

Mass spectrometric characterization and physiological actions of VPNDWAHFRGSWamide, a novel B type allatostatin in the crab, *Cancer borealis*

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Abstract

The neural networks in the crustacean stomatogastric ganglion are modulated by neuroactive substances released locally into the neuropil of the stomatogastric ganglion and by circulating hormones released by neuroendocrine structures including the pericardial organs. Using nanoscale liquid chromatography coupled to electrospray ionization quadrupole-time-of-flight mass spectrometry, we have identified and sequenced a novel B type allatostatin (CbAST-B1), VPNDWAHFRGSWamide, present in the pericardial organs of the crabs, *Cancer borealis*, and *Cancer productus*. We describe the physiological actions of CbAST-B1 on the pyloric

rhythm of the stomatogastric ganglion of the crab, *Cancer borealis*. CbAST-B1 reduces the pyloric network frequency in a dose-dependent manner. The effect of bath-applied CbAST-B1 depends on the preceding physiological state of the preparation. Surprisingly, despite marked amino-acid sequence dissimilarity between the novel CbAST-B1 and the A type allatostatin family of peptides (AST-A), the physiological effects of CbAST-B1 are similar to those of AST-A.

Keywords: allatostatin, crustaceans, ESI-Q-TOF MS, neuromodulation, neuropeptides, pericardial organs.

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Neuropeptides modulate the output of neural circuits in all animals (Nusbaum *et al.* 2001; Marder and Thirumalai 2002). Identification of the complement of the neuropeptides employed by neural circuits and characterization of their actions at the cellular and network levels are crucial to a better understanding of the plasticity of neural circuits. The crustacean stomatogastric nervous system (STNS) provides an excellent system for the study of physiological effects of neuropeptides because the neural circuits of the STNS contain a small number of neurons and display well-characterized rhythmic patterns (Harris-Warrick *et al.* 1992). Networks found in the stomatogastric ganglion (STG) are modulated by neuroactive substances released locally from modulatory inputs (Marder and Bucher 2001; Marder and Thirumalai 2002; Nusbaum and Beenhakker 2002) and by circulating hormones delivered via the hemolymph (Cooke and Hartline 1975; Christie *et al.* 1995; Skiebe 2001; Cooke 2002; Li *et al.* 2003). The pericardial organ (PO) is one of the major hormonal sources in crustaceans. The POs contain and release a plethora of neuropeptide hormones (Cooke and Sullivan 1982; Pulver and Marder 2002). The neuropeptide complements of the

POs from two *Cancer* species have been recently characterized using immunochemical and mass spectrometric methods (Li *et al.* 2002, 2003; Fu *et al.* 2005a,b; Cruz-Bermudez *et al.* 2006). These studies found a number of neuropeptides that had been previously identified in crustacean species, as well as revealing the presence of many neuropeptides that had not been previously identified (Cruz-Bermudez *et al.* 2006).

The allatostatins (ASTs) belong to a part of a major neuropeptide family that is widely distributed in decapod crustaceans. ASTs were first identified in insects for their ability to inhibit the synthesis of juvenile hormone in the

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Abbreviations used: ESI, electrospray ionization; LC, liquid chromatography; LP neuron, lateral pyloric neuron; PD neuron, pyloric dilator neuron; POs, pericardial organs; PY neuron, pyloric neuron; Q-TOF, quadrupole-time-of-flight; STG, stomatogastric ganglion; *stn*, stomatogastric nerve; STNS, stomatogastric nervous system.

corpora allata (Pratt *et al.* 1989; Woodhead *et al.* 1989; Stay *et al.* 1992, 1996; Lorenz *et al.* 1995a; Bendena *et al.* 1999; Stay 2000; Nichols *et al.* 2002). There are three types of ASTs identified: cockroach- or A-type ASTs (with conserved C-terminus F/YXFLGamide) (Pratt *et al.* 1989; Woodhead *et al.* 1989), cricket- or B-type ASTs (with conserved C-terminus WXXXXXXWamide) (Schoofs *et al.* 1991; Blackburn *et al.* 1995; Lorenz *et al.* 1995a, 2000; Hua *et al.* 1999; Predel *et al.* 2001; Williamson *et al.* 2001a; Wang *et al.* 2004), and C-type ASTs (conserved C-terminus pEVRXRQCYFNPI SCF) (Kramer *et al.* 1991; Jansons *et al.* 1996; Williamson *et al.* 2001b) based on their distinct structural differences. Only the A- and B-types ASTs have been identified in crustaceans to date.

