In Situ and In Vitro Identification and Characterization of Cardiac Ganglion Neurons in the Crab, *Carcinus maenas*

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Saver, Michelle A., Jerrel L. Wilkens, and Naweed I. Syed. In situ and in vitro identification and characterization of cardiac ganglion neurons in the crab, *Carcinus maenas*. J. Neurophysiol. 81: 2964–2976, 1999. The aim of this study was to investigate the intrinsic membrane properties and hormonal responses of individual central pattern generating neurons in the cardiac ganglion of the shore crab *Carcinus maenas*. Because the cardiac ganglion in this crustacean species is buried within the heart musculature and is therefore inaccessible for direct morphological and electrophysiological analysis, we developed two novel in vitro preparations. First, to make the ganglion accessible, we established a brief enzymatic treatment procedure that enabled us to isolate the entire cardiac ganglion, in the absence of muscle tissue. Second, a cell culture procedure was developed to isolate individual neurons in vitro. With the use of both isolated ganglionic and neuronal cell culture techniques, this study provides the first direct account of the neuroanatomy of the cardiac ganglion in shore crabs. We demonstrate that cultured neurons not only survived the isolation procedures, but that they also maintained their intrinsic membrane and transmitter response properties, similar to those seen in the intact ganglion. Specifically, we tested the peptides proctolin, crustacean cardioactive peptide, the FLRFamide-related peptide P2, and an amine (serotonin) on both isolated ganglion and in vitro culture neurons. We measured changes in neuronal burst rate, burst amplitude, pacemaker slope, and membrane potential oscillation amplitude in response to the above four hormones. Each hormone either increased neuronal activity in spontaneously bursting neurons, or induced a bursting pattern in quiescent cells. The in vitro cell culture system developed here now provides us with an excellent opportunity to elucidate cellular, synaptic and hormonal mechanisms by which cardiac activity is generated in shore crabs.

INTRODUCTION

Rhythmic behaviors such as respiration (Bullock and Syed 1992; DiCaprio and Fourtner 1984, 1988; Funk and Feldman 1995), locomotion (Getting 1989; Grillner et al. 1995; Kiehn 1991), feeding (Willows 1980), and heartbeat (Arbas and Calabrese 1987; Hartline 1979 for review) are controlled by networks of neurons, called central pattern generators (CPGs) (see Delcomyn 1980; Getting 1989; Kristan 1980; Pearson 1985, 1993). Identification of various CPG neurons and characterization of their intrinsic membrane and synaptic properties is critical for our understanding of the cellular basis of most rhythmic behaviors. A variety of vertebrate (Funk and Feldman 1995; Grillner et al. 1995; Ramirez and Richter 1996) and invertebrate (Calabrese et al. 1989; Harris-Warrick and Flamm 1987; Kristan 1980; Marder 1987; Selverston 1987; Syed et al. 1990, 1992; Turrigiano and Marder 1993) species have been used in the past to understand how CPG neurons initiate, maintain, terminate, and modulate various rhythmic behaviors. In the vast majority of preparations studied to date, however, the precise identity, number, and nature of synaptic connections between CPG neurons have not been fully deduced. This is due to the fact that in most cases a large number of CPG neurons are involved in any given behavior, and their synaptic connections are often complex and difficult to resolve in the intact brain.

The cardiac ganglion (CG) located in the decapod crustacean heart, on the other hand, offers a simpler preparation where a nine-celled CPG is located at some distance from the CNS. Moreover, the neuronal somata are large and physically distant from one another. The decapod CG usually consists of four pacemaker cells (small cells, SCs) and five motor neurons (large cells, LCs) (reviewed in Hartline 1979). The isolated CG reliably produces bursts of impulses in the absence of synaptic activity from the CNS (Welsh and Maynard 1951), and therefore offers an excellent opportunity to investigate the intrinsic membrane and synaptic properties of this rather simple CPG network both in crabs (*Portunus sanguinolentus*) (Tazaki and Cooke 1979a–c) and lobsters (*Homarus americanus*) (Berlind 1985, 1989; Tazaki and Cooke 1986). In these larger crustaceans, the CG is easily discernible under a dissection microscope and can be manually exposed by teasing away the heart muscle tissue surrounding the ganglion (Tazaki and Cooke 1979a). To examine the intrinsic properties of individual ganglionic cells, previous investigators either ligatured (Tazaki and Cooke 1983b), or transected the ganglion to separate neurons from one another within the ganglion (Sullivan and Miller 1984). Alternatively, groups of neurons were pharmacologically isolated by creating a two-pool system in which neurons in each pool were bathed independently (Berlind 1985, 1989; Sullivan and Miller 1984; Tazaki and Cooke 1986). In these studies, both SCs and LCs were found to generate driver potentials, which underlie spontaneous bursting activity in the network (Berlind 1985, 1989; Tazaki and Cooke 1979a–c).

