Regulation of the Crab Heartbeat by FMRFamide-Like Peptides: Multiple Interacting Effects on Center and Periphery

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Fort TJ, Brezina V, Miller MW. Regulation of the crab heartbeat by FMRFamide-like peptides: multiple interacting effects on center and periphery. J Neurophysiol 98: 2887–2902, 2007. First published September 5, 2007; doi:10.1152/jn.00558.2007. We are studying the functional “logic” of neuromodulatory actions in a simple central pattern generator (CPG)-effector system, the heart of the blue crab Callinectes sapidus. The rhythmic contractions of this heart are neurogenic, driven by rhythmic motor patterns generated by the cardiac ganglion (CG). Here we used anatomical and physiological methods to examine the sources and actions on the system of the FMRFamide-like peptides (FLPs) TNRNFLRFamide (F1), SDRNFLRFamide (F2), and GYNRSFLRFamide, an authentic Callinectes FLP. Immunohistochemical localization revealed a plexus of FLP-immunoreactive fibers in the pericardial organs (POs), from which modulators are released to reach the heart as circulating neurohormones. Combined backfill and immunohistochemical experiments indicated that the FLPs in the POs originated in the CNS, from large neurosecretory cells in the B cluster of the first thoracic neuromere. In physiological experiments, we examined the actions of the FLPs on the intact working heart, on the semi-intact heart in which we could record the motor patterns as well as the muscle contractions, on the isolated CG, and in a preparation developed to assess direct actions on the muscle with controlled patterns of motor neuron spikes. The FLPs had strong positive chronotropic and inotropic effects. Dissection of these effects suggested that they were produced through at least two primary actions of the FLPs exerted both on the heart muscle and on the CG. These primary actions elicited numerous secondary consequences mediated by the feedforward and feedback interactions that integrate the activity of the complete, coupled CPG-effector system.

INTRODUCTION

Cyclical, rhythmic behaviors such as breathing, biting and chewing, and various forms of locomotion are produced by central pattern generator (CPG) networks of neurons that distribute patterned activity to a set of peripheral effector muscles (reviewed by Friesen and Stent 1978; Marder and Calabrese 1996; Pearson 1993; Stein et al. 1997). Modification of such motor output to meet varying behavioral demands is often accomplished by neuromodulation (Harris-Warrick and Marder 1991; Katz 1999; Marder and Thirumalai 2002; Nässel 1996; Nusbaum and Beenakker 2002). Typically, the modulation is complex. Most CPGs are regulated by multiple modulators, each with a different but overlapping, sometimes even apparently redundant, constellation of effects on the intrinsic properties of the individual neurons and their synaptic interconnections (Brezina and Weiss 1997; Harris-Warrick et al. 1998; Katz 1999; Marder and Thirumalai 2002; Nusbaum et al. 2001; Skiebe 2001). The same modulators typically also act on the muscles that the CPG drives (Calabrese 1989; Hooper et al. 1999; Kobayashi and Muneoka 1990; Meyrand and Marder 1991; Weiss et al. 1992; Worden 1998). A question of considerable interest is, what is the functional “logic” of this complex modulatory architecture?

In general terms, it is likely that the complexity of the modulation reflects the complexity of the CPG-effector network, with multiple interacting components and feedback loops whose activity must be controlled in an integrated manner. For example, recent experimental and theoretical studies have emphasized the need to tune the properties of the muscle effectors coordinately with the activity of the CPG (Brezina et al. 2000, 2003a,b, 2005; Chiel and Beer 1997). Excessive complexity of the network, and thus of its modulatory architecture, will, however, make study of the issue difficult. For the best chance of success, we should find the very simplest CPG-effector network, where we may be able to understand the complete system in some detail and so grasp the mutual interactions of its elements and the role of the modulation in integrating the system into a coherently acting whole.

Here we study an exceptionally simple CPG-effector network, the cardiac system of the blue crab Callinectes sapidus (Fig. 1; for reviews, see Cooke 1988, 2002; Hagiwara 1961; additional references in Fort et al. 2004, 2007). The crab heartbeat is neurogenic, driven by a rhythmic motor program generated by a simple CPG, the cardiac ganglion (CG), that is embedded within the heart itself. The CG contains only nine neurons: four local premotor interneurons that are thought to act as pacemakers and five motor neurons that send bursts of spikes out of the ganglion to contract the single-chamber heart. Through mutual electrical coupling, all of the motor neurons usually fire in synchrony (Fort et al. 2004; Tazaki and Cooke 1979). Recent studies conducted in isopod and lobster hearts suggest that feedback from the heart muscle can, in turn, modify parameters of the motor program produced by the CG (Mahadevan et al. 2004; Sakurai and Wilkens 2003).

The cardiac system is extensively modulated by cardioactive amines and peptides (reviewed by Cooke 2002; Cooke and Sullivan 1982). These may reach the heart in two ways (Fig. 1). First, neurosecretory neurons in the CNS project to peripheral neurohemal structures, including the pericardial organs (POs) located within the pericardial sinus, where they release the modulator into the bloodstream in a neurohormonal fashion.
(Cooke and Sullivan 1982). Second, regulatory neurons in the CNS project further, through the dorsal nerve, directly into the heart where they release modulators onto the neurons of the CG and possibly the myocardium (Alexandrowicz 1932; Maynard 1960). In two previous papers, we examined the anatomical origins and physiological actions of two of the principal modulators of the *Callinectes* cardiac system, dopamine (Fort et al. 2004) and crustacean cardioactive peptide (CCAP) (Fort et al. 2007). We concluded that dopamine probably reaches the heart both as a circulating neurohormone and by direct release within the CG, whereas CCAP acts as a neurohormone only. Both modulators were generally cardioexcitatory, increasing the frequency and amplitude of the heartbeat. With each modulator, we were able to dissect the overall effects into several direct, primary actions of the modulator that then had multiple secondary consequences mediated by the interconnected nature of the network (see DISCUSSION).

