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# The Effect of Pericardial Organ Hormones on Shore Crab, *Carcinus maenas*, Hearts and Cardiac Ganglia

by

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## Abstract

The precise site(s) and mode(s) of hormonal action on crustacean hearts remain largely unresolved due primarily to the considerable diversity of various species, preparations and hormones used. This study aimed to identify and characterize the precise sites and modes of action of five endogenous hormones on several different preparations from the same species of crab, *Carcinus maenas*. Another aim was to identify excitability and hormonal responses of neurons in a central pattern generator located in the cardiac ganglion.

A multi-level experimental approach was taken to accomplish these goals. A systems level study first surveyed the effects of five endogenous hormones on two types of *in situ* heart preparations. With each successive step taken during this study, from *in situ* to isolated hearts to individual neurons, sites of action were narrowed to fewer possibilities. In the final phase of the study, an *in vitro* cell culture system was developed to study *in situ* and *in vitro* cardiac ganglion neurons.

Each of the hormones tested acted on the cardiac ganglion neurons. Hormone action on these neurons was indirectly measured as changes in heart rate, extracellular electromyograms, and intracellular excitatory junction potentials from *in situ* and isolated hearts. Hormone effects on ganglionic neurons were assessed directly using isolated neurons and intact ganglion preparations. Of several hormones tested (dopamine, 5-hydroxytryptamine, octopamine, proctolin, and crustacean cardioactive peptide), only the peptide proctolin had additional sites of action outside the cardiac ganglion. These included the myocardium (as indicated by disproportionate changes in contractility relative to ganglionic output) and possibly the cardioarterial valves and central nervous system regulatory fibres.

This study provided important clues as to how hormone-induced changes in cardiac contraction rate and force can lead to changes in cardiac output and blood flow that are essential for an animal to meet its metabolic demands under various physiological or environmental conditions. The *in vitro* cell culture system is the most significant development of this study, and will provide exciting opportunities to study the intrinsic and network properties of the crustacean cardiac ganglion network at a resolution unattainable before.

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And finally thanks to Country 105 for the great tunes and the Ranchmans' for some great times.

To my family,
Betty, Vic, and Shawn

And to the memory of Sharon Wilkens

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## List of Symbols and Abbreviations

| | |
|---|---|
| 4-AP | 4-aminopyridine |
| 5-HT | 5-hydroxytryptamine |
| ACh | acetylcholine |
| ABS | antibiotic saline |
| AHP | afterhyperpolarizing potential |
| AP | action potential |
| cAMP | cyclic adenosine 3',5'-monophosphate |
| CCAP | crustacean cardioactive peptide |
| CG | cardiac ganglion |
| CNS | central nervous system |
| CO | cardiac output |
| CPG | central pattern generator |
| DA | dopamine |
| DM | defined medium |
| DP | driver potential |
| EJP | excitatory junction potential |
| EMG | electromyogram |
| EPSP | excitatory postsynaptic potential |
| EWR | eyestalk withdrawal reflex |
| F1 | TNRNFLRFamide peptide |
| F2 | SDRNFLRFamide peptide |
| FaRPs | FMRFamide-related peptides |
| $f_h$ | heart rate |
| FMRFamide | Phe-Met-Arg-Phe-$NH_2$ |
| GABA | gamma-aminobutyric acid |
| $I_A$ | early outward current |
| IBMX | 3-isobutyl-1-methyl-xanthine |
| $I_{K(Ca)}$ | late outward current (calcium dependent potassium current) |
| $I_{K(V)}$ | delayed outward current |
| IPSP | inhibitory postsynaptic potential |
| LC | large cell |
| OA | octopamine |

| | |
|---|---|
| PO | pericardial organ |
| PR | peptide proctolin (RYLPT) |
| $P_{vent}$ | ventricular pressure |
| $R_{input}$ | membrane input resistance |
| SC | small cell |
| SchistoFLRFamide | Schisto Phe-Leu-Arg-Phe-$NH_2$ |
| S.E. | standard error (of the mean) |
| STG | stomatogastric ganglion |
| SV | stroke volume |
| TEA | tetraethylammonium chloride |
| TTX | tetrodotoxin |
| $V_m$ | membrane potential |

# CHAPTER ONE: GENERAL INTRODUCTION

Crustacea are the predominantly marine subphylum of the arthropods that includes crabs, shrimps, lobsters, prawns, barnacles, and their relatives (Waterman and Chace, 1960; Mitchell et al., 1988). These animals successfully inhabit oceans around the world, ranging from coastal zones to deep thermal vents in the ocean floor. The diversity and success of the Crustacea as a group have enticed many investigators to study the anatomical and physiological specializations of these remarkable animals. The most advanced Crustacea, belonging to the order Decapoda, have complex body systems. In particular, the open circulatory system and neurogenic hearts of the decapod crustaceans have received much attention from comparative physiologists over the years. The decapod heart and circulatory system are dramatically different from vertebrates (mammals, in particular) in terms of their anatomy, but the functions of these systems are the same in both animals. In the following sections I will attempt to provide an overview of decapod heart and open circulatory characteristics with appropriate comparisons to mammals, and also introduce the model used in the present study.

## 1.1 General characteristics of the decapod crustacean heart and open circulatory system

Decapod crustaceans have an open circulatory system, composed of a heart, arteries, capillary-like vessels, and several body sinuses that return hemolymph to the pericardial sinus (Fig. 1.1, see review by McMahon and Burnett, 1990). Hemolymph serves as the open system's equivalent of blood and lymph fluids combined. The decapod heart itself is muscular, globular, and consists of a single ventricle (reviewed in Maynard, 1960), as opposed to the multi-chambered heart of mammals. The heart is suspended in the pericardial cavity by the alary ligaments, which are innervated by nerves from the ventral ganglion of the central nervous system and are contractile (Volk, 1988). Due to their contractile nature, the alary ligaments may play an active, rather than passive, role in cardiac filling during the period of ventricular relaxation (diastole) (Maynard, 1960; Volk, 1988). Alary ligament recoil expands the previously relaxed myocardium. Hemolymph can then enter the heart via the ostia, which are valved openings located on the dorsal surface of heart (Maynard, 1960). As a result of these processes, ventricular volume increases, and the heart pumps hemolymph to the body via the arteries.

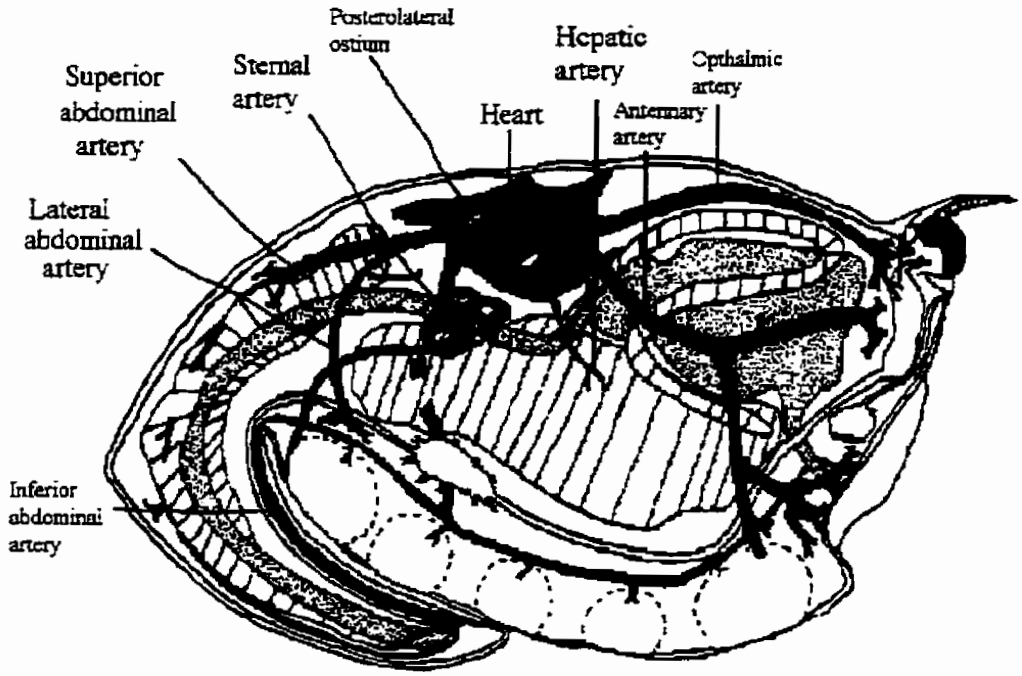



Figure 1.1 Diagram of the open circulatory system of a decapod crab. Approximate mid-sagittal section shows the heart and major arteries. This body plan is not specific to any crab, rather a composite of the features found in several different species of crabs, and is therefore a general anatomical model. Adapted from McLaughlin (1980).

Seven arteries arise from the heart; at the origin of each artery is a muscular cardioarterial valve which prevents hemolymph backflow into the ventricle (reviewed in McMahon and Burnett, 1990). Since crustacean arteries do not possess a layer of smooth muscle like their mammalian counterparts, the muscular contraction of the cardioarterial valves becomes important in the control of hemolymph distribution. In particular, several neurohormones are known to contract or relax these valves, thereby increasing or decreasing outflow to various regions of the body (Kuramoto and Ebara, 1984). While in the past, the open circulatory system has been considered as sluggish and poor in tissue perfusion ability; it has been shown to contain complex capillary beds and is as capable of fine-tuning cardiac output as closed circulatory systems (McMahon and Burnett, 1990). The capillary-sized spaces or lacunae pass hemolymph from arteries into a series of body sinuses that channel hemolymph back to the pericardial sinus surrounding the heart (Maynard, 1960). Hence the role of veins in the mammalian system is replaced in the crustacean by these body sinuses.

The rate and force of cardiac contractions in decapod crustaceans are determined by neural output from a cardiac ganglion (CG), hence, these hearts are termed "neurogenic" (Alexandrowicz, 1932). The CG innervates the myocardium, and bursts of impulses from the CG lead to depolarization of heart myocytes and subsequently, heart contraction (Anderson and Cooke, 1971). In comparison, the hearts of mammals, molluscs, and some lower order crustaceans are termed "myogenic" because muscle or modified muscle cells initiate heart contractions in these animals (Hill and Wyse, 1989). Cardiac pumping is regulated by central nervous system (CNS) activity and by hormonal modulators in decapod crustaceans. Branches of the CNS regulatory fibres are known to synapse on the myocardium, the main trunk of the CG, and on peripheral motoneuron branches (Yazawa and Kuwasawa, 1984a,b) to alter rate and force of cardiac contractions, while several neurohormones are known to affect the bursting activity of CG neurons (reviewed in Cooke, 1988). The cardiac ganglion, myocardium, and modulation of heart contraction by neurohormones and nerves will each be explained in the following sections.

## 1.2 Central pattern generators (CPGs)

The network of neurons in the crustacean cardiac ganglion is considered to be part of a central pattern generator (CPG). The network consists of interactions between the pacemaker cells and motoneurons within the ganglion, and the ensuing motor output to

heart muscle cells leads to cardiac contraction. The interactions between neurons within the ganglion and between these neurons and the heart muscle cells can be modulated in the CG system by CNS regulatory nerves and pericardial organ neurohormones. The CG is only one example of a neural network; a variety of vertebrate and invertebrate preparations have been used to examine the properties of CPGs that underly several rhythmic behaviors. Research on vertebrate models includes the neural networks that coordinate locomotion in lampreys (Grillner et al., 1995), cats, rats and turtles (reviewed in Kiehn, 1991). Invertebrate preparations, in general, contain fewer cells, most of which are identifiable, and are used to study relatively simple rhythmic behaviors. However, the networks underlying these simple behaviors are still complicated (Selverston, 1980; Getting, 1989). The crustacean stomatogastric ganglion (STG) has been intensely studied over the past few decades. This ganglion contains about 30 identified neurons that generate two motor rhythms (Turrigiano and Marder, 1993). The connections between these neurons have been worked out, and several neuromodulators have also been identified in this system (Flamm and Harris-Warrick, 1986; Marder, 1987). Specific examples of these neuromodulators are the peptides proctolin, red pigment-concentrating hormone, and FMRFamide-like peptides (Dickinson and Marder, 1989; Marder and Meyrand, 1989) and the amines dopamine, octopamine and 5-hydroxytryptamine (Flamm and Harris-Warrick, 1986). Some neuromodulators can alter entire network properties and even reconfigure a network into different functional circuits; thus a full understanding of the STG system has not yet been achieved (Marder and Meyrand, 1989; Dickinson, 1995). The synaptic connections of an even simpler network, the three-celled ventilatory CPG of the freshwater mollusc *Lymnaea stagnalis*, have been established *in vivo* and *in vitro* (Syed et al., 1990, 1991). Not only can these synaptic connections be reconstructed in culture, but a transplanted brain cell can make proper connections with its target cells *in vitro* (Syed et al., 1990, 1992).

There are several "building blocks" that are common to all these CPGs, including cellular and synaptic properties and network connectivity patterns (Getting, 1989). The neural network, however, is not simply a summed action of each of these building blocks. Rather, these building blocks are simple patterns that are commonly observed in various networks and seem to be the basis of network function in those CPGs that have been examined thus far (Getting, 1989). Some of the cellular properties include post-inhibitory rebound, plateau potentials, bursting (endogenous vs. conditional), and post-burst hyperpolarization. These cellular properties are due to the intrinsic ion conductances that a

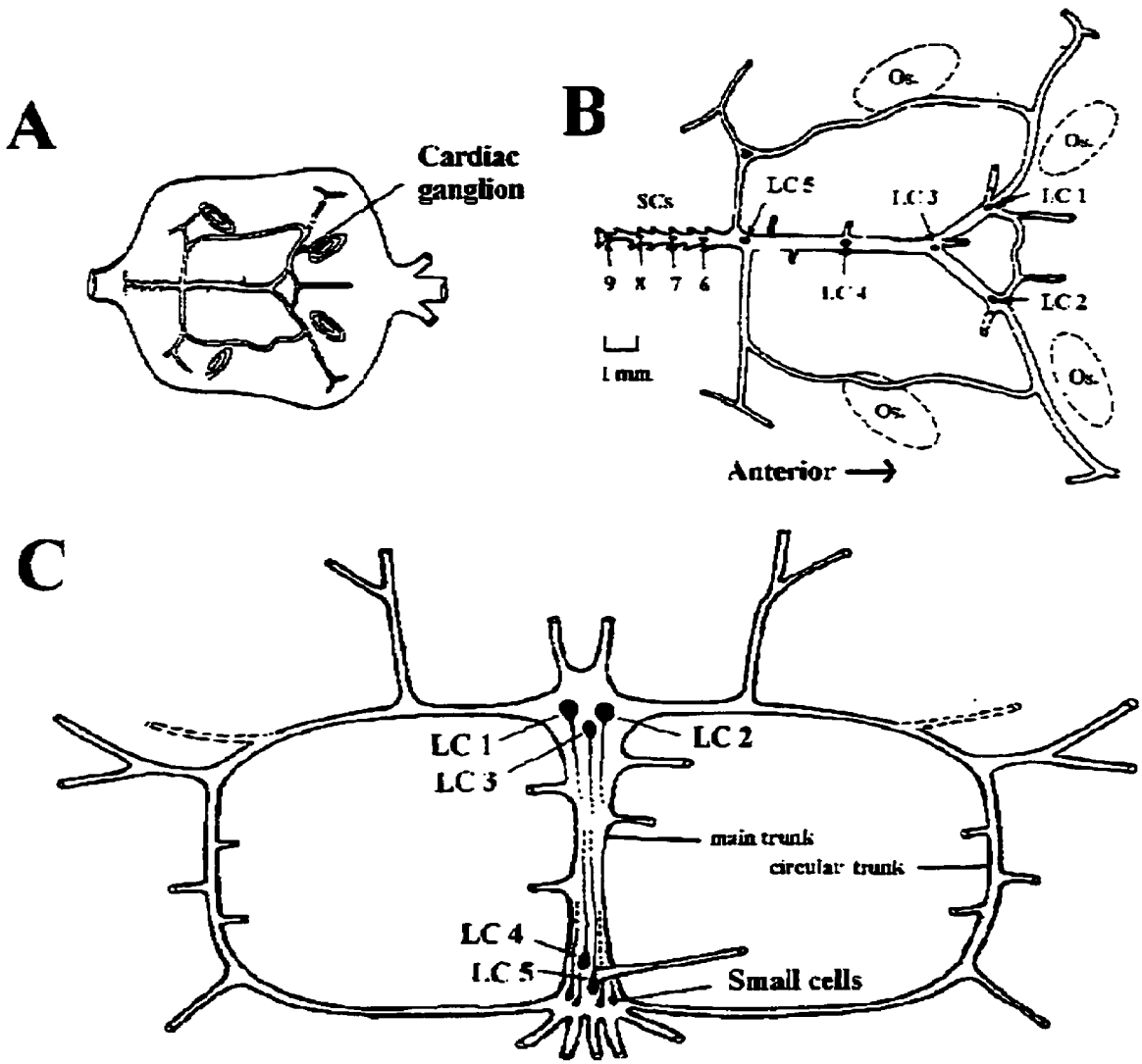
particular neuron is endowed with. Examples of synaptic properties include the sign and strength of the synaptic connection, whether the synapse is electrotonic or chemical, and whether the mechanism of transmitter release is graded or encoded by spikes. Finally, some network connectivity patterns include mutual excitation, recurrent inhibition, and parallel excitation/ inhibition. Since there is a tremendous number of pathways between neurons that become possible as the number of neurons in a network increases, these are just a few network combinations that are frequently encountered, and by no means do these examples explain all the interactions found in the various CPGs examined (Getting, 1989). There are complex interactions between the cellular, synaptic, and network levels, and also modulation from higher centres or from neurohormones that can alter the CPG at any or all of these three levels. Thus, the nerve networks that were once thought to be "hard-wired" are actually very complex, diverse systems that are not yet fully understood (Getting, 1989).

## 1.3 The cardiac ganglion

Several properties of the crustacean cardiac ganglion make it an excellent preparation to study. Welsh and Maynard first introduced this CPG to the field of neurophysiology in 1951 when they showed that the isolated CG continues to produce spontaneous, rhythmic bursts of impulses. In decapods, this Y-shaped "brain" that drives heart contraction is located on the inner dorsal surface of the myocardium. The number of neurons in the CG is variable, ranging from five in shrimp to 15 in crayfish (Kuramoto and Yamagishi 1990). Lobsters and crabs have nine CG neurons; moving from anterior to posterior, cells 1–5 are motoneurons (large cells, LCs) and cells 6–9 are pacemakers (small cells, SCs, Fig. 1.2; Alexandrowicz, 1932). In Maine lobster (*Homarus americanus*) CGs, LC diameter ranges from 80–100 μm, while the SCs are 30–40 μm in size (Sullivan and Miller, 1984). Cell diameters have not been documented for some smaller crustaceans, such as the shore crab *Carcinus maenas*. Anatomically, there is a wide spatial separation between the LCs and SCs in the lobster ganglion, and these neurons are identifiable from ganglion to ganglion. In addition, the LCs but not the SCs can be easily penetrated with microelectrodes (Friesen, 1975a).

The SC pacemakers innervate the LCs, and as their name suggests, their firing is responsible for initiation of ganglionic bursting (Hartline, 1967). There are both electrotonic and synaptic connections between LCs and SCs. In fact, published data show the presence of SC–>SC and SC–>LC synaptic interactions, but not from LC–>SC in

Figure 1.2 Comparison between lobster and crab cardiac ganglion (CG) anatomy. (A) Drawing of the lobster (*Homarus americanus*) heart showing the location of the CG. [Modified from Hartline, 1979]. (B) Enlarged view of the CG, showing the positions of the soma of the large cells (LCs, 1–5) and small cells (SCs, 6–9). Note the wide spatial separation between large cells. Nerve tracts and circular trunk are also shown. Abbreviations: Os. = ostium. [Modified from Hartline, 1967]. (C) Drawing of the crab (*Portunus sanguinolentus*) CG. Note the clustering of 3 LCs at the anterior end of the ganglion, and 2 other LCs beside the 4 SCs at the posterior end of the ganglion. The authors noted that the main trunk is about 5 mm long in 150 g animals. [Modified from Tazaki and Cooke, 1979a].


A
Cardiac ganglion
B
Os.
Os.
SCs
LC 5
LC 3
LC 1
9
8
7
6
LC 4
LC 2
1 mm
Os.
Os.
Anterior
C
LC 1
LC 3
LC 2
main trunk
circular trunk
LC 4
LC 5
Small cells

*Panulirus japonicus* lobsters (Tameyasu, 1976). Electrotonic connections dominate in crabs while chemical synapses dominate lobster ganglionic burst formation (Kuramoto and Yamagishi, 1990). Electronic coupling is strongest in the direction of SCs to LCs, and although the reverse coupling does exist (i.e., LCs->SCs), it is weaker (Hartline, 1967; Mayeri, 1973). SC axons are restricted to the ganglion, whereas LC axons extend from the ganglion into the myocardium to synapse on myocytes (see review in Hartline, 1979). Thus, impulses from the LCs produce depolarization of the myocytes, and heart contraction ensues (Anderson and Cooke, 1971).

### 1.3.1 Large cells

The isolated CG preparation has been used for nearly 50 years to study the burst characteristics of the LCs. Previously, to isolate a particular neuron or group of neurons for electrophysiological study (for instance to compare the effects of hormones or alterations in ion concentrations on either the SCs or LCs) isolated ganglia were ligatured (Tazaki and Cooke, 1983b) or transected (Mayeri, 1973; Sullivan and Miller, 1984). In combination with these techniques, placing the ganglion in a two-pooled chamber in which the neurons are separated by a vaseline gap allowed selective application of hormones to cells in one chamber or the other (Sullivan and Miller, 1984; Berlind, 1989). The two-pooled chamber set-up also allowed bath salines to be changed in one or both chambers, thereby enabling investigators to differentiate between the effects of altered ion concentrations on each group of neurons (Berlind, 1985; Tazaki and Cooke, 1986). The bath ion substitution studies, in particular, have shown that LC bursting consists of several events. The following sections provide first an overall description of each of the events underlying LC bursting, and then a more detailed account of the driver potential depolarization.

#### 1.3.1.1 LC bursting

In both lobsters and crabs, the ganglionic "burst" can be defined as a train of impulses elicited by a non-patterned input (Tazaki and Cooke, 1979c, 1986). Extracellular recordings made with electrodes placed along the trunk of an isolated lobster CG show that there are actually two bursts superimposed on one another (Hartline, 1967; Friesen, 1975a). The long duration burst is due to SC activity (hereafter referred to as the SC burst), while the shorter duration burst occurs after SC activity has been initiated, and is attributed to LC activity (hereafter called the LC burst) (Friesen, 1975a). Furthermore,

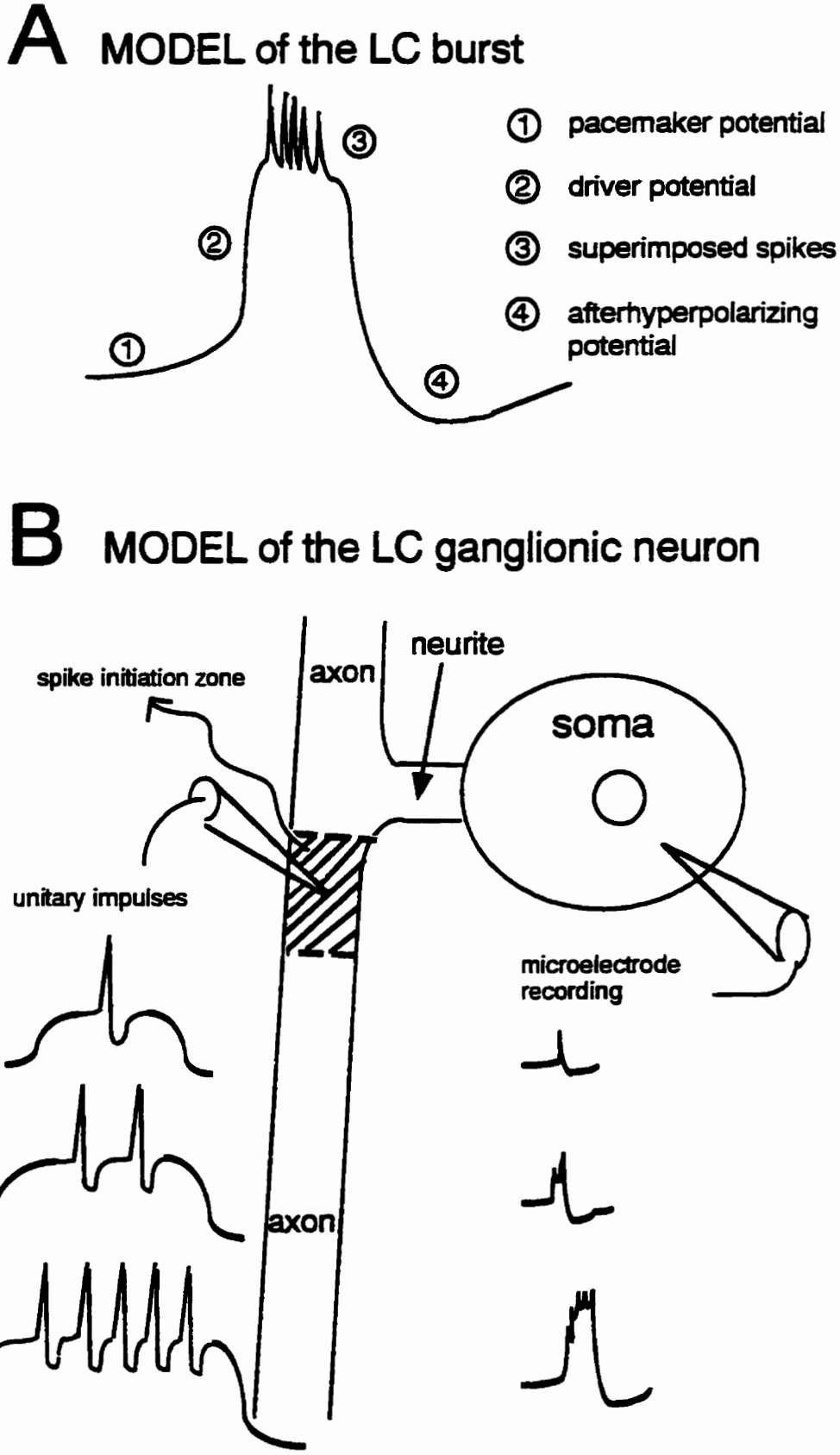
intracellular recordings from LC somata indicate that the LC burst is a postsynaptic response to the SC or LC synaptic and electrotonic inputs that are propogated to the soma.

A model of the decapod crustacean cardiac ganglion LC burst is presented in Fig. 1.3. The following outline of the events occurring in an LC burst is based on descriptions from several sources and several different lobster and crab species (Friesen, 1975b; Tazaki and Cooke, 1979b, 1983c; Sullivan and Miller, 1984; Berlind, 1985, 1989; andreviewed in Cooke, 1988). At the end of the preceding burst, both SCs and LCs are hyperpolarized. The first event in the LC burst is a depolarizing, ramp-like pacemaker potential which acts to depolarize the LC towards its threshold and to initiate the second event in the burst, the driver potential (DP). As SCs are depolarized more quickly than the LCs by this pacemaker ramp potential, the former reach DP threshold prior to LCs. DPs from the SCs in turn provide depolarization for initiation of the LC DP. The DP, which is a gradual depolarization upon which spikes may be superimposed, provides the current to initiate action potentials, or "drive" the axon to action potential threshold. The final part of the LC burst is the afterhyperpolarzing potential (AHP). This AHP actually consists of two events, the first of which decays rapidly while the second decays more slowly (Tazaki and Cooke, 1979b,c). Of all these events, the pacemaker potentials are poorly understood while the DP has received the most attention. Each will be described in detail.

#### 1.3.1.2 Pacemaker potentials

Pacemaker potentials have not been well studied in the crustacean CG system. In fact while they are prominent events in extracellular recordings from SCs, they are absent from LC recordings in some studies (reviewed in Cooke, 1988) while reliably recorded in others (Mayeri, 1973; Benson, 1980). Not only is the presence of pacemaker potentials disputed, but their mode of action has certainly not been examined in crustacean cardiac ganglia. Rhythmic pattern generation in networks as diverse as mammalian thalamocortical neurons, crustacean STG pyloric neurons (Golowasch and Marder, 1992) and leech heart interneurons (Angstadt and Calabrese, 1989) requires an interplay of two currents, $I_h$ and $I_T$, to generate the pacemaker depolarization (reviewed in Pape, 1996). The hyperpolarization-activated current, $I_h$, a mixed cation current that carries both $Na^+$ and $K^+$, is unique in that it is an *inward* current activated by hyperpolarization beyond resting potential. $I_h$ is involved in providing a slow depolarization that reaches threshold for activating a transient, low threshold $Ca^{2+}$ current, $I_T$. The resultant $Ca^{2+}$ entry produces a regenerative response which can trigger a burst of spikes, and also serves to

Figure 1.3 Model of the decapod crustacean LC ganglionic neuron and the events underlying the LC burst. (A) A model of the events underlying the LC burst. In order of onset, these are the pacemaker potential, the driver potential, spikes superimposed on the driver potential, and the afterhyperpolarizing potential. A more detailed account of each of these events is presented in the text. (B) Model of the LC neuron showing soma, neurite, and attached axon. Spike initiation zone indicated by hatched lines in the axon. Unitary impulses recorded by a microelectrode placed in the axon are illustrated, as are the events recorded from the soma. The soma generates driver potentials, which are electrotonically propogated to the spike initiating zone in the axon. At the spike initiating zone, if the driver potential depolarization reaches threshold, an action potential (unitary impulse) is generated.


A MODEL of the LC burst
① pacemaker potential
② driver potential
③ superimposed spikes
④ afterhyperpolarizing potential
B MODEL of the LC ganglionic neuron
spike initiation zone
axon
neurite
soma
unitary impulses
microelectrode recording
axon

deactivate $I_h$. During the repolarization phase, $I_T$ is inactivated, and $I_h$ contributes to an AHP. The AHP, in turn, removes $I_T$ inactivation and activates $I_h$, therefore starting the rhythmic cycle again (reviewed in Pape, 1996). An interplay of $I_h$ and $I_T$ may also be responsible for the pacemaker potentials recorded from crustacean cardiac ganglion neurons, but further work is required to determine the exact nature of the currents involved.

