Regulation of the Crab Heartbeat by Crustacean Cardioactive Peptide (CCAP): Central and Peripheral Actions

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INTRODUCTION

Vital cyclical, rhythmic behaviors such as breathing, biting and chewing, and various kinds of locomotion are produced by central pattern generator (CPG) networks of neurons that distribute their patterned activity to a set of peripheral effector muscles (for reviews see Friesen and Stent 1978; Marder and Calabrese 1996; Pearl et al. 1993; Stein et al. 1997). The patterned cycling of the CPG ensures a basic stereotyped activity in an integrated manner. We are studying how this happens 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). In this study, we used anatomical and physiological methods to examine the sources and actions on the system of crustacean cardioactive peptide (CCAP). Immunohistochemical localization revealed a plexus of CCAP-immunoreactive fibers in the pericardial organs (POs), neurohemal structures from which bloodborne neurohormones reach the heart. Combined backfill and immunohistochemical experiments indicated that the CCAP in the POs originated from a large contralateral neuron in each thoracic neuromere. In physiological experiments, we examined the actions of exogenous CCAP on the intact working heart, on the semi-intact heart in which we could record the motor patterns as well as the muscle contractions, and on the isolated CG. CCAP had strong positive inotropic and chronotropic effects. In physiological experiments, we examined the actions of exogenous CCAP on the intact working heart, on the semi-intact heart in which we could record the motor patterns as well as the muscle contractions, and on the isolated CG. CCAP had strong positive inotropic and chronotropic effects. Dissection of these effects in terms of dose dependency, time course, and the preparation type in which they occurred suggested that they were produced by the interaction of three primary actions of CCAP exerted both on the heart muscle and on the CG. We conclude that CCAP released from the POs as a neurohormone regulates the crab heart by multiple actions on both the central and peripheral components of this model CPG–effector system.

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multiple effects of dopamine on the physiology of the system. We found that a single pair of modulatory CNS neurons very likely delivers dopamine to the heart in both the cardiac system. We examined the anatomy and physiology of its action on the Callinectes cardiac system, with a view toward generating data and insights that will contribute to an eventual full understanding of the modulatory logic in this simple CPG–effector 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

CCAP-LIKE IMMUNOHISTOCHEMISTRY (CCAPli). Specimens were fixed for 1 h in freshly prepared 4% paraformaldehyde. Standard wholemount immunohistochemical protocols were followed (see Miller et al. 1991 for detailed buffer composition, incubation, and wash procedures). Ganglia were washed (five times, room temperature) in a 1:5,000 dilution of the primary CCAP antibody (rabbit polyclonal). After repeated PTA washes (five times, room temperature) in a 1:5,000 dilution of the primary CCAP antibody, the ganglia 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 (Zeiss Pascal LSM5 laser-scanning confocal microscope. Standard confocal 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 ganglionic mass was pinned out near a small Vaseline well that was formed on the Sylgard surface. The segmental nerve of the first thoracic neurome (SN1) was cut near a small Vaseline well that was formed on the Sylgard surface.

FIG. 1. Crab cardiac system. Single-chamber heart (red) is located within the pericardium (not shown), which is flanked by paired neurohemal structures, the pericardial organs (POs; blue). (POs and the nerves linking the heart with the CNS are shown on one side only.) Cardiac ganglion (CG), containing 4 interneurons (purple) and 5 motor neurons (magenta), lies beneath the dorsal surface of the heart and generates the motor patterns that drive the contractions of the heart muscle. Heartbeat is regulated by neurons within the CNS (black) in two ways. Neurons project through segmental nerves such as SN1 (segmental nerve of the first thoracic neurome, green) to the POs, where they liberate into the pericardial sinus neurohormones that then reach the heart via the blood. In addition, neurons project further through the dorsal nerve (green) to directly innervate the heart and, within it, the CG. Structures not drawn to scale. See INTRODUCTION.
and drawn into the well. The crab saline inside the well was withdrawn and replaced with a 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 then removed and the ganglia were washed three to five times, repinned, and fixed in paraformaldehyde as described earlier. 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 Rhodamine600 Avidin D (Vector Laboratories, Burlingame, CA) diluted 1:3,000 in PTA (24–48 h, room temperature). The ganglia were then washed five times with PTA and the quality of the backfill was assessed before further immunohistochemical processing. In the double-labeling experiments (Fig. 4), CCAPli was visualized using the Alexa 488 goat anti-rabbit secondary antibody (see above). A barrier filter (546-nm green interference) was used to eliminate “bleedthrough” of rhodamine when examining and photographing CCAPli 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. 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 (about 0.5 g). Perfusion with saline was maintained at a constant rate (2 ml/min) and pressure. Perfusion rate and pressure were maintained when CCAP trials were performed.