This study examines another family of modulators that is likely to be important in the cardiac system: the FMRFamide-related peptides (FaRPs) or, in the preferred terminology (see Mercier et al. 2003), the FMRFamide-like peptides (FLPs). FMRFamide itself was first isolated and characterized, indeed as a cardioregulatory agent, from a mollusk, the clam *Macrocystis mirabilis*, by Price and Greenberg (1977). However, a rich variety of FLPs, with a broad spectrum of structures and physiological actions, has been found in many invertebrates (for reviews, see Greenberg and Price 1992; Price and Greenberg 1989) including crustaceans. The first crustacean FLPs identified were TNRNFLRFamide (referred to as F1) and SDRNFLRFamide (F2), purified and sequenced by Trimmer et al. (1987) from the lobster *Homarus americanus*, and subsequently found in other crustaceans including the crab *Cancer borealis* (Weimann et al. 1993). From *Callinectes sapidus*, the crab used in this work, Krajniak (1991) purified and sequenced another FLP, GYNRSFLRFamide, which we will refer to as CalFLP. Altogether, 19 crustacean FLPs are now known (Mercier et al. 2003; Weiss et al. 2003). Many of these FLPs were purified from the POs, which in most crustaceans are intensely FLP-immunoreactive and probably contain multiple FLPs (Christie et al. 1995; Cruz-Bermúdez et al. 2006; Fu et al. 2005; Mercier et al. 1993; Skiebe 2001). The FLPs exert numerous physiological actions in crustaceans (for review, see Mercier et al. 2003). They act on the motor patterns produced by the stomatogastric ganglion (Cruz-Bermúdez et al. 2006; Tierney et al. 1997; Weimann et al. 1993), on a number of skeletal muscles (Jorge-Rivera and Marder 1996; Jorge-Rivera et al. 1998; Mercier et al. 1993; Skerrett et al. 1995; Weiss et al. 2003; Worden et al. 1995), and on the heart in several crustacean species (Cruz-Bermúdez et al. 2006; Krajniak 1991; Mercier et al. 1993; Saver et al. 1999; Skerrett et al. 1995; Wlkens et al. 2005; Worden et al. 1995; see DISCUSSION). Here we examine the anatomy and physiology of the action of FLPs on the *Callinectes* cardiac system.

**METHODS**

Specimens of *Callinectes sapidus* (male and female) were captured in the San José Lagoon in the Hato Rey district of San Juan, Puerto Rico. They were housed under ambient light and temperature conditions in water obtained from the collection sites. To reduce fat deposits within the heart, the crabs were not fed. They were typically used within 3 wk of capture.

**Histology**

FLP-LIKE IMMUNOHISTOCHEMISTRY. Specimens were covered in ice (30 min) to achieve immobilization. Tissues were dissected, secured to Sylgard-lined petri dishes with minuten pins, fixed for 1 h in freshly prepared 4% paraformaldehyde, and washed (5 times, room
temperature with agitation) in PTA (0.1 M phosphate buffer containing 2% Triton X-100 and 0.1% sodium azide). After preincubation with normal goat serum (0.8%), the tissues were immersed (48 h, room temperature) in a 1:5,000 dilution of the primary antibody (anti-FMRFamide, Diasorin, Stillwater, MN). Such antisera (rabbit polyclonal) have been shown previously to exhibit high avidity for the extended FLRFamide peptides found in crustaceans (Marder et al. 1987; Trimmer et al. 1987; Weimann et al. 1993). After repeated PTA washes (5 times, ≥30 min each, room temperature), the tissues were incubated in secondary antibodies (1:3,000 dilution) conjugated to a fluorescent marker [Alexa 488 goat anti-rabbit IgG (H+L) conjugate, Molecular Probes, Eugene, OR]. The Alexa 488 was viewed with the G-2A filter block of a Nikon Optiphot system or using the preconfigured FITC channel of a Zeiss Pascal LSM5 laser-scanning confocal microscope. Standard images were captured using the ACT1 (Nikon) software package. Confocal images were reconstructed (AIM Software) from sequential images captured in the z-axis plane of the tissue. Images were transported as TIFF files to Adobe Photoshop (Version 6) for adjusting overall contrast and brightness. Finally, they were imported to Corel Draw 9 for addition of labels, cropping, and arrangement of panels.

NERVE BACKFILLS. The biotin-avidin protocol followed the methods of Fort et al. (2004). The thoracic nervous system was pinned out near a small petroleum jelly well that was formed on the Sylgard surface. The segmental nerve of the first thoracic neuromere (SN1) was cut and drawn into the well. The crab saline inside the well was replaced with saturated aqueous solution (1.6 mg/30 μl) of biocytin (Sigma Chemical, St. Louis, MO). The preparation was covered and incubated overnight at 14°C. The well was removed and the ganglia were washed three to five times, repinned, and fixed in paraformaldehyde as described above. The fixed ganglia were transferred to microcentrifuge tubes, washed five times (30 min each) with PTA solution, and incubated overnight (room temperature, with shaking) in Rhodamine 600 Avidin D (Vector Laboratories, Burlingame, CA) diluted 1:3,000 in PTA (24–48 h, room temperature). The ganglia were washed five times with PTA and the quality of the backfill was assessed before further immunohistochemical processing. In the double-labeling experiments (Fig. 2, B and C), FLP-like immunohistochemistry (FLPli) was visualized using the Alexa 488 goat anti-rabbit secondary antibody. A barrier filter (546-nm green interference) was used to eliminate “bleed through” of rhodamine when examining and photographing FLPli fluorescence.

**Physiology**

**WORKING HEART (WH).** The heart was removed intact; the sternal artery was cannulated with a modified syringe needle and mounted in a 20-ml organ bath (Fig. 1B). The heart was suspended using a fine monofilament nylon thread attached to the force plates of a Grass (astro-Med, West Warwick, RI) FT03 isometric force transducer and placed under a resting load (~0.5 g). Perfusion with saline was maintained at a constant rate (2 ml/min) and pressure. The crab saline composition was based on Puntin’s saline for Cancer pagarus (in mM): 487 NaCl, 13.6 KCl, 13.4 CaCl2, 13.6 MgCl2, 1.4 Na2SO4, and 3 HEPES, adjusted to pH 7.4 with NaOH. Perfusion rate and pressure were maintained when FLP trials were performed.

**SEMI-INTEGRATED WORKING HEART (S-IWH).** The heart was pinned in a Sylgard-lined petri dish in an arrangement as similar as possible to...
that in the intact crab (Fig. 1C). A small incision was made in the ventral wall of the heart to expose part of the connective ring containing the motor neuron axons. The ring was cut, usually across one of the postero-lateral connectives (see Fig. 1), and the severed end proximal to the ganglion was drawn into a polyethylene extracellular suction electrode. Motor neuron spike patterns were recorded with a differential AC amplifier (Model 1700, A-M Systems, Carlsborg, WA). At the same time, the heart was connected to a Grass FT03 isometric force transducer with a hook and nylon thread and placed under a resting load (~0.5 g). The motor neuron spike pattern and the muscle tension signals were simultaneously digitized with a PowerLab (ADInstruments, Colorado Springs, CO) data acquisition system (total sampling rate 100 kHz) running Chart 4 or 5 software (ADInstruments). The preparation was continually internally perfused with saline at a constant rate (2 ml/min) and pressure. Perfusion rate and pressure were maintained when FLP trials were performed.