#### 1.3.1.3 Driver potentials

The DP functions as a source of current to generate action potentials in crab and lobster LC axons (Tazaki and Cooke, 1979b,c; Berlind, 1985, 1989). DPs are characterized as long duration (≈200–250 ms) depolarizations of about 20 mV amplitude (Tazaki and Cooke, 1979b). Evidence for the ability of LCs to form these potentials came from studies on tetrodotoxin (TTX)-treated ganglia (Tazaki and Cooke, 1979b,c, 1983c; Berlind, 1985). Using TTX, synaptic interactions between ganglionic cells are blocked and the ganglion becomes quiescent. A pulse of depolarizing current injected into an LC is required to produce the slow, active DP depolarization during TTX treatment; DPs are not generated spontaneously by the soma (Tazaki and Cooke, 1979b). DPs can be defined as postsynaptic responses to depolarizing input which may arise either from SC excitatory postsynaptic potentials (EPSPs), pacemaker-like depolarization, or electrotonically propagated depolarizations from SCs and other LCs in the ganglion (Tazaki and Cooke, 1983a,b). DPs can also be initiated with depolarizing current injected via a microelectrode and they differ from plateau potentials. Plateau potentials are defined as prolonged regenerative depolarizations resulting from intrinsic membrane properties which contribute to burst generation (Russell and Hartline, 1978). DPs are generated as a result of certain specific ionic conductances present in the soma membrane, and are therefore considered to be "endogenous" to a particular neuron (Tazaki and Cooke, 1983c). However, the fact that these ionic conductances are endogenous to the neuron does not necessarily mean that DPs are spontaneous events, rather they require depolarizing input for their activation. Since generation of DPs relies on synaptic inputs rather than intrinsic membrane properties, DPs can not, therefore, be considered as true plateau potentials.

An LC soma connected by a neurite to its axon(s) and the spike initiation zone located distally down the axon are shown in Fig. 1.3B. In large crabs such as *Portunus sanguinolentus*, this impulse-initiating ("trigger") zone may be as far as 2 mm away from

the soma (Tazaki and Cooke, 1983a). Fig. 1.3B illustrates that unitary impulses (action potentials) can be recorded with a microelectrode from the trigger zone. DPs have never been recorded from axons. Simultaneous recordings from LC somata and their axons show that the soma and its proximal neurite are capable of sustaining DPs and are responsible for integration of inputs (Tazaki and Cooke, 1983a,b). Thus, there appears to be a regional specialization between the soma and the axon; the soma generates DPs while the axon generates action potentials (APs).

#### 1.3.1.4 Ionic basis of driver potentials

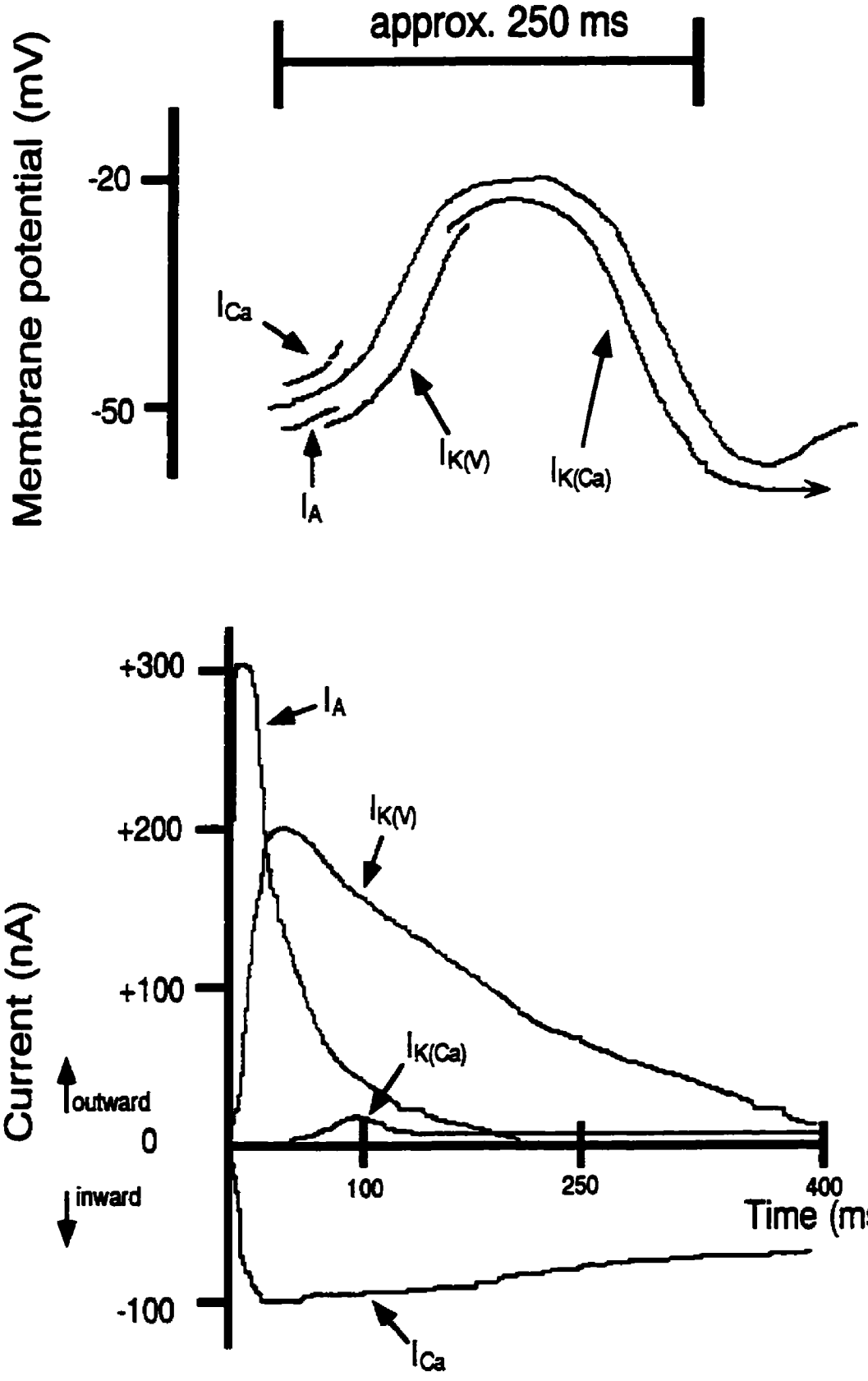
The ionic basis of driver potentials has been investigated, mainly using bath ion substitutions, and involve both inward and outward currents. A model of the currents involved in the LC driver potential are presented in Fig. 1.4. When TTX-treated ganglia are bathed with low $Ca^{2+}$ salines, the rate and amplitude of electrically-evoked DPs are reduced, thereby indicating that DPs are $Ca^{2+}$-dependent (Tazaki and Cooke, 1979c; Berlind, 1985). Using the two-electrode voltage clamp technique and various ion substitutions, Tazaki and Cooke (1986, 1990) have characterized the ionic currents responsible for each part of the LC driver potential. The inward current is $Ca^{2+}$- and voltage-dependent, and inactivates slowly; this inactivation is also thought to be $Ca^{2+}$-dependent (Tazaki and Cooke, 1986, 1990).

There appear to be three types of outward currents, all carried by $K^+$. These are, in order of onset, the early outward current ($I_A$), delayed outward current ($I_{K(V)}$), and late outward current ($I_{K(Ca)}$) (Tazaki and Cooke, 1986). The early outward current reaches its peak at ≈ 5 ms and inactivates quickly. This current is partially blocked by tetraethylammonium chloride (TEA), inhibited by 4-aminopyridine (4-AP), and closely resembles the A-current described in molluscan neurons. The delayed outward current occurs at 40–100 ms, is voltage-dependent, and is unaffected by treatment with 4-AP and manganese. This is followed by the late outward current at 100–400 ms. $I_{K(Ca)}$ is not blocked by TEA, but is $Ca^{2+}$-dependent as evidenced by blockage of this current with cadmium and manganese solutions (Tazaki and Cooke, 1986).

### 1.3.2 Small cells

Studying SCs has proved more challenging due to their smaller somata which are both difficult to visualize and penetrate with sharp microelectrodes (reviewed in Cooke, 1988). As a result, most of the studies have involved extracellular electrodes. In a

Figure 1.4 Model of currents involved in LC driver potentials. The model is based on data obtained from the voltage-clamp study of Tazaki and Cooke (1986). In most experiments, the cell membrane was clamped at -50 mV, which is close to the resting potential. A series of depolarizing voltage steps from this holding potential were applied to elicit membrane currents. For the inward currents, voltage clamp paradigms also included holding membrane potential at hyperpolarized resting levels (e.g., -50 to -80 mV) and stepping the clamp to 0 mV (Tazaki and Cooke, 1986). (A) Drawing of a driver potential. Currents involved in each portion of the DP are indicated. (B) Duration and amplitude of each composite current is diagrammed. In response to a series of depolarizing steps from -50 mV, the inward current, $I_{Ca}$, reached its peak when stepped to -20 mV. $I_{Ca}$ was maximal at 10–30 ms and displayed slow inactivation properties. To elicit $I_A$, test responses to a depolarizing command were applied following hyperpolarizing prepulses applied for increasing time periods. Outward $I_A$ was the fastest current, maximizing at 5 ms and inactivating quickly. The delayed outward current, $I_{K(V)}$, reached maximum at 30 ms, and declined exponentially. A series of depolarizing voltage steps (-30 to +30 mV) from holding potential were used to evoke $I_{K(V)}$. Finally, $I_{K(Ca)}$ was the smallest current (≈ 5 nA); it reached peak amplitude at 100 ms and declined slowly thereafter for nearly 700 ms (not shown on figure). Long (1 s) depolarizing voltage steps (-20 to +10 mV) from holding potential were required to elicit $I_{K(Ca)}$.


approx. 250 ms
Membrane potential (mV)
-20
-50
$I_{Ca}$
$I_A$
$I_{K(V)}$
$I_{K(Ca)}$
+300
+200
+100
0
-100
Current (nA)
outward
inward
100
250
400
Time (ms)

landmark study, Hartline (1967) placed several extracellular hook electrodes along the lobster ganglion to map the complex firing order of cells. He showed that like in the LCs, the SC impulse initiation or "trigger" zones are located at a distance from the soma. As mentioned above, SCs depolarize more quickly than LCs, reach threshold for DP initiation before LCs, and as a result, provide depolarization for the LC driver potential (Tazaki and Cooke, 1983c). Of the pacemakers, SC7 appears to be the first cell to fire in the extracellularly-recorded burst. The first impulse comes from the trigger zone of the SC7 axon and is conducted up to LC5 which, after a delay of ≈ 6 ms, is then triggered to fire. Meanwhile, within 20–30 ms of SC7 firing, all other SCs begin firing (Hartline, 1967, 1979). Following LC5 firing, LCs 3 and 4 fire, followed by cells 1 and 2. The last neuron to fire in the burst is SC8.

A few intracellular studies report SC burst characteristics (Friesen, 1975b, Tameyasu, 1976, 1987; Tazaki and Cooke, 1979a, 1983c) and verify the data obtained from the extracellular electrodes. The intracellular data showed that the membrane potentials ($V_m$) recorded from *P. japonicus* lobster SCs range from -35 to -65 mV (average -45 mV) (Tameyasu, 1976). Values reported from *P. sanguinolentus* crab SCs are also within this range, and are similar to resting $V_m$ recorded from LCs (Tazaki and Cooke, 1979a).

Intracellular studies revealed that SC bursts are distinctly different in appearance from those recorded from LCs. The events underlying the SC burst were the same as those responsible for LC bursting, but the duration and amplitude of these events were not always the same. These differences in bursting behaviour for the two cell types may underly the different functions of the SCs (pacemakers) as opposed to LCs (motoneurons), and will be described briefly here. For instance, the slow pacemaker potential is more pronounced in SCs as compared with LCs. A slow depolarizing potential, matching the description of the DP, is evident, but its duration is prolonged. Finally, there is a train of small spikes riding on the slow potential. These spikes are of smaller amplitude (<5 mV in lobster SCs, Tameyasu, 1976) and fire at a faster frequency than LC spike trains (twice the firing frequency of LCs, Tazaki and Cooke, 1979a). One SC in the lobster preparation showed tonic discharge of spikes. The faster firing frequency and prolonged duration of small cell DPs were thought to provide depolarizing input to the LCs (reviewed in Cooke, 1988). However, the aforementioned studies conflicted in their descriptions of SC DP amplitude, spike amplitude and the role of synaptic versus electrotonic interactions in determining SC firing. Obviously more studies

on SCs are needed to work out these discrepancies and to fully determine SC firing properties, and interactions amongst ganglionic cell types.

### 1.4 The myocardium

Relative to the wealth of information on the crustacean CG, studies on cardiac muscle are scarce. Cardiac muscle is striated (Maynard, 1960; Anderson and Smith, 1971; Anderson and Cooke, 1971; Meyerhöfer, 1993), and individual myocytes branch profusely (Howse et al., 1970; Meyerhöfer, 1993; personal observation). Myocyte diameter ranged from 20–100 μm in Dungeness crabs (*Cancer magister*), 25 μm in the smaller hearts of crayfish (*Procambarus clarkii*, Howse et al., 1970), to 11–39 μm in lobsters (*H. americanus*, Anderson and Smith, 1971). Myocyte lengths of up to 2 mm were reported for these crabs and lobsters, leading one author to conclude that the crab myocytes are "huge" compared with their vertebrate counterparts (Meyerhöfer, 1993). Myocytes are multinucleated and branch and anastomose profusely to form a loosely arranged meshwork of fibres. One study indicated that cells are separated by intercalated discs, while another indicated the absence of these discs (Howse et al., 1970; Meyerhöfer, 1993, respectively). A detailed electron microscopy study reported that "discs" are present, but that gap junctions are absent (Anderson and Smith, 1971). Gap junctions are typically present in mammalian hearts within the intercalated discs and function to spread electrical impulses from one myocyte to the next so that the individual myocytes contract in unison (Berne and Levy, 1992).

The study of heart muscle physiology provides a fuller understanding of how information from the cardiac ganglion is passed to the heart myocytes. Intracellular recordings of single muscle fibres have been made from lobster nerve-muscle preparations (i.e., CG with muscle-attached preparations; *H. americanus*, Anderson and Cooke, 1971; *P. japonicus*, Kuramoto and Kuwasawa, 1980). A nerve impulse recorded from a branch of the CG precedes each myocyte depolarizing potential, and furthermore, myocyte depolarization precedes heart contraction. The transmitter(s) released at these crustacean heart nerve-muscle junctions is/are unknown. Lobster heart muscle contraction is graded and depends upon the myocyte membrane potential (Anderson and Cooke, 1971). These authors provided evidence that each myocyte is polyneuronally and multiterminally innervated. Multiterminal innervation, described as a single axon branch making multiple contacts on a single myocyte, and polyneuronal innervation provide redundancy of information to each muscle cell. Together these phenomena ensure that input from only 5

motoneurons (as found in decapod CGs) is adequate to make the entire heart contract in concert. A synchronous firing of 5 motoneurons also ensures that heart muscle cells contract simultaneously and synchronously. Since there are no regenerative muscle responses in *H. americanus* hearts (Anderson and Cooke, 1971), the functional significance of this type of innervation is to provide a fail-safe mechanism that would ensure coordinated cardiac pumping.

Lobster nerve-muscle preparations show frequency facilitation; as the frequency of stimuli to a CG branch is increased, the amplitude of muscle fibre responses (recorded as excitatory junction potentials, EJPs) increases to a plateau (Anderson and Cooke, 1971). EJPs recorded from *H. americanus* and shore crab (*Carcinus maenas*) myocytes are described as non-overshooting (Anderson and Cooke, 1971; Brown, 1964, respectively). However, evidence from *P. sanguinolentus* crabs shows that some crustacean myocytes *do* produce regenerative, overshooting action potentials (Benson, 1981). There is less information available to date about the properties of shore crab myocytes than there is for other species (namely *H. americanus*). However, one study reported shore crab myocyte resting membrane potential ($V_m$) as -62 mV and EJP burst duration as 250–300 ms. Both of these values are similar to those recorded from lobsters ($V_m$ ranges from -50 to -60 mV, and duration is 400–700 ms, Anderson and Cooke, 1971).

## 1.5 Modulators of heart contraction rate and force

Decapod crustacean heart contractile rate and force are regulated by both extrinsic neuronal and hormonal inputs. Cardioregulatory nerves stemming from the central nervous system (CNS) provide the neuronal modulation of heart contractions, and this will be outlined below. In addition to CNS regulation, the heartbeat is also modulated by neurohormones, mainly those released by the pericardial organs. Several of these neurohormones and their actions on heart rate and contractility will be described below.

### 1.5.1 Neural modulation

Neural inputs consist of two dorsal cardiac nerves which arise from the CNS and enter the heart (Alexandrowicz, 1932). One dorsal cardiac nerve enters each side of the heart, and each nerve contains one cardioinhibitor (CI) and two cardioaccelerator (CA) axons (Maynard, 1953; Yazawa and Kuwasawa, 1984a,b). These nerves enter the cardiac ganglion and run down the main ganglionic trunk (Maynard, 1960; Yazawa and Kuwasawa, 1984a). Nerve fibres branch within the CG and form networks around the

LC cell bodies (Maynard, 1960). The cardioaccelerator axons also make direct synaptic contacts on the myocardium (Yazawa and Kuwasawa, 1984b). In hermit crabs, stimulation of the cardioinhibitory nerve produces inhibitory postsynaptic potentials (IPSPs) in LCs, and increased frequency of CI stimulation leads to reduced LC burst frequency (Yazawa and Kuwasawa, 1984a). Alternatively, increased CA stimulation frequency induces EPSPs in LCs in a one-to-one relationship and increases LC burst rate. Extracellular recordings from the CG trunk revealed that CA stimulation increases small cell EPSPs while CI stimulation abolishes their EPSPs. These data led the authors to conclude that CI axons probably terminate in the region of the SCs and the LCs, while CA axons come into contact with SC axons and transmit SC synaptic excitation to the LCs (Yazawa and Kuwasawa, 1984a). As no EPSPs are observed in the LCs following CA stimulation, the authors suggested that CA axons synapse on LC axons at some distance from the soma.

The identity of the neurotransmitter(s) released from these terminals remains a mystery. One possible candidate for the cardioinhibitor transmitter is, however, gamma-aminobutyric acid (GABA, Maynard, 1961), while acetylcholine (ACh) probably functions as the cardioaccelerator (Welsh, 1939; Sullivan and Miller, 1990). In hermit crabs, however, Yazawa and Kuwasawa (1994) implicate dopamine as being the CA neurotransmitter.

### 1.5.2 Hormonal modulation

The hormones that modulate heart rate and contractility are stored and released into circulation by neurosecretory end-organs, the pericardial organs (POs, Alexandrowicz 1953). The POs are strategically located in the pericardial sinus and they release cardioregulatory agents directly into the path of hemolymph flow to the heart. These neurohormones include the amines 5-hydroxytryptamine (5-HT), dopamine (DA), and octopamine (OA; the amines are reviewed in Cooke and Sullivan, 1983), and the peptides proctolin (PR, Sullivan, 1979), crustacean cardioactive peptide (CCAP, Stangier et al., 1987) and several FMRFamide-related peptides (FaRPs, Trimmer et al., 1987; Krajniak, 1991). Each cardioregulatory agent will be described briefly below.

All three aminergic hormones have been tested on isolated crustacean CGs, and 5-HT was found to increase heart rate and contractility in isolated shore crab hearts (*C. maenas*, Wilkens and McMahon, 1992). Although it appears that ganglionic burst rate is enhanced by 5-HT, the SCs and LCs have different sensitivities to this amine (Cooke and

Hartline, 1975; Lemos and Berlind, 1981; Kuramoto and Yamagishi, 1990). Less is known, however, about the role of DA in crustacean hearts. DA excites semi-isolated crab (*Eriphia spinifrons*) and crayfish (*Astacus leptodactylus*) hearts (Florey and Rathmayer, 1978), but produces only weak tachycardia (increased heart rate) in semi-isolated lobster hearts (Wilkens et al., 1996). One site of action for this amine is the CG (Miller et al., 1984), where it increases burst frequency and the generation of driver potentials in two species of crabs. These two aminergic hormones both appear to exert their effects by action on CG burst output.

Studies on the third amine, OA, show considerable diversity in its effects on various crustacean heart preparations. These effects range from strong (Florey and Rathmayer, 1978) to weak excitation (Grega and Sherman, 1975) to inhibition (Benson, 1984). In each case, OA produces biphasic effects on heart rate where inhibition always precedes excitation. The diversity of these OA effects may be due to species differences in the endogenous levels of this amine found in the POs; crabs generally have lower levels of OA than lobsters or crayfish (Cooke and Sullivan, 1983).

The pentapeptide proctolin (RYLPT) was first described in cockroach hindgut (Brown and Starrat, 1975), and was reported to increase muscle contractility in several insect and crustacean preparations (Mercier and Wilkens, 1985; Bishop et al., 1987; Wilcox and Lange, 1995). This peptide induces contraction of cardioarterial valves (*P. japonicus*, Kuramoto and Ebara, 1984), which in turn affects emptying of the heart. PR has been identified in *C. maenas* POs (Stangier et al., 1986), where it increases beat frequency and force of both isolated and *in situ* hearts from this crab (Stangier et al., 1986; Wilkens and Mercier, 1993, respectively). PR acts on isolated lobster CG preparations not only to increase their burst frequency, but to enhance driver potentials (Freschi, 1989; Miller and Sullivan, 1981, respectively).

The nonapeptide CCAP (PFCNAFTGC) was originally isolated from *C. maenas* POs (Stangier et al., 1987). In contrast to the mainly inotropic effects ascribed to PR, CCAP causes chronotropic responses in crab heart preparations (Stangier et al., 1991; Wilkens and Mercier, 1993). CCAP is ineffective in altering heart rate in some other crabs and in lobsters (McGaw et al., 1994, 1995; Wilkens et al., 1996, respectively). Effects of this peptide have not yet been studied on isolated CGs.

Several FMRFamide-related peptides (FaRPs) have been purified from crustaceans. The physiological effects of FaRPs in crustacean heart preparations are diverse. Two of these FaRPs, SchistoFLRFamide and leucomyosuppressin, decrease

cardiac contractions in crayfish (Mercier and Russenes, 1992). The FaRP F2 (SDRNFLRFamide) was first purified and characterized from lobster pericardial organs (*H. americanus;* Trimmer et al., 1987). In lobster hearts, this peptide moderately increases heart rate, profoundly increases ventricular pressure, reduces sternal artery valve outflow, and increases dorsal abdominal artery flow and pressure (Wilkens et al., 1996). FMRFamide-like immunoreactivity is also found in the POs of crayfish, which leads to the suggestion that FaRPs may target the heart as ONE OF ITS site of action. Indeed, in crayfish hearts the peptide F2 exhibits inotropic effects and also increases heartbeat frequency (Mercier and Russenes, 1992). In blue crab, *Callinectes sapidus*, isolated heart preparations F2 was also FOUND TO BE an effective cardioexcitor (Krajniak, 1991). The closely-related peptide F1 (TNRNFLRFamide) also increases heart rate and contraction amplitude in crayfish and acts directly on lobster skeletal muscle to evoke tonic contractions (Mercier and Russenes, 1992; Worden et al., 1995, respectively). The amino-terminal extensions of F1 ("TNRN") and F2 ("SDRN") appear to enhance the excitatory actions of these peptides on various decapod heart preparations, although their exact sites and modes of action have not been studied (Mercier and Russenes, 1992).

## 1.6 Diversity of crustacean preparations and species in the literature with respect to PO hormones

As reviewed in the above section, various physiological effects of the PO neurosecretory products have been examined in the past, using a variety of preparations and species. Since the PO neurohormonal agents are released into the circulation, and because there are interactions between various components of the system, each substance can operate at multiple sites within the cardiovascular system. For instance, at the heart, these sites could include the CG neurons, the myocardium, the neuromuscular junctions between the CG neurons and the myocardium, and the cardioarterial valves. Other sites include the regulatory nerves stemming from the CNS and the junctions between these nerves and the CG neurons.

Several studies have examined the effects of hormones on the rate and force of heart contraction (Grega and Sherman, 1975; Florey and Rathmayer, 1978; Stangier, 1991; Wilkens and McMahon, 1992; McGaw et al., 1995), the burst patterns of the CG (Lemos and Berlind, 1981; Sullivan and Miller, 1984; Kuramoto and Yamagishi, 1990), cardiac output (Wilkens and McMahon, 1992; Wilkens and Mercier, 1993; McGaw et al., 1994), and cardioarterial valve function (Kuramoto and Ebara, 1984; Kuramoto et al.,

1992). Most of these studies have focused on cardiac responses to only one or two of the PO neurohormones. Additionally, several types of crabs, lobsters, and crayfish have been used in studying hormonal modulation in crustacean hearts. Due to this species and preparation diversity, the precise sites and mechanisms of each hormone's actions are not well defined.

To come to an understanding of hormonal sites and mechanisms of action, I felt it necessary to test hormones on the same animal at different levels. The specific aims of this study therefore were to determine the precise site(s) of action for each hormone and to provide direct and unequivocal evidence for action of hormones at each target site in the shore crab *Carcinus maenas*. The study began with a survey of the general actions of hormones on whole hearts (systems level). Targets of hormone action were subsequently narrowed to fewer sites with each successive level of investigation, finally progressing down to the individual cell level. In order to examine hormonal effects on individual CG neurons, an *in vitro* cell culture system was developed to study these cells in isolation. The findings from this study together with previously published data will provide a new perspective on the mechanisms of action for the respective hormones in *C. maenas* hearts.

## 1.7 Statement of Hypotheses

(1) The pericardial organ neurohormones have multiple sites of action in the shore crab heart, including the CG neurons, the myocardium, and the cardioarterial valves.

(2) Each hormone produces characteristic effects on shore crab heart ganglionic activity and contractile force.

## 1.8 Specific Questions

In this investigation, hormonal action on shore crab hearts were examined at four different levels. To start with, *in situ* hearts were used, then isolated hearts, isolated cardiac ganglia, and finally, individual cultured ganglionic neurons were studied. Specific questions were asked at each level to provide a complete picture of the targets of hormone action.

(1) What is the relationship between CG electrical output and heart contractile force in the *in situ* and isolated *C. maenas* hearts?

(2) At what level in the heart does each hormone act? In other words, does each hormone act at the cardiac ganglion SCs or LCs, or at the myocardium?

(3) At the level of the myocardium, are hormone-induced changes in isolated heart contractility due to changes in myocyte membrane potential or membrane input resistance?

(4) Do CG neurons remain viable and maintain excitability in enzyme-treated isolated cardiac ganglia and in an *in vitro* cell culture system?

(5) Are CG neurons either *in situ* or in cell culture responsive to hormones? If hormones (PR, CCAP, 5-HT and F2) act at the individual cell level, what is the mode of action of each hormone?

# CHAPTER TWO: GENERAL MATERIALS AND METHODS

This section outlines the general materials and methods used for the following three chapters of this investigation. Any specific modifications to these techniques are presented in the appropriate section(s). In Chapter Five, novel methods for isolating the cardiac ganglion and culturing individual neurons from the cardiac ganglion (CG) are presented.

## 2.1 Experimental animals, preparation and surgery

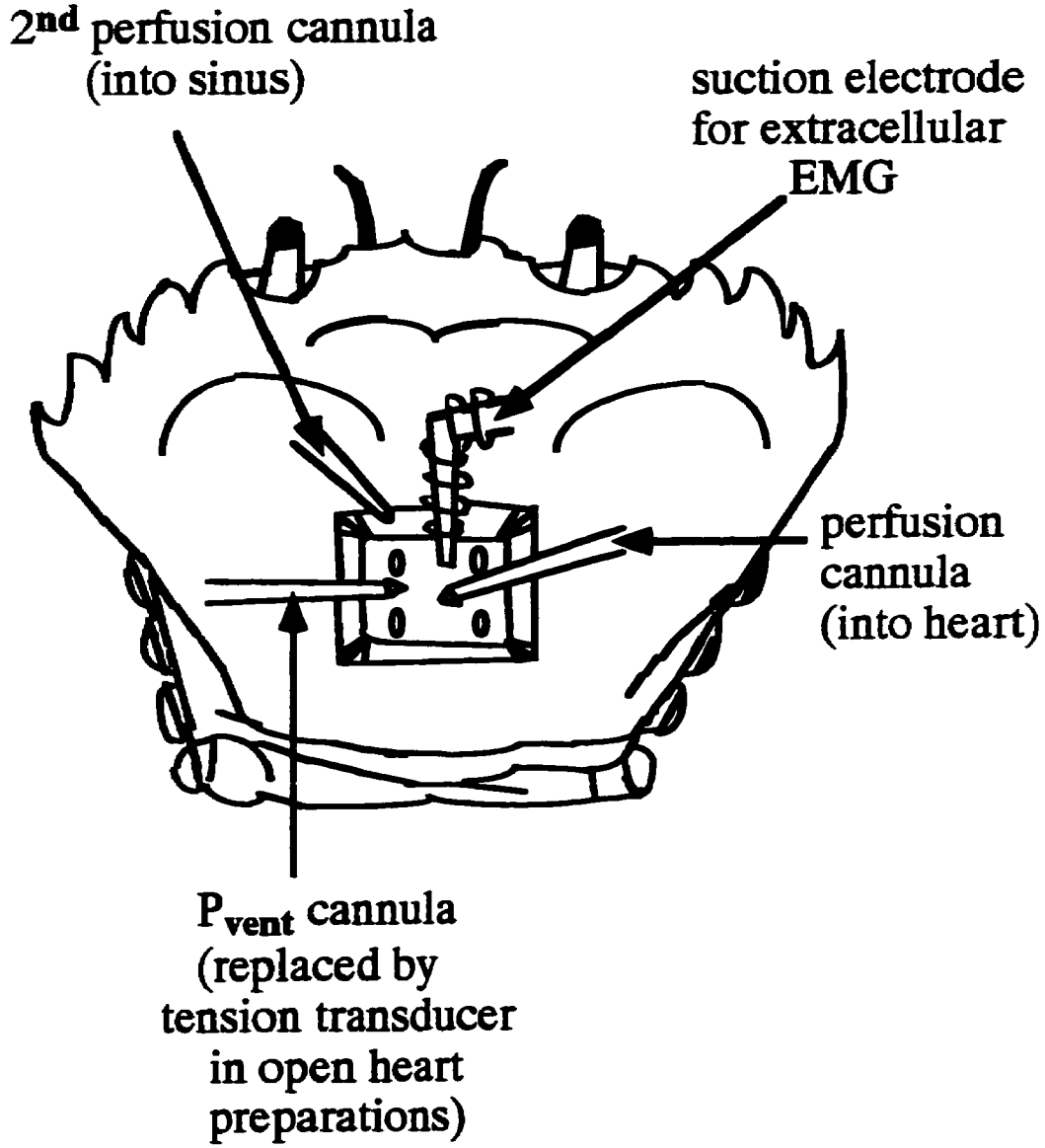
Adult male and female *Carcinus maenas* crabs, in the range of 20–120 g, were used for all phases of this study. Crabs were purchased from a supplier (Ocean Resources, Isle Au Haut, ME) and maintained in artificial sea water (Instant Ocean) tanks at 12–15°C. All experiments were performed at room temperature (20–23°C).

