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RESEARCH ARTICLE

Inter-animal variability in the effects of C-type allatostatin on the cardiac neuromuscular system in the lobster *Homarus americanus*

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SUMMARY

Although the global effects of many modulators on pattern generators are relatively consistent among preparations, modulators can induce different alterations in different preparations. We examined the mechanisms that underlie such variability in the modulatory effects of the peptide C-type allatostatin (C-AST; pQIRYHQCYFNPISCF) on the cardiac neuromuscular system of the lobster *Homarus americanus*. Perfusion of C-AST through the semi-intact heart consistently decreased the frequency of ongoing contractions. However, the effect of C-AST on contraction amplitude varied between preparations, decreasing in some preparations and increasing in others. To investigate this variable effect, we examined the effects of C-AST both peripherally and centrally. When contractions of the myocardium were elicited by controlled stimuli, C-AST did not alter heart contraction at the periphery (myocardium or neuromuscular junction) in any hearts. However, when applied either to the semi-intact heart or to the cardiac ganglion (CG) isolated from hearts that responded to C-AST with increased contraction force, C-AST increased both motor neuron burst duration and the number of spikes per burst by about 25%. In contrast, CG output was increased only marginally in hearts that responded to C-AST with a decrease in contraction amplitude, suggesting that the decrease in amplitude in those preparations resulted from decreased peripheral facilitation. Our data suggest that the differential effects of a single peptide on the cardiac neuromuscular system are due solely to differential effects of the peptide on the pattern generator; the extent to which the peptide induces increased burst duration is crucial in determining its overall effect on the system.

Key words: allatostatin, crustacean, modulation, neuropeptide.

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INTRODUCTION

While it is well established that modulatory inputs, both neuronal and hormonal, can alter the outputs of central pattern generators (CPGs) to modulate rhythmic patterns of movement (Dickinson, 2006; Marder and Bucher, 2001), a number of fundamental questions remain unanswered. Among these are (1) the extent to which such modulation varies both within and among individuals, (2) the mechanisms that underlie such variability, and (3) the role played by modulation of different components of the larger pattern generator–effector system that is responsible for the generation of rhythmic movements.

Variability in responses of CPGs to both modulatory projection neurons and bath-applied neuromodulators is determined in part by the initial state of the network. For example, the anterior pyloric modulator neuron in the spiny lobster (*Jasus lalandii*) increases cycle frequency in networks that are initially slow, but has little effect on cycle frequency in rapidly cycling preparations (Nagy and Dickinson, 1983). Similarly, the modulatory proctolin neuron in the crab stomatogastric nervous system provokes much larger increases in frequency in slowly cycling preparations than in those that are already highly active (Nusbaum and Marder, 1989a). A similar trend was noted in studies of an inhibitory neuropeptide, B-type allatostatin, which decreases activity of the crab pyloric CPG more effectively in preparations that are already cycling slowly (Fu et al., 2007). Interestingly, the functional consequences of this type of state-dependent modulation are that the output of the network tends to converge on a single pattern when modulatory input excites the network, but to diverge when the modulation inhibits a network. Mechanistically, it has been proposed that the larger effect of modulators when network outputs are slower is a simple consequence of the higher average membrane resistance in the neurons of the less active networks (Fu et al., 2007; Thirumalai et al., 2006).

Recent work has demonstrated an additional source of variability that might underlie some instances of state-dependent modulation. Neuronal modeling studies initially suggested that the same pattern of activity in a single neuron could be achieved with many different ion channel numbers and distributions (Prinz et al., 2004). Experimental studies in the crustacean stomatogastric system have confirmed this hypothesis. The densities of specific ionic currents in identified neurons varied widely between individual preparations, even though both neuronal and network patterns of firing were nearly identical (Marder and Goaillard, 2006; Schulz et al., 2006). These differences could be responsible for variation in the responses of individual neurons and entire networks to modulators, as has been suggested by modeling and experimental studies with the *Cancer borealis* stomatogastric nervous system (Goldman et al., 2001).

While state dependence based on network activity level accounts for some of the variability in network responses to modulators, more

complex mechanisms exist in parallel. In the spiny lobster stomatogastric system, for example, the modulatory effects of one neuropeptide (proctolin) can be dramatically altered by interactions with a second peptide (red pigment concentrating hormone) (Dickinson et al., 1997). Similarly, the modulatory effects of octopamine in the moth *Manduca sexta* are altered by exposure to 20-hydroxyecdysone, a phenomenon described as metamodulation (Mesce, 2002). Metamodulation of the crayfish escape response by serotonin likewise results in different animals responding differently to the same neurotransmitter (Edwards et al., 2002). A parallel phenomenon has long been observed in the functioning of hormonal systems in the mammalian reproductive system, where one hormone (e.g. follicle-stimulating hormone) induces the synthesis and insertion of receptors for another (luteinizing hormone) through the cAMP cascade (Chen et al., 1977; Erickson et al., 1979).

Behavioral output and variability in the output of any rhythmic motor system will be determined not only by the effects of the modulator on the pattern generator itself but also by the interactions of the CPG with the muscles, *via* the neuromuscular transform (Brezina et al., 2000a; Brezina et al., 2000b; Brezina and Weiss, 2000), by resultant feedback to the CPG, and by any modulation of these sites that occurs simultaneously, as would be expected for hormonally delivered modulators. All of these components of the pattern generator–effector system are accessible to study in the crustacean cardiac neuromuscular system. The heart of the American lobster, *Homarus americanus*, is controlled by a simple pattern generator, consisting of nine neurons that make up the cardiac ganglion (CG); five of these are motor neurons that synapse onto and cause contractions of the cardiac muscle (Cooke, 2002). Additionally, two feedback systems play an integral role in determining the overall motor output of the system. First, the cardiac muscle contains high levels of nitric oxide (NO) synthase; NO generated in the muscles inhibits the pattern generator, causing a decrease in cycle frequency, and a consequent decrease in amplitude that results from the lower level of facilitation (Mahadevan et al., 2004). Second, stretch-sensitive dendrites on the neurons of the CG are thought to play a generally excitatory role (Alexandrowicz, 1932; Garcia-Crescioni et al., 2010; Sakurai and Wilkens, 2003). While there is considerable evidence indicating that crustacean neuromuscular junction (Kreissl et al., 1999; Stevens et al., 2009) and muscle, including cardiac muscle (Dickinson, 1995; Kravitz et al., 1984; Kreissl et al., 1999; Stevens et al., 2009), are modulated, it is not yet clear whether the feedback pathways themselves are likewise subject to modulation.

Here, we examined the modulatory effects of a recently identified neuropeptide, crustacean C-type allatostatin (C-AST, pQIRYHQ<u>C</u>YFNPIS<u>C</u>F, where the underlines indicate a disulfide bond) (Stemmler et al., 2010). Despite the peptide's identification and molecular characterization in decapod crustaceans, little is known about its physiological effects. Although it consistently caused decreases in heartbeat frequency, its effects on contraction amplitude were highly variable between preparations. We thus asked where the source of this variability lies and what changes in the neuromuscular system might be responsible for not only the consistent effects but also the variable effects of the peptide.