**ISOLATED CARDIAC GANGLION (ICG).** The heart was pinned ventral side up in a Sylgard-lined petri dish (Fig. 1D). A cut was made in the ventral musculature exposing the cardiac ganglion. Dissection was achieved principally by teasing away the adhering muscles. Previous investigators (Tazaki and Cooke 1979) noted that the region within the confluence of the motor roots at each end of the ganglion contains the dendritic endings of the ganglionic neurons. A substantial noncontracting remnant was therefore retained at either end of the ganglion. Extracellular suction electrode recordings were obtained as in the S-IWH preparation from one of the four cut ganglion roots. Intracellular recordings were obtained in the dendritic endings of the ganglionic neurons. A substantial noncontracting remnant was therefore retained at either end of the ganglion. Extracellular suction electrode recordings were obtained as in the S-IWH preparation from one of the four cut ganglion roots. Intracellular recordings were obtained from anterior and/or posterior motor neurons using microelectrodes filled with 2 M KCl (10–30 M). Previous investigators (Tazaki and Cooke 1979) noted that the region within the confluence of the motor roots at each end of the ganglion contains the dendritic endings of the ganglionic neurons. A substantial noncontracting remnant was therefore retained at either end of the ganglion. Extracellular suction electrode recordings were obtained as in the S-IWH preparation from one of the four cut ganglion roots. Intracellular recordings were obtained from anterior and/or posterior motor neurons using microelectrodes filled with 2 M KCl (10–30 M). The preparation was continuously superfused with saline (2 ml/min).

**CONTROLLED STIMULUS (CS) PREPARATION.** The heart was pinned ventral side up in a modified Sylgard-lined petri dish (Fig. 8). A small window was cut in the ventral musculature, anterior to the sternal artery, to expose the musculature of the inner ventral wall. The muscle fibers were carefully teased apart to expose the trunk of the cardiac ganglion. The ganglion trunk was severed at the midpoint and the anterior portion of the ganglion was removed (Fig. 8A). The posterior trunk was drawn into a polyethylene suction electrode for extracellular stimulation. Stimulus voltage pulses were delivered from a Grass S88 stimulator that, for more accurate timing, was driven by an A-M Systems Model 2100 stimulator. The stimulus parameters (usually 10–20 V, 1.5 ms) were adjusted so that each pulse reliably produced a single spike simultaneously in all of the motor neuron axons in the postero-lateral connectives. This was confirmed by the detection of a corresponding compound excitatory junctional potential (EJP) in the muscle that did not grow larger when the stimulus parameters were increased (cf. Benson 1981), and, with bursts of stimuli, a contraction that did not grow larger. Recordings in which some pulses failed to elicit a spike in one or more of the axons, as seen by a quantally smaller EJP or contraction, were not used. The patterns of stimuli applied in this work were modeled on the spontaneous bursting patterns recorded in S-IWH preparations (see Fig. 9 legend). The EJPs were recorded from selected muscle fibers using intracellular microelectrodes filled with 2 M KCl (10–30 M). The fibers for this recording were selected from bundles of the myocardium that were not subjected to excessive motion when contractions occurred. To record the contractions, the anterior portion of the heart was connected to a Grass FT03 isometric force transducer with a hook and nylon thread. The preparation was continuously perfused with saline as in the S-IWH preparation.

**FLP APPLICATION.** In all physiological preparations, FLPs were applied by switching the continuously perfusing saline to FLP-containing saline, and after several minutes, back to control saline for wash. The relatively slow rate and large dead volume of the perfusion system meant that the FLP response often developed only 30–60 s after the switch to FLP-containing solution and washed out over a number of minutes (see Figs. 3A and 5A and C). The preparation was washed for ≥20 min before another FLP application. Typically a series of increasing FLP concentrations, each 10-fold higher than the previous, was tested, separated by washes; no cumulative FLP applications were performed. The dose–response relations in Figs. 3, 4, and 6, and the group data in Figs. 7, B and C, 9C, and 10E were constructed by comparing the parameter values averaged over a period of 30–60 s (~10 spike bursts and contractions) during the strongest response to each FLP concentration to control values averaged over a period of 30–60 s immediately before the application of that FLP concentration.

**TEMPERATURE.** All physiological experiments were performed at room temperature (19–21°C).

**Data analysis**

The four principal parameters measured in this paper (the frequency of the motor neuron spike bursts and heart muscle contractions, the
contraction amplitude, the burst duration, and the number of spikes per burst) all required identification of the successive spike bursts (in the S-IWH and ICG preparations) and/or the resulting phasic contractions (in the S-IWH and WH preparations). This required a precise definition of what constituted a separate burst or contraction, particularly when the burst structure and contraction shape became complex (see Figs. 4A and 6a). Briefly, in the S-IWH and ICG preparations, we always (except in the special analysis in Fig. 7) began with the extracellular electrical recording, from which we extracted a list of the spike times. In the histogram of the successive interspike intervals, there was invariably a well-separated peak of longest intervals, which we defined to be the interburst intervals; all shorter intervals were intraburst intervals. Based on this definition, we partitioned the recording into the successive bursts and interburst intervals. The burst duration was then the time between the first and last spikes in a burst, and, in an S-IWH preparation, the contraction amplitude was the peak tension associated with the burst (often occurring just after the end of the burst, in the following interburst interval) minus the baseline tension at the beginning of the burst. In the WH preparations, where no electrical recording was available, we identified successive (sufficiently pronounced) maxima and minima in the tension waveform itself; the contraction amplitude was then the maximum tension minus the preceding minimum tension. The frequency of the bursts and contractions was expressed in bursts or beats per minute (BPM). For each burst or beat, the “instantaneous” frequency was computed as the reciprocal of the sum of the burst duration and the preceding interburst interval (in the S-IWH and ICG preparations) or of the preceding interval between the peak contractions (in the WH preparations).

The extracellularly recorded spikes were in fact compound action potentials occurring simultaneously in the several motor neuron axons in the connective. Generally all of the spikes in a section of recording were of uniform amplitude, reflecting the firing of different subsets of the motor neurons. The spikes of different subsets were of dissimilar amplitudes, reflecting the firing of different subsets of the motor neurons, they were nevertheless still used in the identification and quantification of the bursts.

Other details of parameter quantification and statistical analysis are given in the figure legends. Initial data processing was performed in Chart 4 or 5; subsequent steps were performed with custom routines written in Mathematica 4 or 5 (Wolfram Research, Champaign, IL) and scripts within the SigmaPlot 10 graphics program (Systat Software, San Jose, CA), which was used to make the physiological figures. Statistical analysis was performed in Mathematica or SigmaStat 3 (Systat Software).

**RESULTS**

Anatomical origins of the cardioregulation by FLPs

First, we used histological methods to identify sources of FLPs in the *Callinectes* cardiac system (Fig. 2). In agreement
with previous observations in Homarus americanus (Kobierski et al. 1987; Trimmer et al. 1987), Procambarus clarkii (Mercier et al. 1993), Cancer borealis (Christie et al. 1995), and Cancer productus (Fu et al. 2005), FLPli was observed throughout the Callinectes pericardial organs (Fig. 2; see also Krajniak 1991). FLPli was present in fine varicose fibers on the surface of all bars and trunks (nomenclature of Alexandrowicz 1953). These superficial fibers appeared to be ramifications of larger nonvaricose fibers that coursed through the interior of the POs (Fig. 2A, arrows). No FLPli fibers were present in the dorsal nerve projecting from the POs to the heart and no FLPli was detected in the cardiac ganglion (4 preparations; data not shown).

