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Characterization of a novel ψ -conotoxin from *Conus parius* Reeve

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Abstract

The M-superfamily of conotoxins currently comprises three major groups of peptides (the μ -, κ M-, and ψ -families) that share a key structural characteristic, the six-Cysteine motif CC-C-C-CC, but differ with respect to their molecular targets. The ψ -family consists of M-superfamily conotoxins that are nicotinic acetylcholine receptor (nAChR) antagonists. To date, only two ψ -conotoxins, PrIIIE and PrIIIF, are known, both of which were isolated from a single *Conus* species, *C. purpurascens*. In this paper, we report the discovery and initial characterization of a ψ -conotoxin from another *Conus* species, *C. parius*, which we designated as PrIIIE. Its amino acid sequence, inferred from a cloned cDNA, differed significantly from those of PrIIIE and PrIIIF. Its bioactivity was investigated by using the synthetic form of the peptide in mice and fish bioassays. At 2.5 nmole, the synthetic peptide induced flaccid paralysis in goldfish in ca. 4 min but did not induce any remarkable behavior in mice (after i.c. and i.p. injection of up to 10 nmole of peptide) and did not block action potential in directly-stimulated frog muscle preparations. Electrophysiological experiments carried out to measure inhibition of ion currents through mouse nAChR receptors expressed in oocytes revealed that PrIIIE (IC₅₀ ~ 250 nM) was significantly more potent than PrIIIE (IC₅₀ ~ 7000 nM) and that PrIIIE showed higher inhibition potency against the adult-type than the fetal type nAChR. In similar electrophysiological assays, PrIIIE showed no inhibitory effects against the mouse muscle subtype Na⁺ channel isoform Na_v 1.4. The discovery of this ψ -conotoxin from a *Conus* species that belongs to the subgenus *Phasmoconus*, which is distinct from and larger than the clade in which *C. purpurascens* belongs, suggests that greater structural and functional diversity of ψ -conotoxins remains to be discovered from the members of this subgenus.

Introduction

Marine snails of the genus *Conus* capture prey principally by envenomation. The bioactive components of their venoms are small disulfide-rich peptides, colloquially known as conotoxins. When injected into prey, *Conus* peptides act as highly selective ligands, binding to target membrane-bound proteins (in particular, ion channels) with high affinity; this binding

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is responsible for causing neuronal malfunction in the prey resulting in such physiological effects as excitotoxic shock or flaccid paralysis.

Owing to the ability of conotoxins to bind to related ion channels in mammals, and particularly their ability to discriminate between similar receptors, these peptides have become useful tools in biomedicine and related fields if not important drug candidates or drug leads (French and Terlau 2004, Terlau and Olivera 2004). However, science and medicine have only just begun exploiting this natural (combinatorial) library of compounds, considering that only a small fraction of the natural diversity of conopeptides have been characterized. Certainly, much remains to be discovered.

In this report, a conopeptide from *Conus parius*, a species in one of the four major clades of fish-hunting *Conus*, subgenus *Phasmoconus*, was identified through molecular cloning of conopeptide genes. We carried out an initial functional characterization of this peptide; which revealed that the peptide is a ψ -conotoxin. The ψ -conotoxins are a family of non-competitive nicotinic acetylcholine receptor antagonists that had previously been identified from a single species of *Conus*. Our results suggest that a potentially much larger number of *Conus* species are likely to have peptides that belong to the heretofore phylogenetically-restricted ψ -conotoxin family; this provides the opportunity to examine other species within this larger clade of cone shells for the presence of ψ -conotoxins and to determine their mechanism(s) of action at the muscle-type nicotinic receptor.

Materials and Methods

Gene cloning, peptide synthesis, and peptide folding

A *C. parius* cDNA library was prepared using protocols described previously. One cDNA clone, encoding a member of the M-superfamily was chosen for further study as representative of a group of undescribed peptides with significant sequence-level dissimilarity from M-superfamily conotoxins already characterized.

