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Multiple modulators act on the cardiac ganglion of the crab, *Cancer borealis*

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Summary

Neuromodulators can change the output of neural circuits. The crustacean cardiac ganglion (CG) drives the contractions of the heart. The CG is a direct target for neurohormones that are released from the pericardial organs and other neuroendocrine sites. In this study, we have characterized for the first time the physiological actions of the peptides red pigment concentrating hormone (RPCH), *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia) and allatostatin III type A (AST-3) on the isolated CG of the crab, *Cancer borealis*. RPCH and CabTRP Ia excited the CG while AST-3 strongly inhibited its motor output. We also studied the actions of other peptides and small molecule transmitters known to be present in *C. borealis*. Dopamine, serotonin, proctolin, crustacean cardioactive peptide (CCAP), a number of extended FLRFamide peptides, and cholinergic agonists increased the activity of the CG, GABA inhibited the CG, while other substances had little or no significant effect on the CG motor pattern. These results demonstrate, in one species, that the CG is multiply modulated. We suggest that multiple modulators may be important to regulate and coordinate the activity of the heart and other organs in response to external stimuli or the endogenous physiological state.

Key words: crustacean, pericardial organ, CabTRP Ia, red pigment concentrating hormone, allatostatin, crustacean cardioactive peptide.

Introduction

Amines, peptides and other molecules play an important role in modulating motor and sensory information of neuronal networks in all animals (Billimoria et al., 2006; Hurley et al., 2004; Marder and Thirumalai, 2002; Matheson, 1997; Nusbaum et al., 2001). Neuromodulatory substances can alter the properties of individual neurons and synapses, and consequently alter the output of neuronal circuits (Harris-Warrick and Marder, 1991; Marder and Bucher, 2007). Neuromodulators can be delivered locally to specific neurons or groups of neurons from terminals within ganglionic neuropils (Nusbaum and Beenhakker, 2002; Nusbaum et al., 2001) or can be delivered systemically as hormones that can reach multiple sites within the animal. In this latter case, it is thought that neuromodulators can globally alter multiple sites within the animal to coordinate behaviors in response to the endogenous state of the animal or environmental stimuli (Dircksen, 1998; Kravitz, 1988).

The cardiac ganglion (CG) of decapod crustaceans, which drives the contractions of the heart, has been used to study the cellular mechanisms and synaptic physiology that underlie the generation and modulation of rhythmic motor patterns (Tazaki and Cooke, 1979a; Tazaki and Cooke, 1979b; Tazaki and Cooke, 1986). It is known that the CG is modulated by substances released locally from regulatory nerve fibers and by hormones released from endocrine sites such as the pericardial organs (POs) (Alexandrowicz and Carlisle, 1953; Christie et al., 1995; Cooke, 2002; Cooke and Hartline, 1975; Li et al., 2003; Li et al., 2002; Pulver and Marder, 2002; Skiebe, 2001). The isolated CG is sensitive to biogenic amines (Benson, 1984; Berlind, 2001; Cooke and Hartline, 1975; Fort et al., 2004; Hashemzadeh-Gargari and Freschi, 1992b; Miller et al., 1984; Saver et al., 1999), GABA (Kerrison and Freschi, 1992), glutamate (Hashemzadeh-Gargari and Freschi, 1992a), cholinergic agonists (Freschi, 1991; Freschi and Livengood, 1989; Sullivan and Miller, 1990), proctolin (Freschi, 1989; Miller and Sullivan, 1981; Saver et al., 1999; Sullivan and Miller, 1984), crustacean cardioactive peptide (CCAP) (Saver et al., 1999), FMRFamide-like peptides (FLPs) (Cruz-Bermúdez et al., 2006; Saver et al., 1999) and nitric oxide (Mahadevan et al., 2004).

Although the aforementioned studies (and others) have provided insights into the mechanisms underlying the modulation of the CG, these experiments were performed with variable experimental conditions such as saline composition or temperature. Such manipulations make it difficult to compare the actions of different substances on CG motor output. For example, recent studies using the isolated heart preparation have shown that cardiac performance in the lobster, *Homarus americanus*, is temperature-dependent (Camacho et al., 2006; Worden et al., 2006). Secondly, studies on the isolated CG have been done using many different species, which poses the question of whether the effect of a single modulator in one

particular species can be generalized to related species. Indeed, there are suggestions that the actions of some modulators may be species specific (Saver and Wilkens, 1998). For instance, octopamine has been reported to decrease the CG burst frequency of the crab, *Portunus sanguinolentus* (Benson, 1984). On the other hand, octopamine was shown to increase the burst frequency and depolarize the CG neurons of the crab, *Limulus polyphemus* (Augustine and Fetterer, 1985). When attempting to reconcile contrasting findings such as these one needs to determine whether apparent discrepancies reported from different studies are a product of different experimental conditions, or reflect true species-specific actions.

To determine how the CG in one species responds to many of the multiple modulators that are known to be present in key neurosecretory structures like the POs (DeKeyser et al., 2007), we decided to measure their physiological actions on the CG of a single species (the crab, *Cancer borealis*) under constant experimental conditions.

In this study we have characterized for the first time in any species the actions of three peptides on the cardiac ganglion. The peptide RPCH (pQLNFSPGW-NH_2) is present in three distinctive neuroendocrine sites in crustaceans (including *C. borealis*): the pericardial organs, sinus gland and eyestalks (Christie et al., 1995; Fu et al., 2005a; Li et al., 2003; Pulver and Marder, 2002; Stemmler et al., 2006). RPCH is also found within the terminals of neurons projecting from anterior ganglia to the stomatogastric ganglion (STG) (Christie et al., 1997a; Nusbaum and Marder, 1988; Thirumalai and Marder, 2002) and it is considered an endogenous modulator of the stomatogastric nervous system (STNS) (Dickinson et al., 1993; Dickinson et al., 2001; Dickinson and Marder, 1989; Dickinson et al., 1990; Nusbaum and Marder, 1988).

The neuropeptide CabTRP Ia (APSGFLGMR-NH_2) was originally isolated from the crab, *C. borealis*, and it is considered to be a member of the invertebrate tachykinin-related peptide family (Christie et al., 1997b). CabTRP Ia has also been identified immunocytochemically in the POs of the embryonic lobster, *H. americanus* (Pulver and Marder, 2002), and with mass spectrometry in the shrimp, *Penaeus vannamei* (Nieto et al., 1998), the crayfish *Procambarus clarkii* and lobster, *Panulirus interruptus* (Yasuda-Kamatani and Yasuda, 2004) and in the anterior commissural organ (ACO) of the crab, *Cancer productus* (Messinger et al., 2005). Physiological studies have shown that CabTRP Ia is a potent modulator of the STG motor output. CabTRP Ia is synaptically released from a pair of modulatory projection neurons into the neuropil of the STG, where it increases the pyloric rhythm frequency and activates the gastric mill motor pattern (Christie et al., 1997b; Thirumalai and Marder, 2002; Wood et al., 2000).