In decapod crustaceans, neurosecretory cells within the CNS project to the pericardial organs through segmental nerves (SNs) located on the dorsal surface of each neuromere of the thoracic ganglion complex (TGC) (Alexandrowicz 1953; Cooke and Sullivan 1982; Maynard 1961a,b). We applied nerve tracing techniques to the first segmental nerve (SN1) of the most anterior TGC neuromere to identify potential sources of the FLPli in the POs (Fig. 2, B and C). Biocytin backfills (see METHODS) labeled a large axon (Fig. 2B1, A), previously shown to originate from the dopaminergic L cell in the commissural ganglion (Fort et al. 2004; Goldstone and Cooke 1971; Maynard 1961b). Also labeled was a large contralateral cell (Fig. 2B1, *) that was previously shown to contain CCAP-like immunoreactivity (Fort et al. 2007). Near the origin of the SN1, the backfills labeled a cluster of three to five ellipsoid cell bodies (Fig. 2B1, B). These cells correspond to the neurosecretory B cells described by Maynard (1961b) in several crab species, including Callinectes. When the ganglia were processed for FLPli after the backfill protocol, double-labeling was observed in two or three of the B cells (Fig. 2B1, B). These cells correspond to the neurosecretory B cells described by Maynard (1961b) in several crab species, including Callinectes. When the ganglia were processed for FLPli after the backfill protocol, double-labeling was observed in two or three of the B cells (Fig. 2B1, B). These cells correspond to the neurosecretory B cells described by Maynard (1961b) in several crab species, including Callinectes.

**Physiological effects of the FLPs: WH**

The WH preparation was essentially the intact heart, with the cardiac ganglion embedded within it, removed from the crab and perfused but not further dissected. This preparation...
allowed us to record the contractions of the heart under minimally perturbed conditions (Fort et al. 2004) (see methods and Fig. 1, A and B).

Bath application of FLPs had both chronotropic and inotropic effects. In the WH preparation, the effects were always in the positive direction, that is, increases in the frequency and amplitude of the contractions. Figure 3A shows a representative recording with 10^{-6} M CalFLP. At this concentration of CalFLP, the heartbeat often accelerated to very high frequencies, in this example to >70 BPM. Nevertheless, each contraction continued to relax almost completely to the baseline before the next contraction, without development of any appreciable tonic contracture. This was possible because the rate of relaxation of the contractions was increased, so that the contractions decayed considerably more rapidly in the presence of CalFLP than under control conditions. This can be seen in the expanded contractions superimposed in Fig. 3A, right.

Figure 3, B and C, shows dose–response relations for the chronotropic and inotropic effects, respectively, pooled from a number of experiments with F1 (gray diamonds), F2 (dark gray squares), and CalFLP (black circles). All three FLPs were effective, but CalFLP had significantly larger effects than F1 and F2 (for statistical comparison, see Fig. 3 legend), particularly on the contraction frequency at the highest concentration tested, 10^{-6} M, and on the contraction amplitude over the entire concentration range. With all three FLPs, the threshold concentrations for both effects were <10^{-7} M at least in some preparations, and in some cases, particularly with CalFLP on the contraction frequency (see Fig. 3B), even apparently <10^{-11} M, the lowest concentration tested. The concentrations from 10^{-11} to 10^{-6} M were applied to each preparation in increasing order, separated by prolonged washes (see methods). Often, the first exposure of the “naïve” preparation to 10^{-11} M elicited a considerably larger effect than did the subsequent exposure to 10^{-10} M. This suggests that, despite the prolonged wash, some persistent desensitization of the effects may have occurred, and the magnitude of the effects at the higher concentrations may therefore have been underestimated. Very long-lasting desensitization has been reported for FLPs including F1 and F2 in other crustacean neuromuscular systems (Jorge-Rivera and Marder 1996; Skerrett et al. 1995; Worden et al. 1995).

Physiological effects of the FLPs: S-IWH

In the S-IWH preparation, the heart was lightly dissected to allow extracellular recording from one of the connectives through which the axons of the cardiac ganglion motor neurons project to the heart muscle (Fort et al. 2004; see methods and Fig. 1C). In this preparation, it was therefore possible to record simultaneously the heart muscle contractions (Fig. 4A, top traces) and the motor neuron spike patterns that produced them (Fig. 4A, bottom traces).

In the S-IWH preparation, as in the WH preparation, the FLPs increased both the frequency and the amplitude of the contractions (Fig. 4, A–C). The increase in the frequency of
The contractions reflected an increase in the frequency of the motor neuron spike bursts that produced each contraction (Fig. 4A). There were no obvious myogenic contractions, occurring independently of the spike bursts, such as are induced by FLPs in certain other crustacean muscles (Jorge-Rivera and Marder 1996; Meyrand and Marder 1991). As in the WH preparation, all three FLPs were effective, but CalFLP had the largest effects, particularly at 10−6 M, and a threshold for both effects, at least in some preparations, even 10−11 M (Fig. 4, B and C).

The effects of the three FLPs differed qualitatively, however, on two parameters with which we quantified the strength of the individual motor neuron spike bursts, namely the burst duration and the number of spikes per burst. Whereas F1 and F2 significantly increased both of these parameters, CalFLP significantly decreased them (Fig. 4, A, D, and E; for statistical comparison, see legend).

**Dynamic modes**

In many of the experiments described thus far, the FLPs acted in the expected straightforward, smooth manner. In these experiments, the heartbeat was relatively regular, in that the parameters that we measured from each beat—the burst and contraction frequency, contraction amplitude, burst duration, and the number of spikes per burst—had values that remained relatively constant from beat to beat under control conditions. The FLP application then shifted these parameters gradually to new values that again remained relatively constant from beat to beat. This was the case, for example, in the experiment in Fig. 3A. In a number of experiments, however, the time series of these parameters had a more complex structure (Fig. 5). Sometimes the parameters merely varied from beat to beat in an irregular manner that did not exhibit any discernible pattern. At other times, the structure was again regular but at a higher level, exhibiting higher-order repeating patterns or cycles comprised of two, three, four, or more successive beats. As the recording continued, the system switched rather abruptly from one such dynamic “mode” of activity to another. This sometimes occurred spontaneously, but more often as the FLPs were applied, particularly at the higher concentrations, or as they were being washed off.