To study the bioactivity of the encoded product of one of the cDNA clones, a 22-residue synthetic peptide representing the predicted mature peptide, AARCCTYHGSLKEKCRKYCCGR, amidated at the C-terminus, was ordered from GenScript Corporation (Scotch Plains, NJ, USA). A single-step glutathione-mediated oxidative peptide folding protocol described previously was used to generate the folded form of the peptide. Purification was by semi-preparative reversed-phase HPLC using a Vydac C₁₈ column; the peptides were eluted from the column using a linear gradient of Buffer B₉₀ (90% acetonitrile in 1% trifluoroacetic acid) from 10% to 40% at a flow rate of 3 ml min⁻¹.

Bioassays—To determine its activity in mice, the purified folded peptide (up to 5 nmole) was dissolved in 10 μ l natural saline solution (NSS) and injected i.c. and i.p. into 15-day old Swiss Webster mice. Potential analgesic property was tested using the mice paw formalin test (Wheeler-Aceto and Cowan 1991): the purified-folded peptide (5 nmole) was injected into the paw and the paw-flinching/licking behavior of the mice after injection was observed over time. The peptide was also tested for its ability to block action potentials in frog (*R. pipiens*) skeletal muscle preparations stimulated using direct stimulation procedures essentially as described previously. To determine its activity in fish, known amounts of the peptide in either purified linear form or purified folded form, dissolved in 5 μ l natural saline solution (NSS), was injected i.p. into goldfish (*Carassius auratus*) (0.55 to 1.2 g weight). The amount of the peptide tested ranged from 0.1 to 2.5 nmole per fish.

Electrophysiology

Electrophysiological recording were made from *Xenopus* oocytes expressing mouse skeletal adult and fetal nAChR subtypes ($\alpha 1\beta 1\epsilon\delta$ and $\alpha 1\beta 1\gamma\delta$ subunits, respectively). Oocytes were clamped at -70 mV with a two-electrode system and gravity-perfused with ND96-solution as previously described (Cartier et al. 1996). Acetylcholine (ACh)-gated currents were elicited with $100\mu\text{M}$ ACh. The toxin was allowed to equilibrate in the static bath for 5 min prior to pulsing with ACh by gravity perfusion. Three oocytes were used for each data point. Dose-response curves were fitted to the following equation:

$$\% \text{ response} + 100 / [1 + (\text{toxin concentration} / \text{IC}_{50})^{n_H}]^m$$

(where n_H is the Hill coefficient).

Results

Cloning of the Pr3.5 gene and sequence analysis

A cDNA library from a *C. parvus* venom duct was prepared; a clone, Pr3.5, that encodes a putative conotoxin precursor of 74AA residues, was chosen for further characterization. The predicted mature peptide (shown in Table 1), which we designate PrIIIIE, is clearly a member of the M-superfamily. The 6 Cys residues of the mature toxin region of PrIIIIE are arranged in a pattern characteristic of members of the M superfamily (i.e., cys pattern #III, [CC-C-C-CC]). The segment between the fourth and fifth Cys residues (the third inter-Cysteine loop in M family conotoxins) of PrIIIIE consists of 4 residues, a distinguishing feature of the m-4 branch of the M-superfamily.

The PrIIIIE precursor exhibits significant sequence similarity to conotoxins belonging to the m-4 branch of the M superfamily. The number of identical residues between PrIIIIE precursor and previously described conopeptides ranged from 76% for the ψ -conotoxin PIIIE precursor to 90% for the μ -conotoxin PIIIA precursor (both the ψ -conotoxin PIIIE and the μ -conotoxin PIIIA are from *Conus purpurascens*). As is generally expected of conotoxins, PrIIIIE shows much lower sequence similarity in the mature toxin region to other conotoxins in the M-superfamily except for the highly conserved Cys residues (Table 1). Hypermutation of non-Cys residues in the mature toxin region is a general feature of gene superfamilies encoding *Conus* peptides.

As noted by Corpuz et al. (2005), members of the m-4 branch are functionally diverse. Some examples of m-4 peptides are shown in Table 1: μ -conotoxins GIIIA and PIIIA are Na channel blockers; κ M-conotoxin RIIK targets K^+ channels, and ψ -conotoxin PIIIE is a non-competitive nicotinic receptor antagonist. The extent of sequence similarity of PrIIIIE within these groups does not provide a clear indication of the potential pharmacological function of this conopeptide. As shown by the alignment in Table 1, the sequence comparison between PrIIIIE and the μ -, κ M- and ψ -conotoxins does not provide an unequivocal assignment of PrIIIIE to any of the pharmacological families.