SEMI-INTACT 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. A small incision was made in the ventral wall of the heart to expose part of the nerve ring containing the motor neuron axons. The ring was cut, usually across one of the posterolateral connectives (see Fig. 1), and the severed end proximal to the ganglion was drawn into an extracellular suction electrode. Motor patterns were recorded with a differential AC amplifier (Model 1700, A-M Systems, Carlsborg, WA) and digitized with a PowerLab (AD-Instruments, Colorado Springs, CO) data-acquisition system (total sampling rate 100 kHz). The heart was then connected to a Grass FT03 isometric force transducer with a hook and nylon thread and placed under a resting load (about 0.5 g). The preparation was continually internally perfused with saline at a constant rate (2 ml/min) and pressure. Perfusion rate and pressure were maintained when CCAP trials were performed.

ISOLATED CARDIAC GANGLION (ICG). The heart was pinned ventral side up in a Sylgard-lined petri dish. 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 from at least one of the four cut ganglionic roots. Intracellular recordings were obtained from anterior and/or posterior motor neurons using microelectrodes filled with 2 M KCl (10–30 MΩ). The preparation was continuously superfused with saline (2 ml/min).

CCAP APPLICATION. In all physiological preparations, CCAP was applied by switching the continuously perfusing saline to CCAP-containing saline, then, after several minutes, back to control saline for wash. The relatively slow rate and large dead volume of the perfusion system meant that the CCAP response developed only 30–60 s after the switch to CCAP-containing solution and washed out over a number of minutes (see, e.g., Figs. 7 and 8, A–C). The preparation was washed for ≥20 min before another CCAP application. Typically a series of increasing CCAP concentrations, each 10-fold higher than the previous, was tested, separated by washes; no cumulative CCAP applications were performed. The dose–response relations in Figs. 6, 9, and 11 were constructed by comparing the parameter values averaged over a 1-min period during the strongest response to each CCAP concentration to control values averaged over a 1-min period immediately before the application of that CCAP concentration. The values were fitted with the equation: % Change = a/[1 + (log [CCAP/EC50])b], where [CCAP] is the CCAP concentration and a, b, and EC50 are parameters of the fit.

RESULTS

Anatomical origins of the cardioregulation by CCAP

First, we used immunohistochemical methods to identify the possible modes and sources of regulation of the Callinectes cardiac system by CCAP (Fig. 2). In agreement with previous observations in Carcinus maenas (Dircksen and Keller 1988), Cancer borealis (Christie et al. 1995), Cancer productus (Fu et al. 2005), and several other arthropods (Dircksen 1998), CCAP-like immunoreactivity (CCAPli) was observed in varicosities and terminals throughout the Callinectes pericardial organs (Fig. 2B). The varicosities, which were located on the surface of all bars and trunks (nomenclature of Alexandrowicz 1953), appeared to originate from fibers that passed through the core of the PO (Fig. 2B, arrows). In contrast to our previous findings with dopamine (Fort et al. 2004), no CCAPli fibers were present in the dorsal nerve that projects from the P0s to innervate the heart and no CCAPli was detected in the cardiac ganglion (four preparations; not shown). These observations suggest that, in contrast to dopamine, regulation of cardiac activity by CCAP is achieved in a purely neurohormonal fashion.

The distribution of CCAPli neurons in the Callinectes nervous system followed the pattern previously observed in other arthropods (reviewed by Dircksen 1998). Symmetric pairs of immunoreactive neurons were located in each of the fused ganglia of the thoracic ganglion complex (TGC) (Fig. 2, A–F). In the anterior neuromeres of the TGC (referred to as the subesophageal or mouthpart ganglia), one neuron in each pair was substantially larger (60–70 μm; arrows in Fig. 2D) than the other (20–30 μm; arrowheads in Fig. 2D). The larger of the two CCAPli neurons (corresponding to the type-1 cell in the nomenclature of Dircksen 1998) was usually positioned slightly posterior to the smaller (type-2) neuron. No consistent differences in their staining intensities were observed. Similar pairs of CCAPli neurons were located in the more posterior (leg) neuromeres (Fig. 2E), but their relative position was more variable, with the smaller neuron frequently being located posterior to the larger. In the most posterior (abdominal) neuromeres, CCAPli neurons also occurred in pairs, but differences in their sizes were less evident (Fig. 2F, arrows). At least one strongly immunoreactive fiber crossed the midline in each neuromere (Fig. 2, C, arrows, E, and F, arrowheads). It was not possible to trace these commissural fibers to their destinations or to their origins.

In decapod crustaceans, neurosecretory cells within the CNS project to the pericardial organs through the segmental nerves...
(SNs) that originate from each neuromere of the TGC (Cooke and Sullivan 1982; Maynard 1961a). To identify potential sources of the CCAPli in the POs, we performed backfills of the first segmental nerve (SN1) of the most anterior TGC neuromere (see Fig. 1). Nerve tracing using biocytin (Fig. 3) revealed a more extensive complement of central neurons projecting into SN1 than was reported previously (Fort et al. 2004; Maynard 1961b). The majority of SN1 fibers originated from two clusters (designated B-cells and C-cells by Maynard 1961b) in the dorsolateral region of the ipsilateral anterior TGC (Fig. 3, A and C). Additional neurons that projected into the ipsilateral SN1 included four neurons near the midline that were aligned in the rostral-caudal direction (Fig. 3D). The most posterior neuron in this group was larger than the others (Fig. 3D, arrow). It was irregularly shaped and gave rise to a very stout axon. Finally, a single neuron in the anterior region of the first ipsilateral leg neuromere was found to project to SN1 (Fig. 3E, arrow). In addition to these neurons, a labeled fiber could be followed the entire length of the TGC (Fig. 3, B, arrow, and E), but it could not be traced to a specific neuron.