Figure 5, A and C, shows examples from two different S-IWH preparations. Panels 1–4 expand some of the modes that are present in these recordings for clearer view. In Fig. 5B we have plotted the colored sections of panels 1–3 in the space...
spanned by three of the parameters measured, namely the burst and contraction frequency, the contraction amplitude, and the number of spikes per burst. In this space, a single point repeated in successive beats represents the simplest, constant mode; a larger, irregular spread of points represents an irregular mode; and a regularly repeating cycle of two or more points represent a higher-order cycle of two or more beats. Thus the recording in Fig. 5A began, under control conditions, in a somewhat irregular mode (mode 1, blue). Application of $10^{-6}$ M CaFLP, which greatly accelerated the heartbeat, at the same time regularized it to a simple, constant mode (mode 2, green). Finally, the system switched to a complex, repeating higher-order cycle of 11 beats (mode 3, red).

Under control conditions, all five motor neurons in the cardiac ganglion usually fire in synchrony, so that all of the motor neuron axons carry essentially simultaneous spikes and a single class of compound spikes is recorded extracellularly in the connectives (Fort et al. 2004; Tazaki and Cooke 1979). This continued to be the case in some of the experiments in which complex modes appeared. In other experiments, however, the appearance of complex modes was accompanied by a differentiation of the extracellular spikes into two or more classes of different amplitudes. This can be clearly seen, for example, in Fig. 5A, panel 3. (Such differentiation was sometimes observed even under control conditions, for example in Fig. 4A, left, but much less frequently.) It suggests that at least some of the complex modes probably had their origin in the interaction of the firing patterns of the individual neurons in the cardiac ganglion after those patterns, under the influence of the neuromuscular junctions or the contractility of the heart muscle itself. To examine this, we developed a fourth preparation, the controlled stimulus (CS) preparation (Fig. 8), in which we could control the pattern of the motor neuron spikes reaching the muscle and therefore, by holding the pattern constant during the application of the FLPs, eliminate any contribution of changes in the burst frequency or other parameters of the pattern.

In developing the CS preparation, we took advantage of the known anatomy of the crab cardiac ganglion (Fort et al. 2004; Mirolli et al. 1987; Tazaki and Cooke 1979) (Fig. 1A). The somata of three of the five motor neurons lie at the anterior end of the ganglion and two at the posterior end. The anterior motor neurons project their axons posteriorly through the ganglionic trunk and out to the muscle through the postero lateral connectives, whereas the posterior motor neurons project their axons anteriorly through the ganglionic trunk and out through the anterolateral connectives. In the CS preparation, we cut the ganglionic trunk, removed the anterior part of the ganglion, and stimulated the posterior part with an extracellular suction electrode (Fig. 8A; see METHODS). This delivered spikes to the muscle through the axons of three anterior motor neurons, which were now otherwise silent because their somata and integrative regions had been removed. At the same time, the
two posterior motor neurons, if their somata were still spontaneously firing, could not deliver their spikes to the muscle because their axons had been cut.

In all of the CS experiments presented here, we also inserted an intracellular electrode into a muscle fiber to record the EJPs that were elicited by the stimulation-evoked spikes. In addition to providing quantification of the strength of the transmission at the cardiac neuromuscular junctions, the EJPs served to confirm that our stimulation succeeded in evoking spikes that reached the muscle, and that each brief stimulus pulse evoked one, and only one, spike in each of the motor neuron axons (see METHODS). Although the CS preparation allowed us to deliver any pattern of spikes, in these experiments we used bursting patterns similar to the spontaneous patterns that we recorded in the S-IWH preparations (see Fig. 9 legend), except now completely constant. Figure 8B shows a typical simultaneous recording of the EJPs (middle) and heart muscle contractions (top) elicited by such a constant bursting pattern (bottom).

FIG. 8. Controlled stimulus (CS) preparation with intracellular recording of excitatory junctional potentials (EJPs) in heart muscle. A: schematic illustration of CS preparation. B: typical simultaneous recording of heart muscle contractions (top), EJPs in muscle (middle), and stimuli delivered to posterior ganglionic trunk (bottom) in CS preparation. Stimulation parameters were as in Fig. 9. The first stimulus burst in this recording was preceded by prolonged rest; considerable facilitation of EJPs and contractions over the first several bursts, then followed by a gradual decline, can be seen. Boxed segment is expanded at right.

FIG. 9. Effects of CalFLP in the CS preparation. In all experiments included here, stimulation consisted of regular bursts of 7 stimuli separated by 30 ms, thus with a burst duration of 180 ms and an intraburst frequency of 33.3 Hz. The bursts were separated by 2.53 s, and thus had an overall period of 2.71 s and a burst frequency of 22.2 BPM. All of these parameter values are similar to physiological values typically observed in S-IWH preparations (see Fig. 3 of Fort et al. 2004 and Fig. 11B of Fort et al. 2007, and Fig. 10E here). The stimulation continued without interruption. When the stimulation first started, amplitudes of the contractions elicited by successive bursts tended to increase (see Fig. 8B); when they had stabilized, 10⁻⁶ M CalFLP was applied. A: representative contractions (top), EJPs (middle), and stimuli (bottom) under control conditions (left) and in the presence of 10⁻⁶ M CalFLP (right). In the raw EJP recording, each stimulus typically elicited a brief, stereotyped stimulus artifact; these artifacts were routinely removed by summing them with an inverted copy of the first stimulus artifact in each burst, which occurred before the first EJP had developed. B: expansion of the two segments boxed in A. Control and CalFLP-modulated contractions, and control and CalFLP-modulated EJPs, are superimposed, with their baselines aligned. C: group data summarizing effect of 10⁻⁶ M CalFLP on the area of EJP waveform and peak amplitude of contraction elicited by the stimulus burst. Means ± SE from 5 preparations (in each preparation averaged over >10 consecutive bursts). EJP area was measured by integrating between the EJP waveform and the baseline, between the time of the first stimulus and 90 ms after the last stimulus in the burst. Statistical significance of each effect, i.e., difference from 0 change, was tested with the Mann-Whitney rank sum test: **P < 0.01 and *P < 0.05.
Physiological effects of the FLPs: CS preparation

Figure 9A shows a recording of the heart muscle contractions (top) and EJPs (middle) elicited by the controlled stimulus bursts (bottom) under control conditions (left) and in the presence of $10^{-6}$ M CalFLP (right). The two boxed segments are expanded and superimposed in Fig. 9B. Even though the pattern of the motor neuron spikes reaching the muscle remained completely constant, CalFLP greatly increased the amplitude of the EJPs and contractions that the spikes elicited. Consistent with the previous description of the electrophysiology of crab cardiac muscle fibers (Anderson and Cooke 1971; Benson 1981), in some of these experiments (Fig. 9, A and B) but not others (Fig. 8B), the first EJP of each burst triggered a clear active response, although usually not an overshooting spike, in the muscle, both under control conditions and in the presence of CalFLP. To avoid this complication, we quantified the area of the entire EJP waveform elicited by each stimulus burst (see Fig. 9 legend). Figure 9C shows the group data from five CS preparations treated with $10^{-6}$ M CalFLP. On average, CalFLP increased the area of the EJP waveform by ~40% and the amplitude of the contraction by ~90%, both in a statistically significant manner (see Fig. 9 legend).

