Peptides 30 (2009) 1660–1668

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Mass spectrometric characterization and physiological actions of novel crustacean C-type allatostatins

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ARTICLE INFO

Article history:
Received 30 March 2009
Received in revised form 25 May 2009
Accepted 27 May 2009
Available online 6 June 2009

Keywords:
Allatostatin
Crustaceans
Neuromodulation
Stomatogastric nervous system
Neuropeptides
Peptide sequencing

ABSTRACT

The crustacean stomatogastric ganglion (STG) is modulated by numerous neuropeptides that are released locally in the neuropil or that reach the STG as neurohormones. Using 1,5-diaminonaphthalene (DAN) as a reductive screening matrix for matrix-assisted laser desorption/ionization (MALDI) mass spectrometric profiling of disulfide bond-containing C-type allatostatin peptides followed by electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) tandem mass spectrometric (MS/MS) analysis, we identified and sequenced a novel C-type allatostatin peptide (CbAST-C1), pQIRYHQCYFN-PISCF-COOH, present in the pericardial organs of the crab, *Cancer borealis*. Another C-type allatostatin (CbAST-C2), SYWKQCAFNAVSCFamide, was discovered using the expressed sequence tag (EST) database search strategy in both *C. borealis* and the lobster, *Homarus americanus*, and further confirmed with *de novo* sequencing using ESI-Q-TOF tandem MS. Electrophysiological experiments demonstrated that both CbAST-C1 and CbAST-C2 inhibited the frequency of the pyloric rhythm of the STG, in a state-dependent manner. At 10^{-6} M, both peptides were only modestly effective when initial frequencies of the pyloric rhythm were >0.8 Hz, but almost completely suppressed the pyloric rhythm when applied to preparations with starting frequencies <0.7 Hz. Surprisingly, these state-dependent actions are similar to those of the structurally unrelated allatostatin A and allatostatin B families of peptides.

1. Introduction

The crustacean stomatogastric ganglion (STG) has been extensively used to study the function of rhythmic neuronal networks and their modulation [39,40,47]. The most abundant and diverse chemical modulators of STG function are neuropeptides, which can be released locally into the neuropil of the STG from the terminals of a number of modulatory projection neurons or delivered to the STG via the hemolymph from neurosecretory organs such as the pericardial organs (POs) or sinus glands (SGs) [38,47,51]. Neural circuits in the STG are extensively modulated by a large number of different neuropeptides [10,39,41,47]. Understanding the role of such a complex set of modulatory substances would be facilitated by knowing the structure and distribution of as many of these substances as possible. The present work adds a new family of neuropeptides to the growing list of those that modulate the STG, and raises interesting questions about the physiological actions of the allatostatin families of peptides.

The allatostatin (AST) peptides were first identified in insects as inhibitors of juvenile hormone synthesis in the corpora allata [44,55]. Subsequently, many ASTs have been identified in insects and crustaceans [12,14,15,43,54]. Chemically, the allatostatins can be subdivided into three distinctly different groups: (1) A-types (cockroach), that possess the common C-terminal pentapeptide motif Y/FXFGL-NH_2 [44,55], (2) B-types (cricket), that possess the C-terminal sequence W$(X)_6$Wamide, with X being variable amino acids [8,35,45], and (3) C-types (*Manduca sexta* or Lepidopteran), that possess a nonamidated, conserved C-terminus – PISCF [23,27,33,53]. The A-, B- and C-type ASTs show little structural similarities; however all three types have been found in a single species (e.g., *Drosophila*; reviewed in [19]).

Immunocytochemical studies demonstrated that A-type allatostatin-like immunoreactivity is widely distributed within the central nervous system (CNS), stomatogastric and peripheral nervous systems of crustaceans [42,46,48]. This highly complex neuronal distribution pattern suggests an important physiological role for the AST-A family. Physiological studies showed that A-type ASTs are inhibitory modulators of the pyloric rhythm of the crustacean STG [48]. Furthermore, these ASTs decrease the amplitude of transmission and movement at several crustacean neuromuscular junctions [25,29] and modulate sensory neuron activity [5,7].

With the use of improved mass spectral techniques, more than 100 crustacean A-type allatostatins have been reported in the past

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0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.05.023

decade [12–15,21,32,37]. More recently, B-type AST was identified in *Cancer productus* POs [14], after which additional isoforms were identified in numerous crustacean species [15,37]. Despite the drastically different sequences of A- and B- type ASTs, their physiological actions on the STG were strikingly similar [16].

We employed a combined bioinformatics, biochemical screening and mass spectrometry approach to search for C-type AST peptides in crabs. Since the discovery of the first C-type AST pQVRFRQCYFNPISCF-COOH in the tobacco hornworm, *M. sexta*, as an inhibitor of juvenile hormone biosynthesis [27], more C-type ASTs have been identified in numerous insect species [1,23,33,34,53]. In contrast to the presence of a large number of isoforms in the A-type and B-type ASTs, C-type ASTs exhibit a remarkably conserved sequence motif of pQXRXRQCYFNPISCF-COOH, with only one or two amino acid substitutions and a highly conserved disulfide bridge between Cys7 and Cys14 [49]. This observation led to the conclusion that there is only a single C-type AST isoform present in any insect species until a recent surge of genomic and transcriptomic information became available for a variety of arthropod species. Sequence alignment of numerous predicted C-type allatostatin precursors revealed the presence of at least another distinct peptide sequence of SYWKQCAFNAVSCFa-mide [52], which was originally reported in a peptidome study of honeybee *Apis mellifera* as an un-annotated peptide [20]. This peptide was also recently predicted via transcriptomics from the crustacean *Daphnia* [17] and identified in the American lobster *Homarus americanus* and other crustacean species by accurate mass measurement [11]. Since this peptide sequence is significantly different from the typical – PISCF C-type AST, it would be interesting to see if both isoforms of C-type ASTs exist in *Cancer borealis*. In the current study, we *de novo* sequenced two novel C-type ASTs from the POs of *C. borealis* and the brain of *H. americanus* using a combination of reductive matrix screening to locate disulfide bond containing C-type ASTs and tandem mass spectrometry. These new C-type ASTs share sequence similarities with those from insect species. Using the synthetic peptides, CbAST-C1 and CbAST-C2, we show that both of these peptides inhibit the pyloric rhythm in the STG of the crab, *C. borealis*, in a state-dependent manner.