Prior to exposing the heart, the crab's legs were removed and the animal was secured in a dissection dish. Sufficient artificial sea water was added to immerse the gill (branchial) chambers but not the dorsal carapace. A Dremel drill was used to cut out a 1 $cm^2$ piece of carapace overlying the heart. The hypodermis and connective tissues were removed to reveal the heart. The contracting heart was fully exposed, allowing perfusion and physiological measurements. Oxygenated *C. maenas* saline (composition in mM: NaCl 433, KCl 12, $CaCl_2$ 12, $MgCl_2$ 20, HEPES 5; adjusted to pH 7.60 with 4 N NaOH) was perfused via two cannulae, one directly penetrating the wall of the ventricle, and the second placed in the open pericardial sinus. A peristaltic pump was used to maintain saline flow rate entering the ventricle at 3.0 mL/min, which is close to the cardiac output of the intact animal (Taylor and Butler, 1978). A leg base was punctured to allow exsanguination and prevent recirculation of hemolymph or perfusate. Hearts perfused in this manner remained viable and continued to pump for several hours.

## 2.2 Intact heart preparation

After the heart was exposed and prior to electrophysiological recordings, the dorsal surface of the heart was cleared of any remaining connective tissues. From the intact hearts CG electrical output was recorded as extracellular electromyograms (EMGs) and heart rate ($f_h$, the frequency of EMG bursts). Contractile force, measured as ventricular pressure ($P_{vent}$), was recorded using a saline-filled cannula that directly penetrated the ventricle wall (Fig. 2.1). The cannula was positioned by a

Figure 2.1 Dorsal view of an intact heart preparation from *Carcinus maenas*. The cannulae used for perfusion and for measurement of ventricular pressure ($P_{vent}$) directly penetrated the ventricle. An additional saline perfusion drip was positioned in the pericardial sinus. The electrical activity of surface myocardial fibres was recorded with a glass suction electrode (for extracellular EMGs). For the open heart preparations, a hole was cut in the antero-lateral wall of the heart for hemolymph drainage (not shown). In open hearts, an isometric tension transducer was hooked to the anterior end of the heart for tension measurements.


2nd perfusion cannula
(into sinus)
suction electrode
for extracellular
EMG
perfusion
cannula
(into heart)
$P_{vent}$ cannula
(replaced by
tension transducer
in open heart
preparations)

micromanipulator and connected to a pressure transducer (Hewlett-Packard 267BC). A glass suction electrode filled with *C. maenas* saline was used to make direct extracellular EMG recordings from dorsal myocardial fibres. EMG recordings from heart myocytes reflect the CG input to the myocytes. EMG is dependent (1:1) on ganglionic output. The EMG frequency is dependent on LC firing frequency, and EMG amplitude is dependent on the summed depolarization from several LCs. EMG signals were amplified with a Grass P15 AC amplifier. To ensure that changes in EMG amplitude were due to hormone treatment alone and not to changes in electrode position, only trials in which EMG signal amplitude remained steady during control readings and then recovered to control levels after experimental treatment were used and analyzed. EMG and $P_{vent}$ recordings were simultaneously displayed on a Tektronix 5031 oscilloscope and a Gould 2400 oscillograph, and stored on tape (Hewlett-Packard 3960) for analysis.

## 2.3 Open heart preparation

Since proctolin (PR) causes cardioarterial valve contractions (Kuramoto and Ebara, 1984) that could influence $P_{vent}$, direct measurements of isometric tenison were made from hearts after the anterolateral wall of the heart was cut open to provide a low resistance pathway for saline outflow. The CG activity of these open hearts was not affected by this opening. A Grass FT.03C transducer was attached by hook to the anterior end of the heart to measure isometric tension. Heart rate and EMG recordings were made as described above for intact hearts.

## 2.4 Isolated heart preparation

As outlined above, the carapace was removed over the area of the heart and the heart was exposed by removing the hypodermis. To free the heart, the arteries and the alary ligaments that suspend the heart in the pericardial sinus were cut. The heart was transferred to a Sylgard-coated dish, the ventral wall was cut open, and the heart was pinned out with the inner dorsal surface of the heart facing upwards. Special care was taken to avoid injury to the region containing the CG. Hearts were perfused with aerated *C. maenas* saline immediately following isolaton.

Membrane potential ($V_m$), membrane input resistance ($R_{input}$), and isometric tension were recorded from the myocytes. CG electrical activity was monitored as spontaneous myocyte excitatory junction potentials (EJPs) and heart rate ($f_h$). Myocyte

$V_m$ and EJPs were recorded using two types of intracellular microelectrodes: (1) conventional sharp microelectrodes, and (2) bent "dog-leg" electrodes (Fedida et al., 1990). The long shaft (1–2 cm) of each dog-leg electrode was bent into shape over the heating element of a microforge. The flexibility of this long shaft allowed electrode movement during heart contractions, thereby permitting long-term recordings from beating hearts. Both types of electrodes were filled with 3 M KCl and those used were in the range of 10–30 MΩ. $R_{input}$ was measured by injecting short (100 ms) hyperpolarizing pulses (0.5 nA) via the recording electrode. Intracellular signals were amplified with a DC amplifier. Isometric tension was measured with a Grass FT.03C transducer, and amplified.

## 2.5 Experimental protocol and hormone application

### 2.5.1 Intact and open hearts

Once the heart rate had stabilized following surgery, control data ($P_{vent}$ or tension, $f_h$, and EMG amplitude) were recorded for several minutes. Following control recordings, solutions of dopamine (DA), octopamine (OA), 5-hydroxytryptamine (5-HT), PR (Sigma Chemical Co., St. Louis, MO) or crustacean cardioactive peptide (CCAP, Peninsula Laboratories, Belmont, CA) were perfused intraventricularly for 30 s, after which time the perfusion was switched to normal saline. Recordings were made continuously during hormone perfusion and for the first 3–5 minutes of the recovery period. Longer hormonal effects ocassionally required longer recordings. Sufficient washing between hormone treatments was allowed for all variables to recover to baseline and new control recordings were obtained prior to each hormone application.

Each hormone was tested at three concentrations ($10^{-8}$, $10^{-6}$ and $10^{-5}$ M). These concentrations were within the range previously tested on this species (Wilkens and McMahon, 1992; Wilkens and Mercier, 1993). Complete dose-response curves are available for CCAP and PR (Wilkens and Mercier, 1993). The normal circulating concentrations of these hormones are not known for any crustacean at this point.

DA was dissolved in saline containing 0.02% sodium metabisulfite to prevent oxidation; by itself, this concentration of sodium metabisulfite in saline had no effect on the heart rhythm. All other hormone solutions were prepared in *C. maenas* saline. Test compounds were presented randomly.

### 2.5.2 Isolated hearts

Intracellular recordings were made from twelve hearts. For each heart, control recordings ($V_m$, $R_{input}$, tension, $f_h$ and EJP amplitude) were obtained from 5 myocytes prior to hormone application.

A single cannula was used alternatively for both saline and neurohormone perfusion. Either PR or 5-HT (diluted to $10^{-6}$ M in *C. maenas* saline) was applied via the perfusion cannula directly onto the heart (in the vicinity of the CG). If both hormones were tested on the same heart, PR and 5-HT were perfused in random order, with saline washing between trials to allow recovery to baseline levels. Once the perfusate was in the line, it took 1.5 minutes to reach the heart. Time zero was considered to be the time at which the hormone arrived at the heart.

Two separate protocols were used for hormone perfusion, depending on which type of microelectrode was employed. For the conventional microelectrode recordings, enough hormone (approximately 60–70 mL) was perfused to fill and exchange the bath three times, thereby replacing the bath volume with hormone solution. An attempt was made to record continuously from one myocyte during the transition from the control period to hormone application; however, it was difficult to maintain the microelectrode in the same myocyte for lengthy periods. Therefore, recordings were made from five more cells during the period of hormone application. Due to the length of hormone perfusion (approximately 20–30 minutes) for these hearts, complete recovery often required over an hour of washing. Cells were never maintained for the entire duration of the perfusion or the recovery, thus several different cells in the same area of the heart were examined for each time period.

During the dog-leg electrode recordings, each hormone was applied for 4–5.5 minutes. Hearts recovered more quickly from such short hormone exposures. As dog-leg electrodes were more flexible than the stiff conventional electrodes, stable long-term recordings were made and often the entire duration of a hormone response could be monitored continuously from one cell.

## 2.6 Data analysis

### 2.6.1 Intact and open hearts

For measuring EMG amplitudes from chart records, the EMG amplitude that corresponded to the peak $P_{vent}$ or tension values was used for each trial. The EMG amplitudes were not necessarily the maximum amplitudes for each trial. Tape-recorded

EMG data were also analyzed on a digital oscilloscope (Nicolet 4094B). $P_{vent}$/EMG ratios were calculated as follows: ($P_{vent}$ drug/EMG drug)/($P_{vent}$ control/EMG control) *100%. The $P_{vent}$/EMG ratios were taken to represent the ratio of mechanical output to CG electrical input. Tension/EMG ratios were calculated in the same manner from open heart data.

Summary data are reported as the mean ± S.E. For each variable, paired *t* -tests were used to determine significant differences between control and hormone treatments. The Mann-Whitney rank sum test was used on data that failed the normality test. Asterisks (*) were used to indicate significant differences ($p < 0.05$) in summary graphs.

### 2.6.2 Isolated hearts

Data were displayed on a 4-channel Gould 2400 oscillograph and simultaneously stored on tape (Hewlett-Packard 3960). From tape, data were replayed and individual traces were saved on Nicolet (model 4094) disk for analysis. Peak PR or 5-HT values were compared to control values and data were reported as a percentage change from control. Paired *t* -tests were performed as for intact and open hearts, and the Mann-Whitney rank sum test was performed on data that failed the normality test. Additionally, significant differences between PR and 5-HT treatments were assessed using a *t* -test and indicated in summary figures with a dagger (†). A value of $p < 0.05$ was used to indicate significance.

# CHAPTER THREE: COMPARISON OF THE EFFECTS OF FIVE HORMONES ON SHORE CRAB INTACT AND OPEN HEARTS

## 3.1 Introduction

The heartbeat of decapod crustaceans is initiated by the 9-celled cardiac ganglion (CG) and regulated by extrinsic neuronal and hormonal factors. In this study the focus was on the hormonal modulation of the heart. In particular, the effects of three monoamines and two of the peptides which are released from the neurosecretory pericardial organs (POs, Alexandrowicz, 1953), were tested on the contractile rate and force of shore crab hearts. Because these products are released into circulation, they can potentially act at several sites in the circulatory system. These sites both within the heart and outside of the heart have been described in detail in Chapter One.

In the past, there has been uncertainty as to the site and mode of hormone action due to the diversity of preparations and species tested in crustacean heart studies. To determine the sites of hormone action, an attempt was made in this study to survey the effects of several hormones on two heart preparations from one species of crab (*Carcinus maenas*). *In situ* intact and open heart preparations were developed for studying myocardial mechanical and CG electrical responses to hormone perfusion.

The intact heart preparation retained normal contractile ability, allowed controlled drug application, and provided direct access to the heart for physiological measurements (which the whole animal system does not). Second, out of concern that PR-induced cardioarterial valve closure in intact hearts may have contributed to the observed changes in contractility as observed in lobsters (Kuramoto and Ebara, 1984), an open heart preparation was developed. Open hearts were created by cutting a hole in the side wall of the heart, thereby allowing hemolymph outflow and minimizing effects that might arise from hormone actions at the cardioarterial valves.

This study was divided into two parts. First, an attempt was made to determine the site of action for each hormone. It was hypothesized that the effects of each hormone could be attributed to action at either the CG neurons or the myocardium. In particular, if the hormone exerted action on the CG neurons, changes in $f_h$ and EMG pattern output were expected. Alternatively, if the hormone acted directly on the myocardium, changes in $P_{vent}$ or tension unrelated to rate and EMG were anticipated. In previous work, PR was reported to have multiple effects on various decapod species and heart preparations, which led to the hypothesis that there are multiple sites of action for this peptide. For

example, in *C. maenas* whole animal preparations, PR causes a transient period of bradycardia (decreased $f_h$) followed by mild tachycardia (increased $f_h$) (Mercier and Wilkens, 1985; McGaw et al., 1995). On isolated *C. maenas* hearts, PR is more inotropic than CCAP (Stangier, 1991; Wilkens and Mercier, 1993), and on isolated CG preparations it modifies burst rate and intensity (*Homarus americanus*; Miller and Sullivan, 1981; Sullivan and Miller, 1984; *C. maenas*, Saver et al., 1996).

Second, an attempt was made to determine the relationship between CG electrical output and mechanical force in these hearts in response to hormone perfusion. The hypothesis was that hormone-induced changes in CG electrical activity would give rise to equal changes in mechanical force for all hormones.

## 3.2 Materials and methods

Animal preparation and surgery, the rationale for using *in situ* intact and open heart preparations, experimental protocols, and data analysis were all presented in Chapter Two. In this chapter, the stability of intact heart preparations was also tested. Unless otherwise stated, all data are mean ± S.E. Paired two-tailed $t$ -tests were performed to determine significant differences from control ($p < 0.05$).

### 3.2.1 Stability tests

The aim was to determine the stability of heart electrical ($f_h$) and mechanical ($P_{vent}$) properties over time in the intact heart preparation. Intact hearts were prepared as described in Chapter Two. Long-term recordings of $f_h$ and $P_{vent}$ were obtained from hydrostatic pressure recordings. The pressure cannula remained undisturbed in the ventricle for the duration of each experiment, which was typically 10 hours. All hearts ($n$ = 7) were perfused with aerated saline; of these, five were perfused with saline containing 5 mM glucose and the remaining two were perfused with saline lacking glucose. The question of whether the hearts perfused with glucose-containing saline showed greater stability than those hearts perfused with saline lacking glucose was asked. The saline perfusion cannula was positioned in the pericardial cavity (not directly in the heart) to minimize damage to heart muscle tissue over the length of an experiment. Heart rate and $P_{vent}$ data were recorded hourly, displayed on both oscilloscope and Gould chart paper simultaneously, and saved on Nicolet disk for analysis.

## 3.3 Results

### 3.3.1 Stability tests

Before the analysis of hormonal actions on the heart could begin, it was important to validate and establish the stability of the intact heart preparation. Stability was tested by making long-term $f_h$ and $P_{vent}$ recordings. Fig. 3.1 shows that in intact hearts with glucose added to the saline, both $f_h$ and $P_{vent}$ declined over time. $P_{vent}$ decreased 67% over 10 hours, and this change was significant ($p < 0.05$). Heart rate decreased by 50% (from 63 to 32 beats per minute, bpm) over the same time, but this was not significant. Heart rate, but not $P_{vent}$, increased initially, remained at or above the starting rate until the second hour, then declined for the duration of the experiment (from 64.5 ± 7.9 bpm at 2 hours to 32.4 ± 7.9 bpm at 10 hours). $P_{vent}$ declined steadily from the start of the recordings, with the greatest decrease between the third and fourth hours (from 30.9 ± 8.3 mV to 20.94 ± 3.28 mV, Fig. 3.1).

The trend of decreasing $f_h$ and $P_{vent}$ over time also occurred in intact hearts that did not receive glucose-supplemented saline; these changes were not significant for either variable (Fig. 3.2). The decrease in $f_h$ was less than that for hearts treated with glucose (30% compared with 48%, respectively), but there was no significant difference between these groups. There was no significant difference between the decrease in $P_{vent}$ in hearts infused with glucose-rich or glucose-free saline (67% in both cases). In glucose-free hearts, $P_{vent}$ increased between the 5th and 7th hours, then declined in the eighth hour. Heart rate remained more stable although it started at a lower level (32.9 ± 15.9 bpm, data shown as mean ± S.D. for these hearts) than that measured in glucose-treated hearts (62.8 ± 12.4 bpm), and there was no progressive decline in $f_h$ as in glucose-treated hearts.

### 3.3.2 Intact heart preparations

The effects of PR, CCAP, 5-HT, DA and OA were surveyed on intact heart preparations. Since the stability tests showed that there were no significant decreases in $P_{vent}$ or $f_h$ over time in glucose-free saline, all hearts were perfused with glucose-free saline. The aim of this part of the study was to determine where the site of action for each hormone was, and whether PR had mulitple sites of action. The changes in mechanical force with respect to changes in CG electrical activity were assessed.

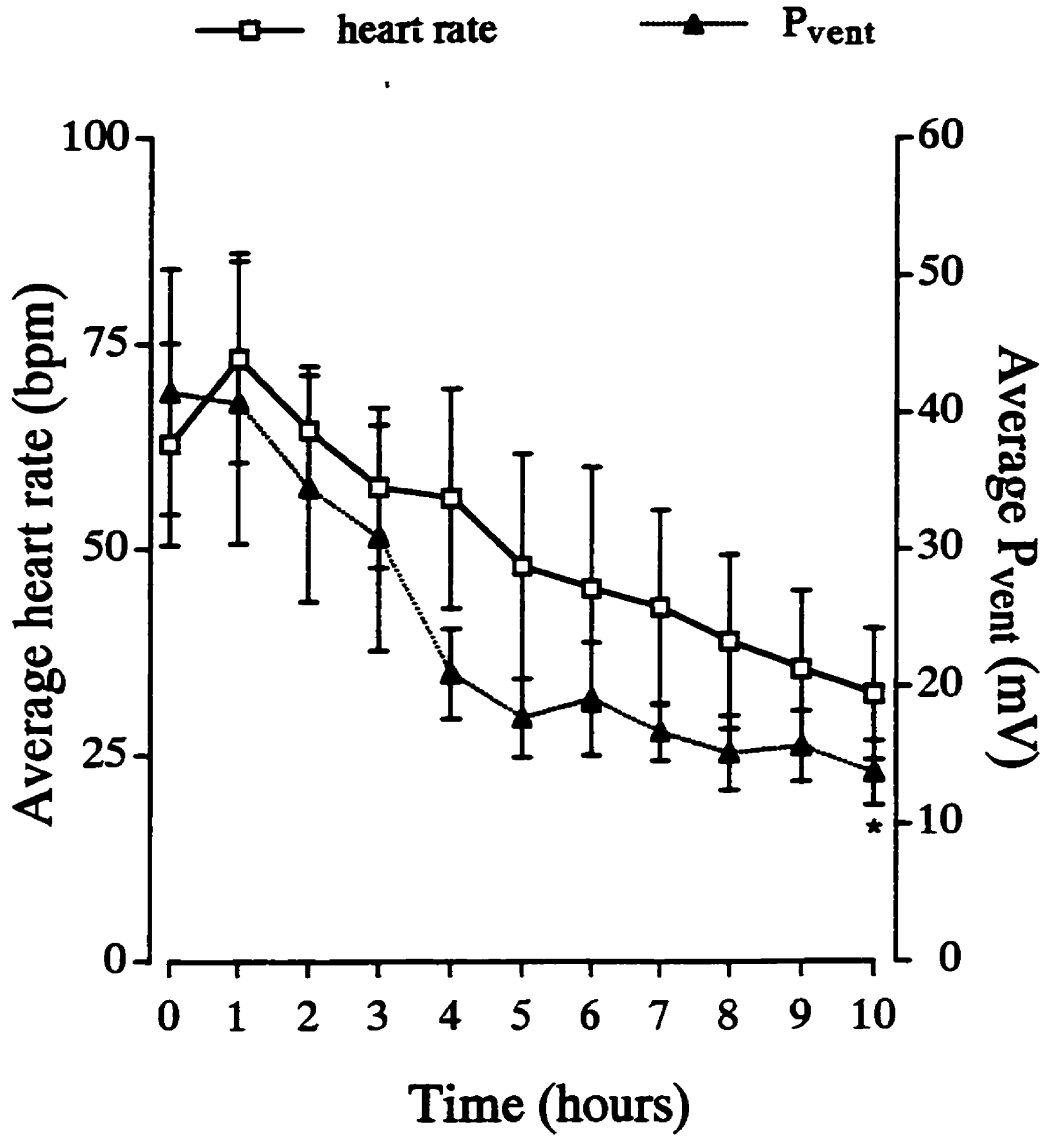



Figure 3.1 Summary data from stability tests. Values shown as mean ± S.E. Data obtained from intact hearts receiving glucose in the saline ($n$ = 5). Heart rate ($f_h$, square symbols) and ventricular pressure ($P_{vent}$, triangle symbols) were shown to decline over 10 hours. The only value that changed significantly at 10 hours from control ($p < 0.05$) was $P_{vent}$ and is indicated with an asterisk (*) on the graph. Since this study was only concerned with absolute changes in $P_{vent}$, these values were not converted to units of pressure, but are merely the raw voltages taken from traces saved on the Nicolet oscilloscope.

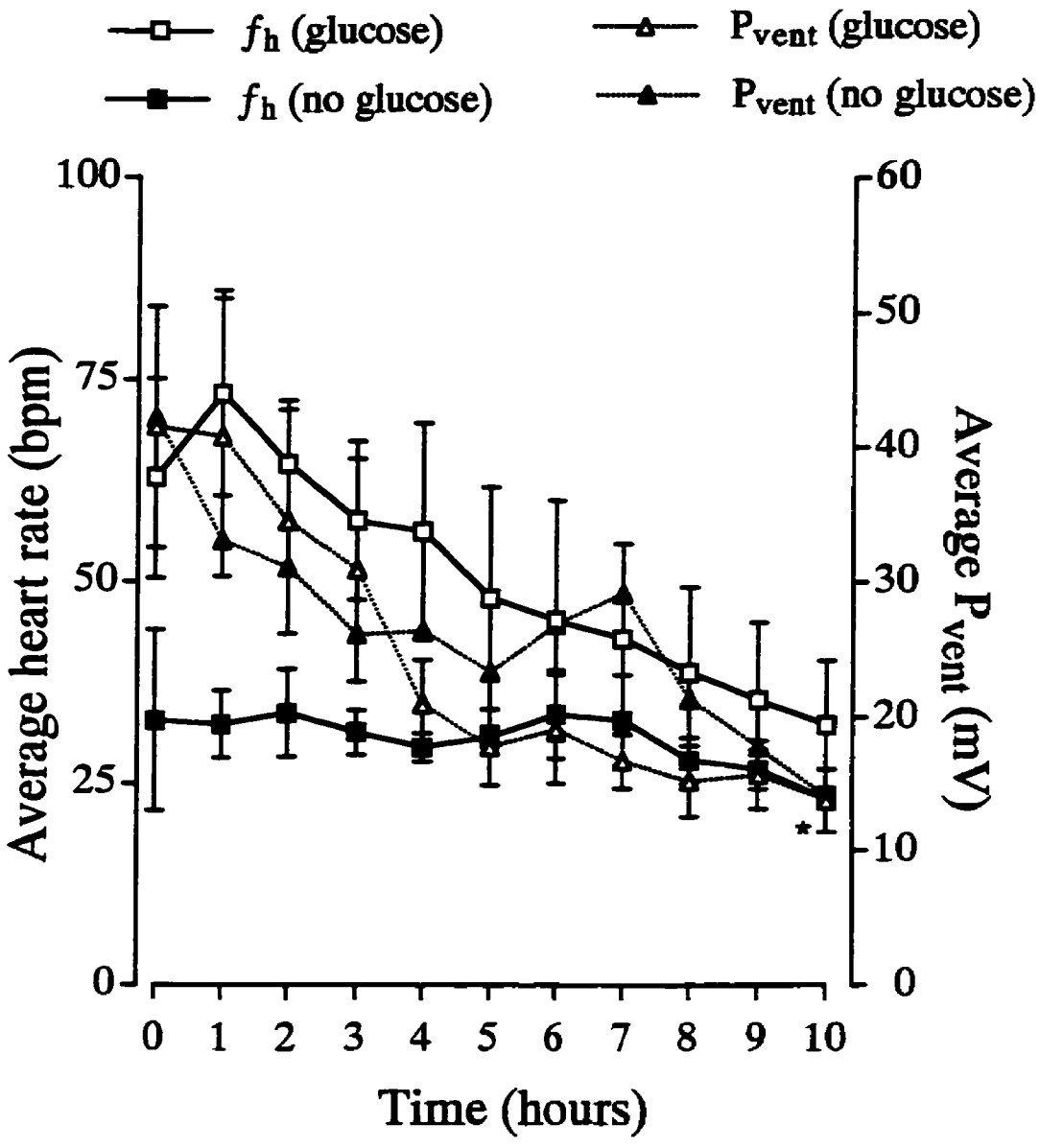



Figure 3.2 Summary data comparing stability of intact preparations for hearts treated with glucose-free ($n$ = 2, open symbols) and glucose-rich ($n$ = 5, solid symbols) saline. Changes in both $f_h$ (data points shown as squares) and $P_{vent}$ (data points shown as triangles) were assessed over a 10 hour time period. Values are mean ± S.E. for glucose-rich saline treatment, and mean ± S.D. for glucose-free hearts. Any significant changes from control ($p < 0.05$) were indicated by asterisks (*) on the graph. The only significant value was for $P_{vent}$ at 10 hours in hearts treated with glucose-rich saline. $P_{vent}$ was uncalibrated.

### 3.3.2.1 Proctolin

The onset of responses to PR perfusion was rapid, with effects first observable within 5 s of hormone application. Maximal responses occurred within 60 s (Fig. 3.3A). All variables returned to control levels following 5–30 minutes of saline wash. In 6 of 10 hearts PR produced a biphasic change in $f_h$, where the rise in rate was interrupted by a period of bradycardia or cardiac arrest. This was followed by a period of tachycardia (Fig. 3.3B). On average, there was a small, but insignificant, increase in $f_h$ for $10^{-8}$ and $10^{-6}$ M PR treatment (9–18%, p = 0.700 and 0.413, respectively, Fig. 3.4). Only PR $10^{-5}$ M significantly increased $f_h$ (56% increase, p = 0.001, Fig. 3.4).

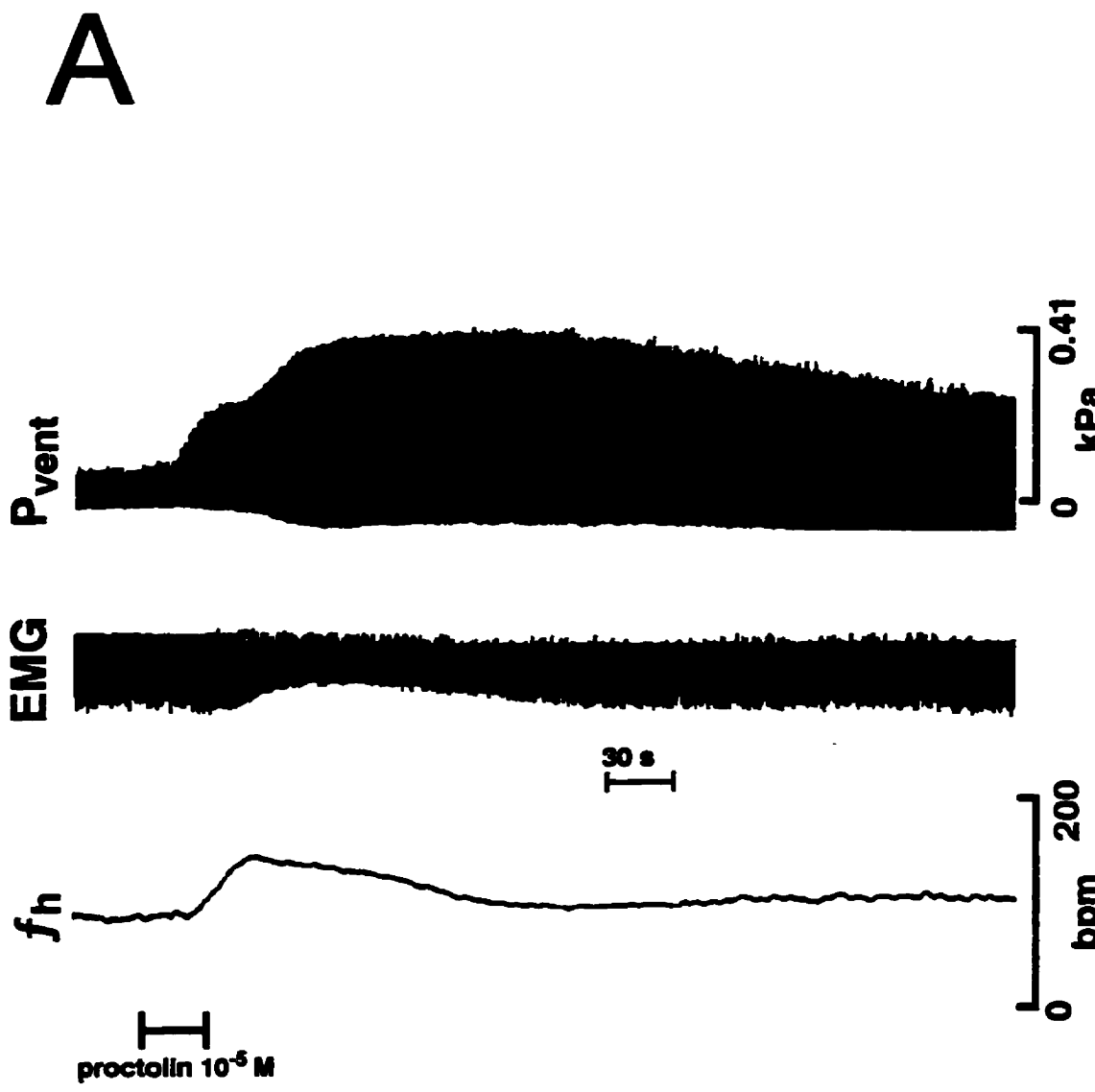
In contrast to its effects on $f_h$, PR caused large and significant increases in $P_{vent}$ (105–477%, p = 0.001 and 0.0005 for $10^{-6}$ and $10^{-5}$ M PR, respectively, Figs. 3.3 & 3.4). PR effects on $P_{vent}$ were longer-lasting than those on EMG; often $P_{vent}$ remained elevated during the saline wash period for more than 4 minutes, especially at higher concentrations, even after the EMG had returned to control levels (typically after about 3 minutes of wash).

