedure as described previously [7]. Partial reduction of the bicyclic pu14a

(final concentration < 10 μ M) was achieved in the presence of 20 mM Tris-2-carboxyethyl-phosphine hydrochloride (TCEP-HCl) in 0.1 M sodium citrate, pH 3.0, for 4–6 min at room temperature. The product was separated by HPLC, and the partially reduced peptide was alkylated with 20 mM of N-ethylmaleimide (NEM) in 0.1% TFA in dark for 2 h at room temperature. The alkylated intermediate product was purified by HPLC, lyophilized, and further fully reduced in 250 mM Tris-HCl, pH 8.6, 2 mM EDTA, and 4 mM DTT for 30 min. Then 500 mM iodoacetamide was added to label the other two free thiol groups in dark for 30 min at room temperature. The final products were fractionated by HPLC and analyzed by mass spectrometry. The fully alkylated peptides (1 mg/ml) were digested with trypsin (10 μ g/ml, Sigma-Aldrich) at a peptide: trypsin molar ratio of 100:1 in 50 mM Tris-HCl, pH 8.0, and 1 mM CaCl_2 . The reactions were incubated overnight at 37°C and the resulting fragments were directly analyzed by MALDI-TOF mass spectrometry.

Due to the limited amount of native ts14a, cyanogen bromide (CNBr) was used to cleave the peptide, the mass spectrum of product fragments was analyzed, and the disulfide connectivity was then predicted. Briefly, approximately 250 pmol of the purified ts14a was dissolved in 1 ml of 70% formic acid, which contains 1 mg CNBr. The cleavage reaction was incubated in dark at room temperature for 24 h and then terminated by direct lyophilization. The CNBr cleaved lyophilized product was redissolved in 20 μ l MilliQ H_2O for MALDI-TOF mass spectrometry analysis.

2.6. Mass spectrometry

The molecular mass analyses of conotoxins were performed on an API2000 Q-trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) in the scan type of enhanced mass spectrometry. The apparatus equipped with a TurboIonSpray source was operated in the positive ionization mode. The monoisotopic mass of ts14a and its CNBr-cleavage products were measured on an ABI Voyager-DE STR matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer.

2.7. Bioassays

A total of 12 Kunming male mice weighing 20 ± 2 g were divided into four groups in order to evaluate possible dose-dependent effects of pu14a. Mice were intravenously (i.v.) injected in the tail vein with 20 μ l of normal saline solution (control group, $n = 3$) or with different doses (10, 15, and 20 μ g) of the synthetic pu14a in 20 μ l of normal saline solution (test group, $n = 3$ for each dosage). Animals were placed in cages and their behaviors were observed for 1 h after pu14a administration.

Freshwater gold fish were injected intramuscularly (i.m.) under the dorsal fin with different doses (10, 15, and 20 μ g) of pu14a dissolved in 20 μ l of normal saline solution. Control fish were injected with 20 μ l of normal saline solution ($n = 3$). The injected animals were observed for 1 h in the aquarium.

2.8. Electrophysiology

Oocytes of *Xenopus laevis* were prepared and injected with capped RNA (cRNA) to elicit expression of mouse fetal skeletal-muscle and various rat neuronal nAChR subtypes as described previously [12, 17]. Oocytes were voltage-clamped at a membrane potential of -60 mV and gravity-perfused with OR2 buffer (96.0 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl_2 , 1.0 mM MgCl_2 , 5 mM HEPES, pH 7.5) at a rate of 5 ml/min using a Warner BPS-8 controller [20]. One micromolar of atropine was added to the OR2 buffer to block the endogenous muscarinic AChRs in all recordings, except for $\alpha 7$ nAChR which is antagonized by atropine [5]. ACh-gated currents were elicited by a 5-s pulse of agonist solution applied at intervals of 2 min to obtain the baseline activity: 10 μ M of ACh for the

muscle subtype and 100 μ M of ACh for the neuronal subtypes. To measure the inhibitory effects, pu14a was applied to an AChR-expressing oocyte in the static bath for 10 min prior to restoration of OR2 perfusion and ACh pulse. The current signals were sampled and filtered at 500 Hz and 200 Hz, respectively. The average peak amplitude of three control responses preceding exposure to toxin was used to normalize the amplitude of each test response to obtain the “% response” and recovery from toxin application. All data were represented as means $\pm$ S.D. from measurements of 3–5 oocytes for each subtype.