When the deduced sequence of the predicted mature (and processed) PrIIIIE peptide is aligned with sequences of μ -conotoxins, although the arrangement of the Cys residues is largely conserved, a major difference is the absence of an Arg residue corresponding to position 13 of μ -GIIIA. This residue is conserved in most μ -conotoxins (conservatively substituted with K in a few examples) and appears to be critical for these conotoxins to interact with the target Na channel, and for blocking Na^+ conductance (Chang et al. 1998). In PrIIIIE, Arg is substituted by an acidic residue Glu, which is immediately flanked, however, by the basic residue Lys on both sides. In addition, positions in the alignment occupied by conserved positively charged

residues in the other μ -conopeptides are occupied by uncharged residues in the new conopeptide. These unusual sequence features of PrIIIE prompted us to directly assess the bioactivity of the peptide.

Oxidative folding of PrIIIE

To characterize the bioactivity of PrIIIE more comprehensively, the peptide was synthesized and folded; except for the C-terminal amidation, no other post-translational modifications were incorporated. Glutathione-mediated oxidative folding was used as described previously; the results, shown in Figure 2, suggest that PrIIIE folded with fast kinetics. Within a few minutes, one major peak appeared in the chromatogram suggesting the formation of a single major folded form. This type of folding is consistent with the 'rapid collapse' model previously described (Fuller et al. 2005). Extending the reactions to 4 h did not result in increased yield (data not shown).

Bioactivity of PrIIIE; electrophysiology

The synthetic folded peptide was purified and used for bioassays. The toxicity of the folded peptide to fish was tested using goldfish as model. Using a range of doses (0.1 – 2.5 nmoles per fish weighing 0.6 to 1.2 g), it was found that the peptide was paralytic. As shown in Table 1, a dose of 0.5 nmole of PrIIIE induced flaccid paralysis in the experimental fish, which took effect about nine minutes after toxin administration. At a dose of 2.5 nmole, paralysis was more rapid, starting at around 4 minutes. The ψ -conotoxin PIIE, which was used as a control (same dose), also elicited a similar effect at around 4 minutes (data not shown).

The peptide was then tested on oocytes expressing the Na_v1.4 voltage-gated Na channel subtype, as well as adult and fetal muscle nicotinic acetylcholine receptors (nAChRs). At a concentration of 5 nM, the peptide did not inhibit the muscle Na channel expressed in oocytes (results not shown). However, the peptide was an antagonist of both the adult and fetal muscle nAChRs. A dose-response plot of the activity of PrIIIE (Fig. 3) gave an IC₅₀ of 245 nM for the adult subtype (95% confidence interval: 212-283 nM) and 3239 nM for the fetal nAChR subtype (95% confidence interval: 2546-4121 nM). This block was quickly reversible (data not shown).

For comparison, ψ -conotoxin PIIE (ψ -PIIE) was assayed on same muscle subtypes expressed in oocytes. ψ -PIIE blocked only the adult skeletal muscle subtype (Fig 3) with an IC₅₀ of 7.4 μ M (95% confidence interval: 6.7-8.3 μ M). At a concentration of 10 μ M, ψ -PIIE failed to significantly inhibit the fetal muscle subtype. Thus, as is shown in Table 3, PrIIIE has a higher affinity for the muscle subtypes of nAChR than ψ -PIIE; the latter has already been shown to be more potent than ψ -PIIF, the only other ψ -conotoxin known to date, which was also isolated from *C. purpurascens*.

Discussion

Fish-hunting cone snails paralyze their prey by using a combination drug therapy strategy to block neuromuscular transmission. A key molecular target in this strategy is the muscle nicotinic acetylcholine receptor. Most conopeptides that block this receptor are α -conotoxins, which have two disulfide bonds and belong to the A-superfamily.