The allatostatins (ASTs) consist of a family of peptides present in various insects including cockroaches (Ding et al., 1995; Vilaplana et al., 1999), moths (Audsley and Weaver, 2003; Kramer et al., 1991), locusts (Skiebe et al., 2006; Veelaert et al., 1996a; Veelaert et al., 1996b), mosquitoes (Hernandez-Martinez et al., 2005) and flies (Duve et al., 1993; Lenz et al., 2001; Williamson et al., 2001). In crustaceans, including *C. borealis*, AST-like immunoreactivity has been detected in the POs and other structures (Christie et al., 1995; Kilman et al., 1999; Pulver and Marder, 2002; Skiebe, 1999; Skiebe, 2001; Skiebe and Schneider, 1994; Yasuda-Kamatani and Yasuda, 2006). AST-3 (GGSLYSFGL-NH_2) is an effective inhibitor of the STG pyloric rhythm (Skiebe and Schneider, 1994). AST-3 also decreases the amplitude of foregut muscle contractions (Jorge-Rivera and Marder, 1997) and modulates sensory information in the STNS (Billimoria et al., 2006; Birmingham et al., 2003).

In addition to RPCH, CabTRP Ia, and AST-3, we studied the action of other neuropeptides and small molecule transmitters and their agonists, whose actions have been described before in cardiac ganglia of other species. This study provides the most comprehensive examination to date of the effects of many of the neuromodulators present in crustaceans directly on the cardiac ganglion. Preliminary results have been previously presented in abstract format (Cruz-Bermúdez and Marder, 2006).

Materials and methods

Animals and dissection

Jonah crabs *Cancer borealis* Stimpson were purchased from Commercial Lobster (Boston, MA, USA) and maintained in artificial seawater tanks at 10–12°C. Before dissection, animals were cold-anesthetized by packing them in ice for 15 min. All dissections were carried out in chilled physiological saline (composition in mmol l^{-1}): NaCl, 440; KCl, 13; $MgCl_2$, 26; $CaCl_2$, 13; Trizma base, 11; maleic acid, 5; pH 7.45). Heart and CG dissections were performed as previously described (Cruz-Bermúdez et al., 2006). Briefly, after removing the stomach and the hepatopancreas, a small piece of carapace with heart attached was separated from the animal. The muscles around the heart were excised and the heart was subsequently removed and pinned ventral side up in a Sylgard™-coated (Dow Corning, Midland, MI, USA) dish. A V-shaped cut was made from the sternal artery on the ventral wall of the heart towards each of the ventral ostia to expose the CG, which sits directly on the dorsal inner wall of the heart. The ganglion was isolated from the muscle by transecting each of the nerve roots at the most distal point at which the root penetrates the muscle. In some preparations, a small non-contracting piece of muscle was left attached to the posterior end of the main trunk. The isolated ganglion was then pinned dorsal side up in a Sylgard™-coated 25 ml dish (60×15 mm) containing chilled (10–12°C) saline.

Solutions

The modulators used in this study were: allatostatin III type A (AST-3), crustacean cardioactive peptide (CCAP) (Bachem, Torrance, CA, USA); *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia; courtesy of Dr M. P. Nusbaum, University of Pennsylvania School of Medicine, Philadelphia, PA, USA); dopamine, γ-aminobutyric acid (GABA), histamine, nicotine, octopamine, pilocarpine, proctolin, serotonin (Sigma, St Louis, MO, USA); red pigment concentrating hormone (RPCH), SDRNFLRFamide, TNRNFLRFamide (American Peptide Company, Sunnyvale, CA, USA); *Cancer borealis* allatostatin type B (CbAST-B1), orcomyotropin-related peptide (OMTR) and NRNFLRFamide (courtesy of Dr Lingjun Li, University of Wisconsin School of Pharmacy, Madison, WI, USA). Peptides were dissolved in distilled water at 10^{-2} or 10^{-3} mol l^{-1}, stored at –20°C, and diluted in *C. borealis* saline

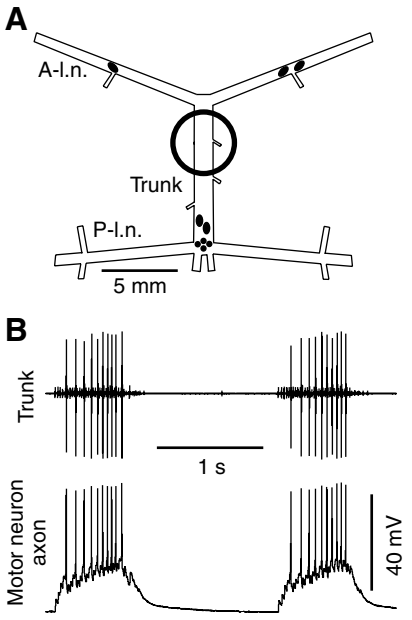

Fig. 1. Anatomy and bursting activity of the *Cancer borealis* cardiac ganglion (CG). (A) Schematic of the CG preparation. Large ovals, motor neurons; small ovals, pacemaker cells; A-l.n., anterolateral nerve; P-l.n., posterolateral nerve. The black ring indicates the petroleum jelly well used to record the activity of the CG. (B) Simultaneous recordings of the CG bursting pattern from the trunk (top trace) and from a motor neuron axon (bottom trace).

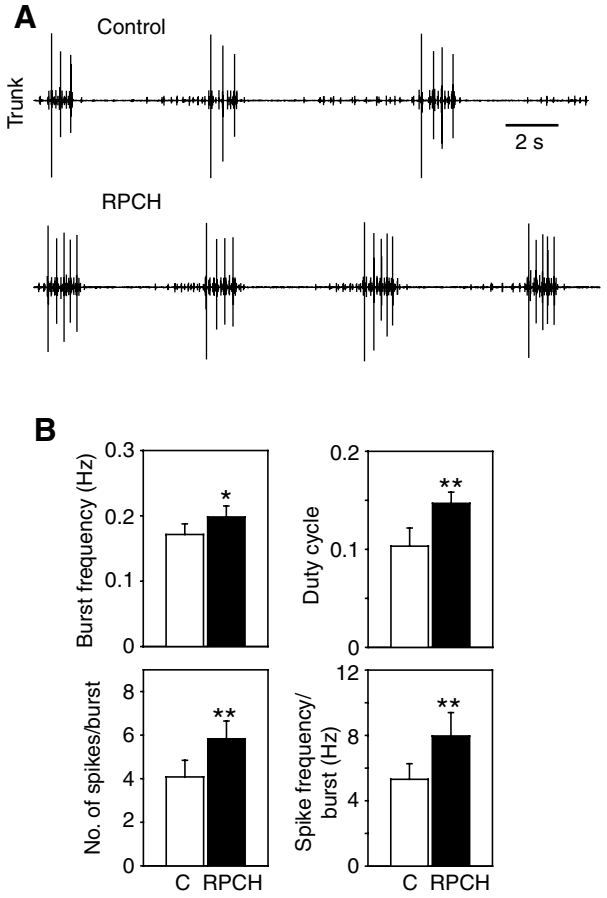

Fig. 2. Effects of red pigment concentrating hormone (RPCH) on the isolated cardiac ganglion (CG). (A) Extracellular recording from the trunk during control (top trace) and in the presence of 10^{-6} mol l^{-1} RPCH (bottom trace). RPCH increased the burst frequency as well as the number of motor neuron spikes (large units). (B) Pooled data bar graphs showing significant increases in burst frequency, duty cycle, number of spikes per bursts and spike frequency in the burst induced by RPCH over control (C) values (Student's *t*-test; N=12; *P<0.05; **P<0.01).

at the desired concentrations immediately before each application.