3. Results

3.1. cDNA cloning of Pu14.1

Using primers corresponding to the conserved sequence of signal peptide and 3' UTR of A-superfamily conotoxins, a novel conotoxin precursor was cloned from *Conus pulicarius* (Fig. 1A). This 69-aa precursor exhibits a considerably conserved organization of coding genes (signal sequence, pro region, and mature toxin) of conotoxins across species. Its 21-aa signal sequence is identical to that of A-superfamily conotoxins, however, its predicted 20-aa mature toxin region surprisingly adopts the 14th cysteine framework in a pattern of CX₁₀CX₁CX₃C which is clearly different from cysteine patterns of other A-conotoxins (Fig. 1B), revealing a new level of conotoxin diversity and a plausible evolution mechanism of *Conus* prepropeptide recombination. According to the conventional nomenclature of conotoxins, the cloned sequence encoding the novel conotoxin precursor in A-superfamily identified from *C. pulicarius* was designated as Pu14.1, with two letters “Pu” represent the *Conus* species from which the clones were identified, numbers “14” and “1” indicate the disulfide framework and order of discovery, respectively. Sequences of the 28-aa pro peptide and the 19-aa mature toxin of Pu14.1 are rather variable from A-superfamily conotoxins and other Cys framework 14 conotoxins. Besides, the deduced mature toxin of Pu14.1 is quite unique with the long intercysteine loop 1 with 10 residues, which includes 4 prolines.

3.2. Peptide synthesis and assignment of disulfide connectivity in pu14a

The sequence of peptide pu14a, encoded by the Pu14.1 clone, was predicted to be DCPHPVPGMHKCVCLKTC, with its C-terminal basic Arg removed during maturation which is common among neurotoxins [21, 27]. Peptide pu14a was chemically synthesized for further characterization. As no disulfide connectivity information was available, pu14a was synthesized with four Cys residues unprotected and then subjected to air oxidation. The predominant product was purified to homogeneity (Fig. 2A and 2B) to determine its disulfide connectivity and biological activity.

The partial reduction and alkylation strategy was used to assign the disulfide connectivity of the folded pu14a. The partially reduced intermediate (Fig. 2C) was modified with NEM (Fig. 2D), and then further reduced and labeled with iodoacetamide. Purification of the final product by HPLC gave two peaks (P1 and P2) with the same molecular mass, each of which was labeled with two NEM and two iodoacetamide groups. Trypsin digestion was adopted to distinguish these two peaks. Molecular masses of the digested fragments showed that the two peaks had different labeling pattern (Table 1), indicating that the partially reduced intermediate had actually two isoforms which were separated when their thio-groups were fully reduced and alkylated. To confirm the disulfide connectivity of the folded pu14a, these two peaks were subjected to MS/MS analysis. For unknown reason, peak 1 gave very low ionization signal, whereas m/z values of the consecutive fragment ions of peak 2 clearly demonstrated that the two disulfide bonds in peptide pu14a were formed between C1–C3 and C2–C4 (Fig. 2E).

3.3. Isolation and characterization of ts14a

The crude venom extract from *C. tessulatus* was initially fractionated using RP-HPLC on a C18 semi-preparative column (Fig. 3A). The ESI ion trap mass value of 2092.0 for the native peak indicated by asterisk in Fig. 3A and 2518.0 for its alkylated derivative suggested the presence of four cysteine residues. This fraction was further purified on an analytical C18 column to homogeneity (Fig. 3B). Edman sequencing showed that this peptide has the same cysteine pattern as that of Pu14.1, which is called ts14a (DGCPPHPVPGMHPCMCTNTC, accession number P86362). The mass value of the native ts14a (Fig. 4A) was consistent with that of the assigned sequence (monoisotopic $[M + H]^+$: calculated, 2091.79; observed, 2091.76), suggesting that this peptide has a carboxyl C-terminus.