In three trials, PR infusion either reduced or had no effect on EMG amplitude. Overall, however, EMG amplitude was increased by PR treatment (ranging from 18% for $10^{-8}$ M to 73% at $10^{-5}$ M; p = 0.700–0.056), but to a much lesser extent than $P_{vent}$ (105–477%, Figs. 3.3 & 3.4). This increase in EMG amplitude was not significant at any concentration tested. PR produced the highest $P_{vent}$/EMG ratios of the hormones tested, and at all concentrations (from 76% at $10^{-8}$ M to 237% at $10^{-5}$ M PR, Fig. 3.5). Both $10^{-6}$ and $10^{-5}$ M PR treatment increased $P_{vent}$/EMG ratios significantly (p = 0.026 and 0.016, Fig. 3.5).

### 3.3.2.2 Crustacean cardioactive peptide

The time course of the CCAP responses were similar to those for PR; maximum responses occurred within 30 s (compare Figs. 3.3 & 3.6), and all variables returned to control levels after 5–30 minutes of rinsing. In 8 of 19 trials, $f_h$ and EMG returned to control slightly before $P_{vent}$ (in 1.5 minutes and 2.0 minutes, respectively), especially at higher CCAP concentrations. This recovery was slightly faster for CCAP than for PR (on average, 2.2 minutes compared to 3.2 minutes, respectively). CCAP increased $f_h$ 10–101%, and this change was significant for $10^{-5}$ M (p = 0.026, Fig. 3.4). Increases in EMG amplitude (8–21%, p = 0.503 to 0.372) were accompanied by equivalent increases

Figure 3.3 Effects of PR perfusion on intact *Carcinus maenas* heart preparations. Traces A and B were obtained from two different animals. (A) Raw data trace from a intact heart preparation showing heart rate ($f_h$), extracellular EMG, and ventricular pressure ($P_{vent}$) responses to a 30 s PR $10^{-5}$ M treatment. This trial showed a typical increase in $f_h$ and $P_{vent}$, and although EMG amplitude decreased transiently, it returned to control levels at the end of the trial. Note also that the $P_{vent}$ amplitude remained elevated even after the EMG amplitude had returned to normal values. In this heart, the eyestalk withdrawal reflex (EWR), an indicator of the viability of the CNS, was absent. Generally, hearts lacking the EWR, and hence lacking CNS regulation, showed excitatory responses to PR application. (B) Effects of a 30 s $10^{-6}$ M PR perfusion on $P_{vent}$, EMG, and $f_h$ measured from an intact heart preparation in a different animal. The EWR was present in this animal, indicating that the CNS was viable. Note the biphasic effect of PR on $f_h$, and the brief period of cardiac arrest.


A
$P_{vent}$
0.41
kPa
0
EMG
30 s
$f_h$
200
bpm
0
proctolin $10^{-5}$ M

B

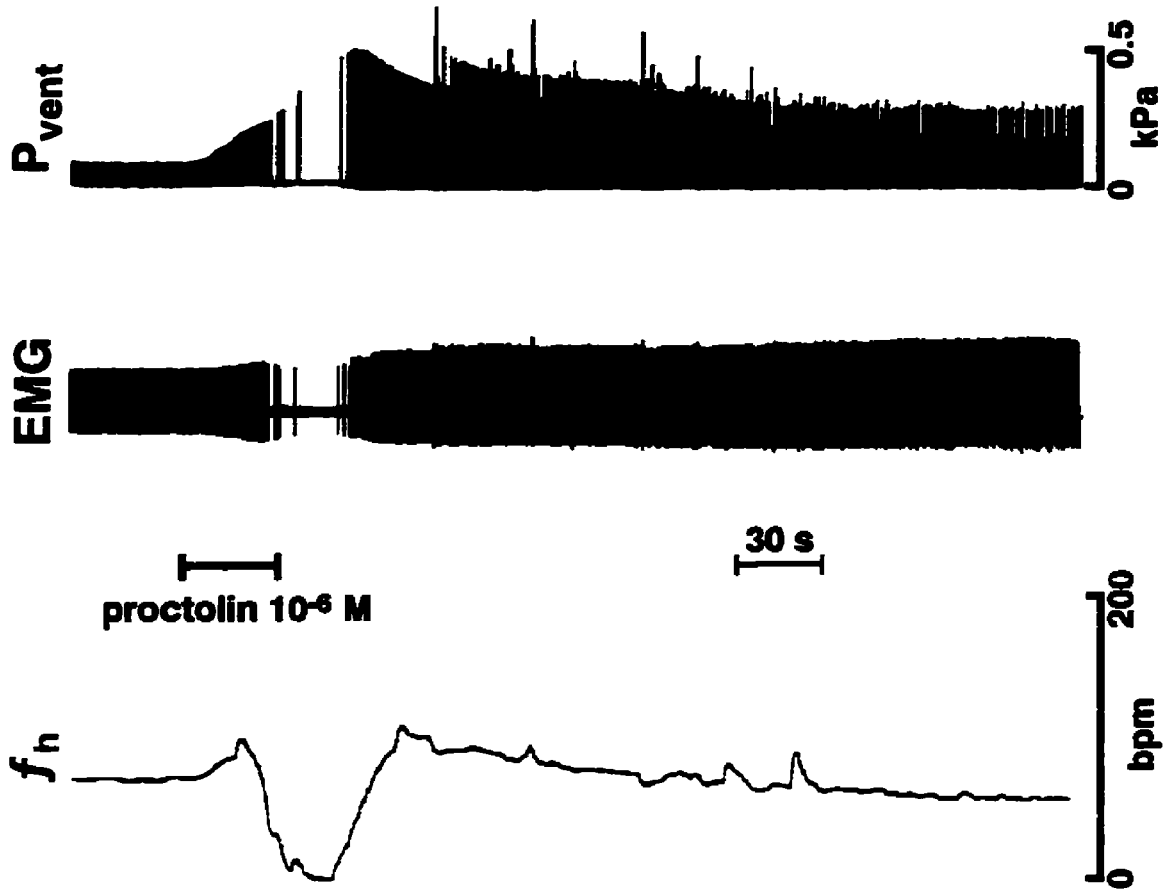
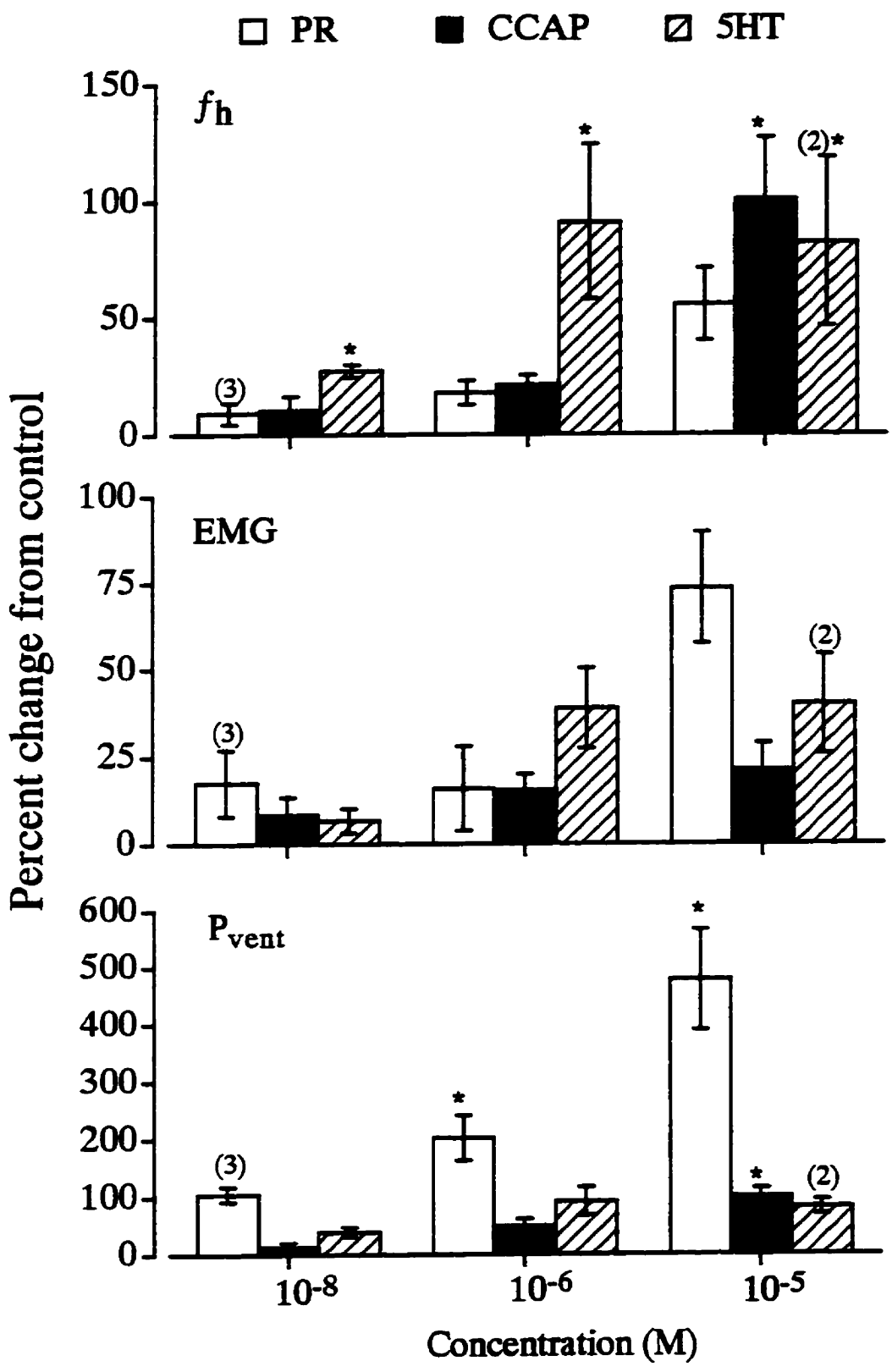


Figure 3.4 Summary of intact heart responses to the peptides and 5-HT. Normalized responses of $f_h$, EMG amplitude, and $P_{vent}$ to three concentrations each of PR, CCAP and 5-HT. PR increases $P_{vent}$ to a greater extent than the other hormones. For each bar N = 2–7 and data are mean ± S.E. Values of N = 2–3 are indicated above their corresponding bar on the graph; all other values are N = 4 or greater. Asterisks (*) indicate significant differences from control ($p < 0.05$).


PR
CCAP
5HT
$f_h$
EMG
$P_{vent}$
Percent change from control
Concentration (M)
$10^{-8}$
$10^{-6}$
$10^{-5}$
150
100
50
0
75
25
600
500
400
300
200
(3)
(2)
*

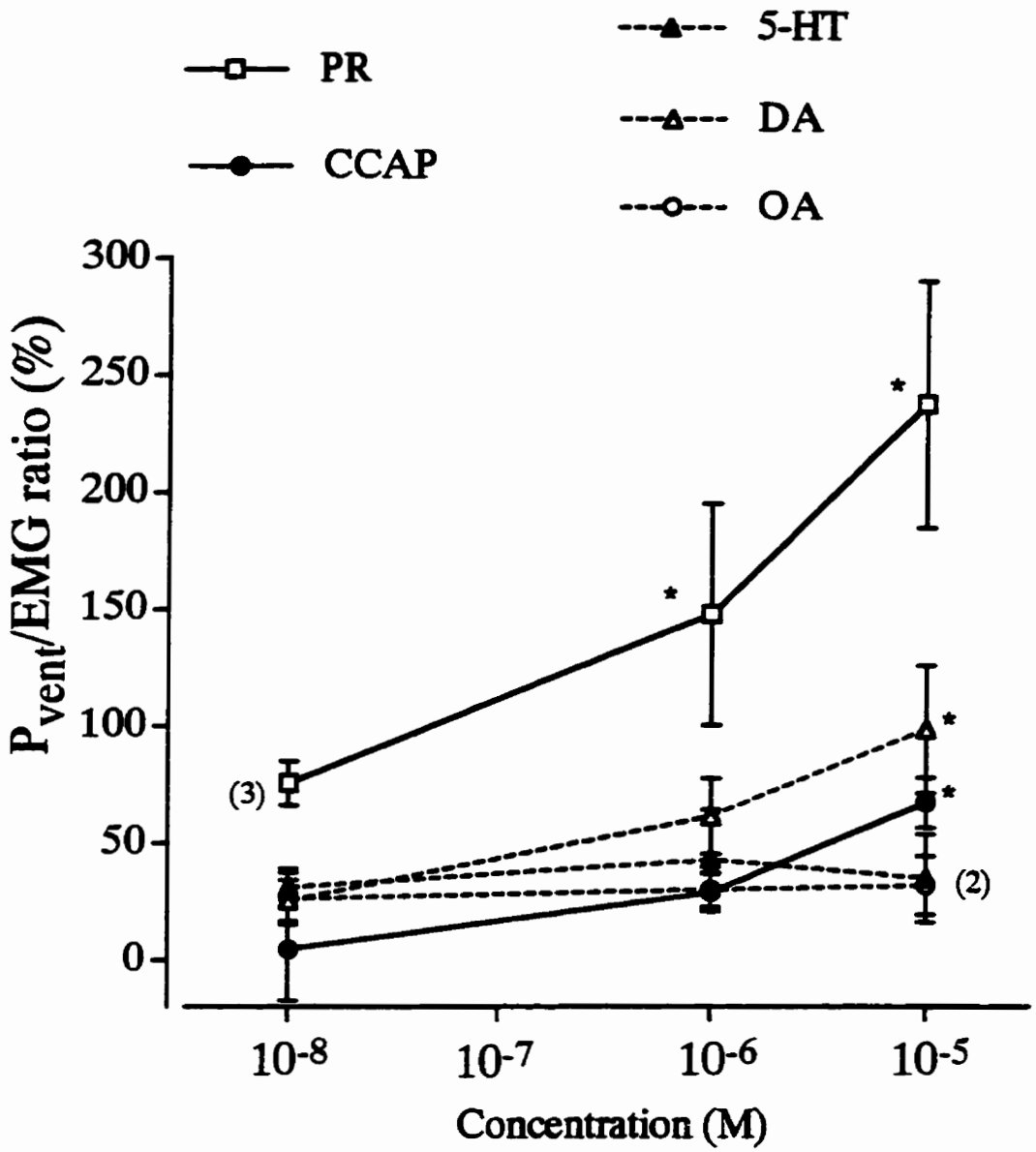



Figure 3.5 $P_{vent}$/EMG ratios for responses to all five hormones. These values indicate the ratio of heart mechanical to electrical properties. PR increases the $P_{vent}$/EMG ratio to a greater extent than the other hormones. Data were collected from intact hearts and are presented as the mean ± S.E. for each hormone. N = 2 to 7 for each point; values of N = 2–3 are indicated on the graph beside their corresponding points. Asterisks (*) indicate significant differences from control ($p < 0.05$).

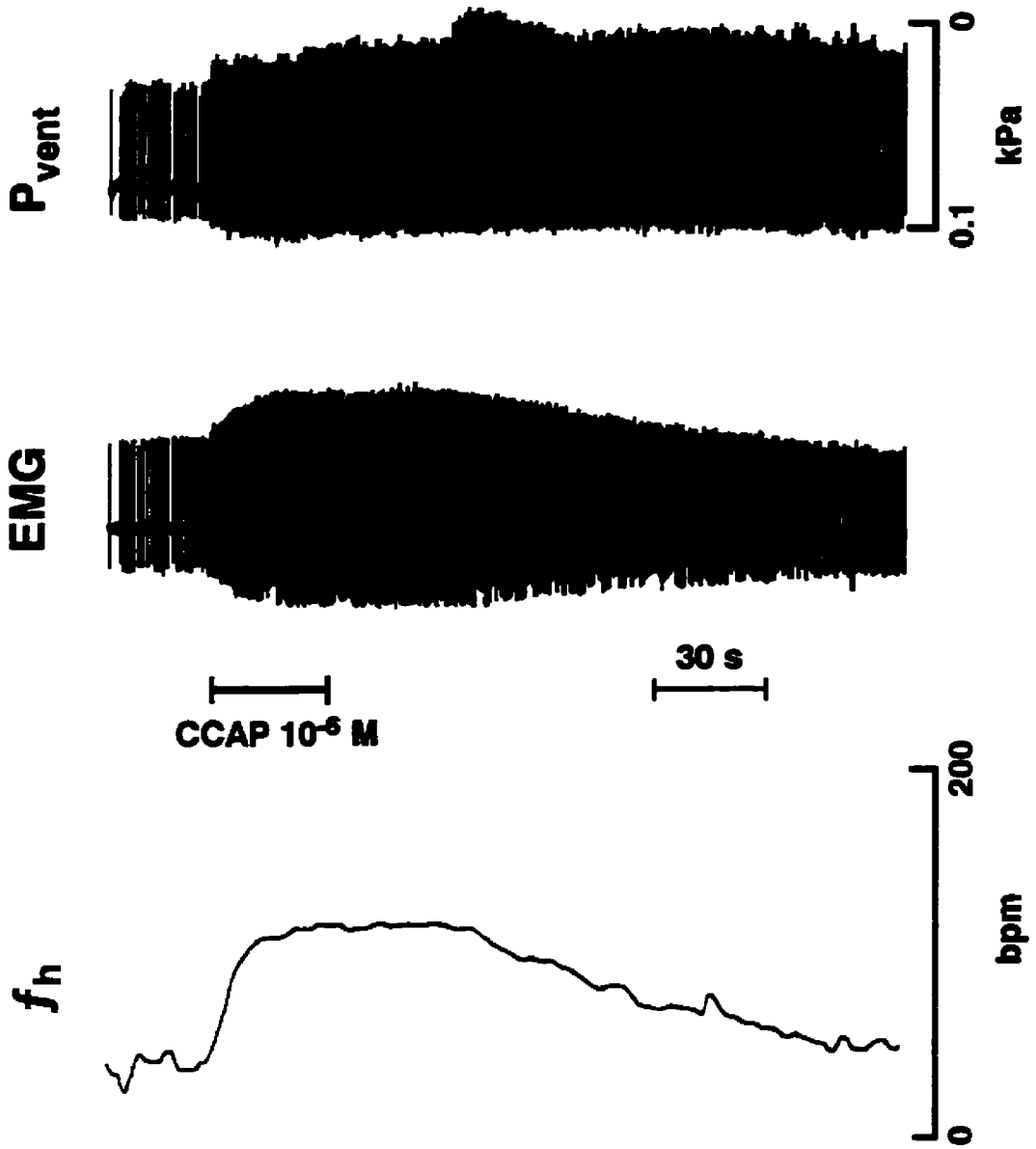



Figure 3.6 Effects of CCAP $10^{-6}$ M on an intact heart preparation. Heart was perfused with CCAP ($10^{-6}$ M) for 30 s. Effects on $P_{vent}$, EMG, and $f_h$ are illustrated. CCAP caused an immediate, dramatic rise in $f_h$. Effects on $P_{vent}$ were longer-lasting than the corresponding time course of changes in EMG amplitude.

in $P_{vent}$ (14–100%, p = 0.456 to 0.021, Fig. 3.5), and the CCAP-induced changes in $P_{vent}$/EMG ratio (5–67%) were smaller than those induced by PR at all concentrations tested (76–237%, Fig. 3.5). However, the $P_{vent}$/EMG ratio was significantly different from control for CCAP treatment at the highest concentration tested (p = 0.039, Fig. 3.5).

### 3.3.2.3 5-Hydroxytryptamine and dopamine

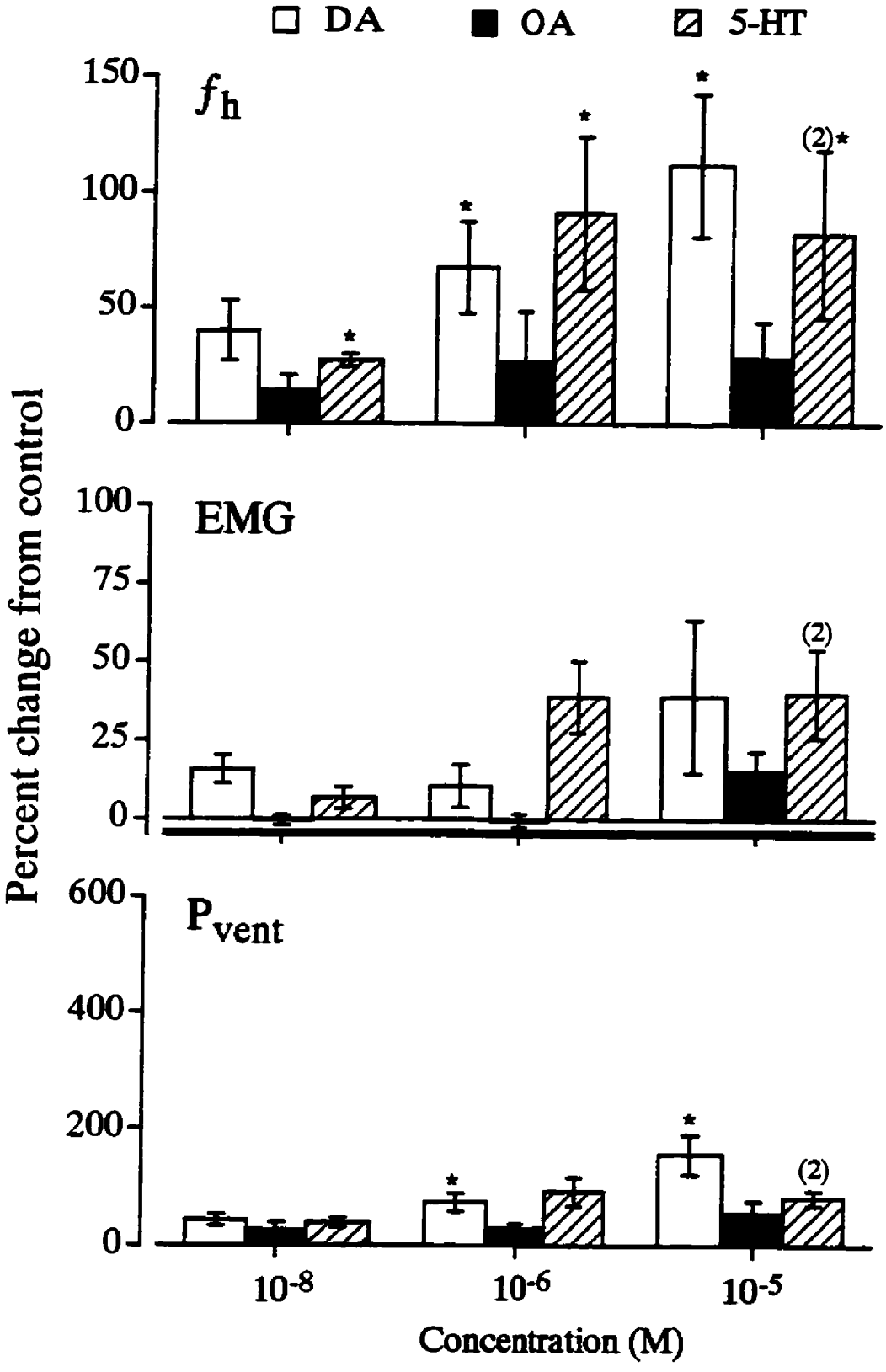
5-HT and DA were strongly cardioexcitatory, but the responses developed more slowly and were more prolonged than those produced by either PR or CCAP. The responses began in 15–30 s, and maximal effects were observed 120–150 s after the start of perfusion (data not shown). Recovery from amine perfusion was generally slower than for the peptides. Heart rate and EMG recovered before $P_{vent}$ for DA perfusions, but $f_h$ usually recovered after $P_{vent}$ for 5-HT trials. Although trials were only tape-recorded for 3–5 minutes, the responses of $f_h$, EMG, and $P_{vent}$ were monitored until they returned to baseline, which took up to 30–60 minutes at the highest concentrations for each amine. The time course of recovery from amine perfusion contrasted that of the peptides, which usually did not exceed 30 minutes at the highest concentrations.

Perfusion with DA or 5-HT increased $f_h$, EMG amplitude and $P_{vent}$. Summary data for these amines are shown in Fig. 3.7. EMG amplitude increased in parallel with $P_{vent}$ for both DA and 5-HT (Figs. 3.5 & 3.7). Only DA $10^{-5}$ M produced a significant increase in the $P_{vent}$/EMG ratio (p = 0.033, Fig. 3.5). 5-HT perfusion significantly increased $f_h$ at all concentrations tested (p = 0.015 at $10^{-8}$ M, p = 0.002 at $10^{-6}$ M, and p = 0.016 at $10^{-5}$ M). DA treatment increased both $f_h$ (28–112%) and $P_{vent}$ (61–99%) significantly above control at the $10^{-6}$ and $10^{-5}$ M concentrations ($f_h$: p = 0.019 and 0.010, $P_{vent}$: p = 0.032 and 0.005, respectively). Neither hormone produced significant changes in EMG amplitude.

### 3.3.2.4 Octopamine

*C. maenas* hearts were minimally responsive to OA. In some hearts, $f_h$ and $P_{vent}$ were decreased or showed no change in response to OA. The time course of OA effects generally followed that of the other amines; however, none of these changes was significant (Fig. 3.7).

Figure 3.7 Summary bar graphs of intact heart responses to the amines DA, OA, and 5-HT. Data are normalized and are mean ± S.E. Note that the scale for $P_{vent}$ remains the same as in Fig. 3.4, to show that the inotropic effects of the amines were lower than for PR. Each bar represents N = 2–7. Values of N = 2–3 are indicated above their corresponding bar on the graph; all other values are N = 4 or greater. Significant differences from control ($p < 0.05$) are indicated by asterisks (*).


DA
OA
5-HT
$f_h$
EMG
$P_{vent}$
Percent change from control
150
100
50
0
75
25
600
400
200
(2)
$10^{-8}$
$10^{-6}$
$10^{-5}$
Concentration (M)

### 3.3.3 Open heart preparations

To ensure that the increases in $P_{vent}$ recorded from intact hearts did not result from failure of the heart to empty due to tonic contractions of the cardioarterial valves (Kuramoto and Ebara, 1984); cardiac responses in open heart preparations were examined. Cardioarterial valve contracture was not measured directly, but rather inferred from observations regarding changes in intact and open heart contractility. Here in the open hearts, as in the intact hearts, the hormones PR, 5-HT and CCAP increased $f_h$, EMG amplitude, and tension (Fig. 3.8). The time course of hormone effects was similar to that observed in intact hearts. The effects of OA on open hearts were not examined since hearts appear to be unresponsive to this amine. For a comparison between peptide and amine effects on open hearts, the amine 5-HT was tested because of its chronotropic effects on *in situ* hearts; DA was not tested.

Only PR caused a large increase in muscle tonus that was maintained even during periods of cardiac arrest (Fig. 3.9). The PR-induced increases in isometric tension were disproportionate to (i.e., much greater than) the concurrent changes in EMG amplitude (Figs. 3.8 & 3.10).

The ratio of mechanical to electrical output is displayed as the tension/EMG ratio in Fig. 3.10. To calculate tension/EMG ratios, maximum tension amplitudes during hormone treatment were averaged and then compared to the average control tension to give the percent change in tension. Maximum EMG amplitudes that occurred at the same time as maximum tension were averaged and compared to the average control EMG amplitude to give the percent change in EMG. Finally, by dividing the percent change in tension by the percent change in EMG amplitude, the tension/EMG ratio was obtained. As for the $P_{vent}$/EMG ratios, the tension/EMG ratios following treatment with PR were significantly greater than those associated with the other tested hormones ($p = 0.014$ and 0.006 at PR $10^{-6}$ and $10^{-5}$ M, respectively, Fig. 3.10).

The average increase in EMG amplitude was slightly greater for CCAP than 5-HT at $10^{-6}$ M (22% and 15%, respectively). However, 5-HT induced greater tachycardia (165%) than did CCAP (133%) at $10^{-6}$ M; in both cases, the changes in $f_h$ were significant ($p < 0.0001$ for 5-HT and $p = 0.0059$ for CCAP, Fig. 3.8). Increases in EMG amplitude were positively correlated with—and proportional to—increases in tension for each of these hormones. Two hearts perfused with CCAP at $10^{-5}$ M showed signs of tonic contraction, but the amplitude of this increase in tonus was much lower than that induced by PR at $10^{-8}$ M (data not shown).

Figure 3.8 Summary data from open heart preparations. Normalized responses of $f_h$, EMG amplitude and tension to perfusions of PR, CCAP, and 5-HT. Only excitatory effects are presented. PR produced the greatest increases in tension. Data are shown as the mean ± S.E.; N = 4–9 for each bar. Asterisks (*) indicate significant differences from control ($p < 0.05$).