MATERIALS AND METHODS

Animals

American lobsters, *H. americanus* Milne-Edwards, were obtained from local fish markets (Brunswick and Harpswell, ME, USA). Lobsters of both sexes, weighing between 250 and 350 g, were used in these experiments; neither the size nor the sex of the animal correlated with the specific response to the peptide. All animals were kept in tanks filled with aerated, recirculating natural seawater (salinity 30–32 p.p.t.), maintained at 10–12°C. Animals were kept for up to 3 weeks before use; those kept longer than 1 week were fed weekly. Prior to dissections, the animals were chilled by packing them in ice for 20–30 min. The heart was removed from the lobster by dissecting out the posterior dorsal region of the lobster's thoracic carapace, leaving the intact heart attached to the carapace. All dissections and experiments were carried out in chilled (8–12°C) physiological saline (in mmol l^{-1}: NaCl 479.12, KCl 12.74, $CaCl_2$ 13.67, $MgSO_4$ 20.00, Na_2SO_4 3.91, Trizma base 11.45 and maleic acid 4.82; pH 7.45).

Chemicals

All chemicals used in making saline were ACS grade or better and were obtained from Sigma-Aldrich (St Louis, MO, USA). C-AST (pQIRYHQ<u>C</u>YFNPIS<u>C</u>F) was custom synthesized by GenScript USA (Piscataway, NJ, USA) and dissolved in deionized water to make a 10^{-3} mol l^{-1} stock solution, which was stored at –25°C in small aliquots, thus minimizing possible peptide degradation due to repeated freezing and thawing. C-AST peptide was diluted in saline to 10^{-7} mol l^{-1}, the concentration at which it significantly and consistently affects heart contraction parameters (P.S.D., unpublished data), just before use.

Physiological recordings

To examine the effects of C-AST on the lobster cardiac neuromuscular system, we applied C-AST to a number of different heart preparations *in vitro*, enabling us to distinguish the effects of the peptide on different sites within the system. All preparations were allowed to stabilize for at least 1 h before C-AST application. The peptide was applied to the preparation for 8–12 min at a flow rate of 5 ml min^{-1}. Because the cardiac system exhibits its maximum output at 10°C (Worden et al., 2006), the temperature was maintained at 9–11°C using an in-line Peltier temperature regulator (CL-100 bipolar temperature controller and SC-20 solution heater/cooler, Warner Instruments, Hamden, CT, USA). After each peptide application, the preparation was washed with fresh saline for 45–60 min, allowing contractions to return to baseline before additional peptide applications at different concentrations. If cardiac activity did not return to approximately baseline, data were not included in the analysis. Responses to repeated peptide application were qualitatively similar in each heart. Although responses varied quantitatively as a function of peptide concentration, they did not vary solely as a function of time. Some hearts did show fatigue over time, with gradually decreasing contraction amplitudes; these data were discarded from the analysis, so that only those hearts in which stable baselines were maintained were used.

Semi-intact heart: heart contraction and neural activity

The thoracic carapace with the heart attached was pinned to the bottom of a small Sylgard 184 (KR Anderson, Santa Clara, CA, USA)-lined dish, with the ventral surface of the heart facing up. The posterior artery was cannulated with polyethylene tubing, providing a constant flow at 2.5 ml min^{-1} of physiological saline to maintain appropriate pressure inside the lobster heart. To help maintain temperature, another inflow tube, also with a flow rate of 2.5 ml min^{-1} was placed above the cannulated artery.

To record heart contractions, the anterior arteries were tied off with 6/0 surgical suture silk (Teleflex, Coventry, CT, USA) and attached to a Grass FT03 force–displacement transducer (Astro-Med, West Warwick, RI, USA). The cannulated artery was stretched in

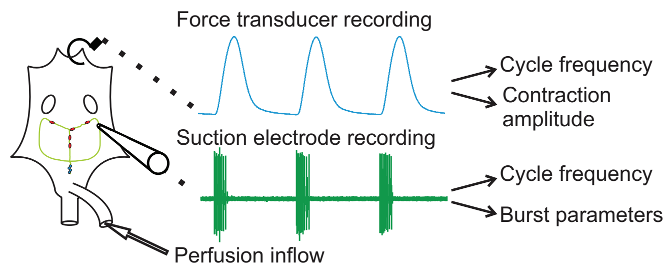

Fig. 1. Diagrammatic illustration of the electrophysiological recording configuration used in these experiments. The posterior artery was cannulated and the preparation was perfused with saline or C-type allatostatin (C-AST). A suction electrode on the anterior motor nerve was used to record neuronal activity extracellularly, allowing us to measure burst parameters (duration, spike frequency, number of spikes per burst) as well as cycle frequency. A force transducer tied to the anterior arteries a with suture silk (shown as a hook in the diagram) was used to record the frequency and amplitude of heart contractions, as well as to stretch the heart to a baseline force of 2 ***g***.

the posterior direction; the force transducer was placed at an angle of 30 deg from the horizontal axis and stretched until the heart exhibited a baseline tension of 2 ***g*** in order to mimic the minimum stretch observed *in vivo* (Cooke, 2002). The output from the transducer was amplified with an ETH-250 Bridge/Bio amplifier (CB Sciences, Dover, NH, USA) and a Brownlee 410 instrumentation amplifier (Brownlee Precision, San Jose, CA, USA). The amplified signal was then recorded onto a computer using a Micro 1401 Plus data acquisition board and Spike2 version 6.09 software (Cambridge Electronic Design, Cambridge, UK).

The neural signal from the CG was recorded simultaneously with heart contraction force using a suction electrode, as illustrated diagrammatically in Fig. 1. A small slit was cut along the ventral midline of the heart to provide an entrance into the heart for the electrode. This incision caused a small decrease (<15%) in contraction amplitude, but did not affect the baseline tension of 2 ***g***. The electrical signal from the CG was recorded extracellularly with an A-M systems Model 1700AC amplifier (A-M Systems, Inc., Carlsborg, WA, USA) and a Brownlee 410 instrumentation amplifier. The signals were further processed by the Micro 1404 Plus data acquisition board and Spike2 version 6.09 software. Under these conditions, we were able to record stable cardiac activity for at least 8 h (Stevens et al., 2009).

Stimulated isolated heart: nerve-evoked contraction

To determine whether the peptides affected the neuromuscular junction and/or the muscle fibers, we used a protocol adapted from Stevens et al. (Stevens et al., 2009). The stimulated isolated heart was prepared as described for the semi-intact heart preparation above, but without a suction electrode. Instead, the pacemaking capability of the cardiac system was ablated, and controlled trains of stimulus bursts were given to one of the remaining motor nerves. To achieve this, we removed the CG, including both the small pacemaker neurons and the motor neurons, to eliminate any spontaneous activity. The central end of one of the remaining motor nerves was then pulled into a suction electrode and impulse trains of 0.5 ms duration at a frequency of 60 Hz, with a burst duration of 300 ms at burst frequencies ranging from 0.2 to 1.0 Hz were applied to the nerve ending to mimic the endogenous bursting activity of the CG. The electrical impulses were generated using the Micro 1401 data acquisition board and Spike2 version 6.09 software with a custom-designed sequencer file. The isolated heart contracted in response to the stimuli; however, the evoked contraction decreased over time. To avoid degraded muscle activity due to continual stimulation, the stimuli were delivered in impulse trains, consisting of 15 bursts each, recurring every 2 min. These conditions were sufficient for contraction amplitude to reach maximum facilitation at these constant frequencies between 0.2 and 1.0 Hz. The nerve-evoked contraction was recorded as described for the semi-intact heart preparation.

Isolated CG: neural activity in isolation

To record the effects of the peptide on the CG in the absence of peripheral effects and feedback pathways, the CG was isolated from the cardiac muscles and pinned to the bottom of a clear Sylgard-lined dish containing chilled physiological saline. Throughout the experiment, the CG was superfused with chilled (9–11°C) physiological saline at a constant flow rate of 5 ml min^{-1}.

Stainless steel pin electrodes were used to record the motor output of the CG extracellularly as described previously (Dickinson et al., 2009). In brief, the neural activity in one of the CG motor nerves was recorded using an electrode isolated from the bath with a petroleum jelly well. The CG neural signal was processed as described above for the semi-intact heart preparation.

