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Identification, by RT-PCR, of eight novel I_2-conotoxins from the worm-hunting cone snails *Conus brunneus*, *Conus nux*, and *Conus princeps* from the eastern Pacific (Mexico)

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ABSTRACT

Marine snails of the genus *Conus* (~500 species) are tropical predators that produce venoms for capturing prey, defense and competitive interactions. These venoms contain 50–200 different peptides ("conotoxins") that generally comprise 7–40 amino acid residues (including 0–5 disulfide bridges), and that frequently contain diverse posttranslational modifications, some of which have been demonstrated to be important for folding, stability, and biological activity. Most conotoxins affect voltage- and ligand-gated ion channels, G protein-coupled receptors, and neurotransmitter transporters, generally with high affinity and specificity. Due to these features, several conotoxins are used as molecular tools, diagnostic agents, medicines, and models for drug design. Based on the signal sequence of their precursors, conotoxins have been classified into genetic superfamilies, whereas their molecular targets allow them to be classified into pharmacological families. The objective of this work was to identify and analyze partial cDNAs encoding precursors of conotoxins belonging to I superfamily from three vermivorous species of the Mexican Pacific coast: *C. brunneus*, *C. nux* and *C. princeps*. The precursors identified contain diverse numbers of amino acid residues (*C. brunneus*, 65 or 71; *C. nux*, 70; *C. princeps*, 72 or 73), and all include a highly conserved signal peptide, a C-terminal propeptide, and a mature toxin. All the latter have one of the typical Cys frameworks of the I-conotoxins (C-C-CC-CC-C-C). The prepropeptides belong to the I_2-superfamily, and encode eight different hydrophilic and acidic mature toxins, rather similar among them, and some of which have similarity with I_2-conotoxins targeting voltage- and voltage-and-calcium-gated potassium channels.

1. Introduction

Marine snails belonging to the genus *Conus* are tropical predators that produce venoms for prey (fish, mollusks, and worms) capture, but also for defense and competition. These venoms comprise a large number of small, highly structured peptides that are generally named "conotoxins" or "conopeptides" [52].

Conotoxins bind to ion channels and neurotransmitter receptors and transporters, generally with high affinity and selectivity, and in many cases they discriminate among related subtypes of their molecular targets [43]. Therefore, several conotoxins have become invaluable pharmacological tools in neuroscience research, for the characterization of ion channels and receptors, and they are promising candidates for the development of new drugs [23,36,37].

It has been estimated that the whole genus *Conus* (~500 species) express ~50,000 different conotoxins [42], which are synthesized in the form of precursors that generally comprise 60–90 amino acids, and that have three well-defined regions: the N-terminal signal sequence, an intervening propeptide region, and the mature toxin region at the C-terminus [24,45]. Conotoxins have been classified into gene superfamilies, based on the sequences of the signal sequences of their precursors [25,28,31,38,42,46,47]. Usually, the conotoxins of each superfamily share a particular arrangement of Cys residues [42,45,52]. Each superfamily can

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include several pharmacological families, which are defined by the molecular targets and how the toxins affect them [29,40,52].

The I-superfamily of conotoxins was defined by excitatory peptides and similar sequences identified, respectively, from the venom and venom duct cDNA of the piscivorous *C. radiatus* [27]. These peptides have eight Cys residues arranged in framework # 11 or XI (C-C-CC-CC-C-C, where "-" denotes one or more non-Cys residue) [44]. Currently, this superfamily also includes members from vermivorous and molluscivorous species, and it has been subdivided into gene superfamilies I_1, I_2 and I_3 based mainly on the signal sequences and the position of the propeptide region [6,53], which in the case of the I_2 superfamily is located at the C-terminus of the precursor. However, I_2-conotoxin precursors that contain two propeptide regions have been recently identified from *C. spurius* [54].

Here we describe for the first time the identification and analysis of I-conotoxin precursors expressed in the venom ducts of three vermivorous species from the Mexican Pacific coast, *C. brunneus*, *C. nux*, and *C. princeps*.

2. Materials and methods

2.1. Biological material

Specimens of *C. brunneus*, *C. nux*, and *C. princeps* were collected near the coast of the State of Jalisco, in the Mexican Pacific Ocean in September, 2008. The venom ducts were dissected from the living snails, immediately added to RNAlater (Qiagen, Hilden, Germany), incubated overnight in the reagent at 4 °C, and then stored at −70 °C.

2.2. Isolation of total RNA

Total RNAs from venom ducts of single specimens (about 10 mg) were isolated and purified using an SV Total RNA Isolation System Kit (Promega, Madison, WI) according to the instruction manual. RNA yields and qualities were estimated by OD_{260}, and by OD_{260}/OD_{280} and OD_{260}/OD_{230}, respectively.

