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Discovery of a new subclass of α-conotoxins in the venom of *Conus australis*

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ABSTRACT

Cone snails (*Conus* sp.) are poisonous animals that can be found in all oceans where they developed a venomous strategy to prey or to defend. The venom of these species contains an undeniable source of unique and potent pharmacologically active compounds. Their peptide compounds, called conotoxins, are not only interesting for the development of new pharmaceutical ligands, but they are also useful for studying their broad spectrum of targets. One conotoxin family in particular, the α-conotoxins, acts on nicotinic acetylcholine receptors (nAChRs) which dysfunctions play important roles in pathologies such as epilepsy, myasthenic syndromes, schizophrenia, Parkinson's disease and Alzheimer's disease.

Here we define a new subclass of the α-conotoxin family. We purified the venom of a yet unexplored cone snail species, i.e. *Conus australis*, and we isolated a 16-amino acid peptide named α-conotoxin AusIA. The peptide has the typical α-conotoxin CC-X_m-C-X_n-C framework, but both loops (*m*/*n*) contain 5 amino acids, which has never been described before. Using conventional electrophysiology we investigated the response of synthetically made globular (I–III, II–IV) and ribbon (I–IV, II–III) AusIA to different nicotinic acetylcholine receptors. The α_7 nAChR was the only receptor found to be blocked with a similar potency by both peptide-configurations. This suggests that both α5/5 conotoxin isomers might be present in the venom gland of *C. australis*. NMR spectroscopy showed that no secondary structures define the peptides' three-dimensional topology. Moreover, the ribbon configuration, which is generally considered to be non-native, is more stable than the globular isomer. Accordingly, our findings show relevancy concerning the α-conotoxin classification which might be helpful in the design of novel therapeutic compounds.

1. Introduction

Venoms from cone snails (genus *Conus*) offer a unique and extensive source of chemical diversity as they are driven by evolutionary pressure to improve prey capture and/or protection of the species. These snails are

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considered as specialized predators that have developed a sophisticated peptide chemistry and neuropharmacology by producing a structural and functional variety of neurotoxins. Therefore, the *Conus* venom gland is recognized as an emerging source of peptide-based therapeutics (Lewis et al., 2012). Their ability to use a diverse array of small disulfide-bridged peptides, called conopeptides or conotoxins, makes them very unique (Fry et al., 2009). Up to date, only 0.1% out of potentially 500,000 venom components has been functionally and structurally investigated. The largest group of characterized *Conus* sp. peptides is the family of α-conotoxins (belonging to the A-superfamily), that are selective antagonists of the muscle and neuronal subtype nicotinic acetylcholine receptors (nAChRs) (McIntosh et al., 1999). They act at the nAChR acetylcholine binding site as competitive antagonists and are among the smallest of the conopeptides (12-20 amino acid residues) (McIntosh et al., 1999; Terlau and Olivera, 2004). Alpha-conotoxins have a characteristic CC-X_m-C-X_n-C framework and these four cysteines can yield three possible disulfide connectivities: globular (I–III, II–IV), ribbon (I-–IV, II–III) and beads (I–II, III–IV). However, naturally appearing α-conotoxins typically exhibit the globular conformation (Janes, 2005). The number of residues included within the two loops (*m*,*n*) of α-conotoxins is the basis for the division into several structural subgroups (*m*/*n*: 3/5, 3/6, 4/2, 4/3, 4/4, 4/5, 4/6, 4/7, 4/8, 5/2 and 5/8) (Akondi et al., 2014). The most commonly reported framework is the 4/7 subgroup (Terlau and Olivera, 2004).

Nicotinic acetylcholine receptors, being a member of ligand-gated cationic channels, mediate fast synaptic transmission. They are broadly distributed throughout the peripheral and central nervous systems of both primitive and evolutionarily advanced organisms (Gotti and Clementi, 2004). Consequently, nAChR dysfunctions are involved in a number of central nervous system (CNS) disorders such as epilepsy, Alzheimer's disease, Parkinson's disease, schizophrenia, nicotine addiction, pain, cancer etc. (Albuquerque et al., 2009; Hurst et al., 2013; Improgo et al., 2010; Tapper et al., 2004).