More than 80 A-type ASTs have been reported in the past decade (Duve *et al.* 1997, 2002; Dirksen *et al.* 1999; Skiebe 1999; Huybrechts *et al.* 2003; Li *et al.* 2003; Billimoria *et al.* 2005; Fu and Li 2005; Fu *et al.* 2005b; Yin *et al.* 2006). A-type ASTs are inhibitory modulators of the pyloric rhythm of the crustacean STG (Skiebe and Schneider 1994), decrease the amplitude of transmission and movement at several crustacean neuromuscular junctions (Jorge-Rivera and Marder 1997; Jorge-Rivera *et al.* 1998; Kreissl *et al.* 1999), and modulate sensory neuron activity (Birmingham *et al.* 2003; Billimoria *et al.* 2006). The B-type ASTs were only recently identified in extracts from the POs of *Cancer productus* and *Cancer borealis* (Fu and Li 2005; Fu *et al.* 2005b). However, to date there are no reports on the physiological actions of B-type ASTs in crustaceans.

In the current study, we *de novo* sequenced a novel B-type AST (CbAST-B1) from the PO of *C. productus* and *C. borealis* using liquid chromatography coupled to electrospray ionization quadrupole time-of-flight tandem mass spectrometry (LC ESI Q-TOF MS/MS). This new 12-mer neuropeptide shares the conserved C-terminal motif of W(X)₆W (where X is variable amino acid residue), which is characteristic of B-type AST peptide family. Using the synthetic peptide, we show that VPNDWAHFRGSWamide can modulate the pyloric rhythm of the crab, *C. borealis* STG.

Materials and methods

Animals and dissection

Cancer borealis were obtained from the Marine Biological Laboratories (Woods Hole, MA, USA) and Commercial Lobster (Boston, MA, USA) and maintained without food in artificial seawater tanks at 10–12°C. *Cancer productus* were collected by hand, ring-trap or dredge in the Puget Sound area and San Juan Archipelago of Washington State (USA). Animals were cold-anesthetized by packing them in ice for 15–30 min prior to dissection. For MS analysis, we isolated the intact STNS from the foregut and the POs from the pericardial ridges located on either side of the heart. All dissections were carried out in chilled physiological

saline (composition in mmol/L: NaCl, 440; KCl, 13; MgCl₂, 26; CaCl₂, 13; Trizma base, 11; maleic acid, 5; pH 7.45).

Tissue extraction

The extraction of pooled tissue was performed using ice-cold acidified methanol solution (90% methanol, 9% glacial acetic acid, 1% water). First, approximately 30 POs were removed from the acidified methanol storage buffer and placed in a 0.1 mL tissue grinder (Wheaton Inc., Millville, NJ, USA) along with 100 µL of the storage buffer. The tissue was then homogenized completely after which the extraction liquid was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 16 100 g for 10 min on an Eppendorf 5415 D microcentrifuge (Brinkmann Instruments Inc., Westbury, NY, USA). The supernatant was retained and the pellet re-extracted with acidified methanol and respun. Supernatants were combined and concentrated to dryness with a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL, USA). Finally, a minimal amount (20–300 µL) of resuspension solution (deionized H₂O w/0.1% trifluoroacetic acid or 0.1% formic acid) was added to the extract. This resuspended extract was then vortexed and briefly centrifuged with supernatant to be used for HPLC separation followed by MALDI FTMS screening or LC MS/MS analysis.

Capillary LC Q-TOF MS/MS

NanoLC-MS/MS analysis was performed using a Waters capillary LC system coupled to a Q-TOF Micro mass spectrometer (Waters Corporation, Milford, MA, USA). Chromatographic separations were performed on a reverse phase capillary column (75 µm i.d. × 100 mm length, 3 µm particle size, Atlantis[®] dC18, Waters Corp.). The mobile phases used were A: deionized H₂O w/5% acetonitrile and 0.1% formic acid; B: acetonitrile w/5% deionized H₂O and 0.1% formic acid; C: deionized H₂O w/0.1% formic acid. A total of 1.4 µL of tissue extract was injected and loaded onto the trap column (300 µm column i.d. × 1 mm, 5 µm particle size, PepMap[™] C18, LC Packings, Sunnyvale, CA, USA) using mobile phase C at a flow rate of 30 µL/min for 3 min. Following this, the stream select module was switched to a position where the trap column became inline with the analytical capillary column and a linear gradient of mobile phases A and B was initiated. A splitter was added between the mobile phase mixer and the stream select module to reduce the flow rate from 18 µL/min to 200 nL/min.

The nanoflow ESI source conditions were set as follows: capillary voltage 3800 V, sample cone voltage 40 V, extraction cone voltage 1 V, source temperature 120°C, cone gas (N₂) 13 L/h. For the reference spray, the same settings were used except that the sample cone voltage was set at 10 V and reference scans were performed every 10 s. A data-dependent acquisition was employed for the MS survey scan and the selection of precursor ions and subsequent tandem MS of the selected parent ions. The MS scan range was from *m/z* 100 to 2000 and the MS/MS scan was from *m/z* 50 to 2000. The MS/MS *de novo* sequencing was performed with a combination of manual sequencing and automatic sequencing by PepSeq software (Waters Corp.).