Discussion

FLPs act as neurohormones

FLPs have been purified from the POs of several crustacean species (Hymenocera americanus: Kobierski et al. 1987; Trimmer et al. 1987; Procambarus clarkii: Mercier et al. 1993; see also Christie et al. 1995; Macrobrachium rosenbergii: Sithigornkul et al. 1998; Cancer products: Fu et al. 2005), including Callinectes sapidus (Krajnak 1991). Our double-labeling experiments indicate that, in Callinectes, the PO FLPs originate from a cluster of large neurons located in the TGC near the origin of SN1. The neurons of this cluster, the B cells, were ascribed a neurosecretory function by early workers (Matsuno 1958; Maynard 1961a,b) using hematoxylin and aldehyde fuchsia staining protocols. Maynard (1961b) specifically described the B cell cluster associated with SN1 of Callinectes (Bsn1 cells) as a group of “four (possibly five) ellipsoid cells” with a coarsely granular cytoplasm indicative of their neurosecretory function. Our observations (Fig. 2B1) are consistent with his illustrations showing the Bsn1 cells projecting toward the midline of the TGC before branching and sending one branch into the nerve.

Our most definitive double-labeling experiments (e.g., Fig. 2C) suggest that not all FLPli cells in the B cluster project to SN1. This conclusion must remain tentative, however, because the biocytin backfill method is imperfect and may not always label every cell that projects into the nerve. Another question not addressed by our experiments is the possible contribution to the PO FLP fiber system by B cells associated with the segmental nerves of thoracic neurones 2 through 7. Our results do, however, enable us to conclude that in Callinectes the PO FLPs are not colocalized with either dopamine or CCAP, which originate from the ipsilateral L cell (Cooke and Goldstone 1970; Fort et al. 2004) and the contralateral Type 1 cell (Dircksen 1998; Fort et al. 2007), respectively. The modulator complement of the L cell is highly variable across species (see discussion in Fort et al. 2004). In Cancer borealis, there is evidence that the L cell contains FLPs colocalized with serotonin and proctolin (Christie et al. 1995), none of which are thought to be present in the L cell of Callinectes. A thorough comparative study of the L cell structure and function should provide insight into this apparent phenotypic variation of its modulator complement.

Based on the high levels of the FLPs in the pericardial organs, it has been proposed that the FLPs act as blood-borne neurohormones (Christie et al. 1995; Fu et al. 2005; Sithigornkul et al. 1998; reviewed by Mercier et al. 2003). Consistent with this role, they are released from the POs in a calcium-dependent manner (Mercier et al. 1993; Trimmer et al. 1987). Moreover, their presence in the eyestalk of the prawn Macrobrachium rosenbergii (Sithigornkul et al. 1998) suggests that, at least in some species, they are associated with another major neurosecretory system, the X-organ sinus gland (see Cooke and Sullivan 1982). In addition to acting as neurohormones, however, the FLPs can also act locally. In the stomatogastric nervous system of lobsters (Kobierski et al. 1987; Marder et al. 1987) and several crab species (Hooper and Marder 1984; Weinnan et al. 1993), the FLPs are present in projection fibers and are thought to act as local modulators of motor pattern generation. In the crayfish hindgut, the FLPs act as modulatory cotransmitters in motor neurons (Mercier et al. 2003). In this study, in contrast to our previous results with dopamine (Fort et al. 2004), we found no FLPLL in the cardiac ganglion of Callinectes. Similarly, Cruz-Bermúdez et al. (2006) found no FLPli in the CG of several Cancer species. We therefore propose that, unlike dopamine but like CCAP (Fort et al. 2007), the FLPs from the POs act on the heart in a purely neurohormonal fashion.

Blood (hemolymph) concentrations of FLPs that have been measured are of the order of 1–5 $\times$ 10$^{-10}$ M (Kobierski et al. 1987; Mercier et al. 2003), and the heart may experience concentrations higher than this as it lies in close proximity to where the FLPs are released from the POs. The thresholds for the FLP effects that we have found, in some cases <10$^{-11}$ M, are well below those blood concentrations. Thus the effects are capable of being expressed in vivo.

Multiple physiological actions of the FLPs

In Fig. 10, A–D, we have superimposed the dose–response relations from Figs. 3, 4, and 6 for the effects of CalFLP on the four principal parameters that we quantified: the motor neuron burst and contraction frequency (A), contraction amplitude (B), motor neuron burst duration (C), and the number of spikes per motor neuron burst (D), in the WH (black diamonds), S-IWH (dark gray downward triangles), and ICG (gray upward triangles) preparations. Comparison of these dose–response relations, together with the results obtained in the CS preparation (Fig. 9), strongly suggests that the effects do not reflect a single unified action of CalFLP, but rather multiple actions exerted at separate loci in the cardiac system. Figure 11 shows our proposed schema of how CalFLP, and the other FLPs that we tested, act. In this model, the FLPs exert primary actions (arrows 1 and 2) that then have numerous secondary consequences mediated by the intrinsic feedforward and feedback coupling mechanisms that very likely operate in the crab cardiac system (arrows 3–8). All of the arrows in Fig. 11 denote the elementary actions and interactions. The overall
effect on a parameter that is actually observed when the FLPs are applied will arise out of the combination of these elementary interactions. The rest of this section describes this in detail.

Figure 11 is a minimal schema, showing just those mechanisms that appear to be necessary to explain the evidence. Even minimally, however, there must be at least two primary actions of the FLPs.

First, in both the WH and S-IWH preparations, the FLPs greatly increased the amplitude of the heart muscle contractions (Fig. 10B). In the CS preparation, the contractions were increased even when the motor neuron spike patterns that elicited them were held completely constant (Fig. 9). There must therefore be a peripheral action of the FLPs. Furthermore, in the CS preparation, the EJPs underlying the contractions were also increased in amplitude. A parsimonious view is therefore that the FLPs act by potentiating the strength of the transmission at the cardiac neuromuscular junctions (Fig. 11, arrow 1). Such action of FLPs, including F1 and F2, is well established at other crustacean neuromuscular junctions (Jorge-Rivera and Marder 1996; Jorge-Rivera et al. 1998; Mercier et al. 1993; Skerrett et al. 1995; Weiss et al. 2003; Worden et al. 1995; reviewed by Mercier et al. 2003). The specific biophysical mechanisms of action that have been documented in these cases include presynaptic potentiation of transmitter release from the motor neuron terminals, postsynaptic modulation of ion currents in the muscle that shape the EJP waveform, and often indeed both presynaptic and postsynaptic mechanisms in combination. Future work will be needed to show which of these mechanisms mediate the action of the FLPs at the crab cardiac neuromuscular junctions.