In the contralateral anterior TGC, the backfills of SN1 produced staining of two neurons (Fig. 3, A and F). One of
these neurons (Fig. 3F, arrow) was substantially larger than the other. The larger neuron had a prominent axon hillock and fiber coursing in the medial–anterior direction. The fiber originating from the smaller neuron was very thin and followed a more irregular trajectory toward the midline.

To determine the source of the CCAP in the POs, we performed the CCAP immunohistochemistry and the biocytin backfills of SN1 in combination (Fig. 4). No CCAPli was observed in any of the ipsilateral backfilled neurons. However, one of the contralateral neurons that were backfilled in the anterior TGC exhibited double labeling (Fig. 4, A–C, arrows). This was the larger of the two backfilled neurons in Fig. 3F, now revealed to be the most anterior of the type-1 CCAPli cells seen in Fig. 2, C and D. The adjacent type-2 CCAPli cell (arrowheads in Fig. 4, B and C) did not correspond to the smaller contralateral backfilled neuron, although its size and position were similar. These observations agree with findings in other species where the CCAP in the POs was shown to originate from a single contralateral type-1 neurosecretory cell in each thoracic neuromere (Dirksen 1998).

**Physiological effects of CCAP: working heart and semi-intact working heart**

In our previous work in the crab cardiac system, we developed two “working heart” preparations (Fort et al. 2004; see Methods). The basic working heart (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 heartbeat under minimally perturbed conditions. In the semi-intact working heart (S-IWH) preparation, the heart was lightly dissected to allow us to record not only the heart muscle contractions (e.g., top traces of each panel in Fig. 5), but also extracellularly from one of the connectives through which the axons of the cardiac ganglion motor neurons project to the muscle, the underlying motor neuron spike patterns (bottom traces of each panel in Fig. 5). When in the previous work we systematically compared the two preparations, we found that the S-IWH preparation retained, as far as could be determined, all of the principal properties of the intact WH (Fort et al. 2004).

**FIG. 5.** Physiological effects of CCAP in the semi-intact working heart (S-IWH) preparation. A, I and 2: representative heart muscle contractions (top traces) and simultaneous sections of extracellular recording from a posterolateral connective (bottom traces), under control conditions (A1) and in the presence of $10^{-9}$ M CCAP (A2). A3: expanded overlay of the contractions and spike bursts boxed in A1 and A2. B: as in $A$, in the same preparation, but with $10^{-7}$ M CCAP.

Effects of CCAP, too, were essentially the same in both preparations (see, e.g., the statistical comparison of the dose–response relations in Fig. 11). We will therefore discuss both preparations jointly, focusing however primarily on the S-IWH preparation that provided more complete information.

Representative recordings of the effects of CCAP in the S-IWH preparation are shown in Fig. 5 and summary dose–response relations pooled from all S-IWH preparations are shown in Fig. 6. Bath application of increasing concentrations of CCAP had both inotropic and chronotropic effects, generally in the positive direction: increases in the amplitude and frequency of the heart contractions. The increase in the frequency of the contractions (top traces in Fig. 5) presumably reflected the increase in the frequency of the motor neuron spike bursts that produced each contraction (bottom traces in Fig. 5). At no CCAP concentration, however, was there any significant change in the duration of the spike bursts or in the number of spikes per burst (Fig. 6, C and D; for statistical analysis see legend).

**Dissociation of the inotropic and chronotropic effects**

The increase in contraction amplitude was observed at lower CCAP concentrations than the increase in burst and contraction
frequency. In the summary dose–response relations, the apparent threshold for the CCAP effect on contraction amplitude was between $10^{-10}$ and $10^{-9}$ M (Fig. 6A), but for the effect on burst and contraction frequency it was between $10^{-8}$ and $10^{-7}$ M, two orders of magnitude higher (Fig. 6B). The dose–response relation for the latter effect then rose steeply, however, reaching near-maximal values within approximately another two orders of magnitude (Fig. 6B). Tests of statistical significance carried out on the summary dose–response relations (see Fig. 6 legend) showed that the effect of CCAP on contraction amplitude was statistically significant (as indicated by the asterisks in Fig. 6) already at $10^{-9}$ M (and at all higher concentrations), but on the burst and contraction frequency only at $10^{-7}$ M (and higher concentrations).