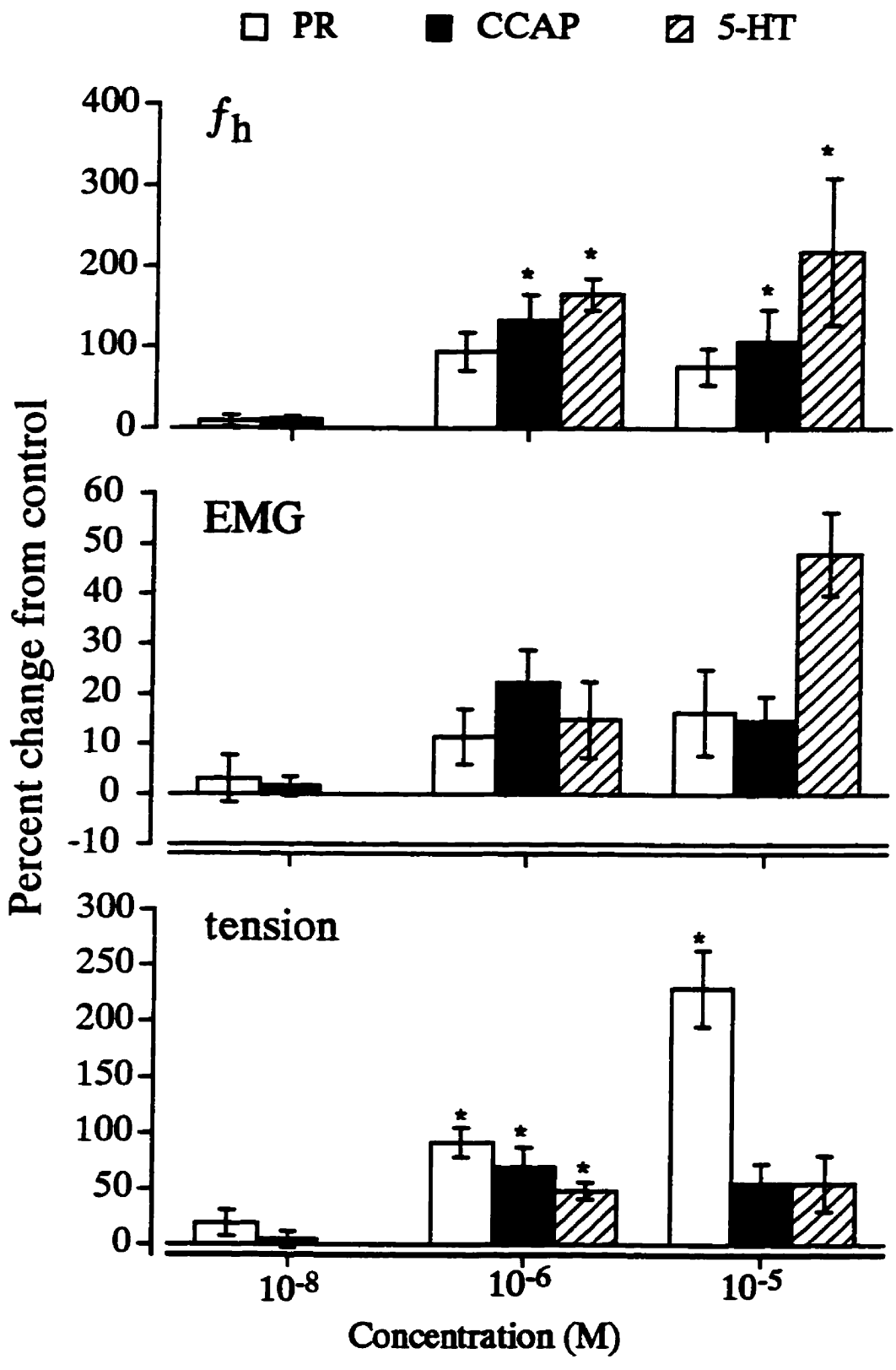
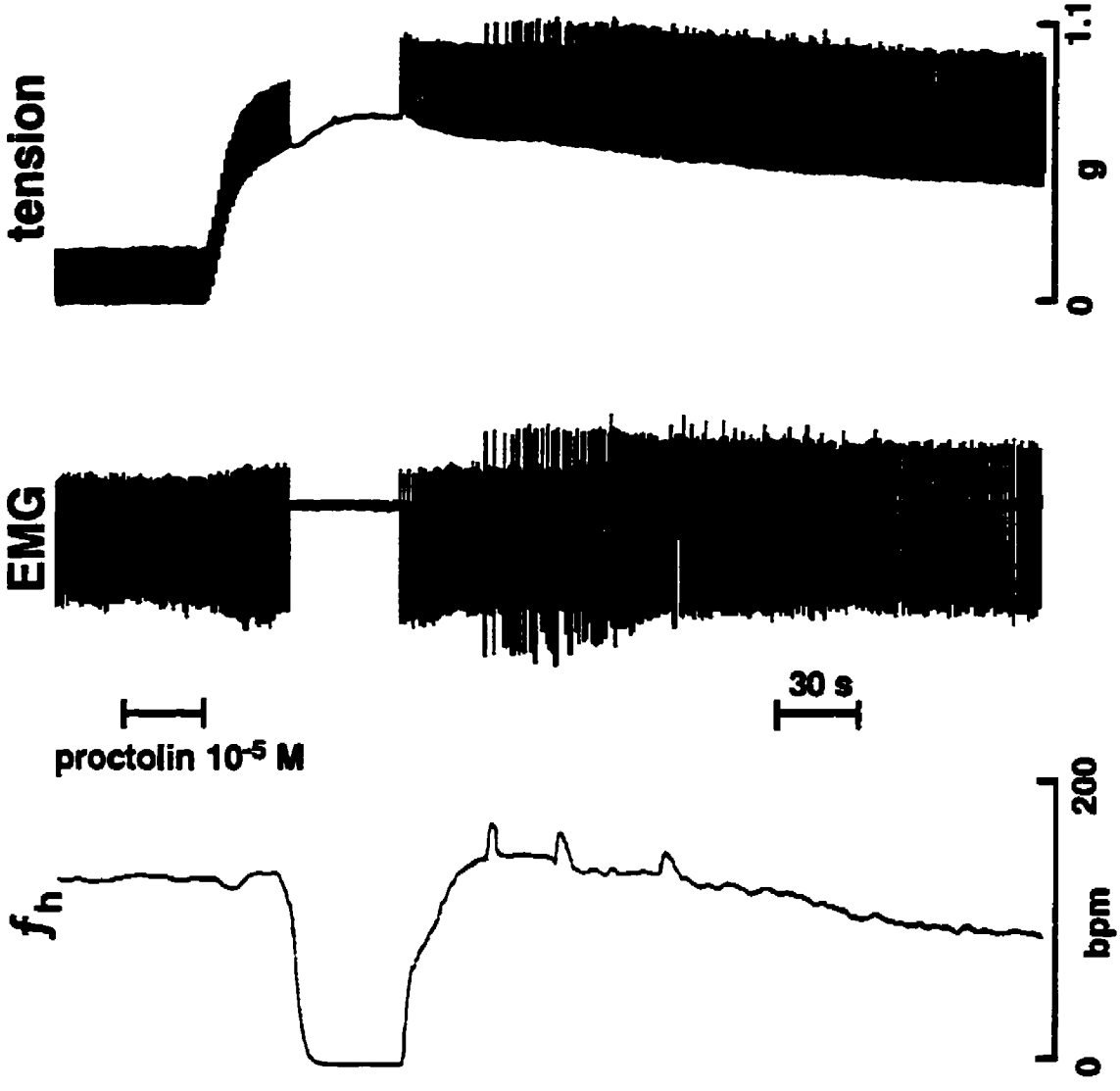

PR
CCAP
5-HT
$f_h$
EMG
tension
Percent change from control
400
300
200
100
0
60
50
40
30
20
10
0
-10
300
250
200
150
100
50
0
$10^{-8}$
$10^{-6}$
$10^{-5}$
Concentration (M)

Figure 3.9 PR perfusion increased muscle tonus during a period of cardiac arrest in an open heart preparation. Effects of PR ($10^{-5}$ M, 30 s) on tension, EMG amplitude, and $f_h$. The heart stopped for about 30 seconds. Note the increase in muscle tonus (indicated by the rise in tension above baseline) immediately after PR infusion that remained elevated throughout the period of cardiac arrest.


tension
1.1
g
0
EMG
30 s
proctolin 10-5 M
fh
200
bpm
0

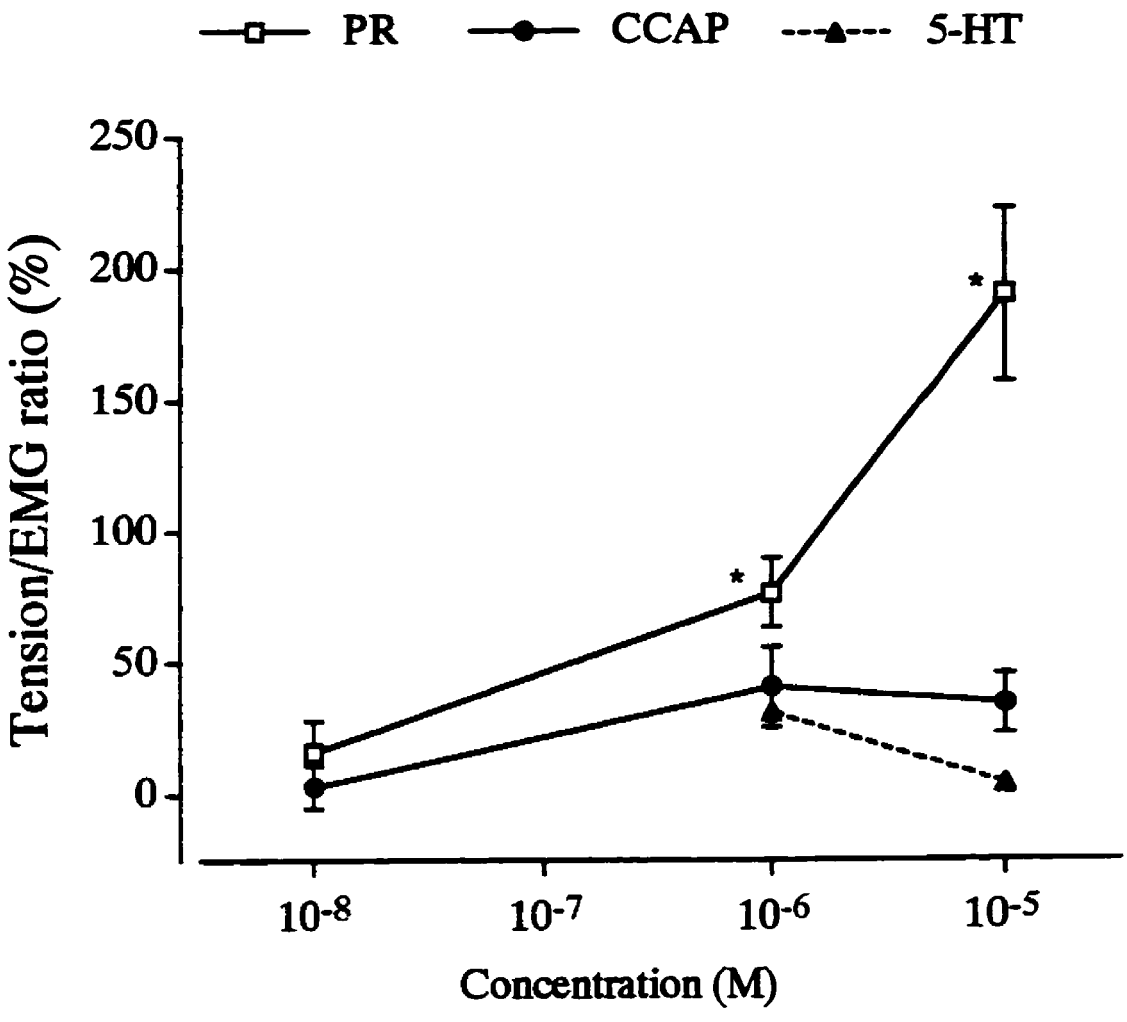



Figure 3.10 Tension/EMG ratios measured from open heart preparations. Only PR, CCAP and 5-HT were tested on open hearts. Of these three hormones, PR produced the greatest increases in tension/EMG ratio. Data are mean ± S.E.; N = 4–9 for each point. Significant differences from control ($p < 0.05$) are shown as asterisks (*).

## 3.4 Discussion

### 3.4.1 Stability of intact heart preparations

The fact that it was feasible to make recordings for 10 hours from intact hearts shows the robustness of this *in situ* preparation. However, these hearts were not stable over a long period of time as indicated by a progressive decline in both the electrical ($f_h$) and mechanical ($P_{vent}$) properties. $P_{vent}$ decreased more than $f_h$ in both glucose-rich and glucose-free saline, but there were no significant differences between glucose treatment groups. Only the decline in $P_{vent}$ in glucose-treated hearts was significant over the full ten hour time period. Thus, even when an attempt was made to replenish the heart with glucose to provide nutrients and energy for the contracting myocardium, there was no change in the magnitude of the decrease in mechanical performance. The conclusion is that heart performance decreased equally in hearts perfused with or without glucose. In *Homarus americanus* hearts there are large stores of glycogen present in the myocardium and heart ostia (J.L. Wilkens, personal communication). Assuming that the *C. maenas* myocardium contains similar glycogen stores as *H. americanus* hearts, then it appears that these endogenous stores provide adequate energy for the contracting myocardium and that glucose does not need to be added into the saline or by other external sources to maintain heart contractility.

Cardioregulatory nerves originating from the central nervous system (CNS) modify contractile rate and force. Central activation drives the cardioregulatory nerves; they show activity only as long as the CNS remains viable. The eyestalk withdrawal reflex (EWR) is used as a measure of the viability of the CNS. Animals with intact CNS activity show withdrawal of the eyestalk in response to its tactile stimulation, while animals without CNS activity do not show EWR. At the same time as the decline in $P_{vent}$ observed between the third and fourth hours of this study, animals no longer showed responsiveness to eyestalk stimulation. Therefore, it is suggested that loss of CNS regulatory activity to the heart may be responsible for the decline in $P_{vent}$ occurring at this time.

The remainder of the data in this chapter were taken from experiments lasting 3–6 hours at most. Thus, there was some decline in heart performance, but the tail end of the decline that occurred between 7–10 hours was not observed. Although $f_h$ and $P_{vent}$ decreased, the effectiveness of each hormone did not change during a trial.

### 3.4.2 Each hormone produces characteristic effects

Each of the five hormones tested in this study produced different effects on the heart of *C. maenas*, and the responses offered some insight to the sites of action of each agent. Chronotropic effects suggested hormone actions that modify the burst rate of the CG. All of the hormones tested increased $f_h$; the relative potencies were DA>CCAP>5-HT>PR>OA, ranked according to the average increase in $f_h$ upon exposure to each hormone at $10^{-5}$ M (Figs. 3.4 & 3.7). Therefore, each hormone exerted at least some effect directly on the CG.

All of the hormones produced inotropic effects, with the most powerful responses seen in response to PR treatment. The relative potencies, in terms of their inotropic effects were PR>>DA>CCAP>5-HT>OA, ranked according to the average $P_{vent}$ responses observed during exposure to each hormone at $10^{-5}$ M (Figs. 3.4 & 3.7). Only PR brought about inotropic effects (revealed by increases in both $P_{vent}$ and isometric tension) not accompanied by parallel changes in EMG. Since all hormones produced increases in $f_h$, a component of the inotropic effect may have been due to facilitation occurring in response to the elevated burst rates (Anderson and Cooke, 1971).

### 3.4.3 Proctolin

#### 3.4.3.1 PR acts at the cardiac ganglion and perhaps at the CNS cardio-regulatory nerves

The cardiac responses to PR were reported previously from a variety of crustacean species and preparations (Miller and Sullivan, 1981; Sullivan and Miller, 1984; Wilkens et al., 1985; Freschi, 1989; Wilkens and Mercier, 1993; McGaw et al., 1994, 1995). The chronotropic effects of PR depends on the type of preparation being used. Injection of PR into intact *C. maenas* is followed by a period of bradycardia or arrest and then by moderate tachycardia (Wilkens et al., 1985), whereas perfusion into semi-isolated hearts produces only tachycardia (Wilkens and Mercier, 1993). In contrast, PR has no significant effect on $f_h$ in intact *Cancer magister* (McGaw et al., 1994, 1995). In the present study, PR often caused biphasic rate responses (Fig. 3.3B) in which hearts exhibited brief periods of cardiac arrest or bradycardia in response to PR, then resumed beating with increased contraction amplitude ($P_{vent}$) and rate. Generally PR increased $f_h$. The possibility that PR may act on the cardioregulatory centers in the central nervous system to produce bradycardia or arrest can not be discounted, since this was observed in hearts that were innervated by the cardioinhibitory nerves. The latent period from PR

arrival to arrest could be explained as the time required to pump the hormone into the CNS. It is suggested that the CNS cardioregulatory nerves may be a source of PR control of heart function in *C. maenas*. This finding fits with the prediction of multiple sites of action for PR.

In both intact and open heart preparations examined in this chapter, PR increased $f_h$ (chronotropic effect) and slightly increased EMG amplitudes (see Figs. 3.4 & 3.8). Since $f_h$ and EMG amplitude are indicators of cardiac ganglion (CG) electrical activity, it is concluded that PR acts at the level of the CG neurons to alter the CG burst frequency and CG burst pattern, respectively. Increased CG burst frequency and duration in response to PR were reported previously for the lobster *H. americanus* (Miller and Sullivan, 1981; Sullivan and Miller, 1984) where PR causes longer-lasting effects on the large CG cells (motoneurons) than on the small cells (pacemakers), and the CG often produces double bursts in response to PR treatment. PR also activates a voltage-dependent $Na^+$ current in *H. americanus* CG motoneurons (Freschi, 1989). Thus, proctolin may change the bursting rate, number of spikes per burst, burst duration and amplitude of CG discharges.

### 3.4.3.2 PR acts at the myocardium

In the present study PR induced inotropic responses in both intact and open heart preparations. In intact hearts $P_{vent}$ increased by up to 477% at the highest concentration of PR perfused ($10^{-5}$ M, Fig. 3.4). In open hearts, the same concentration of PR produced a 229% increase in tension (Fig. 3.8). Since the CG motor neurons send processes to innervate each individual myocardial fibre (Anderson and Cooke, 1971; Kuramoto and Kuwasawa, 1980), the CG electrical activity was expected to give rise to proportional increases in mechanical output. In other words, increased EMG amplitudes were expected to parallel the resultant increase in $P_{vent}$ or tension. However, the PR-induced increases in mechanical force greatly surpassed the increases in EMG amplitudes (Figs. 3.4, 3.8 & 3.10). This suggested that the large increases in $P_{vent}$/EMG ratio were due to the direct action of PR on muscle fibre contractility in addition to modulation of CG bursting pattern.

PR infusion into intact and open hearts often resulted in cardiac arrest during which the CG was silent (as indicated by the absence of the EMG signal) but muscle tonus continued to increase in the absence of ganglionic input (Fig. 3.9). This strengthened the argument that one site of PR action is the myocardium. Based on earlier studies from

*Limulus polyphemus* cardiac muscle (Benson et al., 1981), it is concluded that PR is a myotropic hormone for the heart.

PR appears to act directly on the myocardium. According to Laplace's Law, which states that $\sigma = Pr/w$, where $\sigma$ is wall stress (tension, or force per unit area), P is transmural pressure, r is heart radius, and w is heart wall thickness, heart diameter is determined by the balance between the force developed during a muscular heart contraction and the transmural pressure, which is the force produced when the heart is distended (Berne and Levy, 1992). Thus when comparing small vs. large radius hearts, for each heart to generate the same amount of tension, the small radius heart must generate a greater pressure while the larger radius heart must generate a smaller pressure. Laplace's Law may be a factor in intact hearts, where the heart is generating pressure, but does not apply to open hearts, which are assumed to behave like a strip of muscle. In the intact hearts, as the heart contracts the radius decreases. In the presence of a hormone such as proctolin, tension increases (as evidenced by open heart data) and in combination with A decrease in radius, Laplace's Law states that these changes would lead to increased pressure development by the heart. This was observed in intact hearts, and thus part of the increased myocardial contractility may be explained by the relationship between radius, tension and pressure in Laplace's Law.

### 3.4.3.3 PR may act at the cardioarterial valves

The cardioarterial valves are another possible site of PR action in crustacean hearts. Proctolin causes contracture of the cardioarterial valves in lobsters (*Panulirus japonicus,* Kuramoto and Ebara, 1984; *H. americanus,* Wilkens et al., 1996). This contracture could potentially have prevented perfusate ejection and contributed to the increased $P_{vent}$ observed in the present study. To ensure that the increases in $P_{vent}$ recorded in intact heart preparations were *not* due to an increase in outflow resistance arising from valve contraction, the contractile force produced by hearts with the side wall opened was measured. While possible effects of PR on the cardioarterial valves were not studied directly, two observations provided evidence validating the concern about the cardioarterial valves. The tension/EMG ratios following exposure to PR were not as great as the corresponding $P_{vent}$/EMG ratios measured from intact hearts. Additionally, the increase in force was not as great in open hearts as in intact hearts. Taken together, these differences suggested that one site of PR action may indeed have been on the cardioarterial valves in intact hearts. With the open heart preparation, it was thought that the true effects

of PR on the myocardium were seen since the possibility of action on the valves was minimized by opening the side wall.

### 3.4.4 Crustacean cardioactive peptide

CCAP mainly exerted a chronotropic action on the heart. The effects of CCAP on $f_h$ in the present study (Figs. 3.4 & 3.6), performed at 20°C, were less dramatic than those observed in semi-isolated *C. maenas* hearts at 12°C (Stangier, 1991; Wilkens and Mercier, 1993). Since the control $f_h$ at the higher temperature was already greater than 100 bpm in most animals, the scope for further responses may have been limited. The chronotropic actions of CCAP are species-specific; it causes minimal effects on $f_h$ in *C. magister* (McGaw et al., 1995) and in *C. pagurus*, crabs in which the POs are reported to contain very little CCAP (Stangier, 1991).

It was found that tachycardia occurred almost immediately at all concentrations of CCAP. The increases in EMG amplitudes elicited by CCAP followed the same time course as the increases in $f_h$, and were proportional to the increase in $P_{vent}$, suggesting that all of CCAP's effects were accounted for by actions on the CG. Open hearts responded similarly to intact hearts, ruling out the likelihood that increases in $P_{vent}$ were a result of cardioarterial valve-induced changes in outflow resistance, as was observed during PR treatment.

These data strengthened previous suggestions (Stangier, 1991; Wilkens and Mercier, 1993) that the primary site of action of this peptide is the CG rather than the neuromuscular junction or the myocardium. The moderate inotropic effects of CCAP appeared to arise from the increases in EMG.

### 3.4.5 Dopamine and 5-hydroxytryptamine

The responses to DA and 5-HT (time course, $f_h$, EMG amplitude, and $P_{vent}$) by intact hearts were similar in most aspects (Fig. 3.7). At all concentrations of DA and 5-HT, the maximum responses occurred about 2 minutes after hormone arrival at the heart. When high concentrations ($10^{-5}$ M) were used, up to an hour of washing was required before responses returned to control values. Among several possibilities, this time course suggested that a slowly activated second messenger system with a long half-life may be involved in mediating the response. The only second messenger system examined thus far has been cAMP and investigators did not find changes in cAMP in lobster CGs following exposure to 5-HT (Lemos and Berlind, 1981). Other intracellular

messengers such as inositol 1,4,5-triphosphate ($IP_3$), diacylglycerol (DAG), or arachidonic acid can not be discounted at this point, as they have not been tested.

In intact hearts, 5-HT brought about increases in $f_h$ and $P_{vent}$ which were similar to those reported previously for the isolated hearts of *C. maenas* (Wilkens and McMahon, 1992) and *P. japonicus* (Kuramoto and Ebara, 1984). The chronotropic effect suggested direct action on the CG neurons. The parallel increases in the onset and recovery of $f_h$ and $P_{vent}$ suggested that part of this observed increase in contractility may have been due to facilitation resulting from increased burst rate. Facilitation is likely due to increased neurotransmitter release from the presynaptic terminals at higher impulse frequency (Katz, 1966). For many years, this facilitated transmitter release was thought to be due to residual free $Ca^{2+}$ remaining in the presynaptic terminal and adding to the $Ca^{2+}$ entering in subsequent impulses (Katz and Miledi, 1968). More recently, however, there is less support for this residual (free) $Ca^{2+}$ hypothesis (Kamiya and Zucker, 1994) and more evidence pointing to other mechanisms for facilitation (such as bound $Ca^{2+}$, Blundon et al., 1993; Bertram et al., 1996; or a role for $Ca^{2+}$ buffers, Winslow et al., 1994).

DA perfusion led to parallel increases in $P_{vent}$ and EMG in intact hearts, as illustrated in $P_{vent}$/EMG ratios that were in the same range as those for the other hormones tested (Fig. 3.5). DA produced significant increases in heart rate at $10^{-5}$ and $10^{-6}$ M (Fig. 3.7). Increased frequency and duration of action potential bursts in response to DA were observed in semi-isolated CGs from the crabs *Portunus sanguinolentus* and *Podophthalmus vigil* (Miller et al., 1984). At low concentrations ($10^{-8}$ M threshold), DA stimulated driver potentials in the ganglionic small cells (pacemakers), while at higher concentrations ($10^{-5}$ M) it also affected the large cells (motorneurons). Although intact hearts were not studied, these authors suggested that an increased number of action potentials per burst would increase the amplitude and force of myocardial contraction. Data from the present study were consistent with this interpretation, indicating that DA exerts action at the CG neurons.

### 3.4.6 Octopamine

OA elicited only small, insignificant changes in $f_h$, EMG amplitude and $P_{vent}$ (Fig. 3.4) in intact hearts. These minor cardiac responses to OA agreed with some, but not all, previous findings. OA causes only small $f_h$ increases in *H. americanus* (Grega and Sherman, 1975), but it is a powerful cardioaccelerator in *Astacus leptodactylus* (crayfish) and *Eriphia spinifrons* (crab) hearts (Florey and Rathmayer, 1978). On *E. spinifrons*

and *C. magister* hearts, OA causes inhibitory, followed by excitatory effects on $f_h$ (Florey and Rathmayer, 1978; Airriess and McMahon, 1992). Benson (1984) found that OA decreases the burst frequency but increases the burst duration of isolated *P. sanguinolentus* ganglia. In the present study only mild tachycardia was associated with OA treatment in intact hearts, which suggested that the responses to OA are both species- and preparation-dependent.

### 3.5 Summary

Two novel *C. maenas* preparations, *in situ* intact and open hearts, were used to survey the effects of five endogenous PO neurohormones on heart mechanical and CG electrical properties. Some insights into each hormone's site of action were gained from this study. Although each hormone was shown to produce its own characteristic set of heart responses, all hormones increased $f_h$ and EMG amplitude, changes which were attributed to hormone action on ganglionic output.

Consistent with the hypothesis that PR has multiple effects in *C. maenas* hearts, three or possibly four, sites of action were demonstrated for this peptide. These were the CG neurons, the myocardium, and possibly the cardioarterial valves and the cardio-regulatory nerves of the CNS. Evidence was also shown supporting the prediction that all hormones except PR would increase ganglionic output in proportion to mechanical force. This study provides a better understanding of how PR may regulate cardiac activity in *C. maenas* crabs and in other decapod crustaceans.

# CHAPTER FOUR: THE EFFECTS OF PROCTOLIN AND 5-HT ON ISOLATED HEARTS

## 4.1 Introduction

The cardiac ganglion (CG), a neural rhythmic generator located in the decapod crustacean heart, initiates contraction. Heart rate is regulated by one pair of cardioinhibitor and two pairs of cardioaccelerator nerves from the CNS that synapse on the CG (Florey, 1960; Yazawa and Kuwasawa, 1984). Additionally, contractile rate and force are regulated by hormones released from the nerve terminals of the pericardial organs (POs, Alexandrowicz, 1932) located in the sinus around the heart. Each of these amine and peptide substances can operate at multiple sites in the circulatory system.

The effects of five PO neurohormones were previously investigated on intact and open hearts from *Carcinus maenas* (Chapter Three). Of these neurohormones, the amine 5-hydroxytryptamine (5-HT) and the peptide proctolin (PR) were chosen for this phase of the study because of their contrasting effects on *in situ* heart preparations (Chapter Three). Both PR and 5-HT altered CG burst rate and heartbeat frequency ($f_h$) in intact and open hearts. However, in contrast to the predominantly chronotropic effects evoked by 5-HT on *in situ* hearts, PR caused large inotropic effects. The chronotropic effects of 5-HT were attributed to action on the CG neurons. In contrast, the peptide PR altered CG burst rate and output pattern, myocardial force, cardioarterial valve contracture, and caused periods of cardiac arrest accompanied by contracture. It was not ruled out that some of the PR effects arose from actions at the level of the CNS. To eliminate the possibility of the problem of CNS regulatory neuronal input to the heart, an isolated heart preparation was developed for this investigation. In this preparation, the heart was fully removed from CNS and hormonal inputs, which narrowed the focus to hormonal effects at either the CG neurons or at the myocardium.

In this study, myocardial intracellular recordings were made from isolated *C. maenas* hearts to assess hormone effects on myocyte membrane properties. The aim was to discover whether the action of PR and 5-HT at the myocyte could be accounted for by changes in membrane potential ($V_m$) and membrane input resistance ($R_{input}$). From the excitatory junction potentials (EJPs) and tension development, the relationship between ganglionic output and myocardial tension development, the tension/EJP ratio, was examined to distinguish between the modes of action for PR and 5-HT. The hypothesis tested was that PR has dual sites of action in isolated hearts; at the CG and at the

myocardium, which would result in significant changes in the tension/EJP ratio. For 5-HT, however, significant differences in the tension/EJP ratio were not predicted, since the only site of action for this amine is thought to be at the CG.

## 4.2 Materials and methods

### 4.2.1 Isolated heart preparation

Animals, surgical procedures, and methods to isolate the heart were detailed in Chapter Two. Unless stated otherwise, values are expressed as the mean ± S.E. Paired two-tailed *t* -tests were performed and significant differences from control ($p < 0.05$) are indicated in figures by an asterisk (*). Significant differences between PR and 5-HT treatments were also assessed by *t* -test and indicated in figures with a dagger (†).

## 4.3 Results

### 4.3.1 General characteristics of isolated hearts

This is the first study to detail intracellular recordings from *C. maenas* myocytes, although Brown (1964) reported a resting membrane potential of -62 mV in the same species. The isolated heart preparation itself was very robust; some hearts continued to contract for 6–8 hours, although contractile tension decreased over time.

The resting myocyte $V_m$ was -46 mV (range: -34 to -66 mV). Myocyte $R_{input}$ varied from cell to cell, with the average resting value being 20 ± 3 MΩ. The resting heart rate of these spontaneously contracting hearts was 17 ± 3 beats per minute (bpm), which was slower than resting heart rates in either intact or open *C. maenas* hearts (114 and 61 bpm, respectively, Chapter Three). This difference in heart rate is most likely due to the loss of its regulation by the CNS in the isolated preparation. The typical EJP waveform showed multiple peaks. The first spike of the burst was of the highest amplitude, followed by several small peaks. The first spike of the burst was used for analysis of EJP amplitudes. Overshooting bursts were recorded in only 3 of 12 hearts. Whole-heart tension was also recorded from these spontaneously contracting hearts.

Occasionally small depolarizations, which were characterized as slow deflections of only 3–5 mV above baseline, were recorded from myocytes rather than a regular EJP burst. These non-bursting myocytes had lower membrane potentials ($V_m$ was -50 mV) than average. All of the data presented were taken from myocytes showing normal bursting activity.