Although the above studies on crustacean heart preparations contributed significantly toward our understanding of various neuronal properties underlying CG burst generation, most issues concerning the role(s) of intrinsic versus network properties of neurons still remain unresolved. For instance, neither ligature nor two-pool preparations could create a completely “isolated” environment, and hence the synaptic connections between the neurons may have still persisted in these studies.

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Ganglionic isolation saline (in mM: 433 NaCl, 12 KCl, 12 CaCl_2) was bathed with a few notable exceptions (Cooke et al. 1989; Grau and Cooke 1992; Panchin et al. 1993; Turrigiano and Marder 1993).

This study was designed to understand the intrinsic properties of various CG neurons in the crab *Carcinus maenas* and to determine how these are modulated by various cardiovascular substances. However, as compared with their larger counterparts, the CG in smaller crabs (such as *C. maenas*) is buried within the heart musculature and is therefore not discernible even under a high-powered dissection microscope. To overcome this potential problem, we first developed an enzymatic procedure that allowed us to isolate the entire CG from the rest of the heart. Subsequently, intracellular recordings were made from the isolated CG neurons and various hormones (serotonin, 5-HT; proctolin, PR; crustacean cardioactive peptide, CCAP; and SDRNFLRFamide, peptide F2) were tested for their modulatory roles. Further techniques were developed to isolate individual neurons from the intact ganglion in primary cell culture. The isolated neurons not only survived enzymatic and dissociation procedures, but also exhibited electrophysiological and pharmacological properties that were similar to those observed in the intact ganglia. Moreover, the aforementioned hormones that are known to influence heart rate and contractility in higher order crustaceans (F2: Trimmer et al. 1987; Mercier and Russenes 1992; all others reviewed by Cooke et al. 1988) were tested for their modulatory roles. These responses were also compared with those obtained from the intact ganglia. The ganglionic and primary cell culture techniques developed in this study provide a useful model system in which to explore cellular and synaptic mechanisms by which rhythmic heart activity is regulated in the crab *C. maenas*.

**M E T H O D S**

**Heart isolation procedure**

Adult male and female shore crabs (*n* = 139) were used in this study. All legs were autotomized before exsanguination of the animal. The heart was accessed by removing the overlying carapace and connective tissues. The alary ligaments, which suspend the heart in the pericardial sinus, were cut and the heart was removed. Finally, the ventral heart wall was cut open and the heart was pinned in a silicone elastomer (Sylgard) dish. The isolated heart was bathed with a Sylgard dish containing 3 ml DM. At this stage, the CG could easily be recognized among completely fragmented heart muscle cells. After an additional DM wash, the CG was pinned at its distal branches on a Sylgard dish containing 3 ml DM.

Unless otherwise stated, the following procedures were performed at room temperature (~20°C–23°C). To soften the connective tissue sheath, the CG was bathed in DM containing both protease (type IX, 3.33 mg/ml) and trypsin (type III, 2 mg/ml) for 10 min. Enzyme treatment was followed by three consecutive washes in DM (3 ml each wash). The ganglion was desheathed, first using a pair of fine forceps and then by two sharp microelectrodes. Intracellular micro-electrode recordings were made from the desheathed ganglion.

To aid neuronal visualization, some preparations (*n* = 15) were stained with methylene blue dye. Ganglia were isolated and desheathed as outlined above, and a few drops of methylene blue were added to the preparation dish. Ganglia were then refrigerated overnight, and, subsequently, the positions of labeled somata visible under a dissection microscope were traced on a drawing sheet.

**Single cell isolation**

As for the above protocols, all procedures were carried out under sterile conditions. No further enzyme treatment was required beyond the aforementioned procedures to isolate neurons from the ganglion. Neurons were first identified visually by their position in the ganglion and were subsequently removed by applying gentle suction through a fire-polished and Sigmacote-treated (Sigma cat. no. SL-2) pipette that was held in a micromanipulator (MM-33). The isolated soma with its accompanying axon stump was plated in a poly-L-lysine (MW 300,000)–coated Falcon 3001 dish containing 2–3 ml of DM. A maximum of three identified neurons were plated in any given dish, and their positions were marked on the culture dish. The isolated cells were allowed to adhere to the poly-l-lysine–coated dish overnight and were subsequently penetrated with microelectrodes. Because SCs were often difficult to find, fewer were isolated.

Photographs were taken on a Zeiss Axiocam 135 inverted microscope with visible light at ×10–40 magnification. Tech pan film (50 ASA) was used in a Contax camera.