In the summary dose–response relations, however, the CCAP effects were already normalized and averaged across multiple preparations whose raw values might have been quite different from each other. We therefore sought to confirm that the dissociation seen in the averaged dose–response relations could also be observed in the raw values measured at the same time in the same preparation. We performed experiments in which we directly compared the effects of a low ($10^{-9}$ M) and a high ($10^{-5}$ M) CCAP concentration in the same S-IWH preparation. Figure 5 is drawn from one of these experiments. At $10^{-9}$ M, CCAP had essentially no effect on the burst and contraction frequency, the burst duration, or the number of spikes per burst, yet it clearly increased the contraction amplitude (Fig. 5A; the control and CCAP bursts and contractions boxed in A1 and A2, respectively, are superimposed in A3 for direct comparison). At $10^{-5}$ M, CCAP increased the contraction amplitude considerably more and also increased the frequency of the bursts and contractions, but still had no effect on (or even decreased) the burst duration and the number of spikes per burst (Fig. 5B). In a comparison of 33 control heartbeats and 39 beats with $10^{-9}$ M CCAP in this preparation, there was a significant increase in contraction amplitude (control (mean ± SE) 1.44 ± 0.01 g, CCAP 2.00 ± 0.03 g, increase by 39%, $P < 0.001$, Mann–Whitney rank-sum test) but no significant change in burst and contraction frequency [control 25.37 ± 0.20 bursts and heartbeats per minute (BPM), CCAP 25.92 ± 0.15 BPM, $P > 0.05$], burst duration (control 104.33 ± 3.95 ms, CCAP 108.79 ± 3.42 ms, $P > 0.05$), or the number of spikes per burst (control 5.33 ± 0.13, CCAP 5.49 ± 0.10, $P > 0.05$). In a comparison of 36 control heartbeats and 68 beats with $10^{-5}$ M CCAP, there was an even larger increase in contraction amplitude (control 0.60 ± 0.010 g, CCAP 1.32 ± 0.010 g, increase by 120%, $P < 0.001$), now with a significant increase in the burst and contraction frequency (control 28.46 ± 0.16, CCAP 41.11 ± 0.25 BPM, increase by 44%, $P < 0.001$), but still no significant change in the burst duration (control 55.82 ± 3.40 ms, CCAP 55.85 ± 1.04 ms, $P > 0.05$) and a slight decrease in the number of spikes per burst (control 5.36 ± 0.08, CCAP 4.91 ± 0.03, decrease by 8%, $P < 0.001$). Such a decrease in the number of spikes per burst would have tended to decrease, rather than increase, the contraction amplitude (see, e.g., Fig. 8D).

The inotropic and chronotropic effects of CCAP could also be dissociated in the temporal dimension. Figure 7, A and B, shows the time course of the effects of CCAP on contraction amplitude (red) and frequency (blue) at $10^{-8}$ M (A) and $10^{-6}$ M (B) in a representative WH preparation. When the CCAP was applied, it increased the contraction amplitude similarly at both concentrations, either without (at $10^{-8}$ M) or with (at $10^{-6}$ M) a concomitant increase in the contraction frequency. The onset of both effects was rapid, within several heartbeats, and was almost certainly limited by the speed of access of the CCAP to the tissue. Subsequently, although the contraction amplitude remained elevated, the contraction frequency progressively declined to values even lower than the initial control frequency. The effects on both amplitude and frequency reversed when the CCAP was washed out. Figure 7C shows the averaged time courses from six such experiments, three in WH preparations and three in S-IWH preparations, with $10^{-6}$ M CCAP. The dissociation was robust across the six preparations.
the contraction frequency (blue) declined relatively rapidly to values even lower than the initial control frequency, whereas the contraction amplitude (red) declined much more slowly; the difference was statistically significant (see Fig. 7C legend).

Dynamic modes

The results described to this point might give the impression that the heartbeat was regular: that the parameters with which each beat could be described, such as the contraction amplitude, burst and contraction frequency, burst duration, and the number of spikes per burst, had values that remained relatively constant from beat to beat under control conditions and, then, on application of CCAP, shifted to new values that again remained relatively constant from beat to beat. This was often the case. Frequently, however, the time series of these parameters had a more complex structure (Fig. 8). Sometimes the parameters merely varied from beat to beat in an irregular manner that did not exhibit any discernible pattern. At other times, however, the structure was again regular but at a higher level, exhibiting higher-order repeating patterns or cycles consisting 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 CCAP was applied or as it was being washed off.

Figure 8, A–C shows examples from three different S-IWH preparations. The vertical dashed lines mark the times of the apparent switches between modes. In Fig. 8A, the system switched from an irregular mode to the simplest regular, constant mode. In Fig. 8B, a similar switch occurred, although in this case the initial “irregular” mode in fact exhibited some nonrandom alternation between beats with weaker spike bursts and smaller contractions and beats with stronger bursts and larger contractions.

