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New conopeptides of the D-superfamily selectively inhibiting neuronal nicotinic acetylcholine receptors

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ABSTRACT

The venom of cone snails (*Conus* spp.) is a rich source of peptides exhibiting a wide variety of biological activities. Several of these conopeptides are neuronal nicotinic acetylcholine receptor (nAChR) antagonists and belong to the A-, M-, S-, C and the recently described D-superfamily (αD-conopeptides). Here we describe the discovery and characterization of two αD-conopeptides isolated from the venom of *Conus mustelinus* and *Conus capitaneus*. Their primary structure was determined by Edman degradation, MS/MS analysis and by a PCR based approach. These peptides show close structural homology to the αD-VxXIIA, -B and -C conopeptides from the venom of *Conus vexillum* and are dimers (about 11 kDa) of similar or identical peptides with 49 amino acid residues and a characteristic arrangement of ten conserved cysteine residues. These novel types of conopeptides specifically block neuronal nAChRs of the α7, α3β2 and α4β2 subtypes in nanomolar concentrations. Due to their high affinity, these new ligands may provide a tool to decipher the localisation and function of the various neuronal nAChRs.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric, ligand-gated ion channels. Several α subunits (α2–10) in combination with various β subunits (β2–4) generate a great diversity of nAChRs exhibiting various pharmacological properties (Palma et al., 1999; Khiroug et al., 2002; Karlin, 2002; Nicke et al., 2004). Neuronal nAChRs are involved in pain sensation, memory, learning and development and represent therapeutic targets for the treatment of chronic pain or neurological disorders such as schizophrenia, Alzheimer and Parkinson diseases (Decker et al., 2004; Gotti and Clementi, 2004; Newhouse et al., 2004). In this respect, subtype specific ligands are required to decipher the localisation and function of the various neuronal nAChR subtypes (McIntosh et al., 1999a,b; Nicke et al., 2004; Ripoll et al., 2004; Armishaw and Alewood, 2005).

The venom of cone snails (*Conus* spp.), used to immobilize and capture prey, is a rich source of peptides exhibiting a wide variety of biological activities. Among the small disulfide-rich conotoxins are neuronal nAChRs antagonists. They have been assigned to several superfamilies according to their structural properties (Arias and Blanton, 2000; Terlau and Olivera, 2004; Jimenez et al., 2007): the A- (α- and αA-conotoxins), M- (ψ-conotoxins), S- (αS-conotoxins), C (αC-conotoxins) and the recently described D-superfamily (αD-conopeptides; Loughnan et al., 2006).

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The α-conotoxins consist of 12–15 amino acids and have been divided into four groups based on the number of residues between the second and third cysteine and on the spacing between the third and fourth cysteine in the mature peptide: the α3/5, the α4/3 and the α4/7 subfamily (Nicke et al., 2004). Peptides of these subfamilies target different nAChRs. The α3/5 conotoxins preferably block muscular nAChRs, whereas α4/3 and α4/6 toxins inhibit the neuronal subtypes; the α4/7 toxins also target neuronal subtypes of nAChRs (McIntosh et al., 1999a,b). The αA-conotoxins are larger peptides consisting of up to 30 amino acids and contain three disulfide bonds. They are antagonists of the muscle nAChR subtype. The ψ-conotoxins also block muscle nAChRs, but act as a non-competitive antagonist (Jacobsen et al., 1997; Hopkins et al., 1995; Teichert et al., 2004). The αS-conotoxins inhibit muscle nAChRs as well as various neuronal subtypes (Teichert et al., 2005), whereas αC-conotoxins are highly specific inhibitors of muscle nAChRs only (Jimenez et al., 2007).

The recently discovered conopeptides VxXIIA, VxXIIB and VxXIIC from the venom of *Conus vexillum* represent a new superfamily named αD and they selectively inhibit α7 and β2 containing neuronal nAChR subtypes (Loughnan et al., 2006). Beside several post-translational modifications (e.g. hydroxylation, carboxylation, etc.) these conopeptides show another new feature: they occur as homodimers of two subunits, which are half the size (5135–5741 Da) of the ~11 kDa native peptides and are connected by disulfide bonds. Studies on venoms from *Conus capitaneus*, *Conus miles*, and *Conus mustelinus* indicated that these species also produce αD-conopeptides.

In the present paper, we describe the isolation and characterization of two conopeptides from the vermivorous cone snails *C. mustelinus* and *C. capitaneus*, which both exhibit high affinity to α7 nAChRs.