Data analysis

Heart contraction parameters were analyzed in Spike2 version 6.09 software using locally written scripts and built-in Spike2 functions. Parameters measured included cycle frequency, contraction amplitude and contraction duration. For the semi-intact heart preparation, the extracellular activity of the CG was recorded in concert with the contraction force.

Extracellular recordings of the CG were analyzed for burst cycle frequency, burst duration, spike frequency and number of spikes per burst, using the built-in functions of Spike2 and scripts, available at http://www.whitney.ufl.edu/BucherLab/Spike2_Scripts2_box.htm.

To calculate the percentage change from baseline for each parameter during peptide application, mean values for the 200 s just before peptide application (control) were compared with the 200 s mean at the peak of the peptide effect, 5–8 min after the onset of peptide application. For those preparations in which the contraction amplitude showed a biphasic response (that is, a decrease followed by an increase in amplitude), the 200 s around the peak of either the decrease or increase in contraction amplitude were used to find the mean change in amplitude.

Data were further analyzed and graphed using Prism5 software (GraphPad Software, San Diego, CA, USA). The percentage change from baseline was averaged across all animals to find the mean ± s.e.m. One-sample *t*-tests were performed to determine whether any treatment caused a change in any parameter that was significantly different from a hypothetical value of 0 (α=0.05). To compare changes among groups of animals, unpaired *t*-tests were used, with Welch's correction for unequal variance when necessary.

RESULTS

The effects of C-AST on contraction amplitude differ among preparations

In the *H. americanus* semi-intact heart preparations used in this study, contraction frequency ranged from 0.3 to 1.12 Hz, with a mean frequency of 0.72 Hz – values comparable to those seen in previous studies of the lobster cardiac neuromuscular system (Stevens et al., 2009). When the neuropeptide C-AST was perfused through the semi-intact heart at a concentration of 10^{-7} mol l^{-1}, a concentration previously shown to exert significant effects on heart contraction

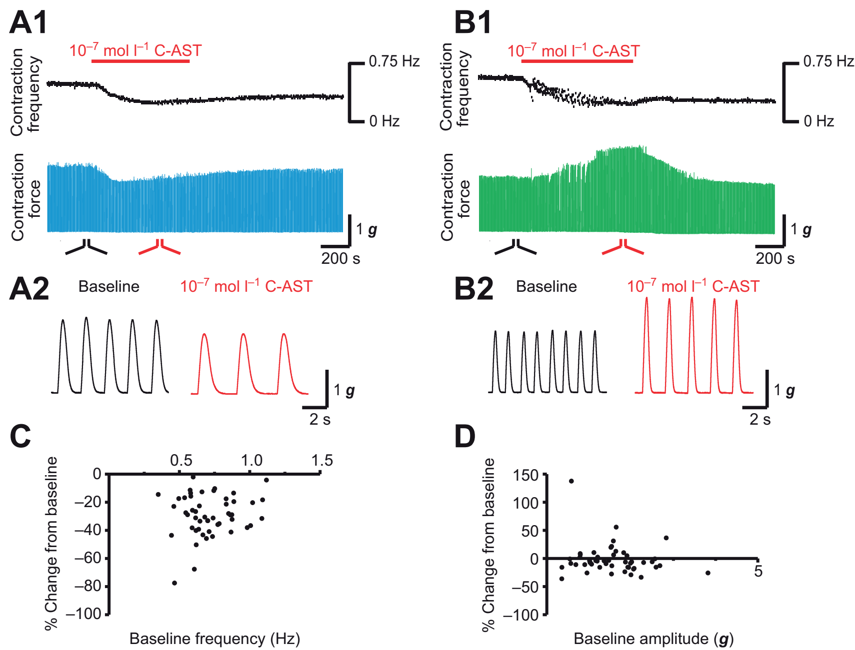

Fig. 2. C-AST consistently elicited decreases in contraction cycle frequency in semi-intact hearts, but its effects on contraction amplitude varied dramatically among preparations. (A1,B1) Contraction frequency is shown graphically as a function of time, on the same time scale as the recorded contraction force. The time during which the peptide was applied is indicated by the red bar above the corresponding recordings. (A2,B2) Recordings taken from the designated regions in A1 and B1, respectively, on an expanded time scale, showing the changes in amplitude and frequency of the heartbeat. Upon application of 10^{-7} mol l^{-1} C-AST, the cycle frequency was reduced in all semi-intact heart preparations (A1 and B1). However, the contraction amplitude decreased in some preparations (A2) and increased in others (B2). Each recording is from a different animal. (C) The extent of the decrease in frequency did not change significantly ($P>0.05$) with initial frequency, but the preparations that showed the largest decrease in frequency were among those with the lowest initial frequencies, suggesting that the effect of the peptide is to some extent state dependent. (D) The change in contraction amplitude, measured at the end of peptide perfusion, was not a function of initial contraction amplitude.

(P.S.D. and T.W., unpublished data), a rapid monotonic decrease of contraction cycle frequency was elicited in all hearts (N=50). These effects were reversed when the heart was subsequently perfused with control saline (Fig. 2A1,B1). Although there was considerable variability in the extent of this decrease in frequency, there was no statistically significant correlation between this and the initial frequency, suggesting that initial frequency was not the main determinant of the extent to which the peptide altered cycle frequency. Nonetheless, the preparations that showed the greatest decreases in frequency were those with the lowest frequencies in control saline (Fig. 2C).

In contrast to the relatively consistent effects of C-AST on contraction frequency, the effects of the peptide on amplitude varied in both magnitude and direction. The effects of repeated application of the peptide were consistent within single preparations (data not shown), but C-AST decreased the contraction amplitude in some hearts, while it increased amplitude in others (Fig. 2A,B). In other semi-intact heart preparations, these peptides elicited a biphasic response. That is, immediately after the onset of perfusion of the peptide, contraction amplitude decreased, but then it slowly increased over the course of the peptide application (Fig. 3). The reverse biphasic change in amplitude (increase followed by decrease) was not observed. The initial amplitude of heart contraction (range 0.35–3.8 ***g***, mean 1.54 ***g***) was not correlated with the magnitude or direction of C-AST's effects, as measured at the end of peptide perfusion (Fig. 2D). Because of the range of changes in amplitude recorded in response to C-AST, we asked whether there were three discrete lobster populations – one that responded to C-AST with an increase in contraction amplitude, one that responded with a decrease in amplitude, and one that showed biphasic responses – or whether there was a continuum of responses to C-AST. A frequency histogram of the distribution of changes in amplitude in response to C-AST perfusion had only one peak, suggesting that C-AST did not have separate and opposing effects on contraction amplitude, but rather that the peptide exerted a continuum of effects on heart contraction amplitude (Fig. 4). The distribution of C-AST's effect on contraction amplitude had positive skew, with the highest value of distribution centered on an amplitude change of –9% from baseline. These data were consistent with the fact that among the lobsters tested (N=50), C-AST elicited a decrease in contraction amplitude in most animals, whereas hearts that responded to C-AST with biphasic and increasing contraction amplitudes were considerably less common (hearts with decreased amplitude, N=36; hearts with biphasic response, N=7; hearts with increased amplitude, N=7). Although the measurements of amplitude change in Fig. 4 were made at the end of the peptide perfusion, when contraction amplitude in biphasically responding hearts had increased, the same general trend was seen when these hearts were measured earlier (during their decrease) or were eliminated from the analysis (data not shown).