ψ -conotoxins are nicotinic receptor antagonists that belong to the M-superfamily. Up to this point, of all conotoxin families that target vertebrate neuromuscular circuitry, ψ -conotoxins have been the most restricted in their phylogenetic distribution. Peptides in this family have been found in only one species, *C. purpurascens*, the purple cone, which belongs to the subgenus *Chelyconus*, comprising new world fish-hunting species. The only other known

species in *Chelyconus* is the Atlantic *C. ermineus*; repeated attempts to find ψ -conotoxin homologs in *Conus ermineus* have failed (M. Watkins and B. Olivera unpublished results).

One problem in advancing the characterization the mechanism of antagonism of ψ -conotoxins is that the available peptides in this family, ψ -PIIIE and ψ -PIIIF have low affinity for the mammalian nicotinic acetylcholine receptor. It would be desirable to have a broader range of ψ -conotoxins before extensive structure/function analyses are carried out. In order to understand binding interactions between ψ -conotoxins and nicotinic acetylcholine receptors, ψ -conotoxin peptides with higher affinities for mammalian nAChRs would be particularly welcome.

The data presented above is consistent with a ψ -conotoxin family member being present in *C. parius* venom. The broader significance of this result is that in contrast to *Chelyconus*, the *Phasmoconus* clade of fish-hunting *Conus* species is a very large one, with probably >40 different species. Thus, the prospects have been significantly improved for obtaining a large, and diverse panel of ψ -conotoxins, based on the results with *C. parius* described in this report. Indeed, a preliminary analysis of M-superfamily member in other *Phasmoconus* species has revealed sequences with considerable similarity to PrIIIE; these are being further evaluated.

Clearly, PrIIIE belongs to the M-4 branch of the M-superfamily. At present, three different pharmacological classes have been characterized in this branch of the M-superfamily: κ M-conotoxins which target K channels; μ -conotoxins which are Na⁺ channel blockers and the ψ -conotoxins. The κ M-conotoxins are excitotoxic to fish and mice; injection of PrIIIE did not cause excitotoxicity (instead, flaccid paralysis of fish was observed). Thus, it seems unlikely that this is a κ M-conotoxin. In addition, the peptide has low sequence similarity with known κ M-conotoxins, lacking the amino acids shown to be determinants for targeting κ M-conotoxins to K channels.

The *C. parius* peptide was also tested on voltage-gated Na⁺ channels; it showed no activity when tested on the mouse Na_v 1.4 Na⁺ channel subtype expressed in *Xenopus* oocytes. As noted above, PrIIIE does not have the conserved Arg residue believed to directly block the ion permeation pathway. Thus, the peptide does not have the canonical features of μ -conotoxins and does not act on the predicted μ -conotoxin target for eliciting paralysis.

However, when the peptide was assayed using either the adult or fetal muscle subtype of mouse nicotinic acetylcholine receptors expressed in *Xenopus* oocytes, the *C. parius* peptide showed inhibitory activity, consistent with its belonging to the ψ -conotoxin family. Preliminary competition experiments with α -bungarotoxin are also consistent with the assignment to the ψ -conotoxin family; preincubation of the *C. parius* peptide had no obvious effect on α -bungarotoxin binding, i.e. the two antagonists bind at different sites (E.L-V, unpublished results). We designate this peptide ψ -conotoxin PrIIIE, the first ψ -conotoxin characterized from the *Phasmoconus* clade of fish-hunting *Conus* species. Thus, the door has been opened by the characterization of the *Conus parius* peptide to the discovery and systematic characterization of ψ -conotoxins from the large *Phasmoconus* clade of cone snails.

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Figure 1.
The shell of *Conus parius*

Figure 2. Oxidative folding kinetics of synthetic PrIII E revealed by the reversed-phase HPLC elution profiles of the folded products sampled at various points during the folding reactions. The X-axis corresponds to the elution time. The experimental conditions are described under Methods.

Figure 3.

Effect of PrIIIIE and ψ -conotoxin PIIE on nicotinic receptors expressed in oocytes. Inhibition curves were obtained by static bath application of various concentrations of PrIIIIE and ψ -PIIE to voltage clamped *Xenopus* oocytes expressing muscle adult and fetal nAChR ($\alpha 1\beta 1\epsilon\delta$ and $\alpha 1\beta 1\gamma\delta$ respectively). ψ -PIIE does not inhibit $\alpha 1\beta 1\gamma\delta$ nAChRs at a concentration of 10 μ M.