In Fig. 8C, the system switched between three quite distinct modes. In Fig. 8E we have plotted sections of the time series from Fig. 8C in the space spanned by the three parameters measured: the contraction amplitude, the burst and contraction frequency, and the number of spikes per burst. In this space, a single point repeated in successive beats represents the simplest, constant mode, whereas regularly repeating cycles of two, three, four, or more points represent higher-order cycles of two, three, four, or more beats. Thus Fig. 8E shows how the system in Fig. 8C progressed, when CCAP was applied, successively from the simplest, constant mode (blue) to a higher-order cycle of five beats (green), and finally to a higher-order cycle of four beats (red).
Regularization of dynamic activity by neuromodulators was recently noted in other systems (e.g., Serrano and Miller 2006; Szücs et al. 2005). In the mode switches seen here, too, CCAP often made the activity more regular and constant from beat to beat (as in Fig. 8, A and B), although sometimes the opposite occurred (as in Fig. 8C). These phenomena were seen not only in S-IWH preparations, but in WH preparations as well. In Fig. 7, A and B, for example, the system appeared to switch, when either concentration of CCAP was applied, from an irregular mode to the simplest regular, constant mode and, then, as the CCAP was washed off, through a brief interval of higher-order cycles of two or four beats (arrows in Fig. 7A) back to a more irregular mode.

In the more irregular modes, the beat-to-beat variation of the parameter values constituted natural experiments in which the relationships between the parameters could be examined. For example, Fig. 8D plots, from all of the beats boxed in Fig. 8B, the contraction amplitude as a function of the number of spikes per burst. The beats varied only in these two parameters; the third parameter measured—the burst and contraction frequency—remained constant. Under these circumstances, the two varying parameters were highly correlated on a beat-to-beat basis. As the number of spikes per burst varied over a nearly fourfold range, apparently randomly from one beat to the next, so did the contraction amplitude. The linear regression fit (solid line in Fig. 8D) had a coefficient of determination $R^2 = 0.96$.

Physiological effects of CCAP: isolated cardiac ganglion

In the isolated cardiac ganglion (ICG) preparation, the cardiac ganglion was dissected completely free of the heart musculature (Fort et al. 2004; see METHODS). The motor neuron spike bursts could then be recorded extracellularly as in the S-IWH preparation (e.g., bottom traces in Fig. 9, A1 and A2) and simultaneously with intracellular voltage recording from the motor neuron cell bodies (top traces in Fig. 9, A1 and A2).

Figure 9A is a representative recording of the effects of CCAP, and Fig. 9, B–D shows summary dose–response relations for the frequency of the motor neuron bursts, the burst duration, and the number of spikes per burst. CCAP had no effect on the burst frequency at any concentration $\leq 10^{-6}$ M (Fig. 9B). It did, however, increase both the burst duration and the number of spikes per burst, at concentrations beginning around $10^{-7}$ M in a statistically significant manner (see Fig. 9 legend and asterisks in Fig. 9, C and D).

The activity of the motor neurons is driven by activity of the other class of neurons in the cardiac ganglion: the four local premotor interneurons. Firing of the interneurons elicits excitatory postsynaptic potentials (EPSPs) in the motor neurons (Miorilli et al. 1987; Tazaki and Cooke 1979, 1983a,b). We did not record directly from the interneurons, but we inferred their spike bursts from the bursts of EPSPs that we could record intracellularly in the motor neuron somata (Fig. 10). The interneuron bursts (e.g., as indicated by the gray bars in Fig. 10B1) were longer, beginning earlier and ending later, than the corresponding motor neuron bursts (black bars in Fig. 10B1) at $10^{-6}$ M, CCAP significantly increased the duration of the interneuron bursts (dashed lines in Fig. 10A1; for statistics see Fig. 10A2 and legend). Because the frequency of the bursts was not significantly changed by CCAP (Fig. 9B), the increased burst duration resulted in a significantly increased duty cycle, defined as the fraction of the period occupied by the burst (Fig. 10B1; for statistics see Fig. 10B2 and legend). CCAP appeared to hyperpolarize the membrane potential of the motor neurons, but only by a modest 2–3 mV (Fig. 10A1), and had no noticeable effect on the motor neuron spike generation threshold. Together, these observations suggest an action of CCAP upstream of the motor neurons, at the level of the premotor interneurons.
DISCUSSION

CCAP acts as a neurohormone

Using immunohistochemistry, we found a characteristic pattern of CCAPli neurons, a larger type-1 cell and a smaller type-2 cell, in each neuromere of the thoracic ganglion of Callinectes. This pattern is consistent with observations in other arthropods examined to date (Dircksen 1998; Dircksen and Keller 1988; Pulver and Marder 2002). The type-1 cell in each neuromere typically gives rise to a fiber that crosses the midline and projects to a contralateral neurohemal structure where it terminates (Dircksen 1998). Projections from the thoracic ganglia of Callinectes extend into the abdomen and undecided regions (Dircksen 1998; Dircksen and Keller 1988). It is possible that the type-1 cells have functions beyond the regulation of cardiac output, but this remains to be determined.

FIG. 9. Physiological effects of CCAP on the activity of the motor neurons in the isolated cardiac ganglion (ICG) preparation. A: representative sections of intracellular voltage recording from a CG motor neuron (top trace) and simultaneous extracellular recording from one of the posteralateral ganglionic roots (bottom trace), under control conditions (A1) and in the presence of 10^-6 M CCAP (A2). B–D: dose–response relations for the effects of CCAP on burst frequency (B), burst duration (C), and the number of spikes per burst (D). Means ± SE from 5 to 7 preparations. Smooth curves are best fits of the data values with a sigmoidal function (see METHODS). Statistical significance of each effect, i.e., the difference from zero of the values in B–D, overall over the entire dose–response relation and then at each individual CCAP concentration, was tested as in Fig. 6 using 2-way repeated-measures ANOVA followed by Holm–Sidak multiple comparisons tests. Overall, the effects in C and D were statistically significant (P < 0.001) but that in B was not (P > 0.05). For the individual concentrations in C and D: ***P < 0.001.