Data acquisition and analysis

Petroleum jelly wells were built around the nerves to monitor electrical activity using stainless steel extracellular pin electrodes. During recordings, preparations were continuously superfused with chilled physiological saline (12°C) by means of a Peltier cooling system and the temperature was monitored using a thermoelectric probe in the bath. Drugs were applied using a switching port on the inflow of the saline. In most of the experiments, we applied 2–6 modulators in randomized order with 40–60 min washout between each application to allow the preparation to return to baseline activity. Intra-axonal recordings were done using 20–40 MΩ glass microelectrodes filled with 0.6 mol l^{-1} K_2SO_4 and 20 mmol l^{-1} KCl and an Axoclamp 2A (Axon Instruments, Foster City, CA, USA). Signals were amplified and filtered using an A-M Systems 1700 Differential AC amplifier (Carlsborg, WA, USA). Data were recorded to a computer hard drive using a Digidata 1322A data acquisition board and pClamp 8 software (Axon Instruments). Data files were analyzed in Spike 2 (version 5; Cambridge Electronic Design, Cambridge, UK). Statistical tests and graphs were performed in SigmaPlot (version 8), SigmaStat (version 3.5; Systat Software Inc., Richmond, CA, USA) and StatView (version 5; SAS Institute, Cary, NC, USA). Figures were made in Canvas (version 10; ACD Systems of America, Inc., Miami, FL, USA). Time stretches of several minutes in which the range of values did not change visibly were assumed to represent the steady state and used to determine the means. We performed paired Student's *t*-tests for statistical significance, indicated in bar plots by asterisks (*P<0.05; **P<0.01), one-way ANOVA or, alternatively Kruskal–Wallis one-way ANOVA when normality tests failed and multiple pairwise comparisons of means (Dunn's Method). All histograms represent the mean ± s.e.m. unless stated otherwise.

Results

The crustacean CG contains nine neurons that rhythmically drive the contractions of the heart (Cooke, 2002). The heart muscle fibers are innervated by five motor neurons (Alexandrowicz, 1932) that make reciprocal chemical and electrical excitatory synapses with four pacemaker neurons that initiate the bursting activity of the whole network (Hartline, 1967; Hartline, 1979; Mayeri, 1973; Tazaki and Cooke, 1979a; Tazaki and Cooke, 1979b; Tazaki and Cooke, 1979c). The CG also receives excitatory and inhibitory inputs from the central

nervous system and the CG is a direct target for neuromodulators released from the POs (Alexandrowicz, 1932; Cooke, 2002). In *C. borealis*, three of the motor neurons are located in the antero-lateral nerves (A-l.n.), 2–4 mm away from the anterior bifurcation (Fig. 1A). The remaining two motor neurons and the pacemaker cells are located in the posterior bifurcation near the junction of the two postero-lateral nerves (P-l.n.). The ganglionic trunk is ~1 cm in length.

The CG generates bursts of motor neuron action potentials that synaptically depolarize the heart muscle fibers. Fig. 1B shows simultaneous extracellular and intracellular recordings of the CG motor pattern. The motor neuron (large units; top trace) and pacemaker neuron (small units; top trace) action potentials were recorded extracellularly from the ganglionic trunk. The intracellular recording from the motor neuron axon (Fig. 1B, bottom trace) was done by removing the connective tissue that wraps the neurons' processes at the anterior junction (Fig. 1A), and shows subthreshold information such as the EPSPs from the pacemaker neurons and the slow wave depolarization that generates the burst. Because the motor neuron spikes are the only impulses that directly elicit contractions, all measurements reported here are from the bursts of impulses generated by the motor neurons (large units in traces; Fig. 1B).

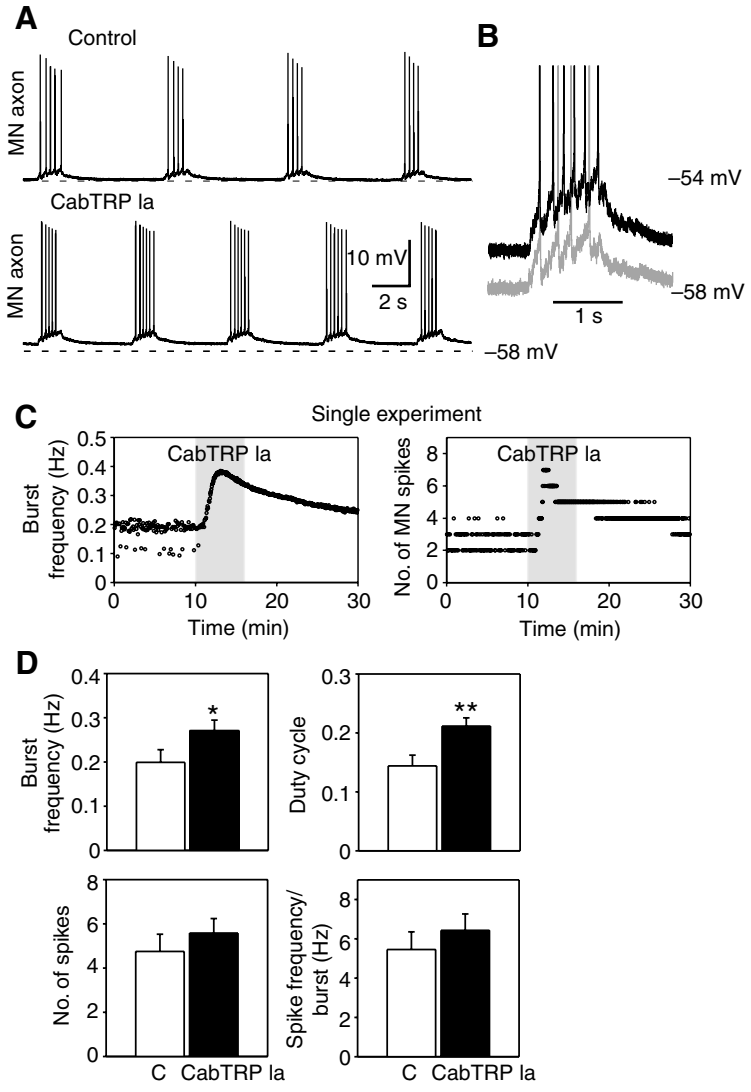

Fig. 3. Effects of *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia) on the isolated cardiac ganglion (CG). (A) Intra-axonal recording from a motor neuron during control (top trace) and in the presence of 10^{-6} mol l^{-1} CabTRP Ia (bottom trace). (B) Superimposed intra-axonal recordings from the same traces in A during control (gray) and after the application of CabTRP Ia (black). CabTRP Ia induced a ~2 mV depolarization. (C) Scatter plots of the instantaneous burst frequency (left plot) and number of spikes per burst (right plot) *versus* time during control, CabTRP Ia (gray bar) and during wash. (D) Bar plots of the pooled data comparing control (C) *versus* CabTRP Ia for the different parameters measured (Student's *t*-test; N=12; *P<0.05; **P<0.01).