**Electrophysiology and hormone applications**

For both the isolated ganglia and cultured CG neuron preparations, sharp microelectrodes filled with a saturated solution of K_2SO_4 (resistance 10–40 MΩ) were used. DM was replaced with regular *C. maenas* saline before intracellular impalments, and this was continuously perfused throughout the experiment. Signals were amplified on a NeuroData (Model IR-283) amplifier, displayed on a Gould 2-channel chart recorder (Model 2200S), and simultaneously stored on a VCR (Sony Model 420 K, A. R. Vetter) for playback and data analysis.

After microelectrode penetration, the cells were immediately hyperpolarized (range of injected current: 0.15–0.5 nA) to reduce spontaneous activity of the intact CG neurons, and this current was maintained throughout the experiment. The above range of hyperpolarizing current was sufficient to completely silence the isolated cells.

The neurohormones 5-HT, proctolin (Sigma Chemical, St. Louis, MO), CCAP (Peninsula Laboratories, Belmont, CA), and F2 (a gift from Dr. Ian Orchard, University of Toronto) were tested. Each neurohormone solution was diluted in 2× Leibowitz L-15 medium to 2× *C. maenas* saline; pH was then adjusted to 7.60 with 2 N NaOH. DM was tested with an osmometer to confirm that the osmolality was around 1,000 mOsm. During enzyme treatment, the tubes were refrigerated (4°C) for 10 min, then removed and shaken at room temperature for a further 10 min. Refrigeration was considered necessary to slow the enzymatic degradation of heart muscle cells. After collagenase treatment, the tube’s contents were added to a fresh Falcon dish containing 3 ml of DM. At this stage, the CG could easily be recognized among completely fragmented heart muscle cells. After an additional DM wash, the CG was pinned at its distal branches on a Sylgard dish containing 3 ml DM.

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RESULTS

Neuronal morphology

Because the cardiac ganglion in *C. maenas* is not discernible in the heart musculature under a dissection microscope, the precise location, position, and number of cells have not been examined to date. In this study, using an enzyme treatment procedure, we were able to isolate the CG from its surrounding heart musculature. Methylene blue staining coupled with visual inspection allowed us to produce a map depicting the precise number, location, and position of CG neurons (Fig. 1). We used methylene blue in the early stages of this study primarily to locate the SCs. After staining several ganglia with methylene blue, we were able to pinpoint the area in which they resided. Once we were more experienced at finding the small cells, we no longer needed methylene blue to pinpoint them but could do it using unstained ganglia. The entire ganglion was surrounded by a thick connective tissue sheath, which was removed via fine forceps to aid neuronal visualization. Rather than a typical nine-celled CG, as observed in other crabs and lobsters, no more than eight cells were ever observed in any given *C. maenas* ganglion. LCs 1 and 2 were located in the left and right “Y” branches, respectively. LC3 was present at the junction of the Y, and was physically distant from LCs 4 and 5, which were usually located side by side at the bottom of the ganglionic trunk. SCs were only observed in a handful of preparations (21 of 139) and were always clustered below LCs 4 and 5. The SCs were encased in a “pocket” of connective tissue that was buried deep within the main trunk of the CG at its distal end. Each LC was covered by a connective tissue sheath surrounding its somata, whereas the SCs were devoid of such a sheath.

About one in every four ganglia showed anatomic anomalies. Sometimes two large cells were located in one Y branch (3/139), whereas in some other preparations LCs 4 and 5 (the “twin cells”) were located anterior and posterior to one another. Although this appearance is typical in lobster CGs, it was not commonly observed in *C. maenas* ganglia (only 20/139 ganglia). In two cases, the ganglia were unbranched and the cells were aligned down the main trunk. In four ganglia, there were two LCs in place of one at the position of LC3. The SCs, however, were always located below the most posterior LCs, and were clustered in a group.

Electrophysiological properties of the isolated CG

To determine the electrophysiological properties of CG neurons in the intact ganglion, direct intracellular recordings were made from freshly isolated ganglia (a total of 23 preparations). Most LCs showed spontaneous spike activity (27 of 30 cells, Fig. 2A), and their membrane potential ($V_m$) ranged from $-12.3$ to $-65$ mV, the average being $-37.4 \pm 4.8$ (SE) mV. Cells with low membrane potentials had a similar morphology, similar physiological activity, and similar responses to hormones as those cells that were more polarized. These low membrane potentials may have resulted from incomplete electrode penetration, but this seems unlikely.

Figure 2A shows the most common LC waveform: small amplitude and short-duration bursts superimposed on a large amplitude driver potential, large afterhyperpotentials, and slow pacemaker potentials (see Table 1 for average burst amplitudes and rates).