Electrophysiology

Experiments were performed with *C. borealis* purchased from Yankee Lobster (Boston, MA, USA). Physiological recordings were

made using routine methods for *in vitro* preparations of the STNS (Goillard *et al.* 2004). All data were collected from preparations consisting of at least one of paired commissural ganglia (CoG), the esophageal (OG), and stomatogastric (STG) ganglia plus their connecting and motor nerves. Preparations with differing starting pyloric rhythms were produced by acutely isolating the STG from the anterior ganglia completely or partially by cutting the stomatogastric nerve (*stm*) or by blocking impulse activity in the *stm* by placing a Vaseline well around the desheathed *stm* and adding 10^{-6} mol/L or 10^{-7} mol/L tetrodotoxin to the well.

Cancer borealis physiological saline was composed of (in mmol/L): NaCl, 440; KCl, 11.3; CaCl₂, 13.3; MgCl₂, 26.3; Trizma Base, 11; maleic acid 5.2, pH 7.4–7.6. Extracellular recordings were made by placing stainless steel pins in Vaseline wells surrounding the nerves. Intracellular recordings of STG neurons were made with sharp microelectrodes (20–50 MΩ) filled with 0.6 mol/L K₂SO₄ and 20 mmol/L KCl using an Axoclamp 2A (Axon Instruments, Foster City, CA, USA). All experiments were performed with a continuously flowing superfusion system (6–10 mL/min), cooled to 10–12°C. Data were collected in Clampex 8.0 (Axon Instruments) and analyzed in Spike 2 v 5.0 and StatView 5.0.1. (SAS Institute, Cary, NC, USA) All statistical tests were two-tailed Student's *t*-tests. The dose–response curve was fit with a four-parameter Hill equation.

Results

De novo sequencing of a B-type allatostatin (AST) by Q-TOF MS/MS

Our previous study of the *C. borealis* PO and the STNS (STG and CoG) revealed the presence of an unknown peptide peak at *m/z* 1470.74 by direct tissue matrix-assisted laser desorption/ionization Fourier transform mass spectrometry (MALDI FTMS) (Kutz *et al.* 2004). Here, we *de novo* sequenced this peptide peak from *C. borealis* and *C. productus* PO extracts using nanoLC ESI Q-TOF MS/MS. The amino acid sequence was determined to be VPNDWAHFRGSWamide, which represents a novel B type AST in *Cancer* crabs. Figure 1 is a representative MS scan from the LC MS/MS analysis of *C. borealis* PO extract showing the presence of this peptide. The precursor ions with

+1, +2, and +3 charges were detected. The in-source fragment ions (Sleno and Volmer 2004), y_{10}^{2+} , y_{11}^{2+} , and y_{11}^{3+} were also detected. These ions exhibited the same retention times as the precursor ions (490.86³⁺, 735.79²⁺, and 1470.60⁺), eluting at 49.85 min. Therefore, these fragment ions were most likely produced from the precursor ions because of the in-source gas-phase fragmentation. It was also noted that three of the A-type AST peptides, AGPYSFGLamide (810.36⁺), pyrRAYSFGLamide (923.41⁺), and ER-PYSFGLamide (967.40⁺), were detected in this MS scan as well. The detection of multiple peptide species in a single MS scan during LC elution reveals the chemical complexity of the PO extract.

Figure 2(a) shows the MS/MS fragmentation spectrum of the novel AST B peptide ion (735.79²⁺, doubly charged precursor ion) from the LC MS/MS analysis of the *C. borealis* PO extract. As shown, several immonium ions indicative of amino acid compositions, such as P (70), V (72, not labeled in the spectrum because of space limit), H (110), and W (159), were detected. Furthermore, complementary series of major sequence-specific fragment ions including *y*-type and *b*-type ions were readily observed. An almost identical fragmentation pattern with the synthesized peptide standard VPNDWAHFRGSWamide (Fig. 2b) was observed. Several proline-induced internal fragment ions, such as PN, PND, and PNDW, were observed in high abundance, providing additional support for the derived amino acid sequence of the novel AST-B peptide. The triply and singly charged precursor ions also displayed similar fragmentation patterns with the formation of several proline-induced internal fragment ions (data not shown). A major proline-induced internal fragment peptide, PNDWAHFRGSWamide (y_{11} ion of the original intact peptide) was observed and selected for tandem MS analysis. Figure 3 shows the MS/MS fragmentation spectra of this in-source fragment ion, y_{11}^{2+} ion (686.28²⁺) of the peptide from the PO tissue extract (panel a) and y_{11}^{2+} ion of the synthesized peptide standard VPNDWAHFRGSWamide (panel b). As seen, identical MS/MS fragmentation patterns were observed. Taken together, these MS/MS