Physiological actions of RPCH, CabTRP Ia and AST-3 on the cardiac ganglion

To the best of our knowledge, the effects of RPCH, CabTRP Ia and AST-3 on the CG of any species have not been previously reported. The effects of RPCH are shown in Fig. 2. Fig. 2A shows extracellular recordings in control saline (top trace) and in the presence of 10^{-6} mol l^{-1} RPCH (bottom trace). These recordings show both the motor neurons' (large amplitude spikes) and pacemaker cells' (small amplitude spikes) activity. In this preparation, RPCH increased the burst frequency and number of motor neuron spikes in the burst from ~3 to 5. On average (N=12), RPCH significantly changed all the CG output parameters (Fig. 2B; Table 1) including burst frequency (0.17±0.02 Hz in control and 0.20±0.02 Hz in RPCH; P<0.05); duty cycle (the burst duration divided by the cycle period) (0.10±0.02 in control and 0.15±0.01 in RPCH; P<0.01); number of spikes per burst (4±0.76 in control and 6±0.81 in RPCH; P<0.01); and spike frequency in the burst (5.31±0.96 Hz in control and 7.97±1.43 Hz in RPCH; P<0.01).

Fig. 3 shows the physiological actions of CabTRP Ia on the CG. The top trace of Fig. 3A shows a recording from a motor neuron. In control saline the frequency of the CG burst was 0.16 Hz with 4–5 spikes/burst (Fig. 3A; top trace). In this preparation, CabTRP Ia increased the burst frequency and number of spikes per burst (Fig. 3A; bottom trace) and depolarized the CG motor neurons (Fig. 3B). Fig. 3C shows plots of the burst frequency (left) and number of spikes per burst (right) against time for another CG in control, CabTRP Ia perfusion (gray bar) and wash. On average (N=12), 10^{-6} mol l^{-1} CabTRP Ia significantly increased the CG burst frequency and duty cycle (Fig. 3D; Table 1). Although CabTRP Ia increased the number of spikes/burst and spike frequency in some preparations, the pooled data across all preparations failed to reach significance (Fig. 3D; Table 1).

Fig. 4A shows intra-axonal representative recordings of the inhibitory effect of 10^{-6} mol l^{-1}AST-3 on the CG motor pattern. In this preparation, AST-3 completely abolished the bursting pattern without changing the baseline membrane potential. Note the few dispersed EPSPs coming from the pacemaker cells during AST-3 bath-application (Fig. 4A, middle trace). In three out of six preparations all motor neuron activity was reversibly

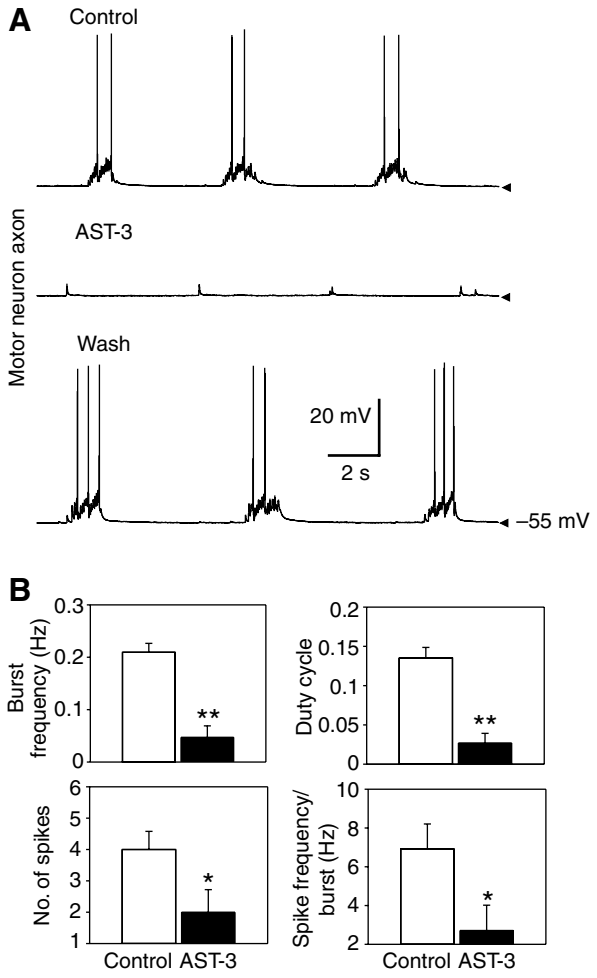

Fig. 4. Inhibitory effect of allatostatin III type A (AST-3) on the isolated cardiac ganglion (CG). (A) Intra-axonal recording from a motor neuron during control (top trace), in the presence of 10^{-6} mol l^{-1} AST-3 (middle trace) and during wash (bottom trace). (B) Pooled data bar graphs showing statistically significant changes on the CG bursting activity with AST-3 (Student's *t*-test; *N*=6; **P*<0.05; ***P*<0.01).

inhibited in AST-3, while in the remaining preparations the motor neuron activity was very substantially reduced. Fig. 4B and Table 1 show pooled data documenting the inhibitory actions of AST-3 on the isolated CG (*N*=6): burst frequency (0.21±0.02 Hz in control and 0.05±0.02 Hz in AST-3; *P*<0.05); duty cycle (0.14±0.01 in control and 0.03±0.01 in AST-3; *P*<0.01); number of spikes per bursts (4±0.58 in control and 2±0.72 in AST-3; *P*<0.05); and spike frequency in the burst (6.92±1.28 Hz in control and 2.71±1.30; *P*<0.05).

Effects of the proctolin, CCAP and FMRF-like peptides (FLPs)

Proctolin, CCAP, and a variety of FLPs have been studied on the CGs and hearts of a variety of species, where they are generally excitatory (Fort et al., 2007; Saver and Wilkens, 1998).