From raw data recordings of LC neurons, either in situ or in vitro, the burst rate, amplitude, pacemaker slope, and oscillation amplitude were measured during control and after hormone treatments. Amplitudes and rates were simply measured from chart records, whereas the pacemaker slope required calculation. The pacemaker potential was arbitrarily defined as the depolarizing phase that began at the end of a postburst afterhyperpolarization, but before the driver potential depolarization of the next burst. Slope was then calculated as the rise/run for this depolarizing pacemaker phase. A model of the events underlying the burst in a LC ganglionic neuron is shown in Fig. 1A.
and pacemaker slopes). This bursting activity, however, varied from preparation to preparation. For instance, some ganglia fired slowly with diffuse bursts that occasionally produced larger spikes (Fig. 2B). Some LCs exhibited larger spikes arising from the driver potential (Fig. 2C), whereas still others had postsynaptic potential (PSP)–like events during the interburst period (Fig. 2D). Because of this variability in burst pattern from cell to cell, some data were presented here as individual raw data traces. A few (5 of 30) LC recordings showed spontaneous membrane potential oscillations in addition to bursting activity. As opposed to driver potentials, which were sustained depolarizations of ~20 mV amplitude and 200–250 ms in duration, spontaneous membrane potential oscillations were smaller (~2–5 mV) and slower (~500 ms) membrane potential fluctuations that occurred during the interburst interval. The values for resting burst rate, burst amplitude, pacemaker slope, and amplitude of membrane potential oscillations as recorded from the LCs are given in Table 1.

Hormonal modulation of CG neurons

To determine whether various cardioactive substances found in crabs affect CG neuronal properties, 5-HT, CCAP, proctolin, and F2 were pressure ejected directly onto the LC somata during the intracellular recordings. In general, all hormones significantly increased burst rate and pacemaker slope ($P = 0.0001–0.010$ for burst rate, $P = 0.005–0.029$ for pacemaker slope, Figs. 3 and 4). The hormone-induced changes in burst amplitude were, however, not significant (Fig. 3). Similarly, in preparations that exhibited spontaneous membrane potential oscillations, hormone treatment did not significantly alter the

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Burst Rate, per/min</th>
<th>Burst Amplitude, mV</th>
<th>Pacemaker Slope, mV/s</th>
<th>Oscillation Amplitude, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated CG</td>
<td>51.71 ± 8.51 (30)</td>
<td>11.14 ± 2.48 (30)</td>
<td>5.35 ± 2.58 (30)</td>
<td>3.55 ± 1.16 (5)</td>
</tr>
<tr>
<td>Cultured</td>
<td>36.42 ± 9.31 (14)</td>
<td>17.40 ± 3.55 (15)</td>
<td>11.31 ± 2.25 (15)</td>
<td>3.02 ± 0.52 (8)</td>
</tr>
</tbody>
</table>

Values are means ± SE with number of observations in parentheses. Electrophysiological recordings were made immediately after microelectrode penetration. For the resting values in this table, the cells were not hyperpolarized. This is in comparison with data obtained during control and hormone applications where cells were silenced by injecting a steady hyperpolarizing current (see values in Figs. 3 and 10, which show lower baseline values as a result of this protocol).
amplitude of these oscillations. Control pressure pulses of saline did not alter any of the above aspects of neuronal excitability (Fig. 5A).

The time course of hormone effects on these burst parameters was similar for all peptides (i.e., the responses were immediate in their onset, and reached peak amplitude within 30 s to 1 min after pressure application). All hormones regularized the rate of LC bursting and augmented the responses of the LCs, especially in slowly bursting preparations. An example of this augmentation effect is shown in Fig. 6, where CCAP treatment induces a change in the burst pattern from PSP-like activity to true bursts with more spikes. Hormonal effects were distinguished from one another, based not only on the time course, but also on changes in burst rate, burst amplitude, pacemaker slope, and amplitude of membrane potential oscillations.

F2 was unique in that it was the only hormone tested that caused desensitization when applied repeatedly to the same cell. As shown in Fig. 7, the first application of F2 caused a 47% increase in burst rate, whereas the second application after a prolonged wash period (17 min) produced a smaller response (24% increase). Desensitization was observed in 5 of 16 cells that received 2 successive F2 treatments. Interestingly, when proctolin was applied to a neuron following an F2 trial, proctolin responses were prolonged; lasting up to 10 min. This was observed in 4 of the 5 ganglionic preparations in which hormones were applied in this order (data not shown).