Because the hearts with biphasic responses yielded an increase in contraction amplitude at the end of C-AST application, when we made most of our measurements, we grouped these hearts with those in which the peptide directly increased the contraction amplitude. When we used the final change as the basis for dividing the lobsters into two groups, we recorded significant changes in contraction

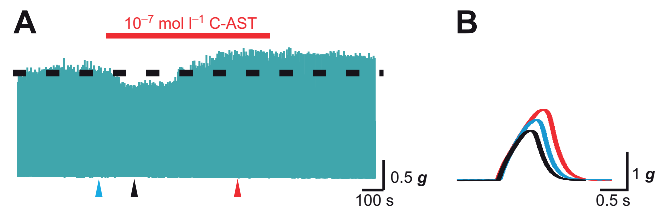

Fig. 3. C-AST elicited biphasic responses during a single application in some lobsters. (A) Recording of the heart showing a biphasic change in amplitude during 10^{-7} mol l^{-1} C-AST perfusion; amplitude initially decreased rapidly, but subsequently increased, so that it was greater than control by the end of C-AST perfusion. The red bar indicates the time of peptide application; the dashed line indicates the baseline contraction amplitude. (B) Overlay of single contractions from A, illustrating the absolute relationship between the amplitude of representative contractions at different times during the experiment: baseline (blue), decreasing (black) and increasing (red) phases. A biphasic response in the reverse order (increase followed by decrease in amplitude) was not observed. The peptide consistently decreased the cycle frequency (not shown). Triangles in A correspond to the times of the corresponding contractions in B.

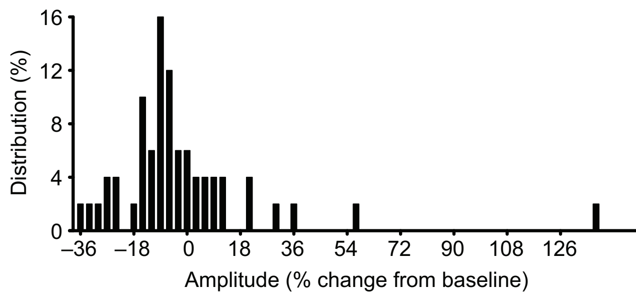

Fig. 4. The modulatory effects of C-AST on heart contraction amplitude show a continuous distribution (bin width 3; *N*=50). Changes were measured at the end of peptide perfusion, when biphasically responding hearts showed an increase in amplitude.

amplitude in both groups (Fig. 5; lobsters with decreased amplitude *N*=36, lobsters with increased amplitude *N*=14, $P<0.05$). In both groups, C-AST significantly decreased contraction cycle frequency and increased contraction duration (Fig. 5).

Mechanisms underlying C-AST's effects on contraction amplitude

To examine the mechanisms responsible for the variability in C-AST's effects on contraction amplitude, we first determined the sites at which the peptide modulated the lobster cardiac neuromuscular system. Specifically, we asked whether C-AST exerted its modulatory effects peripherally, i.e. at the neuromuscular junction or the muscle, and/or centrally, with effects directly on the CG.

C-AST does not directly alter heart contractions *via* effects at peripheral sites

Because C-AST exerted differential effects on contraction amplitude but not on contraction frequency, which is controlled by central output, we initially investigated the peptide's actions on peripheral sites, i.e. the cardiac muscle and neuromuscular junction. We hypothesized that among lobsters in which the peptide increased contraction amplitude, C-AST would increase the response of the cardiac muscle or neuromuscular junction to motor neuronal input, whereas it would decrease the same responses in those preparations that responded to the peptide with a decrease in contraction amplitude.

To address this hypothesis, the effects of C-AST on the semi-intact heart were first determined; then the CG was removed from the heart and the remaining motor nerve was stimulated using trains of electrical impulses. The burst characteristics of the trains of stimuli were held constant: 0.5 ms duration at a frequency of 60 Hz, with a burst duration of 300 ms and a burst frequency of 0.75 Hz. Thus, any change in contraction amplitude upon the application of 10^{-7} mol l^{-1} C-AST could be attributed to the effects of the peptide at the neuromuscular junctions or the myocardium itself. However, in both hearts in which 10^{-7} mol l^{-1} C-AST increased contraction amplitude and those in which the peptide decreased contraction amplitude in the semi-intact heart preparations, there was no significant change in the amplitude of the nerve-evoked contraction in the stimulated preparation (Fig. 6; lobsters with decreased amplitude in the semi-intact heart, *N*=16, lobsters with increased amplitude in the semi-intact heart, *N*=8). Statistical analysis confirmed that the percentage change in the stimulated contraction amplitude before and during peptide application was not different from zero in either group of hearts ($P>0.1$), indicating that C-AST did not directly modulate the lobster cardiac system at peripheral sites.

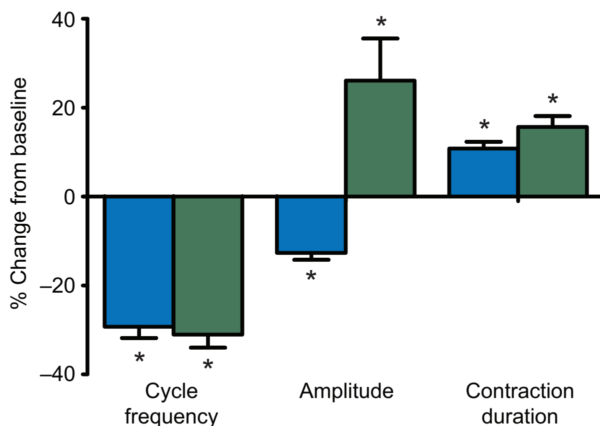

Fig. 5. Contraction amplitude did not correlate with changes in either cycle frequency or contraction duration. Lobsters were divided into two groups based on the change in contraction amplitude at the end of peptide application. Perfusion of 10^{-7} mol l^{-1} C-AST elicited a decrease in cycle frequency and an increase in contraction duration in all animals. However, contraction amplitude differed significantly among lobsters, allowing us to distinguish two distinct groups: one in which the peptide caused a decrease (blue bars; *N*=36) and one in which it caused an increase in contraction amplitude (green bars; *N*=14). As the hearts with biphasic responses showed an increase in contraction amplitude at the end of C-AST application, these preparations were grouped with those showing amplitude increases. *Significantly different from zero (one-sample *t*-test, $P<0.05$). Error bars show s.e.m.

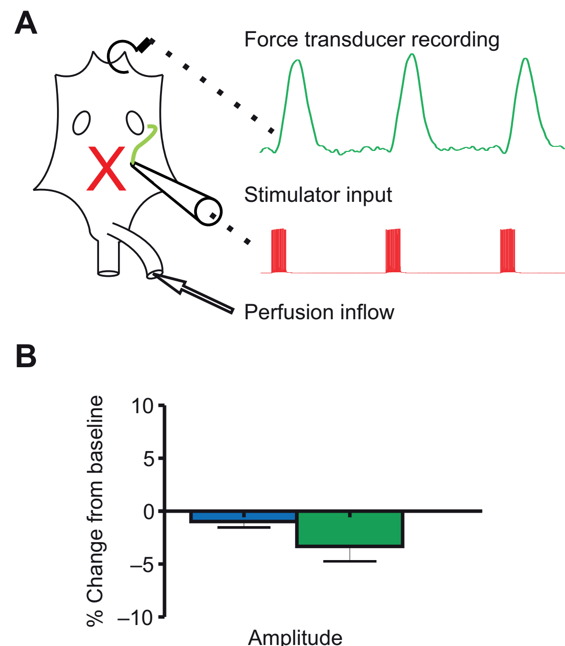

Fig. 6. C-AST did not alter contraction at the neuromuscular junction/muscle level. (A) The cardiac ganglion (CG) was removed, and the remaining motor nerve end was stimulated with impulse trains (Stimulator input; red) through a suction electrode to evoke contraction (Force transducer recording; green), recorded with a force transducer tied to the anterior arteries (hook in figure). (B) Upon perfusion of 10^{-7} mol l^{-1} C-AST, stimulated contraction amplitude did not change significantly either in hearts in which the peptides decreased contraction amplitude (blue bars; *N*=16) or in those in which the peptide increased contraction amplitude (green bars; *N*=8). Single-sample *t*-test, $P>0.1$. Error bars show s.e.m.