Fig. 5 shows intra-axonal recordings from a motor neuron of a single CG to which we applied proctolin and CCAP. Fig. 5A shows the recording in control (top trace) and in the presence

Table 1. *Summary of the modulators applied to the Cancer borealis cardiac ganglion*

Modulator (mol l^{-1})	*N*	Control frequency (Hz)	Duty cycle	No. spikes	Spike frequency/ burst (Hz)	Modulator frequency (Hz)	Duty cycle	No. spikes	Spike frequency/ burst (Hz)
AST-3 (10^{-6})	6	0.21±0.02	0.14±0.01	4±0.58	6.92±1.28	0.05±0.02*	0.03±0.01**	2±0.72*	2.71±1.30*
CabTRP Ia (10^{-6})	12	0.20±0.02	0.14±0.02	5±0.78	5.46±0.90	0.27±0.02*	0.21±0.01**	6±0.66	6.43±0.84
CbAST-B1 (10^{-6})	7	0.23±0.02	0.12±0.03	5±1.13	6.48±1.45	0.24±0.03	0.12±0.03	4±1.02	7.38±2.09
CCAP (10^{-6})	8	0.21±0.02	0.12±0.02	4±0.61	5.77±1.54	0.28±0.02**	0.23±0.02**	7±0.73**	8.52±1.41*
Dopamine (10^{-5})	9	0.19±0.02	0.12±0.03	4±1.20	4.05±0.84	0.23±0.02*	0.19±0.02*	8±1.85*	7.90±1.94*
GABA (10^{-3})	8	0.20±0.02	0.15±0.02	4±0.86	5.61±0.63	0.05±0.03**	0.04±0.03	1±0.84*	1.25±0.82*
Histamine (10^{-4})	6	0.21±0.03	0.13±0.03	4±0.65	6.24±1.14	0.20±0.03	0.14±0.03	4±0.80	5.90±1.16
Nicotine (10^{-5})	8	0.20±0.02	0.13±0.02	5±0.64	5.95±0.66	0.28±0.02*	0.18±0.01*	9±1.38*	11.93±2.74*
NRNFLRFa (10^{-6})	6	0.18±0.04	0.10±0.02	3±0.60	5.48±0.98	0.26±0.04**	0.20±0.02**	7±0.54**	9.02±1.72**
Octopamine (10^{-4})	8	0.18±0.02	0.11±0.03	4±0.85	5.23±1.30	0.17±0.03	0.12±0.02	5±0.78	5.99±1.26
OMTR (10^{-6})	6	0.19±0.02	0.13±0.02	4±0.65	6.72±1.78	0.19±0.03	0.13±0.02	5±0.61	7.13±1.94
Pilocarpine (10^{-5})	8	0.16±0.02	0.12±0.02	5±0.61	5.65±1.13	0.25±0.01**	0.18±0.02**	8±1.16*	10.90±2.03*
Proctolin (10^{-6})	8	0.18±0.02	0.13±0.02	4±0.72	3.97±0.62	0.25±0.02*	0.23±0.02**	8±0.99**	7.28±1.17*
RPCH (10^{-6})	12	0.17±0.02	0.10±0.02	4±0.76	5.31±0.96	0.20±0.02*	0.15±0.01**	6±0.81**	7.97±1.43**
SDRNFLRFa (10^{-6})	9	0.24±0.02	0.14±0.02	5±0.63	6.54±1.02	0.30±0.03*	0.20±0.03	7±0.61**	9.78±1.05**
Serotonin (10^{-6})	7	0.17±0.02	0.09±0.02	4±0.72	4.68±1.09	0.33±0.02**	0.19±0.02*	6±0.81*	9.08±1.20**
TNRNFLRFa (10^{-6})	7	0.22±0.03	0.15±0.03	4±0.65	4.90±0.92	0.30±0.02	0.19±0.03	7±1.25*	8.35±1.13*

All values are means ± s.e.m.; *N*=number of cardiac ganglia. Asterisks indicate values significantly different from control (**P*<0.05, ***P*<0.01).

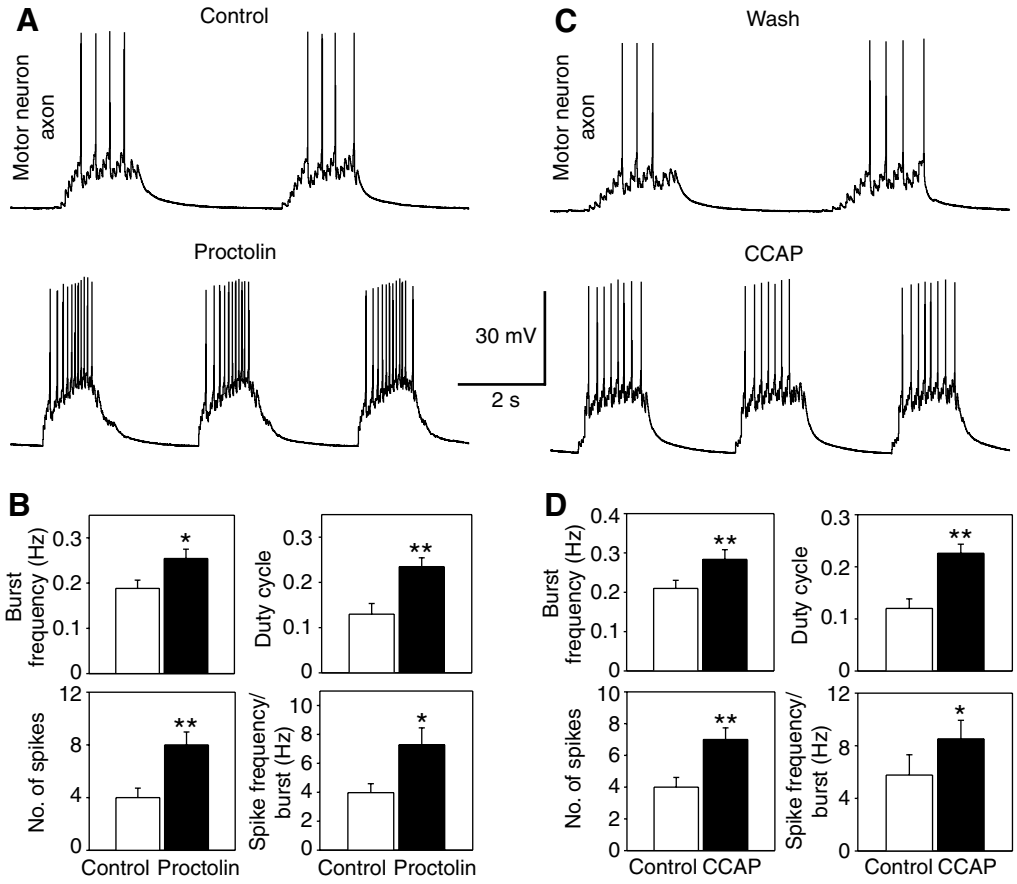

Fig. 5. Excitatory actions of proctolin and crustacean cardioactive peptide (CCAP) on the isolated cardiac ganglion (CG). (A) Intra-axonal recording from a motor neuron in control (top trace) and during 10^{-6} mol l^{-1} proctolin application (bottom trace). Note the increase in the slow wave form depolarization of the burst. (B) Pooled data showing significant effects of proctolin on burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; N=8; *P<0.05; **P<0.01). (C) Same preparation after proctolin was washed out (top trace) and in 10^{-6} mol l^{-1} CCAP (bottom trace). CCAP increased the slow wave form depolarization of the motor neurons. (D) Pooled data showing significant effects of CCAP on burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; N=8; *P<0.05; **P<0.01).