Because of the very limited amount of ts14a, CNBr cleavage combined with mass analysis was applied to determine its disulfide linkage. Peptide ts14a can have two possible disulfide bond assignments: C1–C3, C2–C4, and C1–C2, C3–C4. The disulfide linkage C1–C4; C2–C3 is unlikely to occur as only one residue interspaces between the second and third cysteine which is difficult for forming a disulfide bond. Due to the existence of two Met residues in loop 1 and loop 2, mass spectrometry of the CNBr cleavage product could be applied to deduce the disulfide connectivity of ts14a. The cleavage did not generate the two fragments DGCPPHPVPGMHPCM and CTNTC, which excludes the possibility of disulfide linkage between C1–C2 and C3–C4. And the cleavage product showed a monoisotopic mass value of 2031.45 Da (Fig. 4B), which matches the theoretical mass of a cleavage fragment if ts14a adopting the C1–C3, C2–C4 linkage (Table 2). Thus, we conclude that ts14a has the identical disulfide connectivity to that of the synthetic pu14a.

Identification of the native ts14a firmly supports the predicted mature peptide sequence of Pu14.1. Peptides ts14a and pu14a share a highly homologous primary structure (75% sequence identity), especially in their first long intercysteine loop, as well as the same cysteine framework and disulfide connectivity. Thus, these two conotoxins probably also share the same molecular target, namely nAChRs, although their structures are quite different from α -conotoxins, other A-superfamily conotoxins and cysteine framework 14 conotoxins. We further propose that pu14a and ts14a are defining members of a new subfamily of α -conotoxins in the A-superfamily, which we designated $\alpha 1$ -conotoxins.

3.4. Bioassay

Biological effects of the synthetic pu14a were examined on freshwater goldfish and Kunming male mice (Table 3). At 10 μ g, the intramuscularly injected goldfish appeared to be quiet and did not swim compared to the control fish. Injection of 15 μ g of pu14a induced rapid breathing and seizure, and pu14a caused paralysis symptom by further increasing the dose to 20 μ g. All fish injected with different doses of pu14a died within one hour after injection. Tail vein injection of the same dosages of pu14a elicited unconsciousness in mice, which started a few minutes after injection and lasted for 30–60 min.

3.5. Electrophysiology

The synthetic pu14a was tested for selectivity in various nAChR subtypes expressed in oocytes (Fig. 5A). Peptide pu14a showed the strongest inhibition on the mouse fetal neuromuscular subtype ($\alpha 1\beta 1\gamma\delta$), with 1 μ M toxin blocking ~82% of the ACh-evoked current and the antagonizing effect completely recovering after 4 min of washout (Fig. 5B). At the same toxin concentration, the ACh-evoked current of nAChR was reversibly blocked by ~55% in $\alpha 6\alpha 3\beta 2$ subtype of the rat neuronal nAChR, with a similar dissociation rate. At a higher concentration (10 μ M), the block effect of pu14a was ~51% in $\alpha 3\beta 2$. These results suggest that pu14a is selective for particular nAChR subtypes with fast dissociation kinetics.

The effect of pu14a on voltage-activated K⁺ channels was also investigated. In acutely dissociated mouse superior cervical ganglion (SCG) neurons, 1 μ M of pu14a inhibited nearly 45% of the ACh-elicited inward current, but no blocking effect of the evoked K⁺ response was observed (data not shown).

4. Discussion

Here we report the cDNA cloning and isolation of two highly homologous novel conotoxins from two worm-hunting species *C. pulicarius* and *C. tessulatus*, designated Pu14.1 and ts14a, respectively. Peptides pu14a and ts14a share the 14th cysteine framework with the CX₁₀CX₁CX₃C loop spacing pattern, which is remarkably different from α -conotoxins, other A-superfamily and cysteine framework 14 conotoxins. Based on the fact that the signal sequence and molecular target type of pu14a is identical to that of α -conotoxins, and that pu14a has the unique cysteine framework 14, Pu14.1 and ts14a could be grouped into a new subfamily of α -conotoxins, which we designated as α 1-conotoxins.

Although the 14th cysteine framework (C-C-C-C) was first found in Con 14, a “scratcher” peptide from the piscivorous species *C. geographus* [16], a series of recent reports showed that the cysteine framework 14 peptides may be restricted to worm-hunting cone snails inhabited in a wide variety of unrelated environments [9, 13, 19, 29]. Furthermore, this cysteine framework is also found in other organisms, such as a family of K⁺ channel-binding toxins (κ -hefutoxin, κ -KTx1.3, and Om-toxins) in scorpions [1, 5, 22], suggesting that toxins with the C-C-C-C cysteine pattern may evolve from common ancestral genes which were initially found in scorpions appeared approximately 400 million years ago.