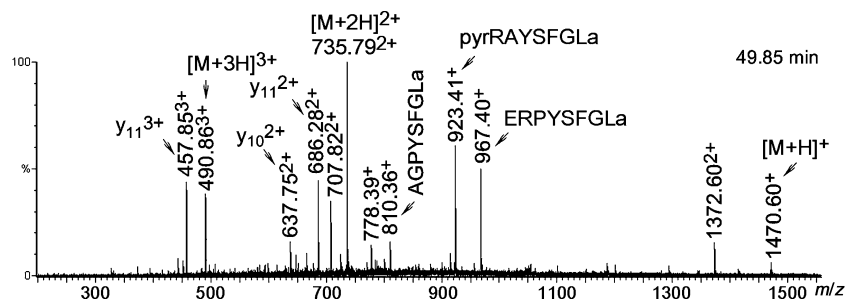


Fig. 1 The LCMS scan at 49.85 min showing the presence of precursor ions at 1470.60 (1+), 735.79 (2+), and 490.86 (3+) for novel peptide CbAST-B1, VPNDWAHFRGSWamide. The fragment ions produced from VPNDWAHFRGSWamide were due to gas phase

fragmentation. Several other A-type allatostatins neuropeptides co-eluted with this new peptide. The singly, doubly, and triply charged ions of VPNDWAHFRGSWamide are labeled in the spectrum.

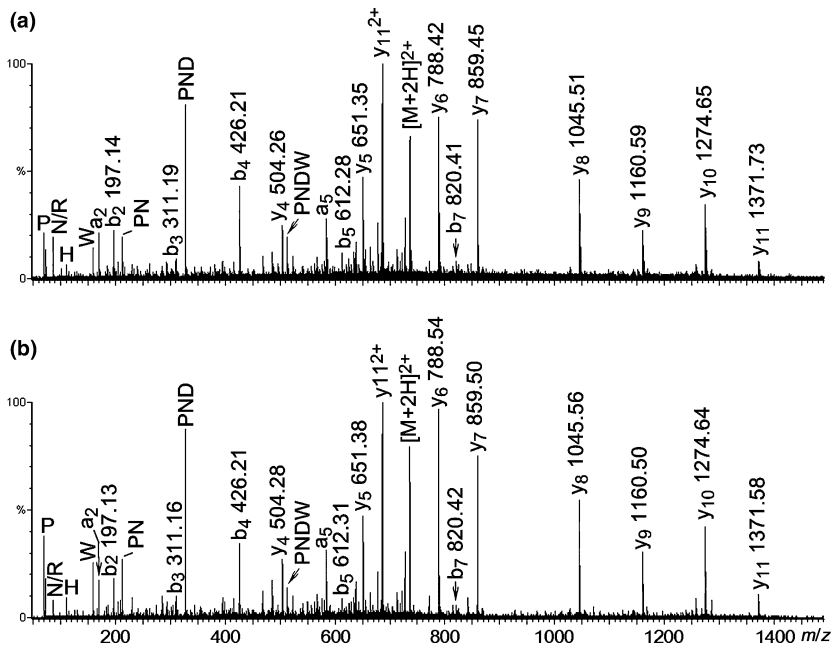


Fig. 2 MS/MS fragmentation spectra of VPNDWAHFRGSWamide from (a) *Cancer borealis* pericardial organ extract, and (b) synthesized peptide standard. All precursor ions are doubly charged. The a-/b- ions, y ions, immonium ions and internal fragment ions are labeled.

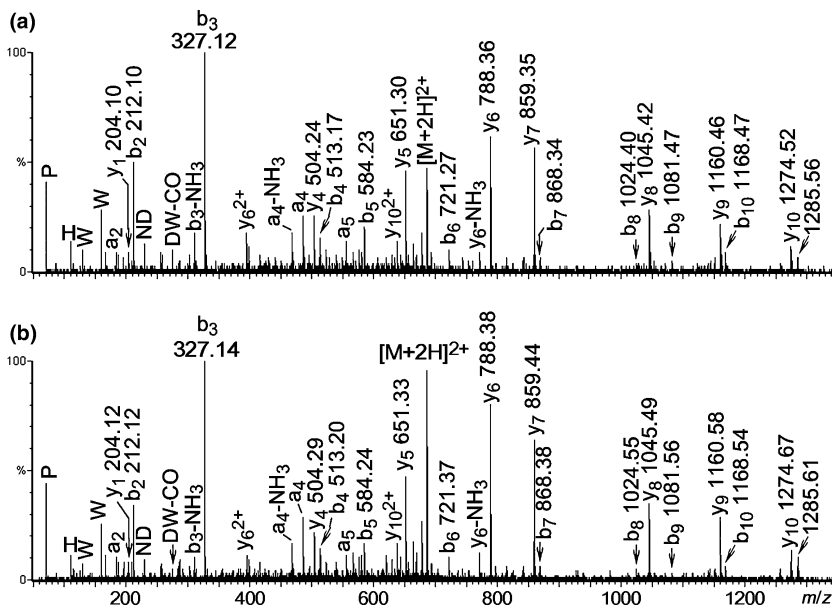


Fig. 3 The MS/MS fragmentation spectra of PNDWAHFRGSWamide from (a) *Cancer borealis* pericardial organ extract, and (b) synthesized standard. All precursor ions are doubly charged. The PNDWAHFRGSWamide is the y_{11}^{2+} product ion produced from VPNDWAHFRGSWamide due to the in-source fragmentation. The 1285.56⁺ and 1285.61⁺ ions in panels (a) and (b) are the $y_{(n-1)} + \text{CO-NH}_3$ ions (Fu and Li 2006).

fragmentation analyses revealed and supported the proposed sequence of VPNDWAHFRGSWamide for the novel B-type AST. We now call this new peptide *C. borealis* AST-B-1 (CbAST-B1).