of 10^{-6} mol l^{-1} proctolin (bottom trace). In data pooled from multiple preparations (Fig. 5B; Table 1), proctolin significantly increased the burst frequency, duty cycle, number of motor neuron spikes and spike frequency in the burst. After proctolin washout from the preparation shown in Fig. 5A, CCAP (10^{-6} mol l^{-1}) was applied (Fig. 5C). Like proctolin, CCAP caused increases in the burst frequency, duty cycle, number of motor neuron spikes and spike frequency in the burst (Fig. 5D; Table 1). Both proctolin and CCAP increased the slow wave depolarization of the motor neurons in the *C. borealis* CG.

The FLP family members SDRNFLRFamide, TNRNFLRFamide and NRNFLRFamide are all found in the *C. borealis* POs (Christie et al., 1995; Li et al., 2003). We recently compared the effects of SDRNFLRFa, TNRNFLRFa and a new family member, GAHKNYLRFa, on the burst frequency of the *C. borealis* CG (Cruz-Bermúdez et al., 2006). In the present study, we have combined data from those experiments with other preparations (new data) to which we have applied FLPs and analyzed additional burst parameters. As expected, SDRNFLRFa, TNRNFLRFa and NRNFLRFa elicited excitatory effects on the isolated *C. borealis* CG (Table 1). On average, SDRNFLRFa and TNRNFLRFa increased the burst frequency to the same value, 0.30 Hz. The three related peptides elicited almost identical changes in other parameters such as duty cycle and number of spikes.

Effects of other newly identified peptides on the cardiac ganglion

We applied two peptides that have been recently identified in *C. borealis* to the CG: orcomyotopin-related peptide (OMTR) (Billimoria et al., 2005) and *Cancer borealis* allatostatin type B (CbAST-B1) (Fu et al., 2007). At 10^{-6} mol l^{-1}, neither OMTR nor CbAST-B1 significantly changed any parameter of the CG bursting pattern (Table 1).

Effects of amines on the cardiac ganglion

There are numerous early and recent studies of the effects of biogenic amines on the CG (Benson, 1984; Berlind, 1998; Fort et al., 2004). In Fig. 6, we show the effects of serotonin and dopamine on the CG motor pattern. Fig. 6A shows extracellular recordings in control (top trace) and during 10^{-6} mol l^{-1} serotonin perfusion (bottom trace). In data pooled from seven

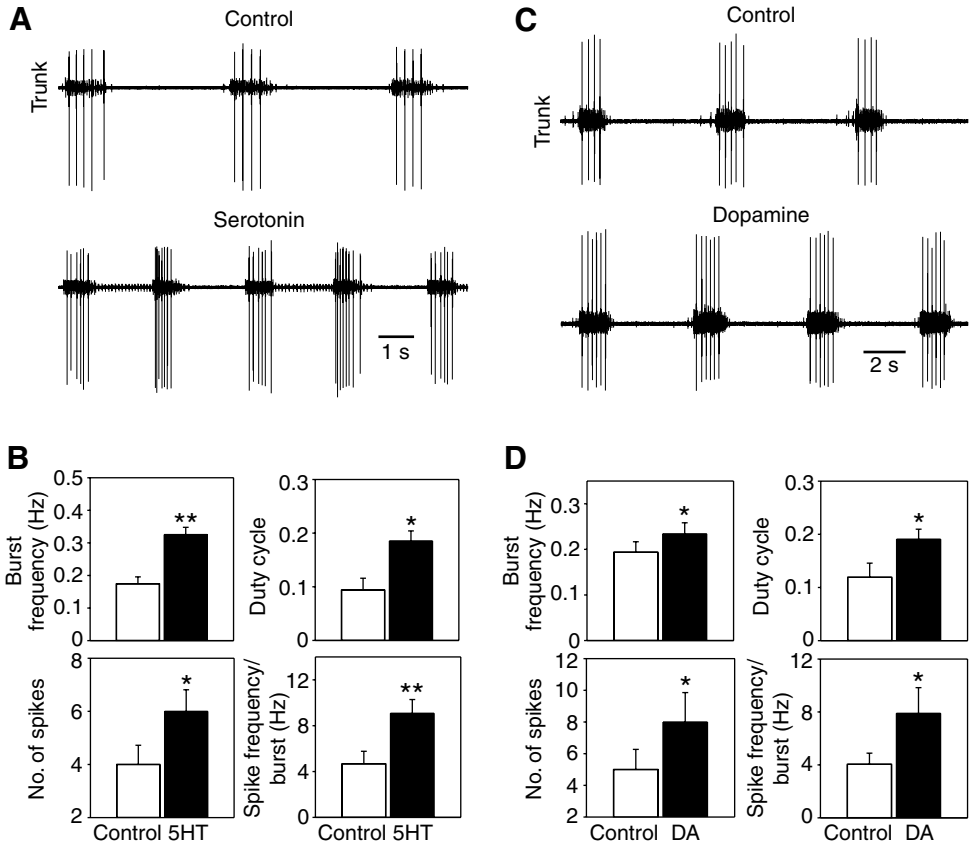

Fig. 6. Excitatory effects of biogenic amines on the isolated cardiac ganglion (CG). (A) Extracellular recordings from the trunk during control and in the presence of the 10^{-6} mol l^{-1} serotonin (5HT). (B) Serotonin significantly increased the burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; N=7; *P<0.05; **P<0.01). (C) Extracellular recordings from the trunk during control and in the presence of the 10^{-5} mol l^{-1} dopamine (DA). (D) Dopamine also significantly increased the burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; N=9; *P<0.05; **P<0.01).

experiments, serotonin significantly increased burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Fig. 6B; Table 1). Fig. 6C shows recordings from another CG in control (top trace) and in the presence of 10^{-5} mol l^{-1} dopamine (bottom trace). Like serotonin, dopamine significantly increased the burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Fig. 6D, Table 1). Similar results have been found in many other CG of different species (Berlind, 1998; Berlind, 2001; Cooke and Hartline, 1975; Fort et al., 2004; Miller et al., 1984; Saver et al., 1999).

In contrast to serotonin and dopamine, neither octopamine nor histamine (Table 1) induced statistically significant changes in any burst parameter measured.