Like many other crustacean muscles, the crab heart muscle is, at best, only a weakly spiking muscle (Anderson and Cooke 1971; Benson 1981) (Figs. 8B and 9). In such muscles, contraction occurs when the summated EJP waveform itself, once it exceeds a certain threshold, opens voltage-gated membrane Ca channels and allows extracellular Ca\(^{2+}\) influx that, usually by further Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from intracellular stores, activates the contractile machinery (Palade and Gyorke 1993; Ruegg 1992; Weiss et al. 2001). It is the integrated area of the EJP waveform that determines the amplitude of the contraction (Orkand 1962) (Fig. 11, arrow 3). However, the quantitative transformation from the former to the latter is highly nonlinear (some indication of this can be seen in Fig. 8B) and is not yet known for the crab heart. In Fig. 9C, we found that CalFLP increased the EJP area by \~40\% and the contraction amplitude by \~90\%. It is quite possible...
that the former fully explains the latter. It is also very possible, however, that part of the latter effect reflects additional primary FLP action (not shown in Fig. 11) downstream of the EJP waveform, directly on the Ca\(^{2+}\) handling within the muscle, CICR, or the contractile machinery itself, such as has been suggested for F\(_2\) in lobster heart muscle by Wilkens et al. (2005) (see also Jorge-Rivera and Marder 1996; Mercier et al. 2003; Weiss et al. 2003). The fact that CalFLP increased the rate of relaxation of the contractions (Fig. 3A, right) might support such additional downstream action, although the increased relaxation rate could also be a secondary consequence simply of the increased contraction amplitude.

In the CS preparation, \(10^{-6}\) M CalFLP increased the contraction amplitude by \(-90\%\) (Fig. 9C), but in the WH and S-IWH preparations it increased it by \(-200\%\) (Fig. 10B). It is likely that the additional effect is a secondary consequence of the CalFLP-elicited increase of the burst and contraction frequency in the WH and S-IWH preparations (Fig. 10A), mediated by frequency-dependent facilitation of synaptic transmission at the cardiac neuromuscular junctions (Fig. 11, arrow 4). As in other crustacean hearts (Anderson and Cooke 1971; Mahadevan et al. 2004), in the Callinectes heart there seems to be strong facilitation of EJPs and contractions operating on the time scale of seconds, that is, from one burst to the next (see Fig. 8B, left), although there is on the contrary depression of EJPs when the spikes occur very close together within a burst (Figs. 8B, right, and 9B). The magnitude of the interburst facilitation will be reduced, however, by the CalFLP-elicited decrease of the number of spikes per burst (Figs. 10D and 11, arrow 5).

In addition to their primary action(s) in the periphery, the FLPs must exert at least one primary action centrally on the cardiac ganglion, because they have effects in the ICG preparation where the periphery has been removed. In the ICG preparation, CalFLP substantially increased the motor neuron burst duration and the number of spikes per burst (Fig. 10, C and D), two parameters that generally covary (Figs. 4, D and E, and 6, C and D) (Fort et al. 2004, 2007) and reflect what we may call burst “strength.” Concomitantly, CalFLP slightly decreased the burst frequency at high concentrations (Fig. 10A). For a minimal schema, it suffices to take the former action, on the strength of the bursts, as the sole primary central action (Fig. 11, arrow 2). Because the motor neuron bursts are driven by the bursts of the premotor interneurons (Hartline 1979; Tazaki and Cooke 1979), which were likewise strengthened (Fig. 7), the specific locus of this action may actually lie at the level of the premotor interneurons. The decrease in burst frequency can then be explained as a secondary consequence of the increased burst strength, through the reciprocal negative interaction between burst strength and frequency (Fig. 11, arrow 6) that has been well described in the cardiac ganglia of lobsters and crabs (Benson 1980; Mayeri 1973; Tazaki and Cooke 1990).

In the S-IWH and (as far as could be determined) the WH preparations, however, CalFLP had just the opposite effects to those in the ICG preparation: it did not increase the burst strength but rather decreased it (compare the ICG and S-IWH dose–response relations in Fig. 10, C and D), and it did not decrease the burst frequency but rather increased it (compare the ICG, S-IWH, and WH dose–response relations in Fig. 10A). We suggest that in the S-IWH and WH preparations, where the cardiac ganglion remains coupled to the periphery, feedback from the periphery comes into play. One relevant feedback mechanism has recently been described in the cardiac system of the isopod crustacean Ligia. There, Sakurai and Wilkens (2003) found that passive stretch or active contractions of the heart muscle powerfully alter the timing and frequency of the CG bursts. This seems to be mediated by beat-to-beat modification of the membrane voltage of the CG neurons, most likely through the mechanosensitive dendrites that the neurons extend into the muscle (Sakurai and Wilkens 2003; see Alexandrowicz 1932). During normal physiological operation of the system, the predominant result is that increasing contraction amplitude phase-advances the CG bursts and so increases their frequency. If this occurs in the crab heart (Fig. 11, arrow 7), it may well explain how CalFLP increases the burst and contraction frequency in the S-IWH and WH preparations, where the increase in frequency is always accompanied by an increased contraction amplitude. Through the reciprocal negative interaction between the burst frequency and burst strength, the large increase in burst frequency may then counteract the direct action of CalFLP on the burst strength and actually bring about a net decrease in burst strength, as we observed in the S-IWH preparation (Fig. 4, D and E).

This interpretation can well accommodate, and is even supported by, the differences that we observed between the effects of CalFLP and those of F\(_1\) and F\(_2\) (Figs. 3, 4, and 6). In terms of the schema in Fig. 11, all of the differences can be explained if simply the peripheral primary action 1 of F\(_1\) and F\(_2\) is weaker than that of CalFLP while the central primary action 2 is stronger. In the ICG preparation, the stronger central action means that F\(_1\) and F\(_2\) increase the burst strength more than CalFLP does, and consequently, through the reciprocal negative interaction 6, decrease the burst frequency more, as we observed in Fig. 6, B–D. In the S-IWH and WH preparations, the weaker peripheral action means that F\(_1\) and F\(_2\) increase the contraction amplitude less than CalFLP does, and consequently, through feedback interaction 7, increase the burst frequency less, as we observed in Figs. 3C and 4B. The small increase in frequency does relatively little, through the reciprocal negative interaction 6, to counteract the large primary increase in burst strength, leaving a net increase in burst strength with F\(_1\) and F\(_2\), as opposed to the net decrease with
CalFLP, as we observed in Fig. 4, D and E. If the network operates in this way, it would well illustrate how, in a coupled system of this kind, merely quantitative differences in the magnitudes of one or two primary effects can result in a qualitative difference in the overall response of the system.