2. Material and methods

2.1. Materials

Specimens of *C. mustelinus* and *C. capitaneus* were collected around Olango Island, Cebu (Philippines). For RNA isolation, venom glands were dissected, immediately placed in RNAlater™ (Sigma–Aldrich, St. Louis, MO, USA) and stored at −20 °C. For protein purification, venom ducts were placed in 10% acetic acid and kept at −20 °C until use. The venom was extracted by homogenizing the ducts. After centrifugation at 3.000 rpm for 15 min the supernatant was lyophilized and stored at −20 °C.

2.2. Isolation of the conopeptides

The lyophilized venom gland extracts from *C. capitaneus* (100 mg) were first separated by gel filtration on a Sephadex G-50 column (80 × 1.5 cm) eluted with 0.1 M ammonium acetate buffer pH 6.8, followed by fractionation on a RP-HPLC Eclipse XDB-C18 semi-preparative column (4.6 × 150 mm; Agilent Technologies, Waldbronn, Germany) which was eluted with a linear gradient of solvent A (0.1% (v/v) TFA in water) to 100% solvent B (60% (v/v) acetonitrile in 0.1% (v/v) TFA in water) over 60 min at a flow rate of 0.5 ml/min using the Agilent 1200 Series HPLC system. The elution of the fractions was monitored at 280 nm (gel filtration) and at 220 nm (HPLC). The venom gland extracts from *C. mustelinus* were directly fractionated by RP-HPLC as described above and the active fraction was further purified by rechromatography. After each separation step the fractions were lyophilized and screened for inhibitory activity on nAChR subtypes expressed in *Xenopus laevis* oocytes using two-electrode voltage-clamp (see below). The purified conopeptides are named αD-Cp (from *C. capitaneus*) and αD-Ms (from *C. mustelinus*), respectively, according to the nomenclature suggested by Loughnan et al. (2006).

2.3. LC-TOF, ESI-MS and ESI-MS/MS analyses

For LC-TOF analysis, the samples were dissolved in 100 μl acetonitrile:water (80:20, v/v) containing 0.1% formic acid. Of this solution, 2 μl were analyzed using the Agilent 1100 Series HPLC system interfaced to an Agilent 1100 Series-TOF system (Waldbronn, Germany) operated in positive electrospray ionization mode (ESI).

For ESI analyses, samples were dissolved in water:-acetonitrile:formic acid (49.8:50:0.2, v/v/v). ESI-MS analyses were performed on a Micromass Quattro micro triple quadrupole mass spectrometer (Waters/Micromass, Milford, MA) for single molecular mass measurements. Reduction of native αD-Cp was carried out using 50 μg of protein in ammonium bicarbonate (100 mM) and dithiothreitol (5 mM) for 3 h at 50 °C. An acidification step and desalting procedure was performed prior to the analysis using a Zip-Tip (Millipore, USA). The reduced mixture was further alkylated with iodoacetamide (10 mM) and digested with trypsin (1% of the substrate mass). The digested mixture was acidified and desalted as described above and ESI-MS/MS experiments were conducted using a Q-TOF micromass spectrometer (Waters/Micromass, Milford, MA). Sequence analysis and attribution of the MS/MS fragments by comparison to the sequencing information from Edman degradation were performed with MassLynx 4.0 (Waters/Micromass, Milford, MA). All molecular masses indicated are average.

2.4. Sequencing by Edman degradation

The reduced and alkylated conopeptides (αD-Cp and αD-Ms) from the venom of *C. capitaneus* and *C. mustelinus* were directly sequenced using the LF 3000 Protein Sequencer (Beckman, Fullerton, CA, USA) or the Procise 492A protein-sequencing system (Applied Biosystems, Foster City, CA, USA). For further sequencing αD-Cp (50 μg) was digested with trypsin (Boehringer, Mannheim, Germany) in 100 μl of 100 mM Tris–HCl buffer, pH 8.5, to which 5 μg of protease was added and left over night at 37 °C. The product was separated by HPLC, using an analytical C18 reverse-phase column (Vydac, Hesperia, CA, USA) run from solution A (0.12% (v/v) TFA in water) to solution B (0.1% (v/v) TFA in acetonitrile) for 60 min at a flow rate of 1 ml/min. Peptide-fragments as indicated in Fig. 1C were sequenced individually.