2. Materials and methods

2.1. Materials

Methanol, acetonitrile, formic acid and glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA), dithiothreitol, iodoacetamide and 1,5-diaminonaphthalene (DAN) were purchased from Sigma–Aldrich (St. Louis, MO). 2,5-dihydroxybenzoic acid (DHB) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Both CbAST-C1 and CbAST-C2 peptides were synthesized at the UW-Madison Biotechnology Center.

2.2. Animal and tissue collection

Jonah crabs, *C. borealis*, were shipped from the Marine Biological Laboratories (Woods Hole, MA) and the American lobsters, *H. americanus*, were purchased from local grocery stores. Both species were maintained without food in an artificial seawater tank at 10–12 °C. Prior to dissection, animals were cold-anesthetized by packing in ice for 15–30 min. They were dissected by removing the stomach section, eyestalks, thoracic ganglia, and pericardial ridges located on either side of the heart. Pericardial organs (POs) were removed from the pericardial ridges. The dissection of *C. borealis* was carried out in chilled physiological saline (composition in mM: NaCl, 440; KCl, 11; $MgCl_2$, 26; $CaCl_2$, 13; Trizma base, 11; maleic acid, 5; pH 7.45), while the dissection of the lobster *H. americanus* was carried out in chilled physiological saline (composition in mM: NaCl, 479.12; KCl, 12.74; $CaCl_2$, 13.67; $MgSO_4$, 20.00; Na_2SO_4, 3.91; HEPES, 5.00; pH 7.4).

2.3. Tissue extraction and off-line HPLC fractionation

Tissues were separately pooled, homogenized, and extracted with acidified methanol (90% methanol, 9% glacial acetic acid, and 1% deionized water). Extracts were dried in a SpeedVac concentrator (Thermo Electron) and re-suspended with minimum amount of 0.1% formic acid. The re-suspended extracts were then vortexed and briefly centrifuged. The resulting supernatants were subsequently fractionated via high performance liquid chromatography (HPLC).

HPLC separations were performed using a Rainin Dynamax HPLC system equipped with a Dynamax UV-D II absorbance detector (Rainin Instrument Inc., Woburn, MA). The mobile phases included: Solution A (deionized water containing 0.1% formic acid) and Solution B (acetonitrile [HPLC grade, Fisher Scientific] containing 0.1% formic acid). About 20–50 μl of extract was injected onto a Macrosphere C_{18} column (2.1 mm i.d. × 250 mm length, 5 μm particle size; Alltech Assoc. Inc., Deerfield, IL). The separations consisted of a 120 min gradient of 5–95% Solution B. Fractions were automatically collected every 2 min using a Rainin Dynamax FC-4 fraction collector.

2.4. Reduction of C. borealis PO extract by 1,5-diaminonaphthalene (DAN)

Ten milligrams of 1,5-diaminonaphthalene were dissolved in 1 ml of 80% acetonitrile in water. After depositing 0.5 μl of *C. borealis* PO crude extract and 0.5 μl of DAN matrix solution on a sample target and drying at room temperature, the mixture was analyzed by MALDI-TOF/TOF.

2.5. Formaldehyde derivatization

An aliquot of 0.3 μl of the *C. borealis* PO crude extract was spotted on the MALDI plate, followed by the addition and mixing of 0.3 μl of 26 mM sodium cyanoborohydride (Sigma–Aldrich, St. Louis, MO), and subsequent addition of 0.3 μl of formaldehyde (20% in H_2O v/v, Sigma–Aldrich). The droplet was left at room temperature for 5 min after which 0.3 μl of 50 mM ammonium bicarbonate solution was added to the reaction mixture. Finally, 0.3 μl of a saturated 2,5-dihydroxybenzoic acid matrix was added to the droplet and crystallized at room temperature.

2.6. Reduction-alkylation of HPLC fraction

An aliquot of 10 μl of the *C. borealis* PO HPLC fraction was mixed with 2 μl of 200 mM dithiothreitol (DTT) and then incubated at 37 °C for 1 h. After 10 μl of 200 mM iodoacetamide was added, the mixture was incubated in the dark at room temperature for 1 h. The reaction solution was then concentrated to dryness in a SpeedVac, and further resuspended in 10 μl of 0.1% formic acid.

2.7. MALDI-FTMS and direct tissue analyses

Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI-FTMS) experiments were performed on a Varian/IonSpec ProMALDI Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0 T actively-shielded superconducting magnet. The FTMS instrument contains a high pressure MALDI source where the ions from multiple laser shots can be accumulated in the external hexapole storage trap before the ions are transferred to the ICR cell via a quadrupole ion guide. A