Actions of cholinergic agonists and GABA on the cardiac ganglion

Acetylcholine (ACh) is thought to be the neurotransmitter that is released from one of the acceleratory fibers projecting from the CNS to the CG to increase cardiac activity (Cooke, 2002), and the sensitivity of the CG to ACh and muscarinic cholinergic agonists has been described in other species (Freschi, 1991; Freschi and Livengood, 1989; Sullivan and Miller, 1990). Fig. 7A shows extracellular recordings in control (top trace) and in the presence of 10^{-5} mol l^{-1} pilocarpine, the muscarinic ACh receptor agonist (bottom trace). Fig. 7C shows recordings from the same CG during wash (top trace) and during 10^{-5} mol l^{-1} nicotine perfusion (bottom trace). Both pilocarpine and nicotine increased the burst frequency and the number of motor neuron spikes in this preparation. On average, both cholinergic agonists elicited excitatory effects on all burst parameters measured (Fig. 7B,D; Table 1).

In contrast, GABA was strongly inhibitory on the *C. borealis* CG (Table 1). This effect is consistent with anatomical and physiological evidence in other species that identified GABA as the neurotransmitter released by the inhibitory fiber projection from the CNS to decrease the activity of the heart (Alexandrowicz, 1932; Cooke, 2002; Delgado et al., 2000).

Discussion

The crustacean stomatogastric ganglion is modulated by a very large number of substances, including many of the same substances studied here (Marder and Bucher, 2001; Marder and Bucher, 2007). Although it is likely that many other neuronal networks are equally richly modulated, there are relatively few systems in which the effects of multiple neuromodulators have been studied on the same neuronal circuit. Previous work on the intact heart has shown that each of five different substances

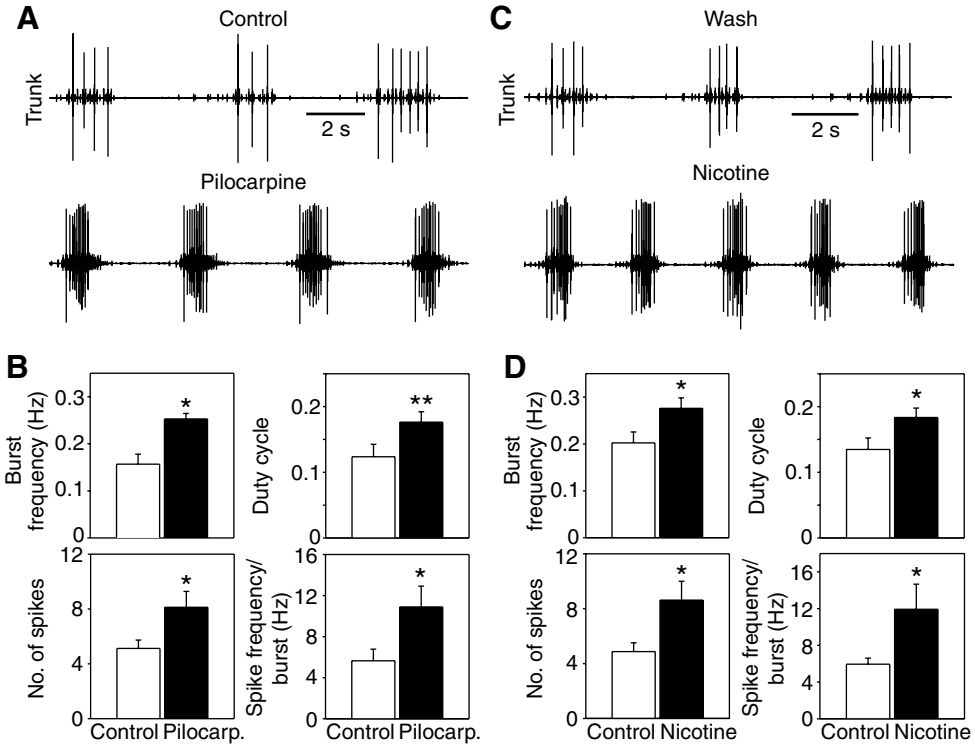

Fig. 7. Excitatory actions of cholinergic agonists on the isolated cardiac ganglion (CG). (A) Extracellular recording from the trunk in control (top trace) and during 10^{-5} mol l^{-1} pilocarpine perfusion (bottom trace). (B) Pilocarpine (Pilocarp.) significantly increased the burst frequency, duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; *N*=8; **P*<0.05; ***P*<0.01). (C) Same preparation shown in A during wash (top trace) and in the presence of 10^{-5} mol l^{-1} nicotine (bottom trace). (D) Nicotine significantly increased the duty cycle, number of spikes per burst and spike frequency in the burst (Student's *t*-test; *N*=8; **P*<0.05; ***P*<0.01).

alters the heartbeat in different ways (Saver and Wilkens, 1998). Given the fact that some of these effects are caused by actions of neuromodulators on heart muscle and neuromuscular junctions (Saver and Wilkens, 1998), and given the pivotal location and function of the crustacean CG, we thought it would be interesting to determine the extent to which it, like the stomatogastric ganglion, is also modulated by a large number of different substances. Fig. 8 summarizes the results of this study, and illustrates that the cardiac ganglion is a direct target of an array of neuromodulatory agents. This must be viewed as an incomplete list, because new mass spectrometry methods are rapidly leading to the identification of additional neuropeptides in neurosecretory structures (Fu et al., 2005a; Fu et al., 2005b; Fu and Li, 2005; Li et al., 2003; Stemmler et al., 2005), and many of these newly identified substances have yet to be studied physiologically.

The actions of RPCH, CabTRP Ia and AST-3 on the CG are described here for the first time in any species. AST-3 joins GABA as a substance with potent inhibitory actions. These results, together with the inhibitory actions of AST-3 described previously in the STG and stomach muscles (Jorge-Rivera and Marder, 1997; Skiebe and Schneider, 1994), make AST-3 a common inhibitory peptide for both the heart and stomach in crustaceans. Similarly, RPCH and CabTRP Ia are generally excitatory on both the CG and the stomatogastric nervous system.

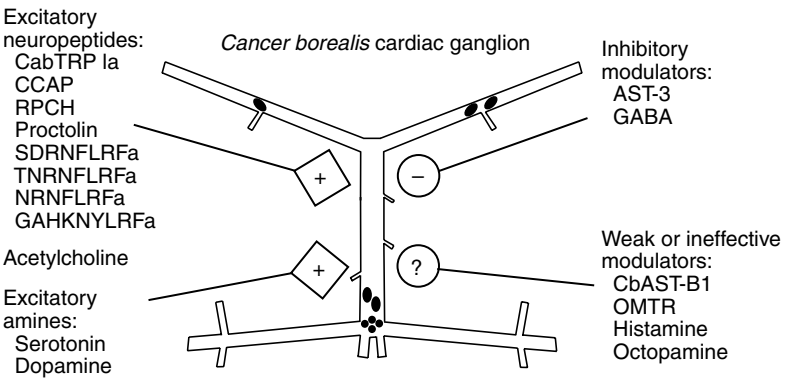

Fig. 8. Diagram of the *C. borealis* cardiac ganglion with neuromodulators present in the pericardial organs. The excitatory actions (+) of peptides, serotonin, dopamine and presumably acetylcholine on the cardiac ganglion [CG; GAHKNYLRFa data were studied in Cruz-Bermudez et al. (Cruz-Bermudez et al., 2006)]. The peptide AST-3 and GABA are both potent inhibitors (–) of the CG output. Other modulators such as histamine and octopamine did not induce statistically significant changes on the CG motor pattern and were classified as weak or ineffective modulators. Large ovals, motor neurons; small ovals, pacemaker cells.