FIG. 10. Effect of CCAP on the activity of the premotor interneurons in the ICG preparation. A1: representative intracellular voltage recording from an anterior motor neuron under control conditions, in the presence of 10^-6 M CCAP, and after a 30-min wash. Bottom traces show the base of the top traces at higher gain (spikes truncated) to facilitate measurement of the durations of the bursts of excitatory postsynaptic potentials (EPSPs, dashed arrows) that reflect the bursts of spikes of the premotor interneurons. Dotted line marks -50 mV and shows that the motor neuron was hyperpolarized by about 2 mV in the presence of CCAP. A2: means ± SE of the durations of the EPSP bursts, and so premotor interneuron spike bursts, measured from 5 preparations (in each preparation averaged over 10 consecutive bursts) as in A1. Statistical significance was tested with Student’s t-test: ***P < 0.001. B1: as in A1, but showing several consecutive bursts under control conditions and in the presence of 10^-6 M CCAP. Black bars indicate the durations of the motor neuron spike bursts and the gray bars the durations of the EPSP and so premotor interneuron spike bursts. Duty cycle was measured as the duration of the latter bursts as fraction of the period. B2: means ± SE of the duty cycle measured from 5 preparations. Statistical significance was tested with Student’s t-test: **P < 0.01.
type-2 cells remain within the CNS. Combining the immunohistochemistry with backfills of SN1, the segmental nerve of the first thoracic neuromere, we confirmed that the type-1 cell, but not the type-2 cell, of the first thoracic neuromere projects to the contralateral pericardial organ. It is thus likely that the entire plexus of CCAPli terminals in the POs derives from a small number of CCAP-expressing central neurons, one type-1 cell per thoracic neuromere.

Dopaminergic terminals in the POs similarly derive from a small number of central neurons, indeed just a single neuron—the L-cell—in each commissural ganglion (Cooke and Goldstone 1970; Fort et al. 2004; Wood and Derby 1996). After arborizing in the PO, however, each L-cell projects further, through the dorsal nerve, directly into the heart to innervate the cardiac ganglion (Fort et al. 2004). We found no such further projection of the CCAPli neurons and detected no CCAPli in the CG. We therefore propose that, whereas dopamine reaches the heart both as a blood-borne neurohormone liberated from the POs and a local neuromodulator released within the CG itself, CCAP acts on the heart in a purely neurohormonal fashion.

If CCAP acts solely as a circulating hormone, the entire cardiac system will be exposed to essentially the same concentration, i.e., that present in the blood (hemolymph) in the region of the heart. How high is this concentration likely to be? Stangier et al. (1988) quantified the CCAP contents of the Carcinus maenas POs and calculated that release of <1% of the total could achieve bulk hemolymph thresholds for cardioactivity (10⁻¹⁰ M) in that species. To date, the most comprehensive measurements of CCAP concentrations in crustacean hemolymph were made by enzyme immunoassay in a study examining the role of CCAP in ecdysis of the crab Carcinus maenas and the crayfish Orconectes limosus (Phlippen et al. 2000). In Carcinus, a peak concentration of 1.4 × 10⁻⁹ M was measured during active ecdysis, when the crab extricates itself from its former carapace. Several considerations, however, suggest that the heart will be exposed to substantially higher levels of CCAP at some point during the exuviation process: 1) hormones released from the POs are initially concentrated around the heart in the restricted volume of the pericardial sinus; 2) the hemolymph samples of Phlippen et al. (2000) were drawn from the hypobranchial sinus, a return vessel that is essentially the furthest point from the heart in the crab circulatory system (see McGaw and Reiber 2002); and 3) the half-life of CCAP in the hemolymph was shown to be very brief, about 6 min (Phlippen et al. 2000), making it difficult to sample the actual peak concentration. We suggest therefore that, although tonically the heart probably experiences only relatively low levels of circulating CCAP, transiently it may experience much higher levels.

Multiple physiological actions of CCAP

In Fig. 11 we have superimposed the CCAP dose–response relations from Figs. 6 and 9, and corresponding dose–response relations in the WH preparation, for the four principal CCAP effects that we quantified, on the contraction amplitude (A), motor neuron burst and contraction frequency (B), motor neuron burst duration (C), and the number of spikes per motor neuron burst (D), in the WH (red), S-IWH (green), and ICG (blue) preparations. Comparison of these dose–response relations strongly suggests that the effects do not reflect a single unified action of CCAP, but rather distinct actions exerted at separate loci in the cardiac system. Our view of how CCAP acts is summarized in the schema in Fig. 12: as the CCAP concentration increases (increasing density of background shading from left to right), different parts of the system are progressively modulated, with progressive changes in the contraction waveform (bottom).