Because C-AST caused a clear and consistent decrease in cycle frequency, we hypothesized that the decreased contraction amplitude could be an indirect effect of this decreased frequency, mediated by a decrease in facilitation at the neuromuscular junction. To address this hypothesis, we delivered trains of stimuli to the heart at the baseline cycle frequency and at the cycle frequency recorded at the peak of the peptide's effect in the semi-intact heart preparation (Fig. 7A,B). Other burst characteristics of the stimuli remained constant. At the lower cycle frequency, the amplitude of the stimulated contraction was lower than that of the heart stimulated at the baseline frequency. When the normalized changes in contraction amplitude of the semi-intact heart and the stimulated isolated-heart preparations were compared, the reduced burst frequency in the stimulated preparations caused a decrease in contraction amplitude comparable to that caused by C-AST in the semi-intact heart preparations (Fig. 7C; ANOVA and Bonferroni's multiple comparison test, *P*=0.004). These results suggest that the decreased facilitation due to decreased CG cycle frequency was sufficient to account for the decrease in contraction amplitude caused by C-AST.

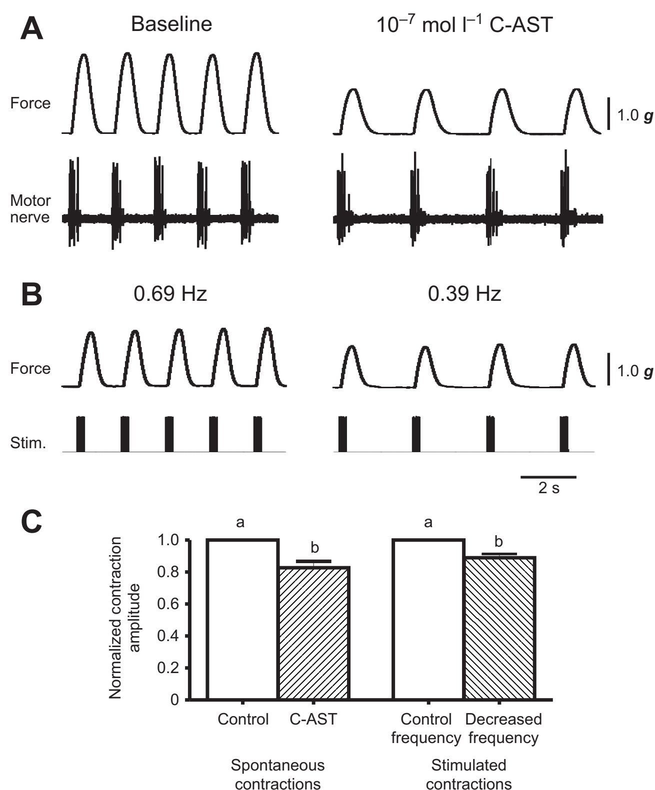

Fig. 7. The decrease in contraction amplitude recorded in some lobster hearts when perfused with C-AST is a consequence of the decreased contraction frequency. (A) Contraction amplitude and cycle frequency in a semi-intact heart decreased upon 10^{-7} mol l^{-1} C-AST application. (B) The same heart was stimulated first at a burst frequency of 0.69 Hz (the frequency recorded in the semi-intact heart control condition), and subsequently at the lower frequency (0.39 Hz) observed in the semi-intact heart during peptide application. Stimulated contraction amplitude decreased at the lower cycle frequency. (C) Pooled data from 10 hearts that responded to C-AST with a decrease in contraction amplitude. Hearts were perfused with C-AST, then the CG was removed and the heart was stimulated, using constant burst parameters, at the cycle frequencies recorded in both control saline and C-AST. The contraction amplitude was normalized to 1 for each control condition. The reduced burst frequency caused a decrease in stimulated contraction amplitude similar to that seen in the semi-intact heart preparation during perfusion with 10^{-7} mol l^{-1} C-AST. The decreases due to both peptide application and stimulation at low frequency are significant (ANOVA followed by Bonferroni's multiple comparison test; *P*=0.004). There was no significant difference in the normalized contraction amplitude between spontaneous contractions and lower frequency stimulated contractions (*P*>0.05). Error bars show s.e.m. Bars with different lowercase letters differ significantly (*P*=0.004).

C-AST modulates heart contraction centrally *via* effects on the CG

To determine whether C-AST differentially modulates contraction amplitude centrally, we again divided lobsters into two subgroups based on C-AST's effects on contraction amplitude in the semi-intact heart preparation (Fig. 8). We simultaneously recorded contraction parameters with a force transducer and motor neuron activity in the CG using a suction electrode. As expected, C-AST caused a clear and consistent decrease in the CG burst cycle frequency in all lobsters. Among the lobsters that responded to the peptide with increased contraction amplitude, the CG burst duration and number of spikes per burst increased significantly, while spike frequency remained unchanged (Fig. 8; *N*=14, *P*<0.05 for burst duration and number of spikes, *P*>0.1 for spike frequency). Somewhat surprisingly, a small, but significant increase in both burst duration and number of spikes per burst was also recorded in those lobsters in which contraction amplitude decreased, again with no change in spike frequency (Fig. 8; *N*=36, *P*<0.05 for burst duration and number of spikes, *P*>0.1 for spike frequency). However, the increases in both burst duration and number of spikes per burst were significantly (~5- to 6-fold) greater in hearts that responded to the peptide with an increase in contraction amplitude than in those that responded with a decrease in amplitude (*t*-test, *P*<0.05).

To determine whether the observed increases in CG burst parameters were direct effects of C-AST on the CG itself, we examined the effects of the peptide on the isolated CG in the absence of the cardiac muscle and, thus, in the absence of feedback from the

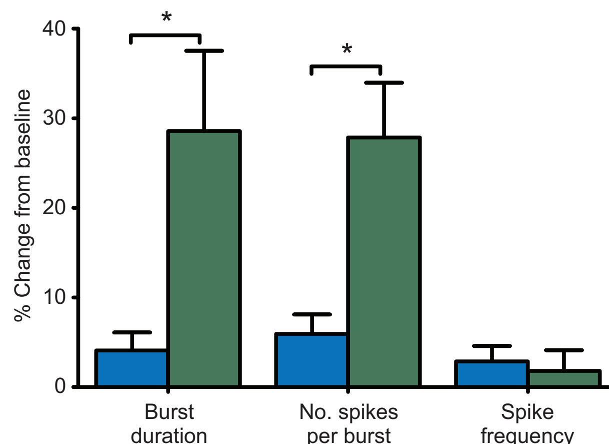

Fig. 8. The excitation of bursting activity in the CG is responsible for the increase in contraction amplitude of the semi-intact heart in response to C-AST perfusion. When CG activity was recorded in semi-intact hearts, 10^{-7} mol l^{-1} C-AST elicited a greater excitation at the level of the CG among lobsters in which the contraction amplitude increased (green bars; *N*=14) than among those in which contraction amplitude decreased (blue bars; *N*=36). Although burst duration and number of spikes per burst increased significantly (*P*<0.05) in both groups of lobsters, the increase was significantly larger in hearts that responded to the peptide with an increase in amplitude (*t*-test corrected for unequal variances, *P*<0.05). There was no change in spike frequency (*P*>0.1) in either group of lobsters. Error bars show s.e.m.

periphery. Spontaneous bursting activity was recorded extracellularly, which allowed us to determine cycle frequency, burst duration, number of spikes per burst and spike frequency within bursts.