Serotonin (5-HT) is found in the pericardial organs of the crab and modulates heart rate (Cooke 1988; Cooke and Hartline 1975; Wilkens and McMahon 1992). To test whether 5-HT also modulated CG neuronal activity, this biogenic amine was tested directly on the CG neurons. Typically, there was a 10- to 30-s delay in the onset of the response to 5-HT, which lasted for 3–4 min (Fig. 4C). Responses to peptide applications were, however, immediate in their onset and lasted for a maximum of 2–3 min (Fig. 4).

The above data were obtained from LCs that were isolated from the surrounding cardiac muscles, regulatory nerves (cardioinhibitor and cardioaccelerators), and endogenous hormones (pericardial organ secretions). Because strong electrotonic and chemical synaptic connections exist between LCs and SCs (reviewed in Hartline 1979) in the intact ganglion, it was therefore difficult to demonstrate unequivocally that the responses to the various cardioactive substances were indeed direct. To study direct hormonal effects on CG neurons, individual somata were carefully microdissected from the intact ganglion and maintained in primary cell culture.

**In vitro isolation and characterization of CG neurons**

Neuronal somata of individually identifiable LCs were isolated and plated in culture (Fig. 8). Electrophysiological recordings were made from cultured neurons (n = 15) at 18–24 h. There was no evidence of growth or sprouting in culture for either the LCs or SCs during this time period. Only those isolated neurons that appeared morphologically healthy (spherical and had longer axon stumps) were used for electrophysiological analysis. The resting membrane potentials of cultured LC neurons (range: −10 to −56 mV, average −34.2 ± 5.6 mV) were similar to those in the isolated ganglion. An example of spontaneous activity in a cultured LC, including pacemaker depolarizations and driver potentials, is illustrated in Fig. 9A. The small deflections riding on top of the driver potential appear to be aborted spikes. The main difference between cultured cells and isolated ganglionic neurons was that over half (8 of 15) of the cultured LCs showed membrane potential oscillations (Fig. 9B); these were observed in only 5 of 30 isolated CG preparations. Table 1 shows the average resting burst rate, burst amplitude, pacemaker slope, and membrane oscillation amplitude recorded from cultured cells.

Because the SCs were difficult to see in the intact ganglion, fewer were isolated. None of the SCs plated exhibited membrane potential oscillations. Moreover, the cellular appearance and spike pattern of SCs were very different from those of LCs (Fig. 9C). For instance, all SCs (n = 3) fired tonically at a faster rate (3.0 ± 0.3 Hz), and their spikes were of larger amplitude than those recorded from LCs. Pacemaker slope was 14.92 ± 3.26 mV/s (n = 2). These values for SC pacemaker slope were slightly greater than the slopes calculated for LCs.
Hormonal modulation of isolated CG neuronal activity in primary cell culture

To determine whether hormonal effects seen in the CG neurons were direct, we tested their effects on the individually isolated cells. As with the isolated ganglion preparation, all data for hormonal effects on cultured neurons were obtained from LCs ($n = 15$). The time course of the onset and recovery of each hormone’s effects were similar to those observed in the isolated ganglia. Before hormone treatment, most of the cultured neurons were quiescent but later exhibited bursting activity in response to hormone application. These changes in burst rate were significant ($P = 0.001–0.008$, Fig. 10). Control saline applications had no effect on the burst characteristics of cultured LCs (Fig. 5B).

On three different occasions, all four hormones were tested on a single neuron (Fig. 11). For oscillating cells, CCAP was the only hormone that significantly increased the amplitude of oscillation (67%, $P = 0.0072$). In fact, CCAP significantly increased all measured variables (rate, $P = 0.001$; amplitude, $P = 0.017$; slope, $P = 0.004$; Figs. 10 and 11). Proctolin, CCAP, and 5-HT significantly increased pacemaker slope ($P =$...
Desensitization was observed in two of four experiments in which F2 was applied twice in succession; no other hormone produced desensitizing responses. All hormones increased burst amplitude; this change was significant for F2 and CCAP ($P = 0.007$ and $P = 0.017$, respectively; Fig. 10).

In comparing Figs. 3 and 10, which summarize the effects of hormones on the isolated ganglion and isolated LCs, respectively, some differences were noted. The results were qualitatively similar in that hormones were excitatory in both preparations, and increased burst rate, burst amplitude, pacemaker slope, and amplitude of membrane potential oscillations. How-

FIG. 6. CCAP modulated the bursting patterns of an LC3 neuron in an isolated ganglionic preparation. The resting membrane potential of this cell prior to any hormonal manipulation was $-18 \text{ mV}$. Exogenous applications of CCAP ($10^{-4} \text{ M}$) regularized the bursting pattern and augmented the response of an intracellularly recorded neuron LC3. $A$: control LC3 recording from a slowly bursting ganglion. $B$: 1 min after the CCAP application, burst rate increased and bursting became more regular. Additionally, there were more spikes per burst and each burst was condensed.

