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A novel alpha conotoxin (α-PIB) isolated from *C. purpurascens* is selective for skeletal muscle nicotinic acetylcholine receptors

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Abstract

The α-conotoxin family is comprised of peptides that share the following arrangement of cysteine residues in the primary amino acid sequence: –CC–C–C–, where each dash represents a variable number of amino acids. The number of amino acids between cysteine residues has been used to group the α-conotoxins into distinct subfamilies. These subfamilies include the α4/7-, α4/3- and α3/5-conotoxins, so named for the number of amino acids between 2nd/3rd and 3rd/4th cysteine residues, respectively. The α3/5-conotoxins antagonize vertebrate-muscle nicotinic acetylcholine receptors (nAChRs), while the α4/7- and α4/3-conotoxins primarily inhibit vertebrate neuronal nAChRs. To date, these three subfamilies are the most extensively characterized of the α-conotoxin family. Here we report the purification and characterization of an unusual α4/4-conotoxin, α-conotoxin PIB (α-PIB), from the venom of *Conus purpurascens*, with the following amino-acid sequence: ZSOGCCWNPACVKNRC (Z = pyroglutamate, O = hydroxyproline). This peptide demonstrates high affinity inhibition of vertebrate-muscle nAChRs, and paralytic effects when injected *in vivo*. Testing of α-PIB against other receptors indicated that the inhibitory effect is specific for skeletal muscle nAChRs. α-PIB shares the key biochemical and pharmacological characteristics of the α-conotoxin family.

Keywords: α-conotoxins; nAChR; *Conus* snails

1. Introduction

Peptides in the α-family of conotoxins were among the first components purified and characterized from *Conus* venoms approximately 30 years ago, including α-conotoxin GI (α-GI) (amino acid sequence: E**C**CN-PA**C**GRHYS**C**) and α-conotoxin MI (α-MI) (amino acid sequence: GR**CC**HPA**C**GKNYS**C**). These peptides were originally purified from fractions of crude venom that caused paralysis and death upon injection into fish or mice. Consistent with other venomous predators, it was not surprising that cone snails had evolved toxins among their venom components that had the ability to paralyze their fish prey. Similar to the first characterized paralytic toxin from snake venom, α-bungarotoxin, both α-GI and α-MI were identified as inhibitors of skeletal-muscle nicotinic acetylcholine receptors (nAChRs), which explained their paralytic effects (Gray et al., 1981; McIntosh et al., 1982).

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Subsequent to the characterization of α-GI and α-MI, many similar conotoxins were discovered from additional *Conus* species, which all shared a common arrangement of cysteine residues in their primary amino acid sequences; an arrangement we now call the Cys pattern. The Cys pattern of the α-conotoxins is the following: –CC–C–C–, where the dash represents a variable number of amino acids. The characterization of the α-conotoxins revealed that a subset, found only among fish-hunting cone snails, with 3 and 5 amino acids, respectively, between Cys residues (CCX_3CX_5C, e.g. α-GI and MI) were antagonists of vertebrate muscle nAChRs. We refer to this subset of the α-conotoxin family as α3/5-conotoxins (McIntosh et al., 1999; Santos et al., 2004). However, a large number of the α-conotoxins were subsequently identified with 4 and 7 amino acids, respectively, between Cys residues (CCX_4CX_7C, e.g. α-MII, amino acid sequence: **G**C**C**SNPV**C**HLEHSNL**C**) (Cartier et al., 1996) and were more broadly distributed across *Conus* clades, including non-fish hunting snails; these comprise a subset of the α-conotoxin family known as α4/7-conotoxins. The characterization of the α4/7-conotoxins has revealed that these primarily are not antagonists of vertebrate muscle nAChRs; rather, many have remarkable selectivity and affinity for a variety of vertebrate neuronal nAChR subtypes (McIntosh et al., 1999; Santos et al., 2004). Other subsets of α-conotoxins have been identified with a limited distribution among *Conus* species, such as the α4/3-conotoxins (e.g. α-ImI, amino acid sequence: G**CC**SDPR**C**AWR**C** and α-RgIA, amino acid sequence: G**CC**SDPR**C**RYR**C**R), which are found in a single clade of worm-hunting cone snails, and appear to be antagonists primarily of homomeric neuronal nAChRs (α7–α10 subtypes) (Janes, 2005; McIntosh et al., 1999; Olivera, 2006) and the heteromeric α9α10 nAChR subtype (Ellison et al., 2006; Vincler et al., 2006).

In this work, we describe the purification and characterization of an unusual α-conotoxin from the venom of *Conus purpurascens*. This peptide, designated α-conotoxin PIB (α-PIB) subsequent to its characterization, is an α4/4-conotoxin (CCX_4CX_4C), which represents a group of mostly uncharacterized conotoxins. The characterization of α-PIB revealed that it is an inhibitor of the neuromuscular nAChR, like the α3/5-conotoxins, based on both *in vivo* and *in vitro* experimental results.

2. Material and methods

2.1. C. purpurascens specimen collection and venom extraction

Venom was acquired by milking *C. purpurascens* specimens collected from the Clipperton atoll in the eastern Pacific and maintained in aquaria as previously described (Hopkins et al., 1995). The snails were milked 1–2 times/week (yielding 5–10 μl/milking) and milked venom was stored at −70 °C for later use. The venom from ~70 milkings (~0.5 ml) was pooled for large-scale purification by preparative Reverse-phase high performance liquid chromatography (HPLC).

2.2. Reverse-phase high performance liquid chromatography (HPLC)

All chromatography was done using either preparative (22 mm × 25 cm, 15 μm particle size, 300 Å pore size) or analytical (4.6 mm × 25 cm, 5 μm particle size, 300 Å pore size) Vydac C_{18} columns. Sequencing grade trifluoroacetic acid (TFA) was obtained from Aldrich and UV grade acetonitrile (ACN) was obtained from Fischer. HPLC buffers consisted of 0.1% TFA in H_2O (A buffer), 0.092% TFA and 60% ACN in H_2O (B60 buffer) and 0.08% TFA and 90% ACN in H_2O (B90 buffer).

2.3. Purification of natural peptide by HPLC

Milked venom was diluted with 0.1% TFA, applied to a preparative HPLC column fit with a guard column (22 × 50.8 mm) and eluted with a 0–80% gradient of B90 buffer over 80 min (flow rate 20 ml/min). Fractions were collected and further purified using an analytical HPLC column with a 25–55% gradient of B60 buffer over 30 min (flow rate 1 ml/min).

2.4. Sequencing and mass spectrometry

Purified, natural peptide was reduced with tris-(2-carboxyethylphosphine), HPLC-purified and then alkylated with 4-vinyl pyridine as described by (Gray, 1993). Pyroglutamate was removed using Pyroglutamate aminopeptidase (PGAP) following protocols by Sigma-Aldrich. The pyridylethylated and the enzyme-treated peptides were purified by HPLC and analyzed by automated Edman degradation on an ABI model 477A sequencer. Positive ion