Upon peptide application, the CG cycle frequency visibly decreased in all preparations (Fig. 9). Moreover, C-AST did not alter bursting parameters in lobsters in which the peptide decreased contraction amplitude. Only CGs from hearts that exhibited an increase in contraction amplitude during C-AST application responded to the peptide with a significant increase in burst duration and number of spikes per burst (Fig. 9; lobsters with decreased amplitude, N=15, P>0.05; lobsters with increased amplitude, N=16, P<0.05). Spike frequency did not change significantly from baseline during C-AST application in either group of lobsters (Fig. 9; P>0.5).

In conclusion, the effects of C-AST on contraction force varied between lobsters, while their inhibitory effect on cycle frequency was consistent. Among those hearts in which C-AST increased contraction force, burst parameters that are important in the control of contraction amplitude, notably burst duration, increased significantly, while the same parameters remained unaltered or changed relatively little among lobsters in which the peptide reduced contraction amplitude.

C-AST's modulation of the CG results in a continuum of effects on heart contraction amplitude

A careful examination of the time course of the peptide's effects showed that hearts with a biphasic response in contraction amplitude were distinct in some respects from those that responded to C-AST with a simple decrease or increase in amplitude. The time course of the effects of C-AST at 10^{-7} mol l^{-1} on cycle frequency, contraction force, burst duration, number of spikes per burst and spike frequency thus allowed us to place the lobsters in this study into three groups based on the heart's response to C-AST: one with increased amplitude, one with decreased amplitude and one with a biphasic change in contraction amplitude (Fig. 10). In this time course analysis, we selected only the preparations in which C-AST was applied for 600 s.

Among the hearts in which C-AST decreased the contraction amplitude, cycle frequency dropped immediately after the onset of peptide application (Fig. 10A1, N=12). The maximum decrease caused by C-AST was at the end of the application. At this time, C-AST had reduced the cycle frequency by –36.1±3.4% (P<0.0001), while the contraction amplitude decreased by –16.0±2.3% (P<0.0001) from baseline. In contrast to those preparations in which an increase in contraction was seen at any point, the CG's burst characteristics remained virtually unchanged throughout the peptide application (Fig. 10A2). Although this result appears to contradict that shown in Fig. 8, in which a small increase in burst duration and in the number of spikes per burst was seen in preparations in which contraction amplitude decreased, this can be explained by the smaller number of preparations in the time course analysis shown here (Fig. 10; 12 compared with 36). At the time of maximum response, around 800–1000 s after the onset of peptide perfusion, the mean increase in both parameters was around 5%, which was not a significant change. In the larger group of animals, the magnitude increased similarly (about 5%), but the larger sample size enabled us to detect the small significant differences in these parameters.

For hearts that responded to C-AST with a biphasic change in contraction amplitude, the amplitude had decreased from the baseline by –9.3±0.6% 4 min after the onset of C-AST application (Fig. 10B1; N=5, P<0.0001). At this time, cycle frequency had decreased by –17.4±2.6% (P<0.01) from baseline. After this initial decrease, the contraction amplitude gradually rose to 10–12% above the baseline, peaking at 12.3±3.4% (P<0.05) around 1000 s after the onset of peptide application. Cycle frequency decreased to –33.6±3.3% below baseline, reaching its lowest frequency around 840 s after the start of peptide perfusion (Fig. 10B1). At the peak of C-AST's effect, CG burst duration and the number of spikes per burst had increased from baseline by 19.3±3.1% (P<0.01) and 19.3±5.2% (P<0.05), respectively (Fig. 10B2). Throughout peptide treatment, spike frequency remained essentially unchanged.

For hearts in which C-AST elicited a simple increase in contraction amplitude, cycle frequency decreased with a time course and amplitude similar to those recorded in the two other groups of lobsters (i.e. a decrease of –26.0±4.5%, P<0.01), peaking around 700 s after the onset of peptide perfusion. The time course of this change was similar to, though slightly faster than, that of the increase in contraction amplitude, which peaked at 40.5±15.7% (P<0.05) about 800 s after the onset of peptide perfusion (Fig. 10C1; N=8). The time courses of response to C-AST of the two burst parameters were similar to those of contraction amplitude, peaking from around the end of the application (600 s) to about 200 s later (Fig. 10C2), with burst duration increasing by 25.0±8.2% (P<0.05) and the number of spikes per burst increasing by 29.2±8.3%

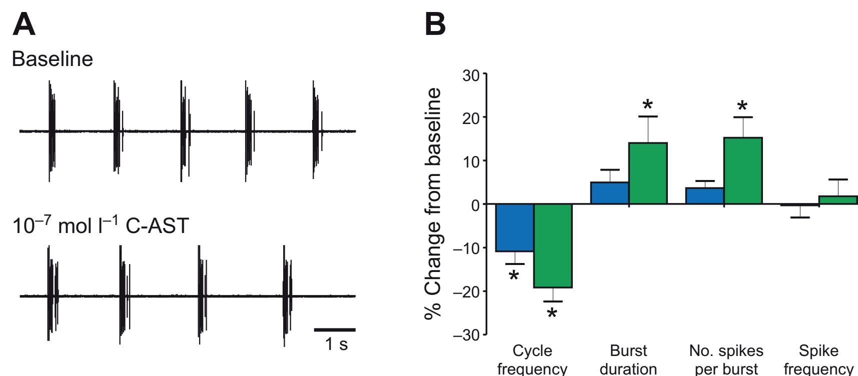

Fig. 9. C-AST decreased cycle frequency but had mixed effects on burst parameters in the isolated CG. (A) CG neuronal output was recorded extracellularly from a motor nerve. (B) Data from lobsters were analyzed according to their response to 10^{-7} mol l^{-1} C-AST (increase in contraction amplitude, green bars, N=16; decrease in contraction amplitude, blue bars, N=15) in the semi-intact heart preparation before the CG was removed for recording. Superfusion of 10^{-7} mol l^{-1} C-AST over the isolated CG caused a decrease in burst cycle frequency in all preparations; means for the two groups did not differ significantly. There was a clear increase in burst duration and number of spikes per burst only among lobsters in which the peptide increased contraction amplitude in the semi-intact heart, while no effect on these two parameters was observed in lobsters with decreased contraction amplitude. Spike frequency did not change in either group of lobsters. *P<0.05, t-test. Error bars show s.e.m.