Two different disulfide connectivities have been observed in characterized Cys framework 14 conotoxins. The J-conotoxin pl14a isolated from *C. planorbis* found in the Philippines [9] and the L-conotoxin lt14a cloned from *C. litteratus* collected from the South China Sea possess the C1–C3 and C2–C4 connectivity [19], whereas conotoxins flf14a–c and vil14a from the western Atlantic (*C. floridanus floridensis* and *C. villepini*) adopt the C1–C4 and C2–C3 pairing [13]. Both the signal sequence and the pattern of disulfide bonds in pu14a are identical to that of the α -conotoxins, namely C1–C3 and C2–C4. The disulfide connectivity of ts14a was also determined to be identical to that of α -conotoxins.

The biological activity of the A-conotoxin pu14a differs from that of the J-conotoxin pl14a. The synthetic pu14a induced a sleeping phenotype in mice, in contrary, pl14a elicited excitatory symptoms, including shaking, rapid circling, barrel rolling, and seizure. The J-conotoxin pl14a was shown to inhibit a K⁺ channel subtype (Kv1.6, IC₅₀ = 1.59 μ M), as well as neuronal (IC₅₀ = 8.7 μ M for α 3 β 4) and neuromuscular (IC₅₀ = 0.54 μ M for α 1 β 1 ϵ δ) subtypes of the nAChR, which is the first observation of a *Conus* peptide selectively inhibiting both a voltage-gated Kv1.6 channel and ligand-gated nAChR subtypes [9]. Previously identified Kv1-channel-blocking peptides from scorpions and sea anemones [1, 3, 4, 22] have two conserved motifs which play a role in blocking Kv1 channels: a dyad structure and the presence of a ring of basic residues [14, 26]. The ring of basic residues in the J-conotoxin pl14a may be an important structural element for its interaction with the Kv1.6 channel (Table 4). Conotoxin vil14a has two potential functional Lys/Tyr dyad pairs in residues Lys23 and Tyr8, and Lys23 and Tyr27. Experiments also indicate that vil14a indeed blocks K⁺ channels in PC12 cells [13]. Peptide as14a has Lys and Tyr residues at the same positions (Table 4), and therefore it might also block K⁺ channels. As there is no Lys/Tyr dyad in pu14a or ts14a; it is not surprising that pu14a specifically targets the nAChR. Furthermore, the inhibition potency of pu14a to the nAChR is similar to that of pl14a, which is lower than that of α -conotoxins probably due to subtle changes in the secondary structure.

In both pu14a and ts14a, there are four proline residues in their first intercysteine loop. Because of the unusual stereo conformation of proline and the unique sequence in the first loop, pu14a and ts14a may adopt a conformation comparable to that of α A-conotoxins with three disulfide bonds (framework IV CC-C-C-C), which also specifically block nAChRs, but not K⁺ channels. Our work thus expands the knowledge of structural diversity of A-superfamily conotoxins, and paves the way for the future structure-function relationship investigation.

Research Highlights

- Pu14a and ts14a represent a novel α 1-family of A-superfamily conotoxin.
- Pu14a and ts14a share homologous sequence with a novel cysteine pattern.
- α 1-conotoxins may also target nAChR.

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ATG GGC ATG CGG ATG ATG TTC GCC GTG TTT CTG TTG GTT GTC TTG GCA ACC
M   G   M   R   M   M   F   A   V   F   L   L   V   V   L   A   T

ACT GTC GTT TCC TTC AAT TCA GAT CGT GCA TCT GAT GGC AGG AAT GCC GCA
T   V   V   S   F   N   S   D   R   A   S   D   G   R   N   A   A

GCC AAT GTC AAA GCG TCT GAC CTG ATG GCC CGG GTC CTC GAA AAA GAC TGT
A   N   V   K   A   S   D   L   M   A   R   V   L   E   K   D   C

CCA CCA CAT CCC GTT CCT GGC ATG CAT AAG TGC GTG TGT TTG AAG ACG TGT
P   P   H   P   V   P   G   M   H   K   C   V   C   L   K   T   C