2.3. cDNA cloning and sequencing

Approximately 200 ng of total RNA from each venom duct was used to generate cDNAs using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) with a 3′-RACE Adapter Primer: 5′-ATTCTAGAGGCCGAGGCGGCCGACATG-$(dT)_{30}$-VN-3′ (Sigma-Genosys, The Woodlands, TX). For PCR amplification of cDNAs encoding I-superfamily conotoxins, forward primer ISF-1 (5′-AGAGAAGTGACGGAGATCAA-3′) and reverse adapter primer ISR-1 (5′-ATTCTAGAGGCCGAGGCGGC-3′) were employed. Primer ISF-1 was designed on the basis of conserved elements in 5′-untranslated regions (UTRs) of known I-superfamily conotoxins, whereas ISR-1 is a short version of the 3′-RACE Adapter Primer (without the last seven nucleotides and the poly dT tail). PCR amplification was carried out under the following conditions: a total volume of 50 μl contained 2 μl of cDNA, 1X Colorless Reaction Buffer, 2 mM $MgCl_2$, each primer at 0.2 μM, 0.24 mM dNTP Mix, and 0.04 U of GoTaq® DNA Polymerase (Promega). The PCR amplifications were performed on a Thermal cycler 2720 (Applied Biosystems, Foster City, CA) programmed for 3 min at 95 °C for initial denaturation, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final step of 72 °C for 5 min. The PCR products were analyzed by electrophoresis on 1.5% low-melting point agarose gels (Promega). Prominent DNA bands were excised from the gel and purified using the PureLink Quick Gel Extraction Kit (Invitrogen). The PCR products were cloned into the pGEM-T-Easy vector (Promega) via TA cloning, and transformed into electro-competent *E. coli* cells XL1-Blue (Stratagene, La Jolla, CA). Clones were randomly selected for cDNA purification and sequencing. Plasmids were purified employing the Wizard *Plus* SV Minipreps DNA Purification Systems (Promega), and they were sequenced with M13 forward primer at the Laboratorio Nacional de Genómica para la Biodiversidad, CINVESTAV-Irapuato (Irapuato, Gto., México), using the dideoxy chain termination method on a 3730xl DNA Analyzer (Applied Biosystems).

2.4. Sequence analysis

The open reading frames (ORFs) corresponding to the precursors encoded in the cloned cDNA sequences were predicted with the ORF Finder server [41]. Only sequences without any ambiguity in the ORFs were analyzed further. Signal peptides were predicted using the SignalP 3.0 algorithm [3] provided within the ConoPrec tool, which is available on the ConoServer website [8,31,32]. ConoPrec was also used to predict the mature toxin regions and post-translational modifications, to identify the gene superfamily of the cloned precursors, and for similarity searches. Protein sequence alignments were carried out using the ClustalW2 [35] server at the EMBL-EBI website with default settings [15], and it was also used to calculate percent sequence identities among the mature toxins identified in this work. For other comparisons of mature toxins, the pairwise alignment EMBOSS Needle server [14,41] was employed to calculate percent sequence identities and similarities. Physicochemical parameters such as theoretical isoelectric point [4] and grand average of hydropathicity [34] were calculated by means of the ProtParam server [20] of the ExPASy website [16].

For sequence comparison and phylogenetic analysis of precursors and mature toxins, we relied on the ConoServer database [8,31]. The search for protein I_1-, I_2- and I_3-superfamily precursors yielded 17, 57, and nine entries, respectively (October 16, 2013). For our analysis, we removed three entries lacking the signal peptide. The search for protein I_1-, I_2- and I_3-superfamily wild type toxins yielded 42, 54 and nine entries, respectively. We kept the names annotated in this database, but we added a prefix (I-1-, I-2-, or I-3-) corresponding to the gene superfamily.

For phylogenetic analyses, the ClustalW2 multiple sequence alignments of whole precursors or mature toxins were entered into MEGA 4.0 [51]; the overall mean p-distance (calculated by MEGA 4.0) of the 93-precursor ClustalW2 multiple alignment is 0.675 (pair-wise deletion), which is within the acceptable range ($\leq$0.8) for obtaining a reliable phylogenetic tree [22]; the corresponding value for the multiple alignment of the 46 mature I_2-conotoxins belonging to the major clade is 0.597 (data not shown). The analyses were performed with the neighbor-joining method [50] using the Jones–Taylor–Thornton (JTT) matrix [30] and pair-wise deletion of gaps. Bootstrap values [18] were estimated from 2000 replicates (95% confidence) [21] with a random seed. For the sequence comparison and phylogenetic analysis of the mature toxins, Gly and the other residues of the "post" region were removed from our sequence dataset.

Basic Local Alignment Search Tool (BLAST) was employed to search the National Center for Biotechnology Information Non-redundant protein sequences database [41]. We employed the protein blast program (Search protein database using a protein query) with the blastp algorithm (protein–protein BLAST) (BLASTP 2.2.28, Nov-23-2013; http://blast.ncbi.nlm.nih.gov/Blast.cgi) [2]), using the default parameters, except that we limited our search to Organism = *Conus* (taxid:6490).

2.5. Nomenclature

The 15 distinct precursor sequences obtained in this work are named as Nx11.1–Nx11.10, Pi11.1–Pi11.3, and Br11.1–Br11.2,