Previous studies of actions of FLPs, dopamine, and CCAP in the cardiac system

Large increases in heartbeat frequency and amplitude, much like those we have observed here, were previously reported with F1, F2, CalFLP, and other FLPs in several crustacean hearts, including that of Callinectes (Krajniak 1991; Mercier et al. 1993; Skerrett et al. 1995; Worden et al. 1995; reviewed by Mercier et al. 2003). Those studies, however, examined only the intact hearts, equivalent to our WH preparation. Other studies used only the isolated cardiac ganglion, equivalent to our ICG preparation. Thus Saver et al. (1999) found that F2 only the intact hearts, equivalent to our WH preparation. Other by Mercier et al. 2003). Those studies, however, examined

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Previously, we conducted similar studies with dopamine (Fort et al. 2004) and CCAP (Fort et al. 2007). Overall, the effects of these modulators were similar to those of the FLPs: in the WH and S-IWH preparations, dopamine, CCAP, and the FLPs all increase the heartbeat frequency and amplitude. The schema of the underlying mechanisms that we inferred for dopamine and CCAP was, however, somewhat different from that in Fig. 11. In part this was simply because the same kind of evidence was not available. In particular, although we suspected that dopamine and CCAP, like the FLPs, increased the contraction amplitude by acting on the periphery, we could not show this directly because the CS preparation was not yet implemented. It will be important now to repeat the experiments in Fig. 9 with dopamine and CCAP. Furthermore, in the case of dopamine and CCAP, there seemed to be evidence that these modulators increased the burst and contraction frequency in the WH and S-IWH preparations by a primary action, presumably on the cardiac ganglion, rather than through feedback from the periphery as in Fig. 11. CCAP, in particular, was apparently able to increase the burst and contraction frequency even when it did not change the contraction amplitude (Fort et al. 2007). Finally, although, like the FLPs, dopamine and CCAP failed to increase the burst frequency in the ICG preparation, we interpreted that in a different way. Because the burst frequency was higher in the ICG preparation than in the WH and S-IWH preparations already under control conditions, and dopamine and CCAP increased the frequency in the WH and S-IWH preparations only about that higher level, we interpreted that level, around 30 BPM, as a ceiling frequency above which the CG could not accelerate (Fort et al. 2004, 2007). To test whether this holds also for the FLPs, in Fig. 10E, we have compared the absolute burst frequencies in BPM from the S-IWH experiments in Fig. 4 (dark gray bars) and the ICG experiments in Fig. 6 (gray bars). Although under control conditions the frequency was indeed significantly higher in the ICG than in the S-IWH preparations (for statistical comparison, see legend), F1, F2, and especially CalFLP increased the S-IWH frequency far above the supposed ceiling set by the control ICG frequency (horizontal dashed line), to >80 BPM with 10^-6 M CalFLP. The ICG frequency itself, in contrast, was actually decreased by 10^-6 M CalFLP (with the absolute frequencies in Fig. 10E, unlike the normalized frequencies in Fig. 6B, the decrease did not, however, reach statistical significance). Thus the ceiling explanation is not applicable to the FLPs and may also need reexamination with dopamine and CCAP.

A striking feature of our findings is that the CG behaves very differently in isolation, in the ICG preparation, than when it remains embedded within the heart musculature, in the S-IWH and WH preparations. This is true with respect to the effects of the FLPs (Fig. 10, A–D), those of dopamine and CCAP (Fort et al. 2004, 2007), as well as the frequency and other parameters of the activity even of the unmodulated system (Fig. 10E) (Fort et al. 2004). We have already suggested that this is a consequence of the existence in the intact cardiac system of numerous feedforward and feedback mechanisms that integrate its activity. Within the CG itself, there is the reciprocal negative interaction between burst strength and burst frequency. Then there is the feedback from the peripheral musculature, which is disrupted when the ganglion is isolated. We already mentioned one kind of peripheral feedback, the positive feedback through the mechanosensitive dendrites of the CG neurons embedded in the heart muscle (Sakurai and Wilkens 2003). Most likely, there is also negative feedback from the periphery. In lobster hearts, Mahadevan et al. (2004) and Goy (2005) have proposed that NO produced by the active heart muscle acts on the cardiac ganglion to reduce the burst frequency. We have included this pathway in Fig. 11 for completeness (arrow 8).

One experimental consequence of this high degree of coupling in the system is that different results will be obtained and different conclusions drawn depending on how complete the experimental system is. Indeed, from the point of view of
in vivo biological function, even the intact heart—our WH preparation—is not the complete system. In the whole crab, McGaw et al. (1995) have reported that $F_1$ and $F_2$ do not increase, but rather decrease, the heart rate and cardiac stroke volume, perhaps because, while they act on the heart as we have described here, they also act on the CNS to activate cardioinhibitory pathways. This must be borne in mind when considering the potential biological significance of the effects in this paper.

Although the cardiac system is exceptionally simple in the number of its elements, the interactions among them have the potential to produce complex dynamical behaviors. That these are indeed produced has been suggested previously. Hokkanen (2000), for example, modeled the data of Benson (1980) on the reciprocal interaction between burst strength and burst frequency essentially as a quadratic map. Depending on parameter values, such a model will express different, discrete attractors. It can settle to a steady state, produce cycles of various periods, or exhibit chaotic dynamics. We suggest that the dynamic modes that we have observed here (Fig. 5) have a similar explanation. Some of the modes may originate entirely within the CG, in some cases, judging by the appearance of more than one class of spikes, perhaps through the interaction of the firing patterns of the individual neurons after those patterns have been uncoupled by the FLPs, or indeed dopamine or CCAP (see Fort et al. 2007). Other modes may depend partially or wholly on the feedback from the periphery.

Because the modulators alter the relationships between the elements of the system on which the modes depend, they will perturb the modes. Because the modes are discrete attractors, some modes will rather abruptly appear and others disappear, as we observed. Indeed, we suggest that much of the task of the modulators in regulating the cardiac system will consist of controlling the mode in which the system operates. To reliably and robustly control the mode that results from the interaction of essentially all of the elements of the system, it is our working hypothesis that the modulators must act simultaneously at multiple sites so as to control many of these elements. This is indeed what we have observed with dopamine, CCAP, and the FLPs. In particular, we have observed that the modulators act simultaneously both on the CG and on the peripheral effector musculature, as has been predicted by theoretical studies (Breznia et al. 2000, 2003a,b, 2005; Chiel 1997; Chiel and Beer 1997). To fully understand the modulatory logic in such a system, it is clear that the relatively simple experiments that we performed here will not suffice. However, such experiments provide data essential for generating mathematical models (Stern et al. 2006, 2007), which, we expect, will provide more global insight into the surprising complexity of this “simple” CPG-effector system.

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