Effects of VPNDWAHFRGSWamide (CbAST-B1) on the pyloric rhythm

We studied the effects of bath-application of CbAST-B1 on the pyloric rhythm of *C. borealis* using both intracellular and extracellular recordings. The control panel of Fig. 4 shows extracellular recordings from two of the motor nerves of the

STG (medial ventricular nerve and lateral ventricular nerve) which contain activity of the inferior cardiac, pyloric dilator (PD), lateral pyloric (LP), and pyloric (PY) neurons of the pyloric rhythm (Fig. 4a). The bottom trace is an intracellular recording from the PD neuron. In control saline, these recordings showed alternating activity of the LP, PY, and PD neurons. When 10^{-6} mol/L CbAST-B1 was bath applied (Fig. 4b, middle panel), the PD, LP, and inferior cardiac neurons all became silent while the PY neurons (the small units seen on the lateral ventricular nerve extracellular recording) fired tonically. The effects of CbAST-B1 were

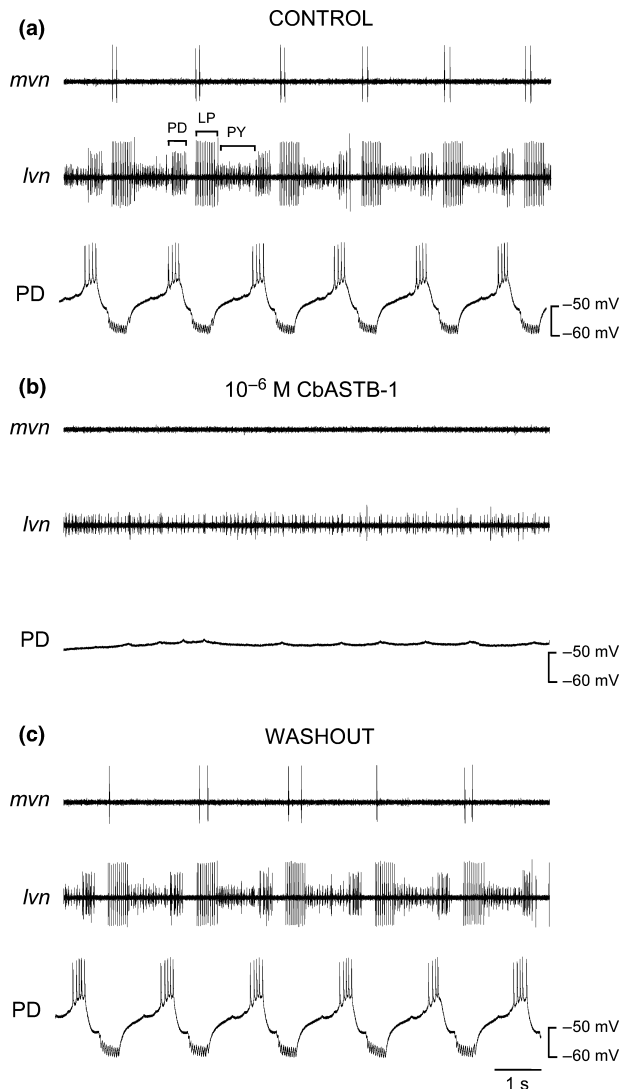


Fig. 4 Inhibitory effects of 10^{-6} mol/L CbAST-B1 on the pyloric rhythm (stomatogastric nerve block). Extracellular recordings from the *mvn*, *lvn*, and one intracellular recording from a pyloric dilator (PD) neuron. (a) *Top Panel*: Pyloric rhythm in control saline condition. PD, lateral pyloric and pyloric alternated in a triphasic sequence (brackets). (b) *Middle Panel*: In presence of 10^{-6} mol/L CbAST-B1, the activity of PD and lateral pyloric neuron was abolished while pyloric became tonically active. (c) *Bottom Panel*: After washout of CbAST-B1, pyloric rhythm fully recovered. *mvn*, medial ventricular nerve; *lvn*, lateral ventricular nerve.

reversible, as rhythmic activity resumed after washout of the peptide (Fig. 4c, bottom panel).