CGT TGA
R   *

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(A)

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Pu14.1      MGMRMMFAVFLLVVLATTVVSFNSDRASDGRNAAANVKASDLMARVLEKDCPPHPVPGMHKCVCLKTCR
κA-SIVA     MGMRMMFTVFLLVVLATTVVSTPSDRASDGRNAAVHERQKSLVPSVITTCGGYDPGTMCPPCRCTNSCG
κA-MIVA     MGMRMMFTVFLLVVLATTVVSIPSDRASDGRNAVVERAPELVVTATTNCCGYNPMTICPPCMCTYSCPPKRKPGRND
κA-SmIVA    MGMRMMFTVFLLVVLATTVVSIPSDRASDGRNAAVNERQTWLVPTITTCGGYDPGTMCPTCMCDNTCKPKPKKSGRND
α-Mr1.1     MGMRMMFTVFLLVVLATTVVSFTSDRASDGRKAAAKDKASDLVAL--TVKGCCSHPACSVNNPDICG
α-AuIB      MFTVFLLVVLATTVVSFTSDRASDGRKDAA----SGLIAL--TMKGCCSYPPCFATNPDCGRRR

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(B)

Figure 1.

cDNA cloning of Pu14.1. (A) The cDNA sequence and predicted translation product of Pu14.1. The signal peptide is underlined and the deduced mature toxin is in shadow. The conserved Cys residues are in bold. The cDNA has been deposited in the GenBank database with the accession number EU912017. (B) Prepropeptide comparison of Pu14.1 with other A-superfamily conotoxins which share the identical signal sequence and apparently conserved pro region. Their signal peptide is in shadow and the mature toxin is underlined.

Figure 2. Disulfide bond assignment of pu14a. (A) Air-oxidized product of the synthetic pu14a. (B) The predominant peak (peak 1) of folded pu14a was further purified by HPLC. (C) Partial reduction of the synthetic and air-oxidized pu14a. (D) The partially reduced product of pu14a was alkylated by NEM and then purified by HPLC. (E) MS/MS analysis of the fully reduced and alkylated product of pu14a.

Figure 3. Purification of ts14a. (A) Separation of the crude venom from *C. tessulatus* on a ZORBAX 300SB C18 semi-preparative column. (B) The fraction indicated by arrow in (A) which contains peptide ts14a was further purified to homogeneity on a PepMap™ C18 analytical column.

Figure 4.

(A) The monoisotopic mass measurement of ts14a. The ionic peak with $m/z = 2091.76$ corresponds to the monoisotopic mass value of ts14a, suggesting that ts14a has a free C terminal. (B) The monoisotopic mass analysis of the fragments of ts14a after CNBr cleavage at Met residues. The ionic peak with $m/z = 2031.45$ indicates that ts14a adopts the C1–C3, C2–C4 disulfide connectivity.

Figure 5.

Electrophysiological assay. (A) The selective blocking effects of pu14a on different subtypes of mouse and rat nAChRs. Each bar indicates the average percent response $\pm$ S.D. after toxin application to *Xenopus* oocytes expressing a variety of nAChRs. (B) Representative current traces for pu14a at 1 μM on the mouse $\alpha 1\beta 1\gamma \delta$ nAChR. The arrow marks the first current elicited after a 10-min exposure to the toxin. Subsequent current traces show the peptide dissociation and washout. Two peaks of the ACh-induced response immediately after the 10-min incubation with pu14a are due to the displacement of salt bridges when the high-speed (5 ml/min) gravity-perfusion is restored.