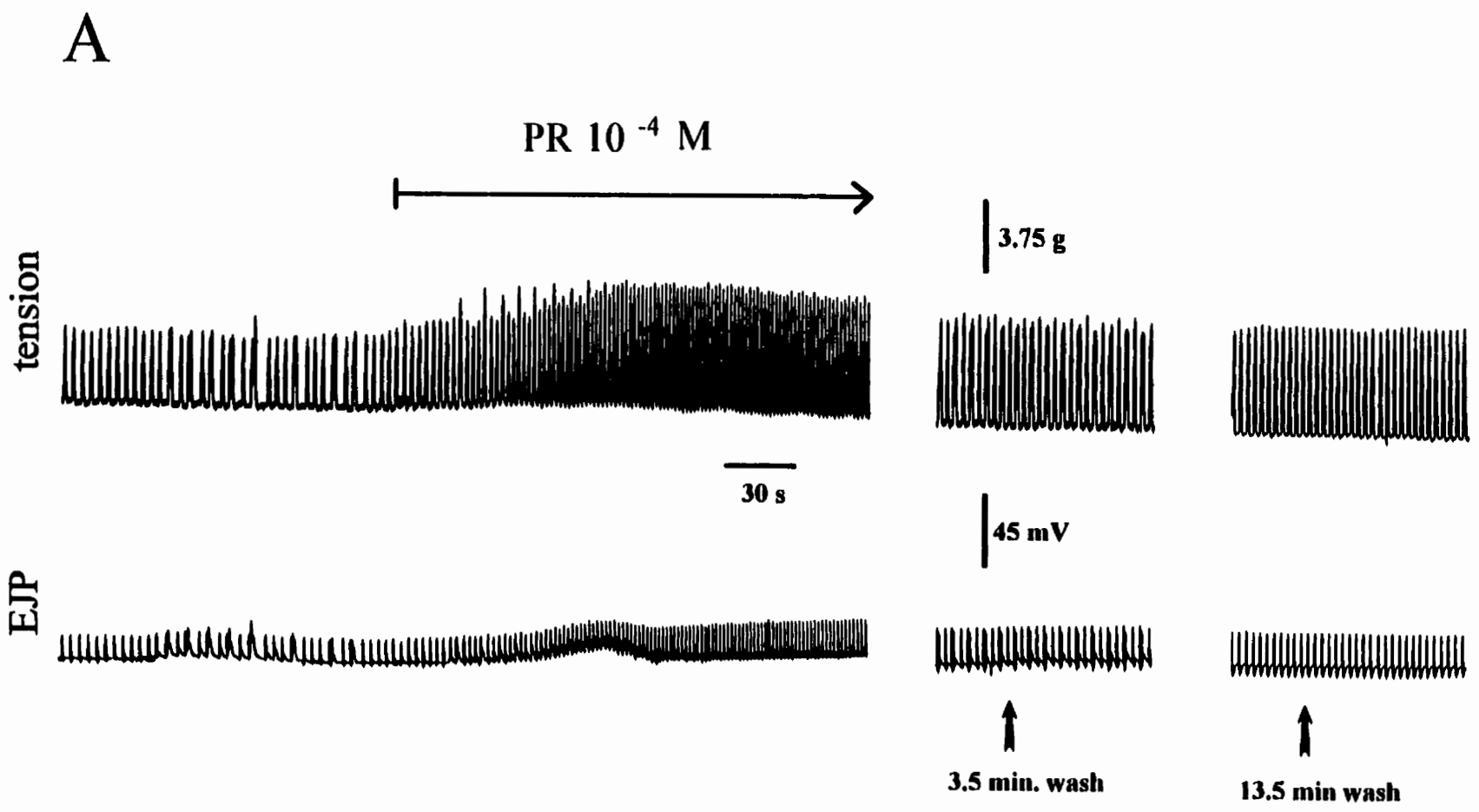
### 4.3.2 Proctolin

PR induced very forceful contractions that often dislodged the microelectrode (either conventional or dog-leg electrode) from the myocyte. In all, PR data were recorded from eleven hearts. In cells where microelectrode penetration was maintained throughout a 5 minute PR perfusion, the time course of PR effects was followed and they were shown to be fast in onset and recovery (Fig. 4.1A). Fig. 4.1A shows the transition from control EJP and tension through a PR perfusion. By 13.5 minutes of saline wash, tension had returned to baseline tonus but amplitude was still elevated. PR arrival at the heart caused immediate changes in three hearts, but there was a delay of 30 s to 2.5 minutes in six other hearts in which cells were maintained through the transition to PR. The time required for the hormone to flow through the line and reach the heart was about 1.5 minutes. Recovery time after washoff varied depending on the duration of the hormone application. For short duration hormone exposures (4–5.5 minutes), heart rate recovered after 12–15 minutes while tension remained elevated for 15–28 minutes. For the longer duration applications, from which conventional microelectrode recordings were made, recovery occurred by 30 minutes for $f_h$ and by 1 hour for tension. In general, heart rate recovered before tension. In fact, for one long duration hormone perfusion, in which PR was applied for 45 minutes, $f_h$ recovered by 15 minutes but tension remained elevated for nearly 3.5 hours of washing.

In some hearts (5 of 11), PR caused two EJPs in quick succession, which were manifested as double beats of the heart (Figs. 4.1A & 4.2A). Fig. 4.2A displays recordings from one heart in which PR caused doublet bursting. In Fig. 4.2A, the raw data traces were recorded from the same cell that was held through a PR exposure. Conventional microelectrodes were used for myocyte recordings in this particular heart.

As shown in Fig. 4.3, PR perfusion increased $f_h$ and isometric tension amplitude nearly two-fold and also increased EJP burst amplitude. These expanded records were taken from the same heart as shown in Fig. 4.1A and were from one cell that was followed through an entire PR exposure. Fig. 4.4 is a bar graph summary of the PR effects on isolated hearts. The average PR-induced increase in tension amplitude (56 ± 12%) was greater than the increase in EJP amplitude (22 ± 5%), and both changes were significantly greater than control ($p = 0.016$ and 0.012, respectively). The ratio of the increase in tension relative to EJP amplitude was 128 ± 8%; this change was significant ($p = 0.011$, Fig. 4.4). PR caused $V_m$ to depolarize by an average of 2 ± 0.5 mV (which

Figure 4.1 A comparison of the time courses of PR and 5-HT effects in isolated hearts. (A) First panel shows transition from control to PR (indicated by bar). Top: tension. Bottom: intracellularly-recorded myocyte excitatory junction potentials (EJPs). Note that tension increased within 30 seconds after arrival of PR in the bath. Second panel shows doublet bursts at 3.5 minutes of saline wash. Third panel shows that at 13.5 minutes of saline wash, rate and EJP amplitude are almost completely recovered but tension is still elevated above control. Resting myocyte membrane potential ($V_m$) and membrane input resistance ($R_{input}$) were -54 mV and 34 MΩ, respectively. (B) First panel shows a 5.5 minute perfusion of 5-HT $10^{-6}$ M (indicated by the bar). Note the delayed onset of 5-HT effects compared to the faster effects of PR in (A). Second panel shows that all values are still elevated above control at 8 minutes of saline wash. Top: tension. Bottom: myocyte EJPs. $V_m$ and $R_{input}$ were -58 mV and 40 MΩ, respectively.


A
PR 10 -4 M
tension
3.75 g
30 s
45 mV
EJP
3.5 min. wash
13.5 min wash

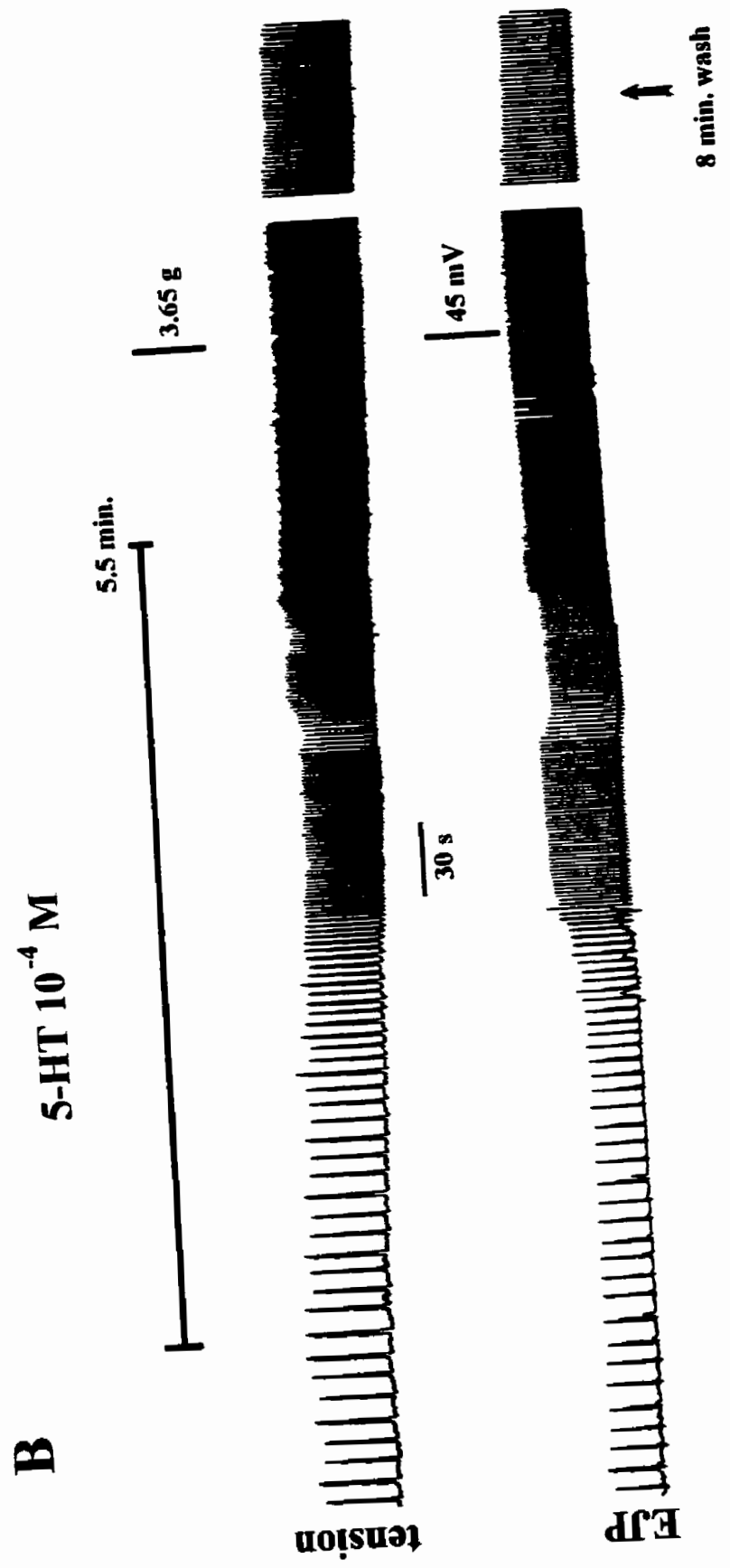

B
5-HT $10^{-4}$ M
5.5 min.
3.65 g
45 mV
30 s
8 min. wash
tension
EJP

Figure 4.2 PR, but not 5-HT, treatment evokes doublet bursting in isolated hearts. Raw data traces A and B from the same heart. (A) Top: Expanded control EJP bursts and tension prior to PR application. Bottom: EJP bursts and tension 2 minutes after PR arrival at the heart. Doublet bursts are predominant. Control $V_m$ and $R_{input}$ were -40 mV and 10 MΩ, respectively. (B) Top: Expanded control EJP bursts and tension prior to 5-HT application. Bottom: EJP bursts and tension after 5-HT treatment. There were no doublet bursts following 5-HT perfusion. $V_m$ was -49 mV and $R_{input}$ was 12 MΩ. Scale bars are the same in both A and B.

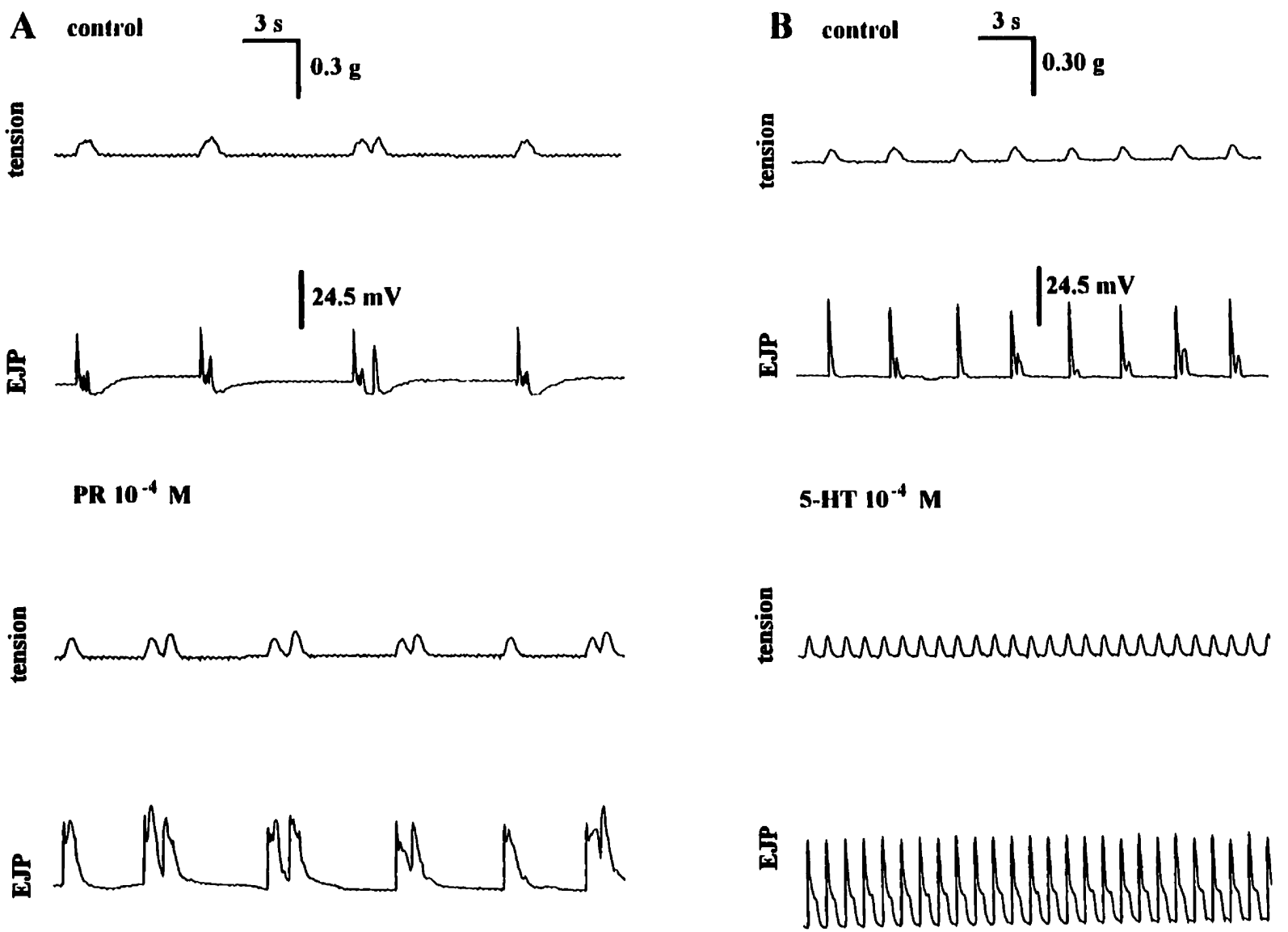

A
control
3 s
0.3 g
tension
24.5 mV
EJP
PR 10 -4 M
tension
EJP
B
control
3 s
0.30 g
tension
24.5 mV
EJP
5-HT 10 -4 M
tension
EJP

Figure 4.3 Raw data traces of an isolated heart response to bath perfusion of PR $10^{-6}$ M. (A) Control tension and myocyte EJPs (recorded with dog-leg electrodes). Resting $V_m$ and $R_{input}$ were -54 mV and 34 MΩ, respectively. (B) Tension and EJP waveforms recorded 5 minutes after PR arrival at the heart. PR induced a large inotropic response, doubled the rate (frequency of EJP bursts), and increased EJP amplitude.

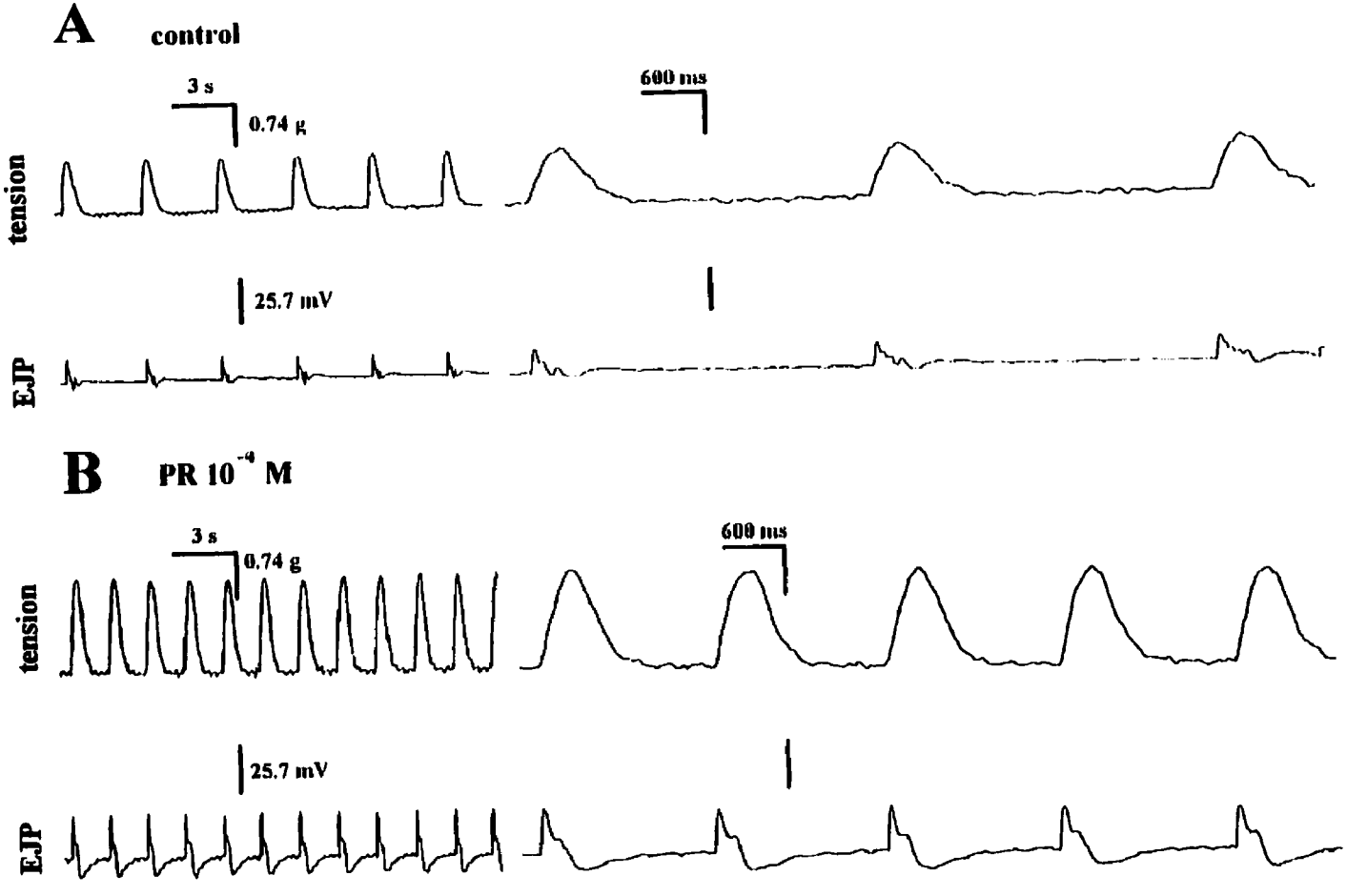
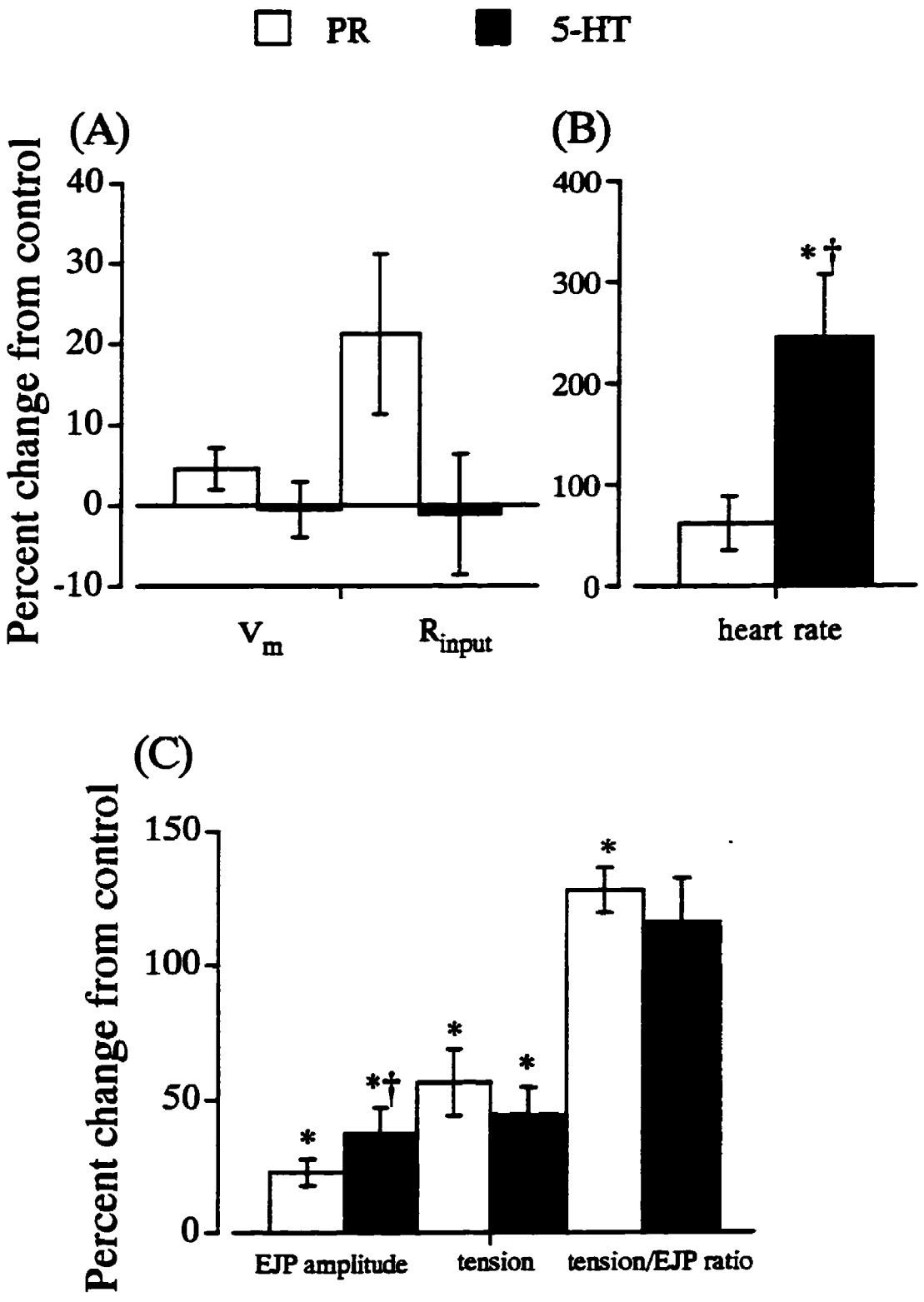

A
control
3 s
0.74 g
600 ms
tension
25.7 mV
EJP
B
PR 10⁻⁴ M
3 s
0.74 g
600 ms
tension
25.7 mV
EJP

Figure 4.4 Summary graphs of the effects of PR $10^{-6}$ M (open bars) and 5-HT $10^{-6}$ M (filled bars) on *C. maenas* isolated heart preparations. Hormone effects on (A) myocyte $V_m$ and $R_{input}$, (B) heart rate, and (C) intracellularly-recorded myocyte EJP amplitude, myocardial isometric tension, and the EJP/tension ratio. All values are percent changes from control, and are shown as the mean ± S.E. Significant differences ($p < 0.05$) from control are indicated by an asterisk (*); significant differences between hormones are indicated by a dagger (†). Neither hormone significantly increased $V_m$ or $R_{input}$. 5-HT increased heart rate and EJP amplitude significantly more than PR. Only PR significantly increased the tension/EJP ratio.


PR
5-HT
(A)
(B)
Percent change from control
40
30
20
10
0
-10
400
300
200
100
0
*†
$V_m$
$R_{input}$
heart rate
(C)
Percent change from control
150
100
50
0
*
*†
*
*
*
EJP amplitude
tension
tension/EJP ratio

was only a 5% change), and increased $R_{input}$ by 21± 10%; neither of these changes were significantly different from control (p = 0.068 and 0.063, respectively, Fig. 4.4).

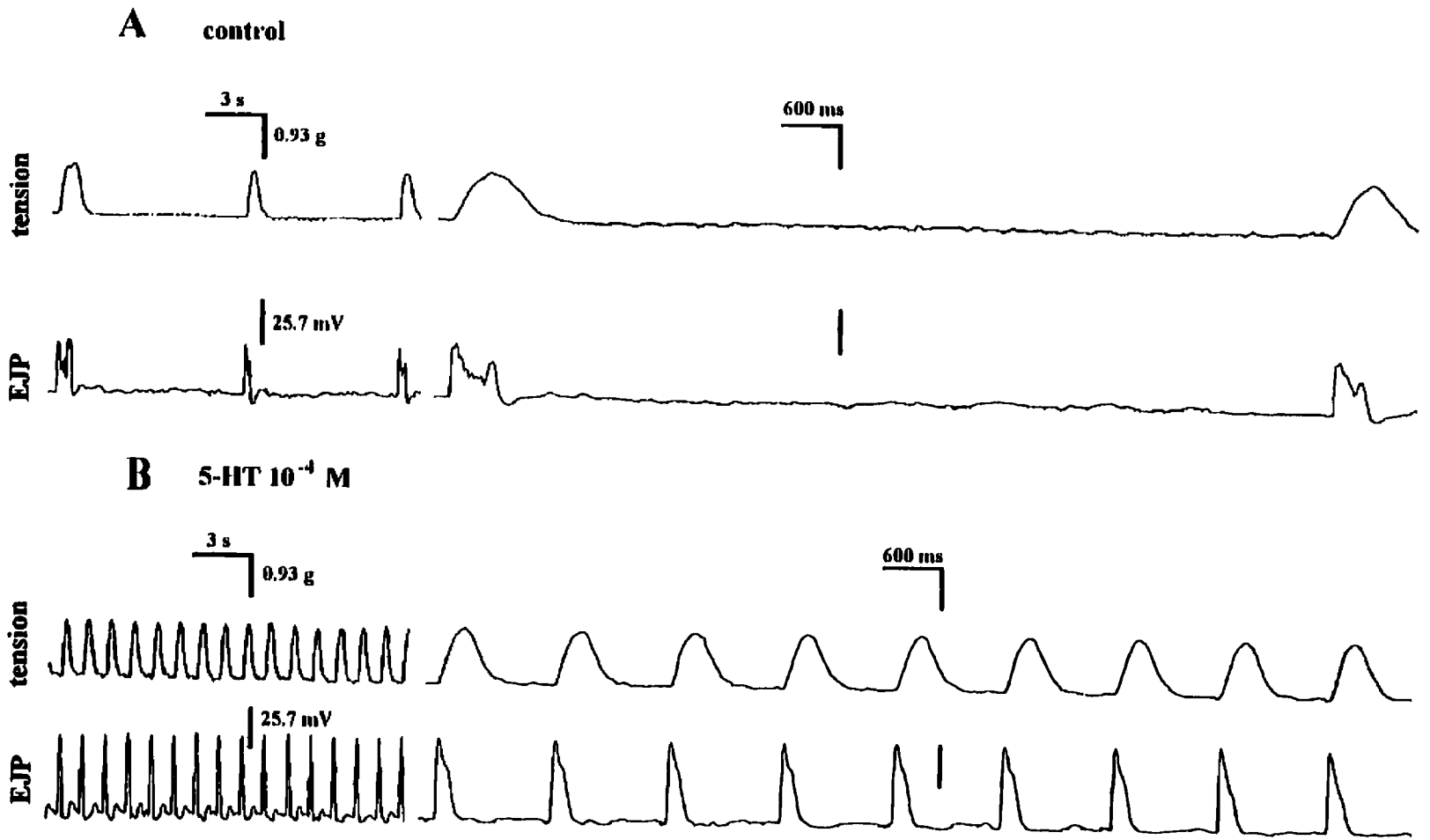
### 4.3.3 5-Hydroxytryptamine

Responses to 5-HT were tested on twelve isolated hearts. The time course of 5-HT responses were described from long-term recordings in 9 cells in which the transition from control to 5-HT was followed. The perfusate generally arrived at the heart 1.5 minutes after starting the perfusion. There was not an immediate response when 5-HT arrived at the heart, but rather a delay of 30 s–4 minutes to onset of the response. Recovery time after washoff varied with the duration of 5-HT perfusion. Heart rate remained elevated for 18–32 minutes and tension was enhanced for 13–25 minutes following short duration perfusions (Figs. 4.1 & 4.5). It took about 1 hour for recovery of $f_h$ and tension following the long-term perfusions. In one heart, rate had recovered by 2 hours but tension was still elevated at this time.

5-HT application increased EJP amplitude and produced a "tighter" EJP burst waveform with less peaks per burst. Generally, EJP appearance changed from wide multi-bursts to tall singlet spikes during 5-HT exposure, and tension amplitude increased in correspondence (Fig. 4.5). 5-HT enhanced or induced overshooting EJPs in 3 of 12 hearts (data not shown). Fig. 4.2B shows that 5-HT application increased EJP amplitude and rate but did not increase doublet bursting; this was only observed in 3 of 12 hearts following 5-HT treatment. The traces in Fig. 4.2B were obtained from two neighboring cells using conventional microelectrodes. The first cell was used for control readings (top traces) while recordings were made from a second, adjacent cell during 5-HT exposure (bottom traces) since the control cell could not be maintained through the 5-HT perfusion. Fig. 4.5 illustrates the mainly chronotropic actions of 5-HT in isolated hearts. During the 5-HT perfusion there was a 2.5 minute lag period after hormone arrived at the heart and before onset of the responses. The rate effects did not recover until after nearly 12 minutes of saline wash (not shown). These expanded traces were taken from the trial illustrated in Fig. 4.1B and this cell was maintained through the 5-HT exposure.