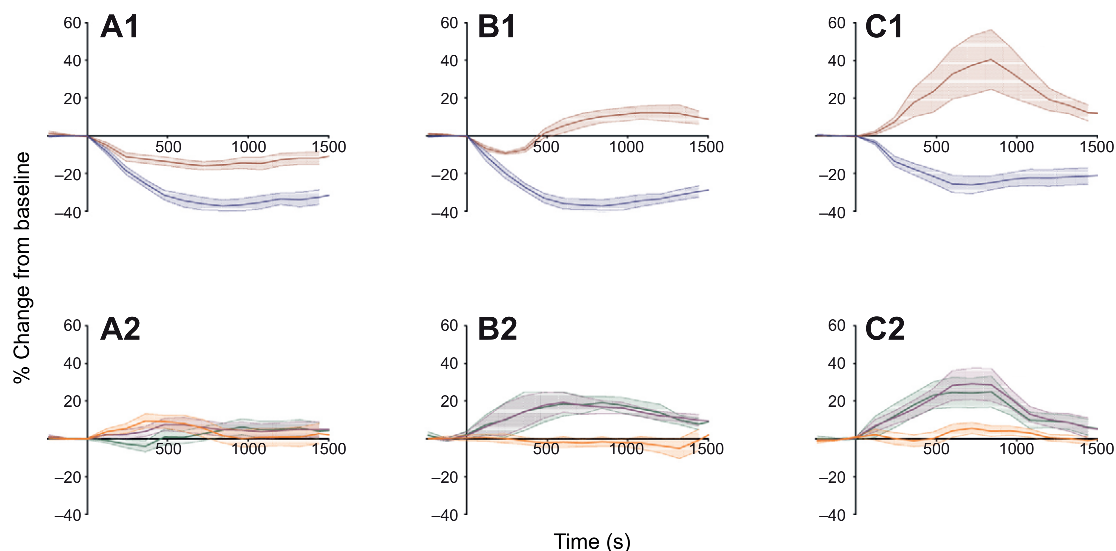

Fig. 10. Changes in burst characteristics of the CG correlate with the overall change – increasing, decreasing or biphasic response – in contraction amplitude during 10^{-7} mol l^{-1} C-AST perfusion. The effects of C-AST on heart contraction (A1, B1, C1; amplitude, red; frequency, blue) and on the burst characteristics of the embedded CG (A2, B2, C2; burst duration, green; number of spikes per burst, purple; spike frequency, orange) differed when data from individual lobsters were analyzed in three groups, based on the peptide's effects on contraction amplitude: (A) hearts in which C-AST decreased contraction amplitude (N=12), (B) hearts that responded to C-AST with a biphasic change in amplitude (N=5), and (C) hearts in which amplitude increased during C-AST perfusion (N=8). Across the three groups of lobsters, cycle frequency decreased similarly. CG burst characteristics did not change significantly among those with decreased amplitude (A2). In lobsters that responded to C-AST with increased amplitude, there was a clear increase in burst duration and number of spikes per burst (C2), while there was a smaller increase in burst duration and number of spikes per burst among lobsters with a biphasic response in amplitude (B2). For lobsters with increased amplitude and those with biphasic changes in amplitude, the peak of the excitation on the CG burst characteristics corresponded to the peak of the increase in amplitude due to peptide application. Data shown are all from preparations in which 10^{-7} mol l^{-1} C-AST was applied for 600 s, from time 0 to 600 s. Bold lines indicate means; shaded regions are s.e.m.

(P<0.01). As was the case for the other two groups, spike frequency within CG bursts did not change throughout C-AST application.

In comparing the responses of the three groups of hearts to C-AST, several clear patterns emerge. First, contraction frequency decreased similarly in all hearts. Second, spike frequency remained unchanged in all cases. Third, the increases in contraction amplitude, seen in the biphasic and increased response heart groups, were correlated with increases in burst duration and consequently in the number of spikes per burst (Fig. 10). In the hearts in which contraction amplitude did not increase, burst parameters changed minimally or not at all. This result suggests that there was a gradient in the magnitude of C-AST's modulatory effect on heart contractions, and that these effects were specifically on the output of the CG.

DISCUSSION

C-AST is a highly conserved neuropeptide family in a number of arthropods. Although it was initially thought to exist solely among holometabolous insects, several recent studies have predicted and/or identified the presence of related peptides from the C-AST family in other insects and crustaceans, including decapod crustaceans (Dickinson et al., 2009; Gard et al., 2009; Ma et al., 2009; Stemmler et al., 2010; Veenstra, 2009; Weaver and Audsley, 2009). Here, we found that the native *H. americanus* C-AST (pQIRYHQCYFNPISCF), like the C-AST-like peptide (SYWKQCAFNAVSCFamide) that was examined previously (Dickinson et al., 2009), modulates rhythmic pattern generation in the *H. americanus* cardiac neuromuscular system. Using the complete yet simple neuromuscular system of the lobster heart, we found that the peptide exerts strikingly different effects on heart contraction amplitude in different individuals, and initiated studies designed to elucidate the mechanisms that underlie these differences.

The effects of a given neuromodulator on a specific pattern generator often vary somewhat among animals, but most studies have focused on mean responses, and have reported similarities in the direction of these effects across the animal population (Christie et al., 2010; Kreissl et al., 1999; Mercier et al., 2003; Richards et al., 2003; Stevens et al., 2008; Stevens et al., 2009). While a number of factors could potentially underlie the quantitative variability in responses across animals, differences in response to a given modulator have most often been attributed to the 'state' of the system; this in turn is often reflected in the starting parameters of the pattern, notably cycle frequency (Dickinson et al., 2001; Fu et al., 2007; Medler and Hulme, 2009; Nadim et al., 2008; Nagy and Dickinson, 1983; Nusbaum and Marder, 1989b; Sargeant, 2007). To explain this correlation, it has been suggested that membrane conductance is, on average, lower in preparations with low cycle frequency, with the consequence that a similar change in currents induced by the modulator will have a larger effect than in more rapidly cycling preparations (Fu et al., 2007). Alternatively, the variability of a modulator's effect might be explained by a differential balance of ion channels within the target neuronal network between animals, as was predicted by modeling studies and shown to be the case for at least some of the neurons in the crustacean stomatogastric nervous system (Goldman et al., 2001).

Despite C-AST's consistent effects on cardiac cycle frequency, its effects on contraction amplitude varied not only in magnitude but also in direction. Upon peptide application, the contraction force decreased in some preparations and increased in others. Moreover, in about 15% of the lobsters tested, the effect of C-AST on contraction amplitude was biphasic (i.e. a decrease followed by an increase in amplitude). C-AST did not show distinct state-dependent effects with respect to the baseline conditions (i.e. cycle frequency

and contraction amplitude) of the heartbeat, suggesting that other factors are responsible for the dramatic differences in the effects of the peptide among preparations.

To determine the factor(s) that might be responsible for C-AST's variable effects on contraction amplitude, we examined a number of possible sites at which C-AST could modulate the cardiac neuromuscular system, and asked whether these modulatory effects varied among preparations such that they could explain the variable response to C-AST. Previous studies of modulation of the crustacean cardiac neuromuscular system have identified a number of potential sites at which transmitters and hormones can alter output of the system as a whole, and have shown that single modulators often affect multiple sites. Modulators act on the pattern generator of the CG itself (Cruz-Bermudez et al., 2006; Cruz-Bermudez and Marder, 2007; Fort et al., 2004; Fort et al., 2007a; Fort et al., 2007b; Freschi, 1989; Krajniak, 1991; Krajniak et al., 1990; Mercier et al., 2003; Miller and Sullivan, 1981; Saver and Wilkens, 1998; Saver et al., 1998; Saver et al., 1999; Stevens et al., 2008; Stevens et al., 2009). Several of these same modulators, including proctolin (Wilkens et al., 2005), and a number of different FMRFamide-like peptides (Fort et al., 2007a; Stevens et al., 2009; Wilkens et al., 2005), also exert effects at the neuromuscular junction or directly on crustacean cardiac muscle. Some have likewise been shown to affect other aspects of the circulatory system, including blood vessels and vascular resistance (Wilkens et al., 2008; Wilkens and Taylor, 2003). Moreover, several recent studies have shown that the effects of the same modulator differ either quantitatively or qualitatively when applied to the semi-intact heart and the isolated CG (Fort et al., 2004; Fort et al., 2007a; Fort et al., 2007b; Stevens et al., 2008; Stevens et al., 2009), suggesting the possibility that these modulators exert effects at other sites, potentially including feedback pathways within the cardiac neuromuscular system.