FIG. 7. F2 applications modulated the intracellular activity of a cardiac ganglion neuron. To test for the effects of F2 on the neuronal activity, intracellular recordings were made from LC5 (resting $V_m = -27 \text{ mV}$) in an isolated ganglion, and the peptide was applied exogenously. $A$: the 1st application of F2 ($10^{-4} \text{ M}$) caused nearly a 2-fold increase in the burst rate of LC5. Insets: waveforms from the control period before F2 application, and at 30 s after F2 application. $B$: after 17 min of saline wash, a 2nd pressure application of F2 ($10^{-4} \text{ M}$) to the same neuron, however, elicited a smaller response, suggesting that the desensitization may have occurred. Inset: individual waveforms from control and 30 s after F2 treatment.
ever, greater increases in burst rate, amplitude, and pacemaker slope were recorded in response to these same hormones in cultured cells, which appeared more responsive to hormonal actions.

**DISCUSSION**

**In vitro cell culture system**

In the past, attempts to examine basic electrophysiological characteristics of the CG neurons from smaller crabs (such as *C. maenas*), have been hampered by the fact that their ganglia are almost impossible to visualize under a dissection microscope. This, however, can now be easily achieved by using a simple enzymatic technique that was developed during this study. To further characterize the intrinsic membrane and hormonal response properties of the CG neurons, we developed an in vitro isolation technique, where individually identifiable CG neurons were extracted from the intact ganglia and maintained in primary cell culture. This, to the best of our knowledge, is the first study to accomplish these goals in a crustacean preparation.

Techniques to isolate individual neurons from invertebrate species were originally developed in mollusks (Dagan and Levitan 1981; Wong et al. 1981) and leeches (Fuchs et al. 1981). Specifically, some of these culture techniques were described for molluscan neurons as early as the 1970s and 1980s (Haydon et al. 1984; Kaczmarek et al. 1979; see review by Bulloch and Syed 1992). Not only have molluscan, insect, and annelid neurons been shown to survive in cell culture, they also exhibit neurite outgrowth and reestablish specific synapses (Bulloch and Syed 1992; Fuchs et al. 1992; Syed et al. 1990; Thomas et al. 1987; Wong et al. 1981). Generally, the above cell culture models have proved highly beneficial for understanding mechanisms underlying learning, memory, growth cone behavior, synaptic plasticity, regeneration, and the formation and function of synapses (Bulloch and Syed 1992; Moffett 1995, 1996).

Cell culture techniques for crustacean neurons are, however, still in their infancy. Only crab (*Cardisoma carnifex*) and lobster (*Panulirus marginatus*) eyestalk X-organ peptidergic neurons (Cooke et al. 1989; Grau and Cooke 1992), and lobster stomatogastric (STG) neurons (*Panulirus interruptus*) (Panchin et al. 1993; Turrigiano and Marder 1993) have been examined morphologically and electrophysiologically in primary cell culture. Grau and Cooke (1992) alluded to problems with adhesion of STG neurons (but not X-organ neurons), due to the thick glial sheath surrounding each neuron. Some difficulties with cellular adhesion and microelectrode penetration...
In the present study, neurons appeared both morphologically and electrophysiologically healthy despite the enzymatic treatments. For instance, the membrane potentials recorded from the enzymatically treated LCs (range: $-12$ to $-65$ mV in isolated ganglia, vs. $-10$ to $-56$ mV for cultured cells) were similar to those recorded from lobster and crab CG neurons that were isolated without the enzymatic treatments. Values were reported to range from $-42$ to $-60$ mV in *Homarus americanus* lobsters (Miller and Sullivan 1981) and were documented as $-54$ mV in portunid crabs (Tazaki and Cooke 1979a). Furthermore, the bursting activity of the LCs, which is another measure of excitability, was similar in the present study to that of previously published data (Benson 1980; Berlind 1985; Miller and Sullivan 1981; Tazaki and Cooke 1983b). Finally, as detailed below, cell responses to hormone applications also matched previous studies (Cooke and Hartline 1975; Freschi 1989; Lemos and Berlind 1981; Miller and Sullivan 1981). Because we could not isolate the CG from *C. maenas* hearts without enzymatic treatment, the effects of enzymes on hormonal responses could not be compared. Taken together, the above data clearly validate the utility of both isolated ganglia and cultured cells for further studies on intrinsic membrane and network properties of this simple CPG network.