5-HT significantly increased $f_h$ (245 ± 63%, p = 0.0002, Fig. 4.4). 5-HT produced significant increases in EJP amplitude (37 ± 10%, p = 0.021) and tension (44 ± 10%, p = 0.017); however, 5-HT application did not significantly change the tension/EJP ratio (p = 0.681, Fig. 4.4). 5-HT perfusion slightly hyperpolarized membrane potential below control (-0.1 ± 0.2 mV, which was only a 0.5% decrease). $R_{input}$ decreased

Figure 4.5 Raw data traces of an isolated heart response to bath perfusion of 5-HT $10^{-6}$ M. (A) Control tension and dog-leg EJP recordings prior to 5-HT perfusion. Resting $V_m$ and $R_{input}$ were -58 mV and 40 MΩ, respectively. (B) At 5.5 minutes of exposure to 5-HT, all variables are elevated. Rate increased five-fold, but the changes in tension and EJP amplitude were not as great.


A
control
3 s
0.93 g
600 ms
tension
25.7 mV
EJP
B
5-HT 10 -4 M
3 s
0.93 g
600 ms
tension
25.7 mV
EJP

slightly (-1 ± 7%) in response to 5-HT. Neither of these changes were significantly different from control values ($V_m$: p = 0.949, $R_{input}$: p = 0.946, Fig.4.4).

### 4.3.4 Comparison of PR and 5-HT effects on isolated hearts

In terms of time course of onset and recovery, the two hormones produced different effects. In all but two hearts, the onset of 5-HT action was later than that for PR. Isolated hearts also recovered more quickly from PR than from 5-HT treatment (Fig. 4.1). Both hormones significantly increased tension and EJP amplitude, however, only PR significantly increased the tension/EJP ratio (Fig. 4.4). The increases in $f_h$ and EJP amplitude induced by 5-HT were significantly greater than those produced by PR ($f_h$: p = 0.004, EJP amplitude: p = 0.027, Figs. 4.3–4.5). In fact, 5-HT increased $f_h$ nearly four times as much as the same concentration of PR (245 ± 63% vs. 62 ± 27% increase, respectively, Fig. 4.4). Neither 5-HT nor PR had any significant effects on the myocyte membrane potential or $R_{input}$ (Fig. 4.4).

## 4.4 Discussion

### 4.4.1 General characteristics of isolated hearts

The average resting membrane potential of isolated *Carcinus maenas* heart myocytes was -46 mV. Brown (1964, cited in Anderson and Cooke, 1971) reported a resting membrane potential of -62 mV and non-overshooting action potentials (i.e., action potentials in which the upstroke does not reach zero mV) in the same species. The results from the present study were consistent with both of those observations. Only 3 of 12 hearts showed overshooting action potentials in this study, and overshooting was never recorded from more than 4 cells per heart (usually 20 cells were monitored in each heart). The range of membrane potentials recorded in the present study was -34 to -66 mV, which includes the value of -62 mV documented by Brown (1964).

### 4.4.2 Proctolin effects on isolated hearts

Biphasic $f_h$ responses to PR application were recorded in some intact and open heart preparations in Chapter Three, and also in intact *C. maenas* whole animal preparations (Wilkens et al., 1985). The biphasic response was characterized by a 30 second latent period followed by cardiac arrest and then a rise in $f_h$. The latent period was assumed to be the time required for the heart to pump PR into the CNS and trigger the cardioinhibitory output to the heart. However, in other *in situ* preparations, PR

application induced excitatory $f_h$ responses (Chapter Three). These excitatory rate effects were correlated with loss of eyestalk withdrawal reflex, which is one indicator of loss of CNS activity (Sandeman, 1967). This evidence indicated that CNS cardioinhibitory activity in the whole animal and in *in situ* hearts may have masked the true chronotropic actions of PR.

To study the true actions of PR on $f_h$, a preparation that removed the heart from CNS regulatory inputs was needed. Isolated hearts were used for this purpose. It was expected that in isolated hearts, there would be no interference of cardioinhibitory central nervous effects on PR responses. Indeed, PR had only acceleratory effects in 8 of 11 hearts. In the remaining 3 isolated hearts perfused with PR, there were only mild inhibitory responses (2) or no change in rate (1). Similar findings were reported previously in semi-isolated *C. maenas* hearts that were also removed from CNS inputs; PR does not cause bradycardia or arrest in these hearts either (Wilkens and Mercier, 1993).

Data from this study supported previous observations (Chapter Three) that PR has a strong inotropic effect on *C. maenas* hearts. The underlying mechanisms appear to be two-fold. The increases in EJP amplitude, although not significant (in some cases, EJP amplitude decreased), could have arisen from increases in CG spike frequency or in quantal transmitter release from the motoneurons. A second mechanism could include action of PR on the myocytes. However, if PR affected influx or efflux of ions at the myocyte membrane, changes in $V_m$ or $R_{input}$ would be expected. Only a small depolarization and a modest increase in $R_{input}$ were recorded from isolated heart myocytes, and neither of these changes were significant. Thus it appears that PR did not have direct effects on myocyte membrane properties. Subsequent steps in the excitation-contraction coupling process are more likely to be the targets for PR modulation in the myocyte, including changes in $Ca^{2+}$ conductance or activation of second messenger pathways.

PR increases muscle tension and activates second messenger systems in other crustacean muscle preparations. Previously, Bishop et al. (1991) found that PR increases $Ca^{2+}$ channel activity in crayfish skeletal muscle. However, changes in membrane properties were not the only method by which PR affected muscle tension. Instead, the authors suggested that PR may activate an intracellular messenger (cyclic adenosine 3',5'-monophosphate, cAMP, in particular) to increase $Ca^{2+}$ channel activity. In the marine isopod *Idotea baltica*, PR also enhances muscle contraction (Erxleben et al., 1995). PR

does not affect resting $V_m$ in isopod muscles, but increases $R_{input}$, and also reduces the number of active non-voltage-dependent $K^+$ channels. These effects were mimicked by the addition of both a cAMP analog, dibutyryl-cAMP, and a cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX), suggesting to the authors that PR acts via an intracellular cAMP-mediated protein phosphorylation pathway to close $K^+$ channels (Erxleben et al., 1995).

In the present study, changes in $Ca^{2+}$ conductance and second messenger activation were not studied directly, but indirect evidence seems to implicate second messenger involvement. For example, the period of increased contraction following PR exposure lasted from 30–60 minutes, and in some instances (long-duration perfusions) lasted for several hours, while elevated EJP amplitude and heart rate rarely lasted longer than 30 minutes. At the peak of the PR response the tension/EJP ratio was significantly elevated. These long-term changes in myocardial contractility, combined with data showing that there were no significant effects of PR on myocyte membrane properties, led to the speculation that PR may modulate second messenger systems in myocytes to produce the large increases in contractility observed.

### 4.4.3 5-Hydroxytryptamine effects on isolated hearts

In contrast to the dual sites of action of PR, all of the responses to 5-HT in isolated hearts could be accounted for by action on the CG burst pattern. As in intact and open hearts (Chapter Three), 5-HT caused mainly chronotropic responses in isolated hearts. In fact, 5-HT increased $f_h$ nearly four times as much as PR (245% vs. 62% increase, respectively); this difference between hormone treatments was significant ($p = 0.004$). The strong chronotropic effects and moderate inotropic effects of 5-HT were also reported in semi-isolated hearts from the lobster *Homarus americanus* (Wilkens et al., 1996). Additionally, 5-HT increases ganglionic burst rate in isolated *H. americanus* CGs (Lemos and Berlind, 1981). In semi-isolated hearts of crayfish (*Astacus leptodactylus*) and crabs (*Eriphia spinifrons*), where EJPs and tension were also recorded from heart muscle, 5-HT is a potent cardioaccelerator (Florey and Rathmayer, 1978).

In the present study, 5-HT significantly increased EJP amplitude, again reflecting action on CG burst output. Increased EJP amplitude is not due to recruitment of more CG motoneurons or myocytes. Rather, the observed increases in EJP amplitude can be interpreted as a greater depolarization of the motoneurons. This may lead to a steeper pacemaker depolarization, a larger driver potential, or possibly a larger spike train

superimposed on the driver potential, but the ultimate output is a greater EJP amplitude. Following 5-HT application the first peak of the EJP bursts was enhanced and more singlet bursts were observed; PR application also increased first peak amplitude, but this was not usually as dramatic as the effect of 5-HT (compare Figs. 4.4 & 4.5). In 3 of the 12 hearts examined, 5-HT induced overshooting EJPs or increased the occurrence of this event in cells that were already overshooting. In comparison to PR treatment, which induced doublet bursting in nearly half of the isolated heart preparations (45%), 5-HT application produced doublet bursting in only 25% of hearts. Using isolated *H. americanus* ganglia, Lemos and Berlind (1981) found that 5-HT decreases burst duration and number of spikes per burst. Although there were no direct recordings from the CG in the present study, the changes in intracellularly-recorded EJPs indicate that similar changes in CG output occurred.

5-HT had virtually no effects on myocyte membrane properties. It slightly hyperpolarized membrane potential and decreased $R_{input}$, but neither of these changes were significant. Like PR, 5-HT significantly increased isometric tension but unlike PR, 5-HT did not significantly increase the tension/EJP ratio. It appears that the increased tension may have arisen from facilitation of transmitter release from the CG motoneurons resulting from the increased burst rate, rather than direct action on the myocardium. The lack of a direct effect of 5-HT on myocardial force or myocyte membrane properties, in addition to the largely chronotropic role of this amine, led to the conclusion that for 5-HT, changes in CG output can fully account for the observed changes in tension. A mode of action for 5-HT in crustacean heart preparations has not been fully established. However, there is evidence that 5-HT action on isolated lobster CGs is not mediated by the second messenger cAMP (Lemos and Berlind, 1981). Further studies are required to fully elucidate the 5-HT mode of action.

## 4.5 Summary

The isolated heart data presented in this study were consistent with earlier findings from *C. maenas* intact and open heart preparations (Chapter Three). The responses of isolated hearts clearly demonstrated that PR acted at both the myocardium and CG, while 5-HT acted solely at the CG; findings that supported the hypothesis. PR caused significant increases in tension and a high tension/EJP ratio, while 5-HT produced mainly chronotropic responses. For both hormones, the increases in EJP amplitude may have arisen as a consequence of motoneuron synaptic facilitation. One aim of this study was to

determine whether changes in the membrane potential or membrane input resistance could account for PR action on myocytes. Neither hormone produced significant changes in $V_m$ or $R_{input}$, although there was a modest (20%) increase in $R_{input}$ in response to PR. These findings suggested that PR action was not via changes in $V_m$ or $R_{input}$. Rather, the effects of PR may have been related to either changes in the calcium dynamics of the cardiac myocytes or second messenger activation. Further work is required to fully elucidate the PR mode of action.

# CHAPTER FIVE: NEURONAL EXCITABILITY AND HORMONE RESPONSES OF GANGLIONIC NEURONS

## 5.1 Introduction

Rhythmic behaviours such as ventilation (Bulloch and Syed, 1992; DiCaprio and Fourtner, 1984, 1988), locomotion (Getting, 1989; Kiehn, 1991; Grillner et al., 1995), feeding (Willows, 1980) and heartbeat (Hartline, 1979 review; Arbas and Calabrese, 1987) have been studied in a variety of vertebrate and invertebrate preparations. In most of these animals the rhythmic behaviours are controlled by networks of neurons, which are termed central pattern generators (CPGs). The underlying neuronal circuits are often complex, and the majority of neurons remain unidentified in most of these preparations. However, some invertebrate neural networks have been examined in detail, including the freshwater pond snail respiratory CPG (*Lymnaea stagnalis*, Syed et al., 1990, 1992) and the lobster stomatogastric ganglion system (STG, Harris-Warrick and Flamm, 1987; Marder, 1987; Selverston, 1987). While the STG system has fewer cells (about 30 in all, Turrigiano and Marder, 1993) than vertebrate preparations, some investigators have questioned whether CPGs are even understandable in such simple models (Selverston, 1980).

Activity of neurons in the cardiac ganglion (CG), the CPG located in the decapod crustacean heart, initiates heart contractions. The decapod CG usually consists of four pacemaker cells (small cells, SCs) and five motorneurons (large cells, LCs; reviewed in Hartline, 1979). The isolated CG reliably produces bursts of impulses in isolation (Welsh and Maynard, 1951), thereby enabling researchers to extensively study the intrinsic and network properties of CG neurons in crabs (*Portunus sanguinolentus*, Tazaki and Cooke, 1979a,b,c) and lobsters (*Homarus americanus*, Berlind, 1985, 1989; Tazaki and Cooke, 1986). In these larger crustaceans, the CG is easily accessible and can be manually exposed by teasing away the heart muscle cells surrounding the ganglion (Tazaki and Cooke, 1979a). To examine the intrinsic properties of ganglionic cells, previous investigators either ligatured (Tazaki and Cooke, 1983b), or transected 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 (Sullivan and Miller, 1984; Tazaki and Cooke, 1986; Berlind, 1985, 1989). From such studies, both SCs and LCs were found to generate driver potentials (DPs),

which are spontaneous depolarizations that 'drive' impulse formation at distal spike-initiation sites (Tazaki and Cooke, 1979b,c; Berlind, 1985, 1989).

The main problem with such ligature or two-pool studies is that synaptic inputs to the individual cells may persist; that is, it cannot be reliably determined whether the cell is indeed truly 'isolated' (Panchin et al., 1993; Turrigiano and Marder, 1993). In addition, tight ligatures around the CG may alter the physiological and/or pharmacological properties of these so-called 'isolated' cells (Sullivan and Miller, 1984). In the *Carcinus maenas* heart in particular, the CG neurons are not easily accessible as the ganglion is buried in heart muscle and is not discernible under a dissection microscope.

In an attempt to overcome the problems outlined above, a novel enzymatic dissociation technique was developed to isolate the CG from the rest of the heart, and then an *in vitro* cell culture system was developed to completely isolate the individual ganglionic cells. This chapter outlines these novel techniques and provides data showing that the LC neurons were viable in culture and showed electrophysiological properties similar to those in the isolated ganglion. Additionally, this cell culture system was used to directly examine CG neuronal responses to neurohormones. Of the known cardioexcitors released by the pericardial organs (POs), one amine and three peptides were tested.

The amine 5-hydroxytryptamine (5-HT) and the peptide proctolin (PR) have been shown to affect isolated CG burst properties. 5-HT increases ganglionic burst frequency (Cooke and Hartline, 1975; Lemos and Berlind, 1981; Kuramoto and Yamagishi, 1990). PR also increases burst rate, and application of this peptide to TTX-silenced ganglia induces depolarizations and driver potentials (Freschi, 1989; Miller and Sullivan, 1981, respectively). 5-HT has been shown to cause chronotropic effects while PR, in contrast, is mainly an inotropic hormone (Chapters Three and Four). Two other biogenic amines; namely dopamine and octopamine, are present in crab POs in addition to 5-HT. However, the amine 5-HT was used for comparison with the peptide hormones because of its chronotropic nature and for the sake of consistency it was studied throughout the four stages of this study (i.e., *in situ* hearts, isolated hearts, *in situ* and *in vitro* CG neurons). The PO peptides crustacean cardioactive peptide (CCAP) and the FMRFamide-related peptide, F2, have never been studied on isolated CGs.

CCAP and F2 were chosen in this study for their actions on *in situ* and *in vitro* CG neurons because they have been shown to produce chronotropic effects in other crustacean heart preparations. CCAP increases heart rate in intact (Chapter Three) and isolated (Stangier et al., 1991; Wilkens and Mercier, 1993) *C. maenas* crab hearts. This

chronotropic action was attributed to modulation of the CG burst pattern output. F2 excites heart rate in crayfish (*Procambarus clarkii*, Mercier and Russenes, 1992) and lobster (*H. americanus*, Wilkens et al., 1996) heart preparations, indicating possible action at the CG for this peptide as well. All four hormones that have been described (5-HT, PR, CCAP, and F2) elevated heart rates. These chronotropic actions were thought to arise from increased frequency of CG burst output; therefore, it is predicted that each of the hormones tested in this study will increase CG burst frequency. Evidence is provided in support of this prediction.

## 5.2 Materials and methods

### 5.2.1 Heart isolation procedure

Adult male and female shore crabs ($n$ = 139) were used in this study. All legs were autotomized prior to 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. The heart was removed, the ventral wall was cut open and the heart was pinned in a Sylgard dish. The isolated heart was bathed with *C. maenas* saline (433mM NaCl, 12mM KCl, 12mM $CaCl_2 \cdot 2H_2O$, 20mM $MgCl_2 \cdot 6H_2O$, 10mM HEPES, adjusted to pH 7.60 with 2N NaOH).

### 5.2.2 Isolated CG protocol

The following steps for ganglion isolation were carried out under sterile conditions in a cell culture hood. The heart was rinsed in 3mL antibiotic saline (ABS= 150μg/mL gentamycin sulphate added to regular *C. maenas* saline) for two, ten-minute washes, each in a fresh Falcon 3001 plastic dish. The heart was then placed in a Falcon Blue-Max 15mL tube containing collagenase (type II, 1mg/mL) in 3mL defined medium (DM= Leibowitz L-15 medium added to regular *C. maenas* saline; pH adjusted to 7.60 with 2N NaOH). During enzyme treatment, the tubes were refrigerated (4°C) for 10 minutes, then removed and shaken at room temperature for a further 10 minutes. Refrigeration was considered necessary to slow the enzymatic degradation of heart muscle cells. Following collagenase treatment, the contents of the tube were added to 3mL DM in a fresh dish and the CG was isolated from pieces of heart muscle. Following a further wash in 3mL DM, the CG was pinned at its distal branches on a Sylgard dish containing 3mL DM.

To soften the connective tissue sheath, the CG was bathed in DM containing both protease (type IX, 3.33mg/mL) and trypsin (type III, 2mg/mL) for 10 minutes. Enzyme

treatment was followed by 3 consecutive washes in DM (3mL each wash). The ganglion was desheathed, first using a pair of fine forceps and then using two sharp microelectrodes. Intracellular microelectrode recordings were made from the desheathed ganglion.

Fifteen ganglia were not used for electrophysiological studies, but were stained with methylene blue to aid visualization of the ganglionic neurons. Ganglia were isolated and desheathed as outlined above, then a few drops of methylene blue were added to the *C. maenas* saline bath. Only enough methylene blue was added to tint the saline to a light sky blue color. Ganglia were then refrigerated overnight. The next day, drawings were made of the stained CG. The blue dye was selectively taken up by cell somata and axons.

### 5.2.3 Cultured CG neuron protocol

As for the above protocols, all of the following procedures were carried out under sterile conditions. Each neuron was first identified by its position in the ganglion and subsequently removed using gentle suction with a fire-polished Sigmacote (Sigma cat. no. SL-2) pipette, positioned in a micro-manipulator. Each identified neuron and its accompanying axon was plated onto a poly-L-lysine (MW = 50,200) coated Falcon 3001 dish containing DM. Up to 3 identified neurons were plated separately in each poly-L-lysine dish and located by their positions relative to a reference mark on the outside of the dish. Isolated cells were maintained overnight to adhere to plates, and the following day cells were penetrated with microelectrodes. SCs were more difficult to find in the desheathed ganglion than LCs and hence, fewer were isolated.

Photographs were taken on a Zeiss Axiovert 135 inverted microscope with visible light at 10X or 20X magnification. Tech pan film (50 ASA) was used in a Contax camera.

### 5.2.4 Intracellular recordings, neurohormone application procedures and data analysis

For both the isolated ganglia and cultured CG neuron preparations, sharp microelectrodes filled with saturated $K_2SO_4$ solution (resistance 10–40 MΩ) were used. DM was replaced with regular *C. maenas* saline and the bath was continuously perfused with saline for recordings from either preparation. All recordings were made from somata. Signals were amplified on a NeuroData (Model IR-283) amplifier, displayed on a

Gould 2-channel chart recorder (Model 2200S) and simultaneously stored on VCR (Sony Model 420 K, A.R. Vetter Co.) for later playback and data analysis.

The neurohormones 5-HT, PR (Sigma Chemical Co., 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 *C. maenas* saline to a final (pipette) concentration of $10^{-4}$ M. Pipettes were fire-polished and back-filled with hormone. Each hormone was applied directly onto an individual cell soma using pressure application (WPI PV800 pneumatic PicoPump). Each pressure pipette was placed adjacent to the cell under investigation and hormones were tested at least 2–3 times on each cell, with sufficient washing in between the applications to allow recovery to control conditions.

Electrophysiological recordings were made from cells immediately after microelectrode penetration and the cells were subsequently hyperpolarized (range of injected current: 0.15–0.5 nA) to reduce spontaneous activity which facilitated a greater scope of response to hormone application. This hyperpolarization was maintained throughout control and hormone testing, and did not usually silence spontaneous activity. For the cultured cells, however, the above range of hyperpolarizing current was often sufficient to completely silence the neurons.

From raw data recordings of LC neurons *in situ* or *in vitro*, the burst rate and amplitude, pacemaker slope and oscillation amplitude were measured during control and hormone treatments. Amplitudes and rates were simply measured from chart records, while pacemaker slope required a calculation. The pacemaker potential was arbitrarily defined as the depolarizing phase beginning at the end of an afterhyperpolarization following the previous burst and prior to the driver potential depolarization of the next burst. Slope was then calculated as the rise/run for this depolarizing pacemaker phase.

## 5.3 Results

### 5.3.1 Morphology

*C. maenas* isolated CG preparations have not been previously examined, therefore it was necessary to describe the anatomy of the CG. From methylene blue staining and visual inspection of the unstained isolated ganglia, a drawing of the typical *C. maenas* CG (as observed in about 75% of a total of 139 preparations) is presented as Fig. 5.1. Rather than a typical 9-celled CG, as observed in other crabs and lobsters, no more than 8 cells were ever observed in one *C. maenas* ganglion. LCs 1 & 2 were located in the left and

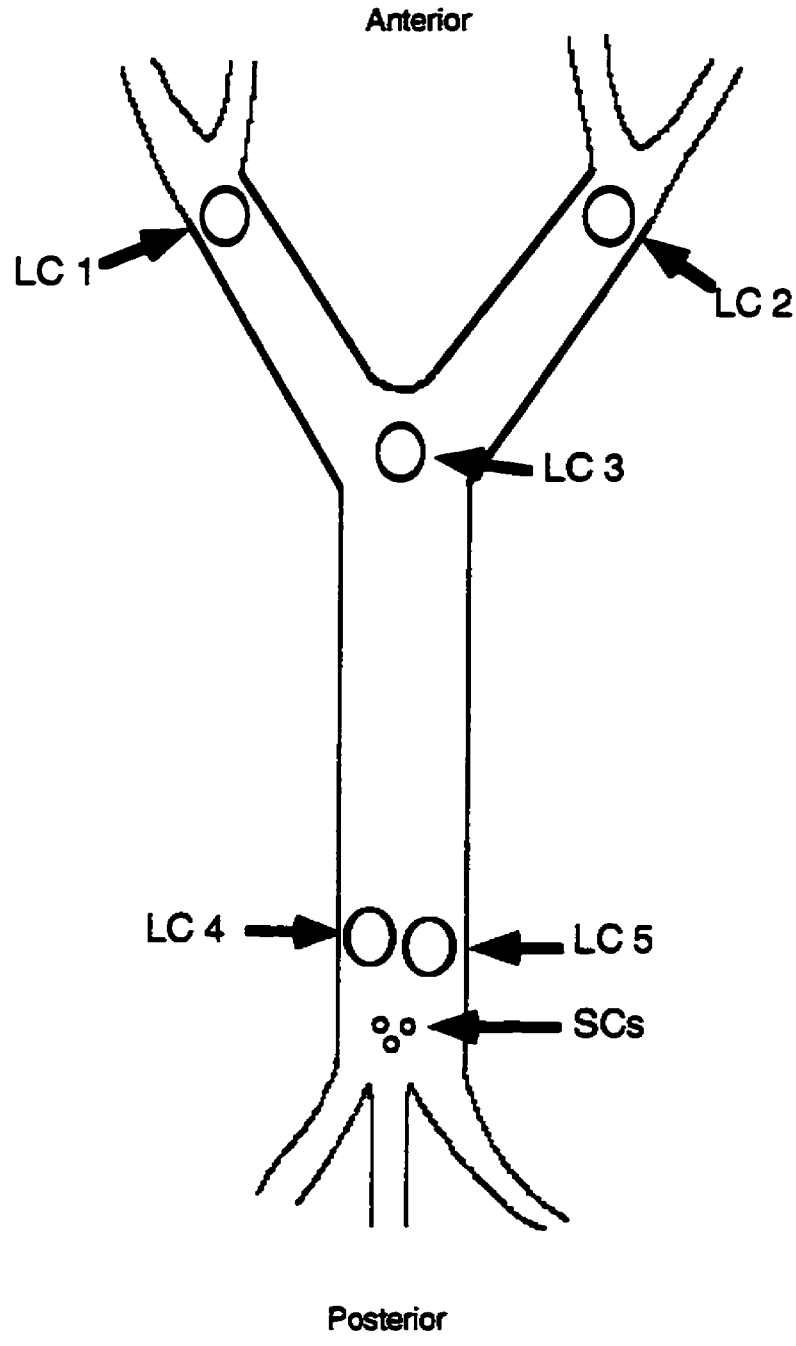



Figure 5.1 Drawing of the *Carcinus maenas* CG anatomy. Cells are numbered according to Alexandrowicz (1932). Only 8 cells, rather than the typical 9 documented in other crabs and lobsters, were observed. LCs were easy to identify because of their size, while SCs were more difficult to locate.

right "Y" branches, respectively. LC3 was present at the junction of the "Y", and there was a wide spatial separation between it and LCs 4 & 5. LCs 4 & 5 were usually located side by side at the bottom of the ganglionic trunk. SCs were only observed in a handful of preparations (21 out of 139). SCs were always clustered below LCs 4 & 5, and were encased in a "pocket" of connective tissue burried deep within the CG. The entire ganglion was surrounded by a thick connective tissue sheath. Each individual LC was covered by its own "personal sheath", as has been observed in lobster STG neurons (P. Dickinson, personal communication). In contrast to this, once the connective tissue "pocket" surrounding the SCs was opened, the individual SCs were found to be devoid of an individual connective sheath.

About 1 in 4 ganglia showed anatomical anomalies. Sometimes two large cells were located in one "Y" branch (3/139), while in some other preparations the "twin cells" LCs 4 & 5 were located anterior and posterior to one another, rather than side by side. 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 not branched, but instead all cells were aligned down the main trunk. In 4 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.

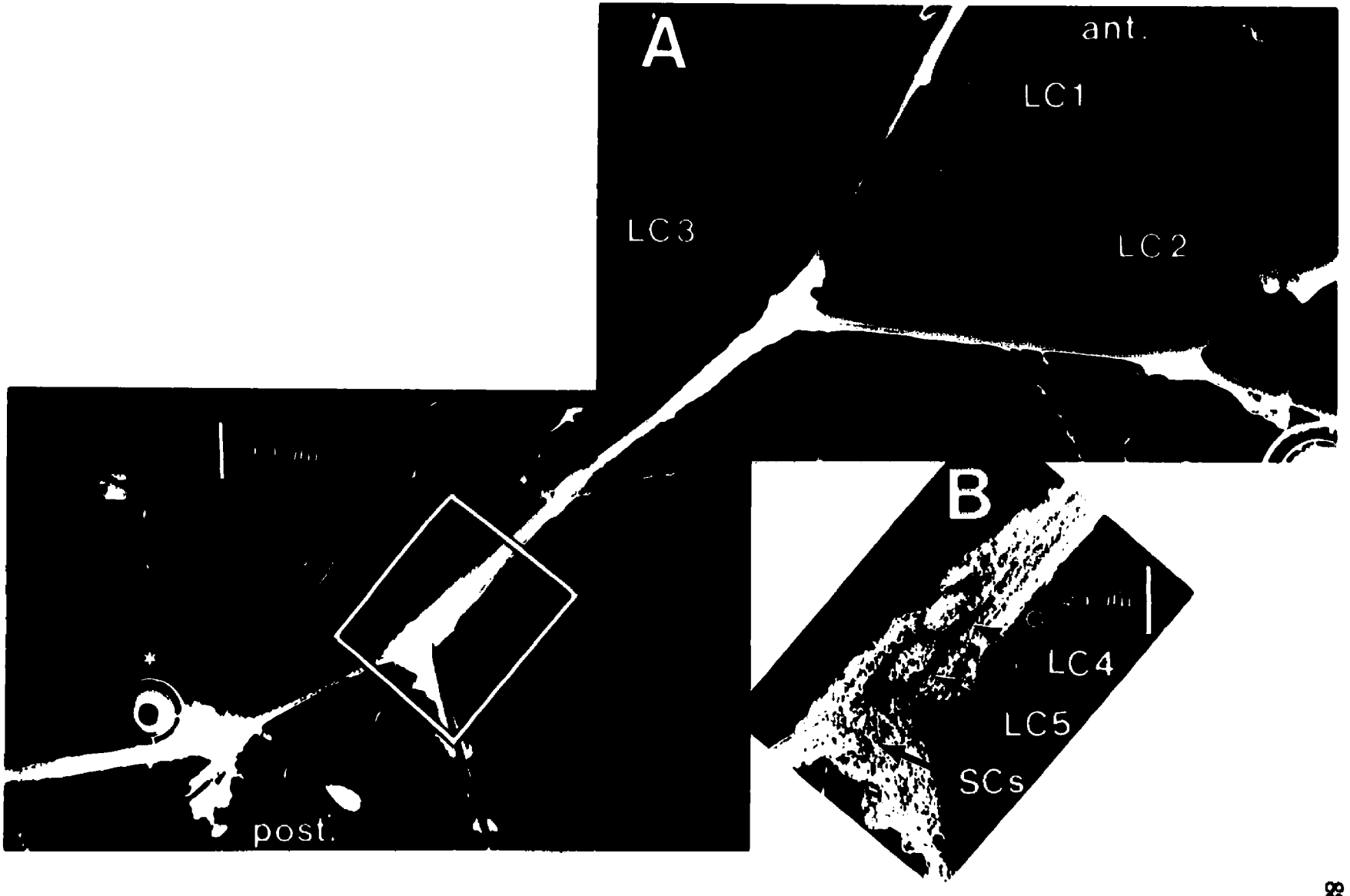
Cells looked similar in culture as they did in isolated ganglia (Figs. 5.2 & 5.3). LC somata having a granular appearance were physiologically inactive and possessed no resting membrane potentials (i.e., granulated cells were dead; Fig. 5.2C). No data were obtained from these cells. All viable LCs had a thick neurite and often contained two or more axons.