First, at the lowest concentrations (10⁻¹⁰ to 10⁻⁸ M) in the WH and S-IWH preparations, CCAP modestly but significantly increases the contraction amplitude (Fig. 11A). This occurs without any change in the burst and contraction frequency, burst duration, or the number of spikes per burst, either
in the pooled dose–response relations (Fig. 11, B–D) or when all of the parameters are examined simultaneously in individual heartbeats (Fig. 5A). We thus propose that the increase in contraction amplitude reflects an action of CCAP on the peripheral heart musculature (middle of Fig. 12). If so, then CCAP should be able to increase the amplitude even of contractions elicited by completely controlled, invariant stimulation of the motor neuron axons in the connectives leading from the CG to the heart muscle. It will be important to test this prediction in future experiments. The detailed mechanism of this peripheral action of CCAP could include potentiation of transmission at the cardiac neuromuscular junctions or an increase in contractility of the heart muscle itself.

Second, at somewhat higher concentrations (>10⁻⁸ M) in the WH and S-IWH preparations, CCAP increases the burst and contraction frequency (Fig. 11B). In our experiments, we observed no dissociation of bursts and contractions, such as might be caused, for example, by CCAP-induced myogenic rhythmicity (see Meyrand and Marder 1991). Because the bursts drive the contractions, we propose that CCAP accelerates the bursts by acting centrally on the CG (Fig. 12, right). An alternative possibility is that the increase in burst and so contraction frequency is a feedback consequence of the peripheral increase by CCAP of the contraction amplitude (see Sakurai and Wilkens 2003; and below). However, the effects of CCAP on amplitude and frequency can be dissociated. At the low CCAP concentrations and also with longer exposure to the higher concentrations (Fig. 7), the increase in amplitude occurs without the increase in frequency. Thus increased amplitude does not automatically produce increased frequency. We suggest, rather, that CCAP increases the frequency by acting centrally, on the CG. Within the CG, CCAP most plausibly acts on the premotor interneurons that initiate each burst (Hartline 1979; Tazaki and Cooke 1979).

A potential difficulty in locating this second action of CCAP in the CG is that the increase in burst frequency is not seen at all in the ICG preparation (Fig. 11B, blue). (This, indeed, strengthens the case for the peripheral feedback mechanism.) There is, however, a likely straightforward explanation. The burst frequency in the ICG preparation is significantly higher than that in the S-IWH (or, presumably, the WH) preparation. Indeed, the frequency in the ICG preparation under control conditions is about as high as that in the S-IWH preparation modulated by 10⁻⁶ M CCAP (see Fig. 11B, insets). This maximal frequency, around 30 BPM, may thus constitute a ceiling above which the cardiac ganglion cannot accelerate (see also Fort et al. 2004).

The increase in burst and contraction frequencies at CCAP concentrations >10⁻⁸ M is accompanied by a further large increase in contraction amplitude (Figs. 5B and 11A). In part this presumably reflects the more fully developed peripheral action of CCAP. Some part of the increase in contraction amplitude, however, is likely to be a consequence of the increased burst and contraction frequency (see Mahadevan et al. 2004; and below), arising, at least in part, from frequency-dependent facilitation of synaptic transmission at the cardiac neuromuscular junctions (Anderson and Cooke 1971; Mahadevan et al. 2004).

There is still a third primary action of CCAP. At concentrations >10⁻⁸ M, CCAP increases the burst duration and the number of spikes per burst (Fig. 11, C and D) in the ICG preparation (blue), but interestingly not in the S-IWH preparation (green). Because it is seen in the isolated ganglion, this is necessarily a central action of CCAP. The increase in burst duration, at least, is seen not only in the motor neurons but also upstream in the premotor interneurons (Fig. 10), where the locus of this action of CCAP may therefore lie. It is a separate primary action: it is not a consequence of the increase in burst frequency, which does not occur in the ICG preparation. Nor, conversely, is it a cause of the increase in burst frequency because it does not occur in the S-IWH preparation where the increase in burst frequency is robust.

Comparison with dopamine

In our previous work (Fort et al. 2004), we observed essentially the same constellation of effects with dopamine. With dopamine, however, the increase in contraction amplitude occurred only at higher concentrations (>10⁻⁸ M), those that also increased the contraction frequency. Consequently it was not clear whether the increase in amplitude reflected a separate primary action of dopamine or merely a secondary consequence, through the mechanism of frequency-dependent synaptic facilitation, of the increase in frequency. With CCAP, the increase in frequency similarly occurs at >10⁻⁸ M, but the increase in amplitude already occurs at >10⁻¹⁰ M (Fig. 6, A and B). Thus with CCAP, the increase in amplitude clearly can occur without the increase in frequency. Functionally, as the cardiac system becomes exposed to increasing concentrations of the modulator, CCAP will initially increase the contraction amplitude and only later increase the contraction frequency (Fig. 12), whereas dopamine will have rather the reverse effect.
These differences in the sequential expression of the two effects will be enhanced by the different concentration profiles that are likely to be achieved by the different modes of delivery of the two modulators. Any activity of the L-cell that releases hormonal dopamine from the POs will also release dopamine in the CG to achieve (presumably) higher local concentrations there and so bring about a coordinated, simultaneous expression of all of the effects of dopamine. The effects of CCAP, in contrast, will probably be expressed differentially and in a graded fashion. Low activity of the CCAP-containing neurons will sufficiently elevate hormonal levels of CCAP to express just the peripheral action to increase contraction amplitude. Higher activity of the neurons, higher levels of CCAP, and the added expression of the central action on contraction frequency may occur only transiently during periods of intense demand for increased cardiac output in behaviors such as ecdysis.