Table 1
Predicted Sequence of *Conus parius* Peptide (Comparison to M-Superfamily Peptides)

The translated open reading frame from a *Conus parius* cDNA library clone. The predicted polypeptide sequence encoded by the open reading frame was aligned with *Conus* peptide precursors encoding the conotoxins shown: μ -conotoxins PIIIA and GIIIA from *C. purpurascens* and *C. geographus*, respectively, κ M-conotoxin RIIIK from *Conus radiatus* and ψ -PIIIE from *Conus purpurascens*. The sequences were aligned so that the Cys residues are in register; shaded residues are identical to the *Conus parius* sequence. The arrow indicates the when protease processing between the prepro- and mature toxin regions occurs; none of precursor sequences are shown with post-translational modifications. The predicted appropriately modified mature peptide sequences are shown below the precursor sequences (# = C-terminal amidation; O = 4-transhydroxyproline).

<u>Species:</u>		<u>Conopeptide:</u>	↓
<i>C. parius</i>	pr3e	MSKLGVLLTICLLFPITALPVDGQPADRPVERMQDNISSEQHPFFEKRAARCCTYHGS-CLKEK-CRRKYCCGR	
<i>C. purpurascens</i>	μ -PIIIA	MSKLGVLLTICLLFPITALPMDGQPADRLAERMQDNISSEHP-FEKRQLCCGFPS-CRSRQ-CKPHRCCGR	
<i>C. geographus</i>	μ -GIIIA	MSKLGVLLTICLLFPITALPMDGDEPANRPVERMQDNISSEQYPLFEKR-RDCCTPPKK-CKDRQ-CKPQRCCAGR	
<i>C. radiatus</i>	κ MRRIIK	MSKLGVLLTICLLFPITALPMDGQPVDRLAERMQDNISSEQHTFFEKRLPSCCSLNLRLCPVPA-CKRNPCCGTG	
<i>C. purpurascens</i>	ψ -PIIIE	MSKLGALLTICLLFPITALLMDGQPADRPAERMDYDISSEVHRLLERRHPPCCMYGR--CRRYPGCSSASCCQGG	

<u>Mature Peptide Sequence:</u>	
pr3e	AARCCTYHGS-CLKEK-CRRKYCC#
μ -GIIIA	RDCCTOOKK-CKDRQ-CKOQRCCA#
κ M-RRIIK	LOSCCSLNLRLCPVPA-CKRNOCCCT#
ψ -PIIIE	ROOCCMYGR--CRRYPGCSSASCCQG#

Table 2

Assay of pr3e activity by i.p. injection into goldfish.

Peptide Injected [*] (nmole)		Observations
NSS (control)		Normal swimming behavior
2.5	A	After 3-6 min of normal swimming, fish floated on one side or sank and lay on one side at the bottom, with no obvious body, mouth/gill cover, or fin movements; this motionless state was occasionally interrupted by brief (1-2 second) jerky body movements/twitches within the next 30 min (in one replicate) to 1.5 h (another replicate); no obvious body, mouth/gill cover, or fin movements were subsequently observed.
0.5	F	Fish stopped swimming 9-14 min after injection and floated on one side. Generally no body/fin movements were observed, although twitching / jerky movements occurred occasionally; movement of mouth and gill cover weaker than in control but noticeable; no recovery observed within two hours but in one replicate, fish recovered after 2.5 h.
0.1	N	Normal swimming behavior

^{*} The peptide was dissolved in 5µl of NSS; 5µl NSS was used as control. Observations were made for two hours. Three replicates were used per treatment.

Table 3

ψ -Conotoxin	nAChR	IC ₅₀ (nM)
pr3e	$\alpha\beta\gamma\delta$	3239
pr3e	$\alpha\beta\epsilon\delta$	245
PIIIE	$\alpha\beta\gamma\delta$	>10000
PIIIE	$\alpha\beta\epsilon\delta$	7470