Burst characteristics of both in situ and in vitro isolated neurons recorded in this study were similar. In the past, it has been suggested that there may be functional diversity among the large ganglionic neurons, including the speculation that LC5 may have some impulse-generating ability in the Japanese spiny lobster (Kuramoto and Kuwasawa 1980). However, in the data collected in the present study, no individual differences were noted in the physiological activity or responses to hormones among LCs 1–5. There were, however, two main differences between in vitro and in situ preparations. First, at rest, most in vitro neurons were quiescent (i.e., not firing spikes), whereas in situ cells fired spontaneously. Second, in vitro neurons were more likely to show spontaneous membrane oscillations than in situ neurons. There were some qualitative differences in hormonal responses (Figs. 3 and 10). For instance, hormonal applications to neurons in the isolated ganglia coordinated the bursting patterns and resulted in augmented cellular responses. These responses might be attributed to hormonal effects on synaptic interactions between the neurons in the intact ganglia. Because individually cultured cells were devoid of axonal and dendritic branches, gap junctions, and chemical synaptic connections to other neurons, these features may serve to decrease $R_{input}$ and hence enhance cell excitability. It was, however, interesting to note that neurons in vitro exhibited more dramatic changes in their bursting behavior than the in situ preparation in response to direct hormone application. These data suggest that hormones may directly alter specific ion conductances that underly neuro-
nal excitability. Thus not only did hormones appear to alter synaptic properties, as indicated by more coordinated and regular bursts in the isolated ganglion, they also appear to have modulated the neuronal excitability of individual cells.

**Morphology and electrophysiology of small cells**

This study provides the first anatomic description of the SCs in *C. maenas*. All SCs observed here were clustered below LCs 4 and 5, were encased in a pocket comprised of connective tissue, and were buried deep within the CG. However, in vitro isolated SCs showed no evidence of the thick glial sheath that covered individual LCs; rather, these neurons were devoid of any covering (see Fig. 8). Although only four SC recordings were made from both in situ and in vitro preparations, some generalizations can be drawn about their firing characteristics. In both preparations, the SC firing pattern was very different from that of LCs and of previously reported SC activity. In short, SCs fired tonically, in single spikes occurring at regular intervals, whereas rhythmic bursting was recorded from LCs in the present study and in SCs reported in other studies (Tameyasu 1976; Tazaki and Cooke 1997a). The SC pacemaker potentials recorded in the present study were faster and steeper (Fig. 9C) than those observed in SCs from other species (Tameyasu 1976; Tazaki and Cooke 1997a). Moreover, we showed that SCs fired tonically and did not generate slow driver potential depolarizations; this contrasts with other studies that have recorded prolonged SC driver potentials with superimposed spikes (Tameyasu 1976; Tazaki and Cooke 1997a). Our data are consistent with previous studies on the rate of spike trains in SCs, which was twice (or more) that of the LC firing (Tazaki and Cooke 1997a). Because cultured SCs fired tonically, in the absence of any synaptic inputs, our data clearly show that contrary to previous thinking, these cells have the intrinsic ability to fire. In cell culture, this spontaneous activity is tonic, whereas, in the intact ganglion, the SCs fire in bursts (Tameyasu 1976; Tazaki and Cooke 1997a), implying that in the intact ganglion network interactions must coordinate SC activity.

**Hormonal modulation of large cell activity**

**PROCTOLIN (PR).** The peptide proctolin is present in shore crab pericardial organs (POs) (Stangier et al. 1986), where it exerts myotropic effects on the heart contractility (Saver 1997). Specifically, proctolin was shown to increase heart rate, electromyogram amplitude, and intracellularly recorded excitatory
junction potentials in intact, open, and isolated *C. maenas* heart preparations (Saver 1997). Our previous findings (Saver 1997; Saver and Wilkens 1998; Saver et al. 1998) suggested that one site of action for proctolin is at the CG. In isolated lobster CGs, proctolin depolarizes LCs and increases their burst frequency and pacemaker potentials (Freschi 1989; Miller and Sullivan 1981). In tetrodotoxin-silenced lobster cardiac ganglia, proctolin application evoked depolarization and repetitive driver potentials (Miller and Sullivan 1981). Doublet bursts were often observed with proctolin treatment. Proctolin-induced effects on isolated ganglia and cultured cells observed in the present study were consistent with earlier literature. For instance, the present study showed that proctolin increased all burst parameters and induced immediate bursting activity in previously silent cells. The main difference between the present findings and those of previous studies was the time course of proctolin action. We recorded rapid responses to proctolin (within 0–10 s), whereas slower onset (60–90 s) and longer-lasting effects (10–20 min) were reported elsewhere (Freschi 1989; Sullivan and Miller 1984). This differential time course for peptidergic responses can be attributed to different methods and amounts of peptide application. In the current study, small amounts of proctolin (estimated to be 0.5–1 μl) were pressure applied (“puffed”) directly onto the LC soma under investigation. In other studies, proctolin was either bath applied for 2–3 min, or small pulses (aliquots of 50–100 μl) were delivered “upstream” of the preparation (Miller and Sullivan 1981; Sullivan and Miller 1984). Another likely explanation for the faster onset and recovery could be the fact that in our present study the ganglion was stripped clear of its connective tissue sheath (although each neuron soma was still surrounded by its own sheath). This in turn may have facilitated quicker access for all solutions to the preparation.