Dopamine and CCAP thus converge on a common set of effects, but they activate them in a different balance. This is a common theme in neuromodulation (Brezina and Weiss 1997; Nusbaum et al. 2001), well documented for example in the crustacean stomatogastric system (Marder and Thirumalai 2001), well documented for example in the common theme in neuromodulation (Brezina and Weiss 1997; Nusbaum et al. 2001), well documented for example in the crustacean stomatogastric system (Marder and Thirumalai 2001; Skiebe 2001) and in the accessory radula closer neuromuscular system of *Aplysia*, where it was proposed that the different balance of effects of convergent, simultaneously acting modulators serves to maximize the range of responses of the system to diverse behavioral demands (Brezina et al. 1996, 2003a, 2005).

**Modulation of the integrated CPG–effector system**

A striking feature of our findings is that the cardiac ganglion behaves 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 CCAP (Fig. 11), the effects of dopamine (Fort et al. 2004), and the frequency and other parameters of the activity even of the unmodulated system (Fig. 11B, insets; Fort et al. 2004). We propose that this is because the intact system incorporates multiple feedforward and feedback mechanisms that integrate its activity (as schematized by the arrows in Fig. 12), which are disrupted when the ganglion is isolated. One feedforward mechanism—the facilitation at the cardiac neuromuscular junctions that increases contraction amplitude as burst frequency increases (Anderson and Cooke 1971; Mahadevan et al. 2004)—was in previous mentioned text. There are several additional mechanisms.

First, there are local mechanisms operating within the cardiac ganglion itself. In particular, the burst duration and the number of spikes per burst are often found to decrease as the burst frequency increases (Tazaki and Cooke 1990; but see Benson 1980; Hokkanen 2000). We propose that this mechanism may explain why, when CCAP acts to increase the burst duration and the number of spikes per burst, a net effect of CCAP on these two parameters is seen in the ICG preparation (Fig. 11, C and D, green) but not in the S-IWH preparation (blue). In the S-IWH preparation, unlike in the ICG preparation, CCAP also increases the burst frequency, which will tend to decrease the spike duration and the number of spikes per burst, oppose the increasing action of CCAP, and keep these two parameters approximately constant.

Second, two recent studies suggested that there are feedback mechanisms by which the state of the heart muscle can modify the parameters of the motor pattern produced by the cardiac ganglion. In the isopod *Ligia*, Sakurai and Wilkens (2003) showed that passive stretch or active contractions of the muscle alter the timing and frequency of the CG bursts. This appears 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 heart muscle (Sakurai and Wilkens 2003; see Alexandrowicz 1932). In lobster hearts, Mahadevan et al. (2004) and Goy (2005) proposed that nitric oxide (NO) produced by the active heart muscle acts to reduce the CG burst frequency. The presence of high levels of NO synthase in the heart of *Cancer productus* and the sensitivity to NO of neurons in the Cancer CG (Scholz et al. 2002) suggest that the NO-mediated feedback may also operate in the cardiac systems of crabs. If such feedback mechanisms do indeed operate in *Callinectes*, they might explain two of our findings in this study. Disruption of negative feedback might explain why the cardiac ganglion bursts faster in isolation than in the intact heart (Fig. 11B, insets). Negative feedback could also be responsible for the secondary decrease in contraction frequency during a maintained CCAP-induced elevation of contraction amplitude (Fig. 7). The gradual time course of this decrease makes it more likely to be mediated by a diffusible messenger such as NO than by direct mechanosensitivity.

**Dynamic modes and system complexity**

The crustacean cardiac system is one of the simplest CPG–effector systems. Nevertheless, it is clear from the preceding discussion that it has a sufficient number of interacting elements to produce complex dynamical behaviors. That these are indeed produced was suggested previously. For example, studying just the unmodulated cardiac ganglion—not even the complete system—Benson (1980) provided experimental data on the relationship between the duration of a spike burst and the following interburst interval and, conversely, between an interburst interval and the duration of the following burst, and so between one interburst interval and the next. Hokkanen (2000) then modeled these data 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 have a similar explanation.

Because 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 modulators such as CCAP and dopamine 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, as we have indeed observed with both dopamine and CCAP. Clearly, to fully understand such a modulatory logic, the relatively simple experiments that
we performed here will not suffice. However, such experiments provide data essential for generating mathematical models (see Stern et al. 2006a,b), which, we expect, will provide more global insight into the complexity of this central pattern generator–effector system.

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