In mammals there are 16 different nAChR subunits: 9 α-subunits (α_{1-7}, α_9 and α_{10}), 4 β-subunits (β_{1-4}), as well as γ, δ and ε subunits. Five of these subunits combine to form muscle nAChR subtypes ($\alpha_1\beta_1\gamma\delta$ and $\alpha_1\beta_1\delta\varepsilon$) which are found exclusively at neuromuscular junctions, whereas the other subunits (α_2-α_{10}, β_2-β_4) assemble in numerous homomeric (exclusively α-subunits) or heteromeric (α- and β-subunits) neuronal nAChR subtypes (Albuquerque et al., 2009). The assembly of different pentamers forms a complex variety of nAChR subtypes with different pharmacological and biophysical properties (Grishin et al., 2010; Hurst et al., 2013; Indurthi et al., 2014).

Here we report the isolation of a novel 16-amino acid α-conotoxin from the venom of the vermivorous marine snail *Conus australis* and its electrophysiological screening against six different nAChRs. To the best of our knowledge this is the first conotoxin to be characterized from this species found in the Indian Ocean near Tamil Nadu, India. The peptide, called AusIA belongs to a new subclass of the α-conotoxin family (i.e. α5/5) which has never been described before. We investigated the effect of globular (I–III, II–IV) and ribbon (I–IV, II–III) AusIA on different nAChRs from which α_7 nAChR was the only receptor found to be blocked with a similar potency by both peptide-configurations. NMR spectroscopy shows that no secondary structures define the peptides' three-dimensional topology. Moreover, the ribbon configuration, which is considered to be non-native, is more stable than the globular isomer.

2. Materials and methods

2.1. Cone snail specimens and venom extraction

Specimens of *C. australis* (identified by Holten in 1802 and classified by Tucker and Tenorio (Tucker and Tenorio, 2009)) were collected from the Indian Ocean near Tamil Nadu, India. The venomous apparatuses (venom bulbs and venom ducts) were extracted from the specimens as previously described (Olivera et al., 1985). The collected tissue was preserved in RNAlater solution (Ambion) and stored at −20 °C. The venomous apparatuses were used for peptide/protein extraction with 50% acetonitrile (ACN)/water.

2.2. Peptide fractionation and purification

Sample fractionation occurred by reversed-phase high performance liquid chromatography (RP-HPLC, Gilson, Middleton, WI, USA). Two steps were followed for the separation of the venom compounds. In the first step, the lyophilized crude venom powder was solubilized into 50% acetonitrile (ACN)/water and aliquots were loaded on a Gel filtration Superdex™ Peptide 10/300 GL column with 50% ACN/water as mobile phase (flow rate 0.5 ml/min) to separate the peptides and proteins based on their size. Three sample collections were made that were stored overnight at −80 °C, freeze-dried and finally solubilized in 0.1% trifluoroacetic acid (TFA)/H2O. For the second step, an analytical Vydac C_{18} column (218MS54, 4.6 × 250 mm, 5-μm particle size; Grace, Deerfield, IL) with a two solvent system was used: (A) 0.1% trifluoroacetic acid (TFA)/H_2O and (B) 0.085% TFA/ACN. The sample was eluted at a constant flow rate of 1 ml/min with a 0–80% gradient of solvent B over 90 min (1% ACN per minute after 10 min of solvent A). The HPLC column elutes were monitored by a UV/VIS-155 detector (Gilson) scanning both 214 nm and 280 nm.

2.3. Peptide sequencing

Isolated AusIA was collected and freeze-dried for direct peptide sequencing and molecular mass analysis (MALDI-TOF). A Protein Sequencer PPSQ-31A/33A (Shimadzu, Japan) was used to determine the amino acid sequence of the separated compound. In this Edman degradation method, the sample was loaded onto a polybrene-pretreated, precycled glass fibre disk, and was Edman sequenced for 24 residue cycles.

2.4. Peptide synthesis and folding

AusIA(g) (globular) and AusIA(r) (ribbon) were synthesized by GenicBio Limited (Shanghai, China). Formation of