The frequency of the pyloric rhythm is dependent on neuromodulatory inputs from the anterior ganglia (Russell 1979). As shown in Table 1, when the modulatory inputs from the anterior ganglia were left intact, these preparations generated higher frequency pyloric rhythms (1.15 ± 0.32 Hz; $n = 11$) than those preparations with reduced modulatory inputs (0.54 ± 0.20 Hz; $n = 8$). Interestingly, the effects of

Table 1 Pyloric cycle frequency in 10^{-6} mol/L CbAST-B1

	<i>stn</i> intact	<i>stn</i> (partial block)
Control	1.15 ± 0.32	0.54 ± 0.20
10^{-6} mol/L	$0.79 \pm 0.55^*$	$0.01 \pm 0.04^{**}$
	$n = 11$	$n = 8$

* $p < 0.05$; ** $p < 0.001$.

Values (means \pm SD) are statistically significant when compared with control saline, as indicated.

stn, stomatogastric nerve.

CbAST-B1 on the PY neurons were found to be dependent on the starting frequency of the pyloric rhythm. In Fig. 5(a), we plot the normalized effects of 10^{-6} mol/L CbAST-B1 as a function of the initial frequency of the rhythm in control saline. When the initial frequency was less than ~ 0.8 Hz, CbAST-B1 application resulted in complete suppression of the pyloric rhythm in almost all preparations (0.01 ± 0.04 Hz; $p < 0.001$, $n = 8$). In contrast, when the initial frequency was higher than ~ 0.8 Hz, CbAST-B1 was much less effective in decreasing the frequency of the rhythm ($0.79 \pm .55$ Hz; $p < 0.05$, $n = 11$). The raw data for these same experiments are shown in Fig. 5(b), showing the state dependence of the actions of CbAST-B1.

Because the effects of CbAST-B1 are frequency-dependent and show relatively little modulation of the pyloric frequency in faster running preparations, we studied the dose-dependence of CbAST-B1 effects on preparations that were slowly cycling under control conditions (partial blocks of the *stn* were used to produce these rhythms). In Fig. 6(a), extracellular recordings from the pyloric dilator nerve obtained from the same preparation for concentrations from 10^{-9} to 10^{-6} mol/L CbAST-B1 show that PD activity continuously decreases as a function of increasing concentration of CbAST-B1 and is eventually silenced (10^{-7} mol/L and 10^{-6} mol/L in this particular example).

Figure 6(b) shows the dose-dependence of CbAST-B1's actions in pooled data from four preparations. In one out of four preparations, CbAST-B1 reduced the network frequency by 25% at a concentration of 10^{-10} mol/L. The effective concentration at which CbAST-B1 reduced the network frequency by 50% is approximately 10^{-8} mol/L (pyloric frequency during control conditions: 0.41 ± 0.10 Hz; pyloric frequency during 10^{-8} mol/L CbAST-B1: 0.20 ± 0.08 Hz; $p < 0.05$).

Discussion

Over the years a large number of different allatostatin family peptides have been identified and characterized in a variety of arthropod species. Many of the physiological studies of these peptides have focused on their endocrine actions, and relatively little is known of their direct actions on the nervous