We initially postulated that the range of effects exerted by C-AST was a consequence of differences among preparations in its direct modulatory effects on the myocardium. One possibility was that peripheral C-AST receptors that act to enhance contraction are present only in those preparations that respond to C-AST with increased amplitude. We thus examined C-AST's effects directly on the cardiac muscle using a stimulated isolated-heart preparation in the absence of inputs from the CG. In contrast to our predictions, the peptide did not alter contraction in response to constant stimulation in any of the preparations, indicating that C-AST does not directly modulate either muscle contraction or the neuromuscular junction. This contrasts with the effects of A-ASTs, which have been shown to modulate muscle contraction both pre- and post-synaptically (Jorge-Rivera and Marder, 1997; Kreissl et al., 1999). Although there were no direct effects of C-AST on muscle contraction, we found that C-AST's ability to decrease contraction amplitude was in fact due to an indirect effect at the neuromuscular junction; namely, a decrease in facilitation resulting from the decreased cycle frequency, as has been seen in other studies of this system (Mahadevan et al., 2004) as well as other crustacean neuromuscular systems (Jorge-Rivera et al., 1998; Mercier and Wilkins, 1984; Morris and Hooper, 1998). Interestingly, because C-AST consistently decreased cycle frequency in all lobsters, a decrease in facilitation was certainly also present in the preparations in which contraction amplitude increased. This in turn suggests that the factors leading to an increase in contraction amplitude were sufficient not only to cause a direct increase in contraction amplitude but also to overcome the expected decrease due to de-facilitation. Conversely, we noted that even in those preparations that responded to the peptide with a decreased contraction amplitude, burst duration and the number of spikes per burst increased slightly. These factors would normally be expected to result in an increased contraction amplitude. Thus, we postulate that the decreased facilitation in these preparations was sufficient to overcome the effects of enhanced bursting, resulting in the observed decrease in contraction amplitude. Taken together, our data suggest that the gradient of C-AST's effect on contraction amplitude is a function of the balance between the excitation of CG bursting and the decreased level of facilitation at the neuromuscular junctions.

No data are currently available on the cellular mechanisms by which C-AST modulates neuromuscular systems. The allatostatins are best known in insects for their inhibition of juvenile hormone synthesis, a function common to all types of allatostatins, but it has been hypothesized that their original function was neuro- or myo-modulation, with endocrine control being a later evolutionary adaptation (Stay and Tobe, 2007). Such a neuromuscular function for an A-type allatostatin has been elucidated in both isopods and crabs (Jorge-Rivera and Marder, 1997; Kreissl et al., 1999). In these species, AST modulates neuromuscular activity both pre-and post-synaptically. The modulatory effects of C-AST in the lobster cardiac system clearly differ in detail from these effects, as they involve changes in the cycle frequency and duration of action potential bursts in pattern-generating neurons of the lobster CG, but not changes at the muscle or neuromuscular junction. Inhibitory effects of all three AST types (A-, B- and C-types) have been documented in the pyloric pattern generator of the stomatogastric system, but details of the mechanisms used to cause the observed decreases in cycle frequency have not yet been examined (Dickinson et al., 2009; Dircksen et al., 1999; Fu et al., 2007; Ma et al., 2009; Szabo et al., 2011). Additionally, inhibitory effects of A-type allatostatins have been recorded in the crab CG (Cruz-Bermudez and Marder, 2007). In this case, cycle frequency decreases as it does in the presence of C-AST in the lobster; however, although it does not change the membrane potentials of motor neurons, A-AST causes a decrease in both the frequency and number of spikes per burst, changes that do not take place in response to C-AST. Thus, while it is well known that the patterned motor output of the CG results from the interactions of pacemaker potentials and driver potentials in the neurons of the CG (reviewed in Cooke, 2002), we do not yet know the specific mechanisms used by any of the ASTs, including C-AST in this study, to modulate the output of the CG.

Several other instances in which a single modulator exerts opposing effects in different animals have been identified and examined in crustaceans, notably in the responses of identified synapses to aminergic modulation. In the crayfish, serotonin has been shown to cause both increases and decreases in the escape response to controlled sensory stimulation. Synaptic responses of the lateral giant (LG) neuron, which acts as a command neuron in the escape circuitry, can either increase or decrease, depending on the animal's social status (Yeh et al., 1996; Yeh et al., 1997) and on the temporal pattern and concentration of serotonin application (Teshiba et al., 2001). In both crayfish (*Procambarus clarkii*) and crabs (*Eriphia sipinifrons*), octopamine enhances the amplitude of post-synaptic currents and potentials at the neuromuscular junction in the leg opener muscle of some animals, but decreases it in others (Djokaj et al., 2001). Although the underlying trigger for these differential responses has not been identified, as was the case for the LG neuron, this difference in modulation would result in an effect similar to that observed here, with the same modulator eliciting increased contraction in some animals and decreased contraction in others. However, the site at which contraction amplitude is modulated by octopamine differs from that affected by C-AST.

We do not yet know what factors are responsible for triggering the changes in the response of the CG to C-AST among different lobsters. A number of possibilities exist, including metamodulation (Edwards et al., 2002; Mesce, 2002), in which another modulator or modulators might influence the effects of C-AST on the CG. Such interactions are known to occur in crustaceans. For example, octopamine exerts an antagonistic effect on serotonin modulation at the neuromuscular junction in crayfish and crabs; when applied together, the increase in synaptic potential amplitude that is evoked by serotonin is decreased, regardless of whether octopamine increases or decreases the same post-synaptic potential when applied alone (Djokaj et al., 2001). Interactions among peptide neuromodulators have likewise been documented: in the spiny lobster (*Panulirus interruptus*), previous application of red pigment concentrating hormone (RPCH) results in a qualitative change in the response of the cardiac sac pattern generator to proctolin (Dickinson et al., 1997). The lobster heart is modulated by a myriad of substances, so numerous possibilities for such metamodulation exist.

Additionally, we have found that the proportion of lobsters responding with increased *vs* decreased amplitude varies, albeit irregularly, over the course of a year (data not shown); one possibility is thus that the differences in response are related to the molt cycle of the lobster. It has been shown in other systems, such as the moth *M. sexta* (Mesce, 2002; Miller and Levine, 2006), that 20-hydroxyecdysone, a major molting hormone, can alter not only the expression of other neuromodulators but also the response of a pattern generator to a modulator. Both 20-hydroxyecdysone (Bacque-Cazenave et al., 2010; Cheng and Chang, 1991; Styrishave et al., 2008) and the peptide hormone CCAP (Phlippen et al., 2000) fluctuate with the molt cycle in lobsters, making them potential candidates for a role in triggering changes in the response of the lobster cardiac ganglion to C-AST.

In conclusion, a conserved peptide from the C-AST family can modulate the motor output of the *H. americanus* cardiac neuromuscular system. The peptide consistently decreased the CG burst cycle frequency, but exerted differential effects on the contraction amplitude. Among the lobsters in which C-AST increased contraction amplitude, there were significant increases in the CG burst characteristics, suggesting that the peptide's central action was within the lobster CG. However, because we did not observe any decrease in burst characteristics among the lobsters that responded to C-AST with decreased amplitude, and there was instead a very small increase in these parameters, we conclude that the decreased contraction amplitude was likely a consequence of the decrease in facilitation at the neuromuscular junction due to the decrease in burst cycle frequency. In addition, the fact that there was a moderate level of excitation at the CG level among lobsters with a biphasic change in contraction amplitude suggests that C-AST does not exert two diametrically opposed effects – a decrease or an increase in contraction amplitude – in different preparations, but rather that it exerts a continuum of effects on heart contraction in different lobsters.

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