In *H. americanus*, proctolin may decrease K⁺ conductance (Sullivan and Miller 1984) or activate a voltage-dependent Na⁺ current (Freschi 1989). Although cellular mechanisms underlying the mode of proctolin action on LCs were not examined in the present study, in light of the significant increases in burst rate and pacemaker slope produced by proctolin, we postulate that it might increase pacemaker currents in these cells.

**CCAP.** Previously, CCAP was tested on intact or isolated *C. maenas* hearts, where it produced chronotropic responses (Saver 1997; Stangier 1991; Wilkens and Mercier 1993). The chronotropic nature of CCAP’s effects suggests that its site of action is exclusively at the CG. Interestingly, other crustaceans such as the lobster *H. americanus* and the crayfish *Procambarus clarkii*, show negligible heart rate responses to this non-peptide (Wilkens, unpublished observations). These species-specific effects of CCAP are attributed to different concentrations of CCAP in the POs of each species. In *C. maenas*, the POs contain greater amounts of CCAP than other crustaceans (Stangier 1991; Stangier et al. 1987), which confirms the notion that these animals utilize CCAP and have receptors for it.

In this study, CCAP proved to be unique in that it was the only hormone that significantly increased the amplitude of membrane potential oscillations in cultured cells. Weimann et al. (1992) used CCAP to activate the pyloric rhythm of the STG in the crab *Cancer borealis*. Bath application of CCAP (2×10⁻⁸ M) to pharmacologically isolated lateral pyloric neurons caused membrane potential oscillations. Although the CCAP concentrations used in the present study differed from that of Weimann et al. (1992), similar responses were nevertheless observed in each system.

**5-HYDROXYTRYPTAMINE (5-HT).** The amine 5-HT was tested previously on isolated CGs from the lobsters *H. americanus* (Cooke and Hartline 1975; Lemos and Berlind 1981) and *Panulirus japonicus* (Kuramoto and Yamagishi 1990), where it increased neuronal burst frequency. In this study, 5-HT application to LCs (both in situ and in vitro) significantly increased burst rate and pacemaker slope. Most 5-HT applications produced large increases in burst rate that lasted for 3–4 min. Burst duration and number of spikes per burst were not calculated in this investigation.

Cooke and Hartline (1975) found that the area between the cell and its axon, but not the soma, was the most sensitive site for 5-HT effects. This, however, is not likely the explanation for the observed delay of 10–30 s recorded in our current study in response to 5-HT application to both the isolated ganglia and cultured cells. The electrotonic spreading of the impulse generated at this sensitive site would occur within milliseconds. Taken together, the delayed onset and the longer-lasting responses for 5-HT than for the peptides observed in the present study suggests that 5-HT may exert its effects via a second-messenger pathway requiring more time than peptidergic responses.

**PEPTIDE F2.** The actions of FMRFamide-related peptides (FaRPs) have been examined on various heart preparations in several different crustacean species (blue crabs, Krajniak 1991; crayfish, Mercier and Russenes 1992; lobster, Trimmer et al. 1987). In particular, the peptide F2 (SDRNFLRFamide) was first isolated and sequenced from lobster, where it was found in high concentrations in the POs, and was shown to function as a cardioexcitor (*H. americanus*) (Kobierski et al. 1987; Trimmer et al. 1987). F2 increases spontaneous contraction rate and amplitude in isolated crayfish hearts (*P. clarkii*) (Mercier and Russenes 1992). In the present study, F2 increased LC burst rate and pacemaker slope in both intact ganglia and cell culture preparations. These data are therefore consistent with other functional observations and suggest a role for this peptide at the neuronal level.

In summary, two novel techniques were developed in this study: one to obtain the isolated ganglia from a small crab heart, and the other to isolate individual neurons in culture. These preparations were subsequently used to identify the intrinsic membrane properties and to test the effects of various neurohormones that function at the heart level. The in vitro cultured neurons can now be used to determine cellular and biophysical mechanisms underlying neuronal excitability and synaptic function. Moreover, cultured neurons can be used to study neurite outgrowth and specific synapse formation between the CG neurons, and this network of neurons can be reconstructed in cell culture to further study the network properties of this rather simple CPG.

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