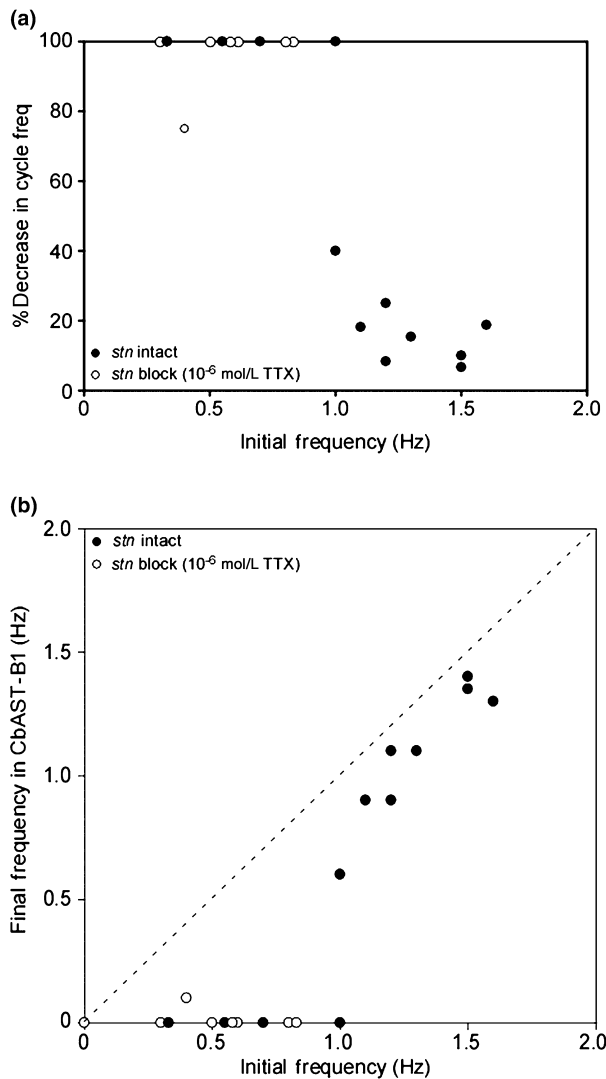


Fig. 5 (a) Percentage of decrease in cycle frequency induced by 10^{-6} mol/L CbAST-B1 as a function of the initial frequency of pyloric rhythm in both stomatogastric nerve (*stn*) intact (filled circles) and *stn* blocked (open circles). *Filled circles*: in *stn* intact conditions, 10^{-6} mol/L CbAST-B1 had little effect at starting frequencies > 0.8 Hz. *Open circles*: in *stn* blocked (partial), 10^{-6} mol/L CbAST-B1 significantly reduced the pyloric network frequency. (b) Final frequency of the pyloric rhythm in CbAST-B1 as a function of initial frequency in both *stn* intact (filled circles) and *stn* blocked (open circles) preparations. Dashed line represents condition in which there was no difference between the cycle frequency in CbAST-B1 and the cycle frequency in control.

system. To the best of our knowledge, this paper constitutes both the first identification of a novel member of the AST-B family of peptides, as well as the first set of electrophysiological studies of any of the AST-B family of peptides in crustaceans.

VPNDWAHFRGSWamide (CbAST-B1) shares significant homology at the C-terminal end of the amino acid sequence

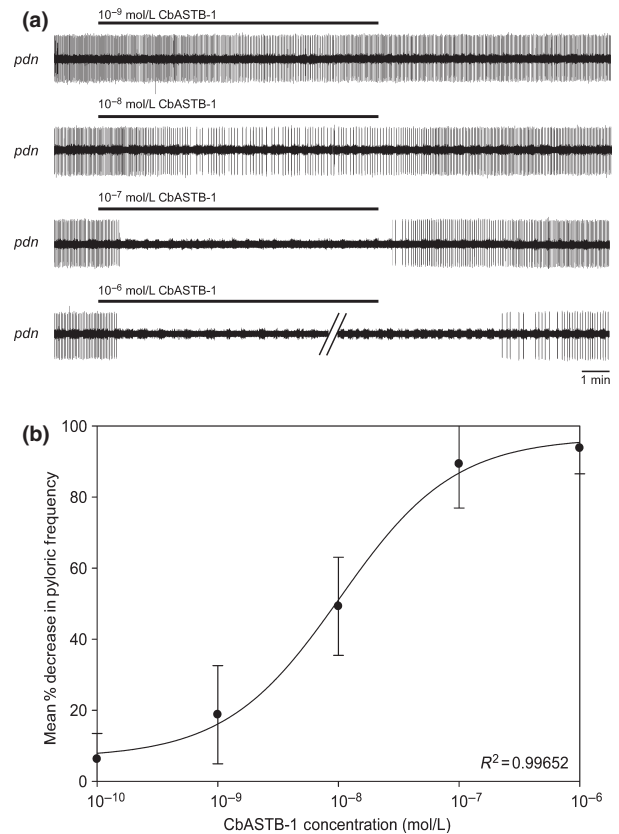


Fig. 6 (a) Dose-dependence of CbAST-B1 effects on slowly cycling pyloric rhythms (partial block with 10^{-6} mol/L tetrodotoxin). All traces were extracellular recordings from the pyloric dilator nerve (*pdn*) in the same preparation. Dark horizontal bars represent the duration of CbAST-B1 application except for bottom trace where CbAST-B1 was applied for an additional 10 min (not shown, hashed marks). PD neuron activity decreased continuously with increasing concentration of CbAST-B1. Preparations were washed extensively between applications. (b) Dose-dependence of CbAST-B1 on pyloric frequency. All preparations chosen for dose-response curve were cycling at a starting frequency < 0.8 Hz during control conditions ($n = 4$). Increasing concentrations of CbAST-B1 reduced the pyloric frequency relative to control. The error bars were \pm SE.

with those of *Drosophila* AST peptide Drm-AST B2 (pEAQGWNKFRGAWamide) (Williamson *et al.* 2001a) and Grb-AST B4 (AWERFHGGSWamide) (Lorenz *et al.* 1995b). Within the conserved W(X)₆W C-terminal sequence motif, Phe is flanked by a basic amino acid residue on both sides, yielding the detection of both doubly and triply charged precursor ions. A truncated peptide PNDWAHFRGSWamide was also identified and sequenced in this study. This truncation is likely due to gas-phase fragmentation of the full-length peptide, VPNDWAHFRGSWamide, because the truncated and longer peptides share identical LC retention times. With only one N-terminal amino acid valine removed from the sequence of VPNDWAHFRGSWamide, the shorter peptide displays identical fragmentation.

Since the early identification of the ASTs, it has become evident that these families of peptides contain very large numbers of members. Not only do different species show various forms of these peptides, but within a given species many different forms are present. For example, there are more than 30 ASTs in *C. borealis* and *C. productus*, including sixteen newly sequenced A-type ASTs and seven B-type ASTs (Fu and Li 2005; Fu *et al.* 2005b).