Table 1

Molecular mass of trypsin-digested fragments of the fully reduced and alkylated pu14a

Fragments		Sequence	Theoretical MW (Da)	Determined MW (Da)
P1	Full-length	DC _{NEM} PPHPVPGMHKC _{IAA} VC _{NEM} LKTC _{IAA}	2429.9	2429.2
	P1-1	DC _{NEM} PPHPVPGMHK	1439.7	1438.6
	P1-2	C _{IAA} VC _{NEM} LK	747.0	747.8
P2	Full-length	DC _{IAA} PPHPVPGMHKC _{NEM} VC _{IAA} LKTC _{NEM}	2429.9	2429.1
	P2-1	TC _{NEM}	347.4	348.0
	P2-2	DC _{IAA} PPHPVPGMHK	1371.6	1371.5
	P2-3	C _{NEM} VC _{IAA} LK	747.0	747.8

Table 2

Molecular mass of the CNBr cleavage fragments of ts14a with presumed disulfide connectivity

Connectivity	Fragments after CNBr cleavage	Theoretical MW (Da)	Determined MW (Da)
DGCPPHPVPG M HPC M CTNTC	2031.7864	2031.3887
DGCPPHPVPG M HPC M CTNTC	1493.6298 538.1672	-
High energy barrier for the disulfide bond in "CMC" which is much unlikely to form		

M: C terminal homoserine lactone. Bolded residues represent N-termini.

Table 3Biological activity of the synthetic pu14a from *C. pulicarius*

	Saline solution (20 μ l) (n=3)	pu14a (10 μ g/20 μ l) (n=3)	pu14a (15 μ g/20 μ l) (n=3)	pu14a (20 μ g/20 μ l) (n=3)
Kunming male mice (i.v. injection)	Normal activity	Sleeping	Sleeping	Sleeping
Freshwater goldfish (i.m. injection)	Normal activity	Quiet, no swimming, death	Rapid breathing, seizure, death	Unbalanced body, paralysis, death

Table 4
Comparison of Cys framework 14 conotoxins from different gene superfamilies

Superfamily	Peptide	Species/Diet Type	Signal Sequence	Mature Toxin	Disulfide Connectivity	Selectivity	Ref.
A	pu14a	<i>C. pulicarius</i> v	MGMRMMFAVFLVVLATTVVS	DCPPHPVPG-MHKCVCLKT--C	1-3, 2-4	α 1 β 1 γ 6, α 6 α 3 β 2, α 3 β 2	This work
	ts14a	<i>C. tessulatus</i> v	-	DGCPPHPVPG-MHPCMCNTNT--C	1-3, 2-4	-	This work
	pl14a			FPRPRICNLAC RAG-IGHKYPFCH--CR *	1-3, 2-4	α 1 β 1 ϵ 6, α 3 β 4, Kv1.6 channel	
J	pl14.1	<i>C. planorbis</i> v		GPGSAICNMACRLG-QGHMYPFCN--CN *			[9]
	pl14.2		MPSVRSVTCCCLLWMMFSVQLVTP	GPGSAICNMACRLG-HGHLYPFCH--CR *			
	pl14.3			GPGSAICNMACRLG-HGHLYPFCN--CD *	-	-	
	fe14.1	<i>C. ferrugineus</i> v		SPGSTICKMACRTG-NGHKYPFCN--CR *			
	fe14.2			SSGSTVCKMMCRLG-YGHLYPSCG--CR *			
L	lt14a	<i>C. litteratus</i> v	MKLSVMFIVFLMLTMPMTCA	MCPPLOCK-----PSCTN-C *	1-3, 2-4	Muscle and neuronal nAChRs	[19]
	vil14a	<i>C. villepini</i> v		GGLGRCIYNCMNSGGGLSFQCKTMCY	1-4, 2-3	K ⁺ channel blocker	[13]
	flf14a			WDVNDCHHFCLIGVVVERSYTECHTMCT	1-4, 2-3		
	flf14b	<i>C. floridanus floridensis</i> v		WDVNDCHHFCLIGVVGRSYTECHTMCT	1-4, 2-3	-	
	flf14c			WDAYDCTQFCMRPEMRHTYAQCLSICT	1-4, 2-3		
Others	as14a	<i>C. austini</i> v		GGVGRCIYNCMNSGGGLNFIQCKTMCY			[29]
	as14b			RWDVDQCTIYYCLNGVVGYSYTECQTMCT	-	-	
	Con14	<i>C. geographus</i> p		KFLSGGFKYIVCHRYCAKG--IAKEFCN--CPD		-	[16]

p, piscivorous; v, vermivorous; γ , gcarboxyglutamate

* amidated C-terminus. Cys residues are in bold. Residues potentially involved in K⁺ channel targeting (functional Lys/Tyr dyads in vil14a and as14a, as well as the basic loop in pl14a) are in bold and underlined.