The effects reported here for modulators that have been previously described in other species were, in many cases, consistent with previous studies. For example, in *C. sapidus* CCAP and dopamine increased duty cycle and the number of spikes/burst much as we found (Fort et al., 2004; Fort et al., 2007). In *H. americanus* histamine activates a chloride conductance in the motor neurons, but the burst frequency is unaffected (Hashemzadeh-Gargari and Freschi, 1992a). Therefore, it is possible that the apparent lack of histamine action on overall burst parameters in *C. borealis* may nonetheless be associated with the presence of receptors that can be revealed with more detailed biophysical analyses.

Comparisons with previous work on other species are complicated by the fact that modulators affect different features of the heart output at different concentrations. For example, in *C. sapidus* low concentrations of CCAP can influence contraction amplitude, while higher concentrations increase burst duration and the number of spikes/burst (Fort et al., 2007). This also makes it difficult to quantitatively compare the effects of multiple modulators on any of the burst parameters we measured in a meaningful manner. Although we used modulator concentrations that were suggested by previous work on crustacean systems to be saturating, or close to saturation, in the absence of complete dose–response curves for each substance it is difficult to determine unambiguously the extent to which modulators may differentially act to alter specific parameters of the cardiac ganglion output. Initially we had hoped to determine if some neuromodulators affected frequency more than burst duration or duty cycle, and *vice versa* (Table 1). Thus far, statistical comparisons across the modulators showed no significant differences on these parameters among those substances that excited the cardiac ganglion. Because many, if not all, of the modulators that act on the cardiac ganglion may also act on the cardiac muscle or neuromuscular junctions, it is very possible that modulators that have qualitatively similar actions when studied on the cardiac ganglion alone may influence cardiac output, and other aspects of heart performance differentially (Fort et al., 2004; Fort et al., 2007; Saver and Wilkens, 1998).

Unfortunately, the 'simple' cardiac ganglion has a complex anatomical structure that makes it difficult to determine easily whether neuromodulators act on the pacemaker neurons, the motor neurons, or both. Because the pacemaker cell axons run in the trunk of the ganglion in which the motor neurons are found, and because the motor neuron axons run through the region containing the somata of the pacemaker cells (Fig. 1), it is not possible to simply cut the two regions apart, nor to apply neuromodulators only to one class of neurons. Moreover, the extensive electrical coupling further complicates the isolation of neurons. These problems are acute in *C. borealis* because the pacemaker cells are closer to the most posterior motor neurons than is the case in other species. Consequently, we were unable to determine whether any or all of the modulators studied have receptors that are restricted to either the pacemaker or motor neurons.

Do neurohormones act alone?

In this study we assayed the action of multiple modulators by applying each one at a time followed by long washes to return to baseline. However, it is very likely that *in vivo* structures like the POs might release two, three or more of these modulators simultaneously. Such hormonal corelease suggests a few possible pharmacological scenarios. Some modulators might occlude each other's actions (Swensen and Marder, 2000), or synergistically enhance physiological responses elicited by their joint actions.

In the lobster *H. americanus*, CG, proctolin and cholinergic agonists activate a voltage-dependent Na^+ current (Freschi, 1989; Freschi and Livengood, 1989) that is probably the same voltage-dependent inward conductance activated by proctolin (Golowasch and Marder, 1992), pilocarpine, RPCH, CabTRP Ia, CCAP and TNRNFLRFamide (Swensen and Marder, 2000; Swensen and Marder, 2001) in STG neurons. Because of the structural dissimilarity of these agonists, it is unlikely that any of them except for the FLP family members activate the same receptor (Cruz-Bermúdez et al., 2006). Thus, convergence of their actions on the same membrane channel likely occurs at some point in the signal transduction pathway between receptor and channel. Consequently, in a network as 'simple' as the cardiac ganglion, it may not be surprising that many of the excitatory neuropeptides have similar actions on the output of the network. Of course, if the receptors for some neuromodulators are preferentially found on the motor neurons, and others on the pacemaker neurons, this would influence the extent to which the neuromodulator affected frequency or duty cycle, etc. of the motor neuron burst. If several neuromodulators are coreleased or are circulating at the same time, their responses will depend on their concentrations as well as on the state of the signal transduction pathways in their target neurons.

Do hormonal modulators coordinate the activity of multiple systems in the animal?

Most of the modulators studied here have strong physiological actions on the CG. Excitatory modulators could be hormonally released to increase activity not only of the heart, but also of other organs when the overall internal activity of the animal is low. For instance, substances released from the POs including dopamine and octopamine are involved in ionic regulation and branchial exchange in crabs (Morris, 2001). In the digestive system, peptides such as proctolin, CCAP, FLPs and RPCH have excitatory effects on the STG motor patterns, on identified synapses within the circuit and on many stomach muscles (Hooper and Marder, 1984; Jorge-Rivera and Marder, 1996; Jorge-Rivera et al., 1998; Nusbaum and Marder, 1988; Nusbaum and Marder, 1989a; Nusbaum and Marder, 1989b; Weimann et al., 1997). At the behavioral level, increased amounts of serotonin in the lobster, *H. americanus*' circulatory system are correlated with aggressive behaviors (Huber et al., 1997a; Huber et al., 1997b; Kravitz, 1988; Kravitz, 2000; Kravitz and Huber, 2003). Differences in circulating levels of serotonin, dopamine and octopamine between winners and losers after confrontation have been also reported in the crab, *C. maenas* (Sneddon et al., 2000).

The sensitivity of the CG to multiple substances may be a reliable mechanism to increase its cardiac activity and deliver neurohormones released from the POs and other structures in a particular physiological context. One might imagine that if it is important that a hormonally released substance quickly reach

distant tissues, it would be advantageous for that substance to act directly on the heart to enhance cardiac performance, and the delivery of the substance. This could explain why it is important for the heart to respond to so many of the neuromodulatory substances found in the animal.

List of abbreviations

ACh	acetylcholine
A-l.n.	anterolateral nerve
AST-3	allatostatin III type A
CabTRP Ia	*Cancer borealis* tachykinin-related peptide Ia
CbAST-B1	*Cancer borealis* allatostatin type B
CCAP	crustacean cardioactive peptide
CG	cardiac ganglion
CNS	central nervous system
FLPs	FMRF-like peptides
OMTR	orcomyotropin-related peptide
P-l.n.	posterolateral nerve
POs	pericardial organs
RPCH	red pigment concentrating hormone
STG	stomatogastric ganglion
STNS	stomatogastric nervous system

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