Given the fact that there is no apparent sequence homology between the A-type ASTs and the B-type ASTs, it is quite remarkable that the physiological actions we report here for CbAST-B1 are quite reminiscent of the effects of the A-type ASTs previously studied on the crab pyloric rhythm (Skiebe and Schneider 1994). Both classes of peptides strongly inhibited the pyloric rhythm when it was already slow under control conditions, but were quite ineffective on robust rhythms. In crabs and other crustaceans, the A-type ASTs also act on neuromuscular junctions and sensory neurons (Jorge-Rivera and Marder 1997; Kreissl *et al.* 1999; Birmingham *et al.* 2003; Billimoria *et al.* 2006). Therefore, it would be interesting to determine if the actions of CbAST-B1 and other B-type ASTs on these other targets also resemble those of the A-type ASTs.

CbAST-B1 had very pronounced state-dependent actions, in that the effects were strongly inhibitory only when the rhythm was initially weak. CbAST-B1 is not the first example of a modulator reported to have state-dependent actions in the STNS. For example, proctolin (Nusbaum and Marder 1989) and octopamine (Goaillard *et al.* 2004) strongly excite preparations with relatively weak rhythms but have relatively little effects on the period of preparations with high frequency pyloric rhythms. However, to date, the underlying mechanisms of state-dependent actions have not been elucidated. Several hypotheses can be proposed to explain the state dependence of CbAST-B1 actions. (i) State-dependent modulation could be a simple consequence of the input conductance of the neuron. For example, in the *stn* intact condition, the input conductance of strongly bursting neurons is higher than when neuromodulatory inputs are partially or completely blocked. Therefore, the activation of conductances by neuromodulators would be less effective in changing the firing pattern of the neuron, because the ratio between the modulator-activated conductance and the total input conductance of the neuron is low. This could explain why CbAST-B1's effect is stronger when the *stn* is partially blocked and the rhythm is weaker, as is the case in our study. (ii) State dependence could arise from a more complex consequence of the global biochemical state of the neuron. Specifically, there could be a direct interaction between CbAST-B1 and one or more of the other intracellular signal transduction pathways responsible for maintaining a robust pyloric rhythm in the absence of the descending modulatory inputs. In this case, the occlusion of effects between modulators would be responsible for the dependence of

neuromodulator effect on network activity (Nusbaum *et al.* 2001; Nusbaum and Beenhakker 2002).

CbAST-B1 produces physiological actions on the pyloric rhythm at concentrations usually considered consistent with hormonal actions (Keller 1992; Weimann *et al.* 1997). Many peptides found in the POs are also found in modulatory descending projection neurons to the STG (Marder and Bucher 2001; Nusbaum and Beenhakker 2002). Our direct tissue MALDI FTMS analysis (Kutz *et al.* 2004) and LC ESI MS analysis revealed the presence of CbAST-B1 in both the STG and the CoG along with other previously identified and unknown putative neuropeptides (data not shown). These observations suggest the intriguing possibilities that CbAST-B1 could be co-localized in modulatory neurons with other neuropeptides and small molecule transmitters, and that its actions might be synergistic or coordinated with other neuromodulatory substances. Further investigations would be necessary to determine whether CbAST-B1 is indeed co-localized with other neurotransmitters in modulatory projection neurons.

Given that animals must be able to respond appropriately to changing environmental and internal demands, the nervous system must be both robust and yet still flexible. Functionally, it would appear that state-dependent modulation can provide both stability and flexibility for a nervous system. From the viewpoint of stability, it is known that the state-dependent effects for excitatory modulators can 'converge' on the same signal transduction cascades and/or their effectors (Swensen and Marder 2000). As a consequence, convergence can serve as an upper bound that constrains the pyloric rhythm from operating outside its target regime. In terms of flexibility, it is well-established that neuromodulators can reconfigure circuits so that the same ensemble of neurons can provide a variety of behaviorally relevant outputs (Marder and Thirumalai 2002). However, much less is known about how the same ensemble of neurons can be differentially modulated by the same neuromodulator in different network 'states.' Presumably, these different network 'states' represent the different behavioral and physiological states of an animal and that these different 'states' are functions of changing environmental and internal demands subject to both neural and endocrine control. In this context, state-dependent peptide action could be an important factor in mediating physiological processes, such as the interaction between neural and endocrine control, as has been reported in the molting behavior of locusts (Zilberstein *et al.* 2006).

Here, we show that CbAST-B1 exhibits state-dependent actions on the motor patterns of the pyloric rhythm and can act at hormonal concentrations under certain conditions. These features suggest that CbAST-B1 may belong to a growing class of peptides with state-dependent actions that help mediate neural and endocrine control of the STG. Although little is known about the functional role of the allatostatin type peptides in crustacean behavior, it would be

interesting to see if state-dependent peptides, such as CbAST-B1, mediate behaviors that require a proper sequence of motor pattern events such as those needed during molting. Behavioral studies in conjunction with more detailed mechanistic studies may provide a link between the biophysical basis of state-dependent neuromodulation and behavior.

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