### 5.3.2 Isolated ganglia

#### 5.3.2.1 General characteristics of neurons

Out of 41 animals from which ganglia were isolated, only 23 ganglia showed electrical activity, therefore the survival rate was 56% for this preparation. Of these 23 isolated ganglia preparations, thirty LC recordings were made. Microelectrode recordings were made from cells on the same day that the ganglion was removed from the heart. Membrane potential ($V_m$) ranged from -12.3 to -65 mV, the average was -37.4 ± 4.8 mV. Most LCs (27 of 30) showed spontaneous bursting activity (Fig. 5.4A). Alternatively, a few preparations (3 of 30, $V_m$ range: -12.5 to -45 mV) were quiescent initially, and subsequently showed spontaneous bursting.

Figure 5.2 Photographs of LCs in the isolated ganglion and in culture. (A) Collage of two photographs of an enzymatically-dissociated isolated cardiac ganglion (CG). The large cells (LCs) were located anterior to the small cells (SCs). The ganglion was desheathed mechanically via a fine pair of forceps or electrodes for identification and penetration of cells. (B) An enlargement of LCs 4 & 5. Although these cells are typically arranged side by side in a ganglion, in this particular ganglion, LC4 was located slightly above and to the left of LC5. The approximate area of the SCs is indicated by an arrowhead, however, they can not be visualized in this particular ganglion. [ant. = anterior, post. = posterior, asterisk (*) is directly above a bubble in the Sylgard dish] (C) Photograph of healthy cultured LCs 4 & 5 (arrow points to LC4). LC4 had a healthy $V_m$. Photograph in (D), taken some time later, illustrates the granular, swollen appearance of the same LC4 (arrow). At this stage, no $V_m$ was present. Scale bars are the same in both C and D.


A
ant.
LC1
LC3
LC2
post.
B
LC4
LC5
SCs


C

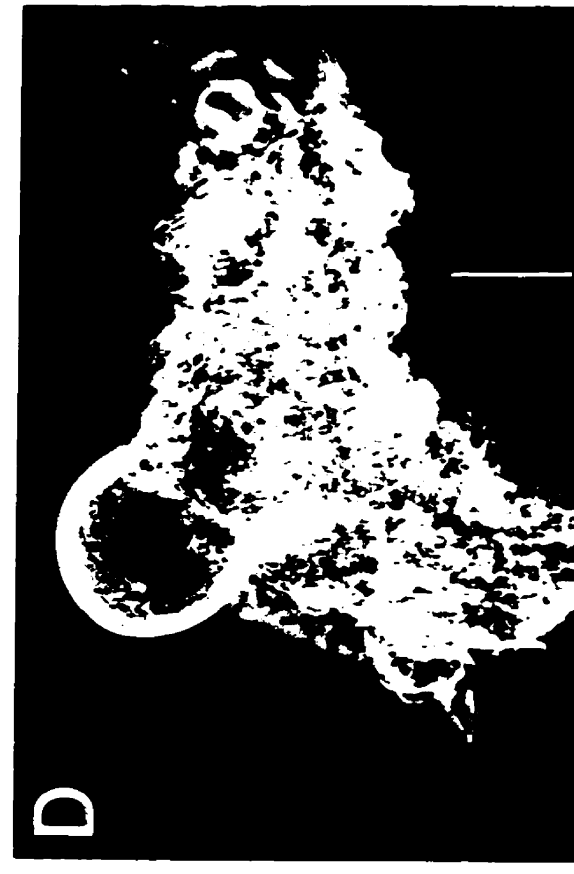

D

Figure 5.3 Photographs showing cultured LCs with long axons. (A) Photograph of cultured neuron LC5 showing its long axon stumps (a = axon). Mark underneath the soma (s = soma) represents scratches on the bottom of the plastic dish. (B) Cultured LC2 also had long axons, and the soma was located to one side of the axon. Both scale bars are 50 μm.

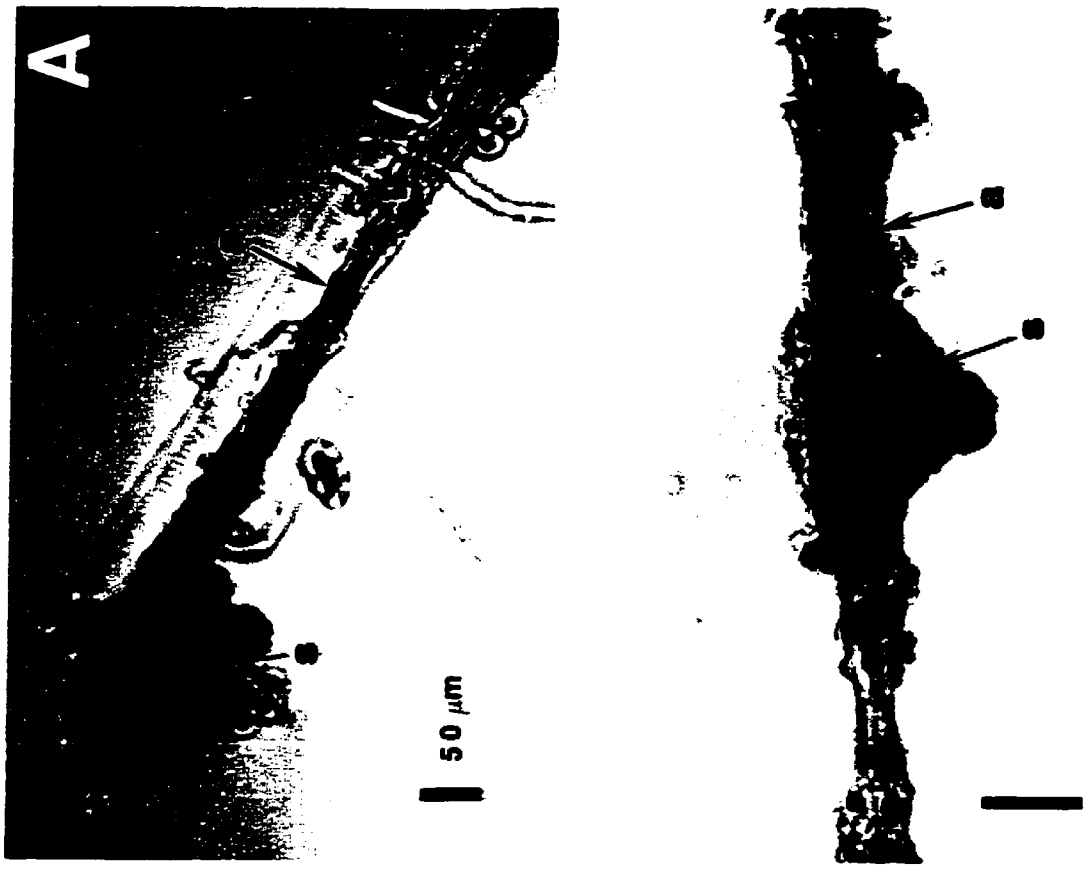

A
50 μm

Figure 5.4 A comparison of LC bursting activity in the isolated ganglion and cultured cell preparations. (A) Spontaneous LC5 phasic bursting recorded from an isolated CG. (B) Cultured cells, as exemplified by LC1 in this trace, typically showed spontaneous membrane potential oscillations in addition to bursting activity.

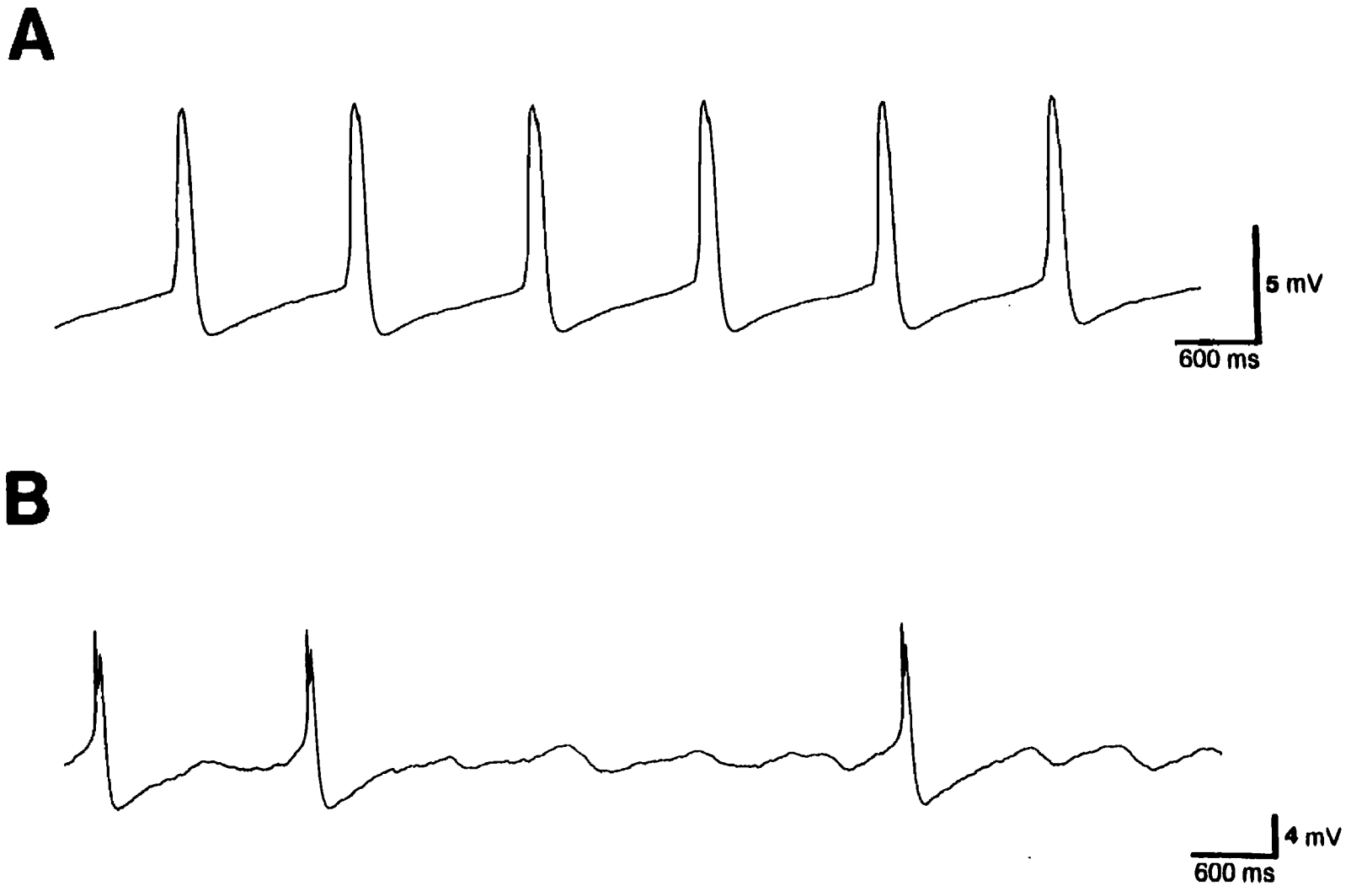

A
5 mV
600 ms
B
4 mV
600 ms

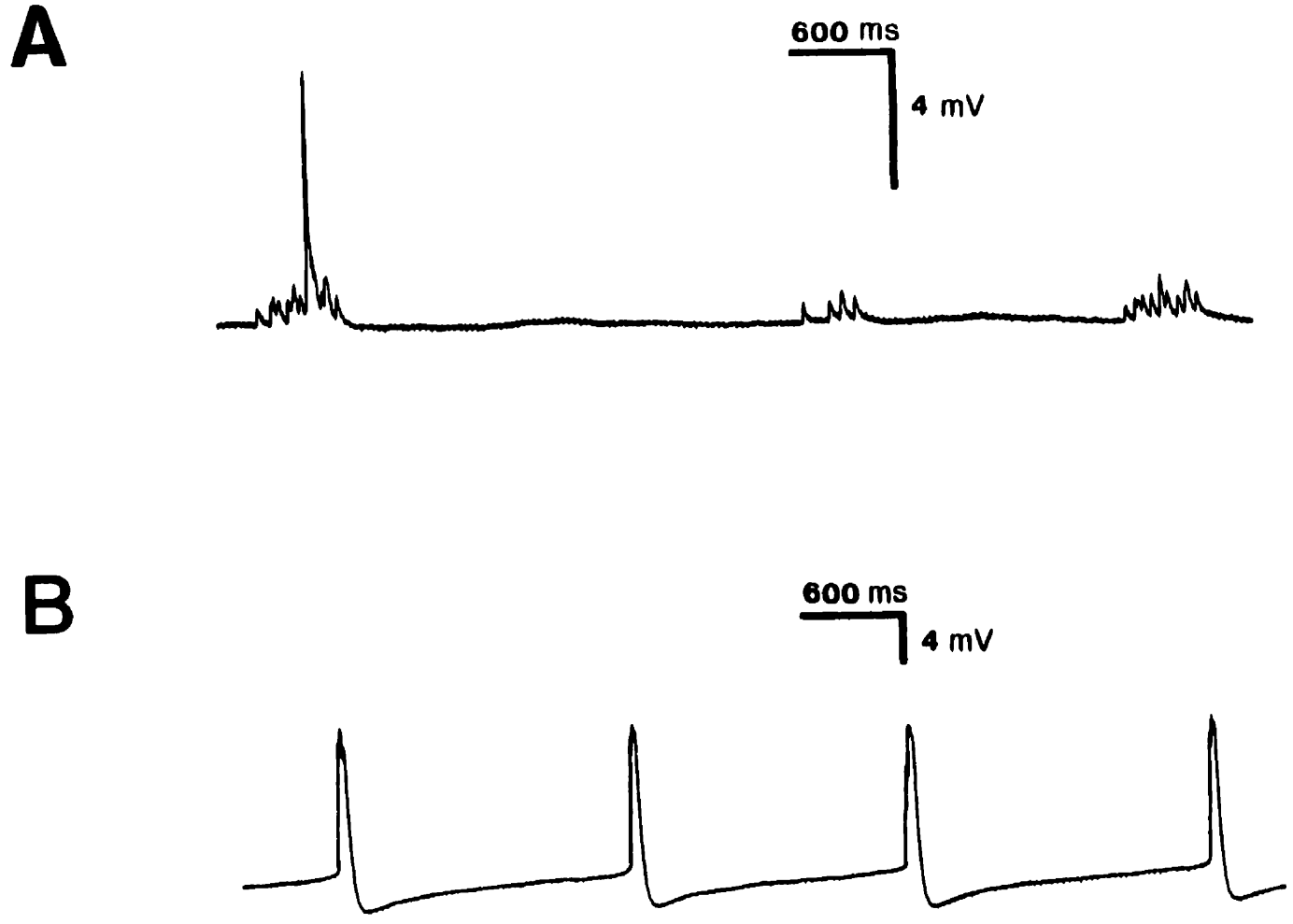
Figure 5.5 shows the variability in burst appearance from ganglion to ganglion. Differences in burst appearance were attributed to variable electrode placement nearer to or further from the neurite. Some ganglia fired slowly, with diffuse bursts that occasionally produced large spikes (Fig. 5.5A). Figure 5.5B shows the most common LC waveform: small bursts superimposed on a tall driver potential (DP), large afterhyperpotentials (AHPs) and slow pacemaker potentials. Other recordings showed larger spikes arising from the DP (Fig. 5.5C), whereas still others had small events during the interburst period (Fig. 5.5D). Because of this variability across cells, some data were presented as individual raw data traces. However, for the hormone treatments, data was also summarized in bar graphs. 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 about 20 mV amplitude and 200–250 ms in duration, spontaneous membrane potential oscillations were small (about 2–5 mV), slow (≈500 ms) fluctuations in the membrane potential that occurred in the interburst interval. Table 5.1 shows the values for resting burst rate, burst amplitude, pacemaker slope, and amplitude of membrane potential oscillations.

Due to the difficulty in locating SCs, data were obtained from only one cell. Recordings from SCs are exceedingly rare so these data are described, but since this finding has not been repeated, please note its preliminary nature. No hormones were tested on this SC. However, from the one SC examined, tonic firing was recorded; firing rate was much faster than the rate of LC bursting (Fig. 5.6). No driver potentials were evident. Additionally, SC spike amplitude was larger than that recorded from LCs (Fig. 5.6). The ganglion itself was bathed in lobster saline which only differed from crab saline by 3 mOsm, while pH was 7.6 and 7.8 for crabs and lobsters, respectively. These differences between crab and lobster saline were negligible and were not expected to affect cell excitability.

### 5.3.2.2 Effects of hormone application

Hormone responses were recorded in 25 of the 30 LCs examined in 18 of the 23 isolated CG preparations. These experiments provided data showing responses of LCs when connected in a ganglion, thereby allowing comparison to the effects of these same hormones on cells isolated *in vitro*. In general, all hormones when applied to the somata increased burst rate in spontaneously active ganglia. All hormones tested significantly increased LC burst rate and pacemaker slope ($p = 0.0001$ to 0.010 for burst rate,

Figure 5.5 An illustration of the different burst appearances recorded from various LCs in several isolated ganglion preparations. Each trace shown was taken from a LC in a different ganglion. (A) Poorly bursting ganglion showed small, prolonged bursts. Occasionally, a spike arose from the burst. Recorded from LC5. (B) LC3 showing large afterhyperpotentials (AHPs), a slow pacemaker potential, and small spikes at the top of the driver potential (DP). (C) Another LC3 showed large AHPs, a slow pacemaker potential, and large spikes on the DP. (D) LC5 recording showing small events between the bursts. The small events re-set the pacemaker slope.


A
600 ms
4 mV
B
600 ms
4 mV

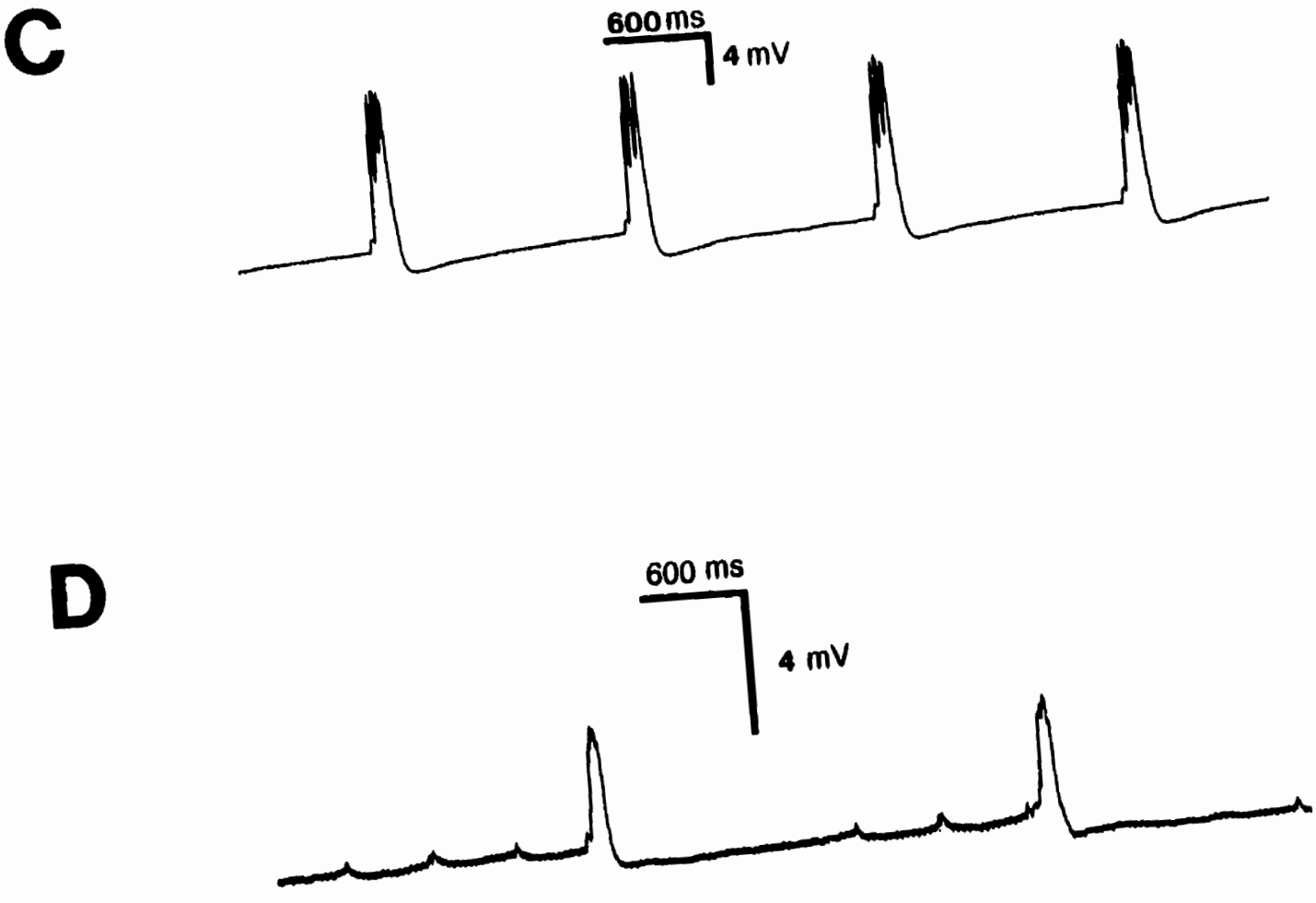
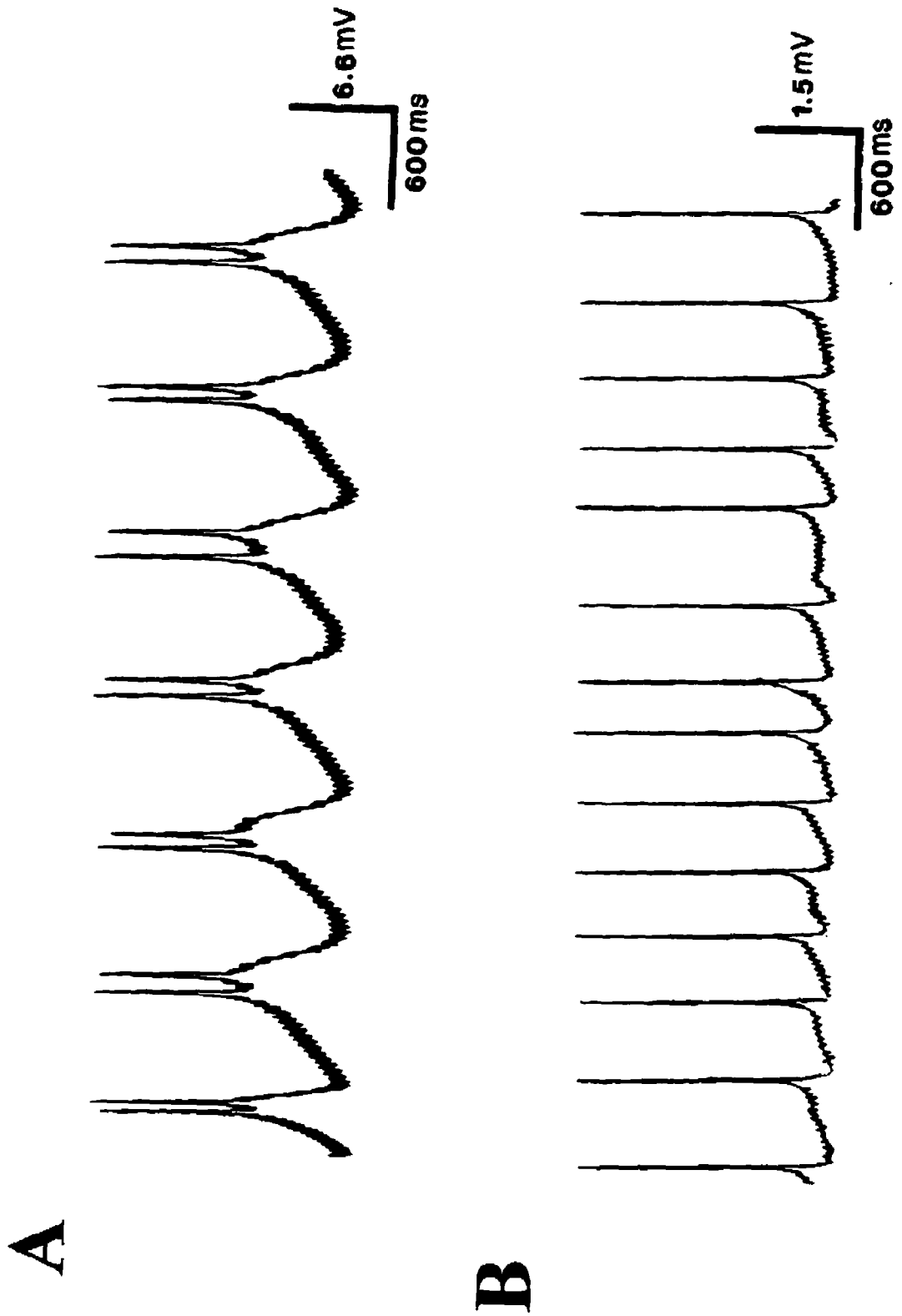

C
600 ms
4 mV
D
600 ms
4 mV

Table 5.1 Resting burst characteristics for LC neurons in the isolated CG and cultured cell preparations. 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. 5.7 and 5.16, which show lower baseline values as a result of this protocol). Data presented as mean ± S.E., with the number of observations in parentheses below each value.

| Preparation | Burst rate (/ minute) | Burst amplitude (mV) | Pacemaker slope (mV/s) | oscillation amplitude (mV) |
|---|---|---|---|---|
| isolated CG | 51.71 ± 8.51 | 11.14 ± 2.48 | 5.35 ± 2.58 | 3.55 ± 1.16 |
| | (n = 30) | (n = 30) | (n = 30) | (n = 5) |
| cultured | 36.42 ± 9.31 | 17.40 ± 3.55 | 11.31 ± 2.25 | 3.02 ± 0.52 |
| | (n = 14) | (n = 15) | (n = 15) | (n = 8) |

Figure 5.6 Comparison of the burst characteristics of an LC and an SC recorded in intact ganglion preparations. (A) LC1/2 activity consisted of bursts with a long pacemaker slope and large AHP. (B) SC record showing tonic firing, faster rate of discharge, and smaller amplitude of waveforms. Time scale is the same in both traces. Only one observation was made of SC firing in the intact ganglion, and the ganglion was bathed in lobster saline.


A
6.6mV
600ms
B
1.5mV
600ms

p = 0.005 to 0.029 for pacemaker slope, Figs. 5.7–5.8). None of the hormone-induced changes in burst amplitude were significant (Fig. 5.7). In preparations that exhibited spontaneous membrane potential oscillations, hormone treatment did not significantly alter the amplitude of these oscillations.

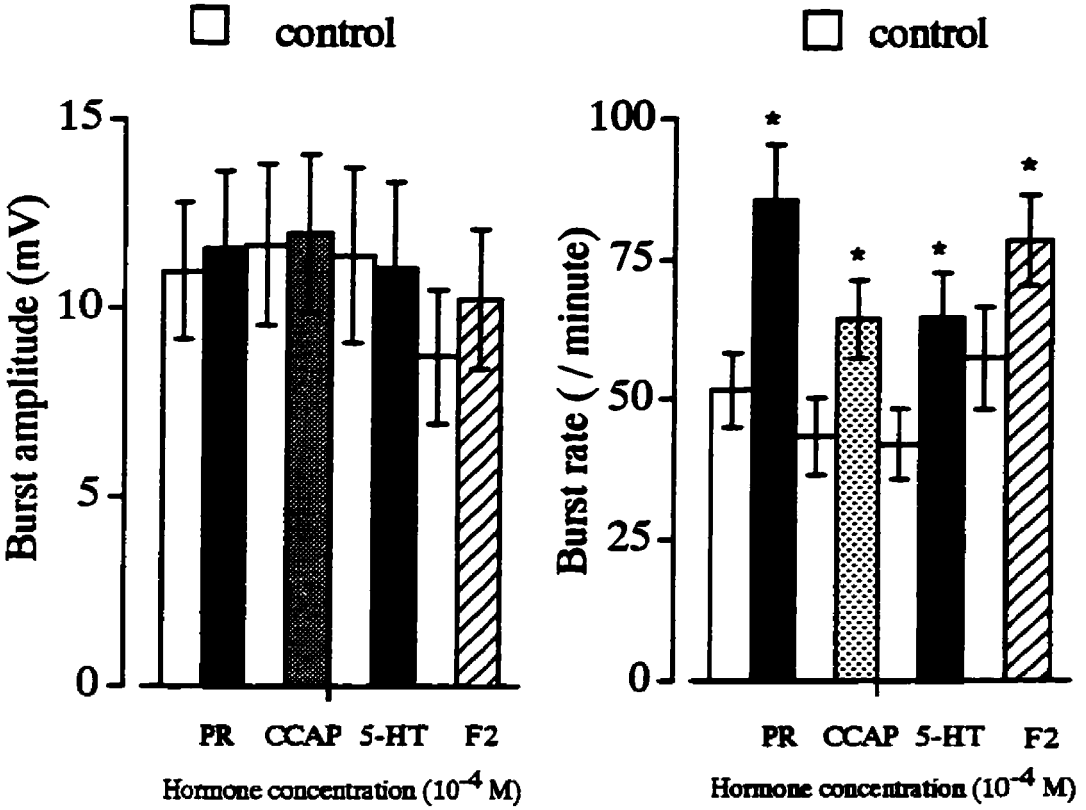
The time course of hormone effects on these burst parameters was similar for all peptides. Responses were immediate in their onset, and responses were saturated within 30 s to 1 minute after pressure ejection. Interestingly, when PR was applied to a neuron following an F2 trial, PR responses were prolonged; lasting up to 10 minutes. This was observed in 4 of the 5 ganglionic preparations in which hormones were applied in this order.

To test whether peptidergic responses were similar to or different from those of the biogenic amines, 5-HT was also tested on isolated CG neurons. Typically, there was a 10–30 second delay in the onset of the response to 5-HT. In comparison to the peptides' effects, which were washed away by 2–3 minutes, 5-HT produced longer-lasting effects. Most preparations recovered from 5-HT application after 3–4 minutes, however, there were some ($n$ = 4) responses that lasted for 8–12 minutes. Control saline applications did not alter the firing rate and had no effects on the LC activity (Fig 5.9).

All hormones regularized the rate of LC bursting and organized diffuse bursts into more compact bursts, especially in slowly bursting preparations. Two examples of these burst-organizing effects are shown for CCAP treatments (Fig. 5.10). Hormone effects were distinguished from one another based not only on the time course, but by different changes in each of the measured burst properties for each hormone. As mentioned above, the time course of 5-HT action was slower than that of the peptides. 5-HT treatment induced what appeared to be inhibitory post-synaptic potentials (IPSPs) in two preparations. As this finding was unusual and unexpected, one such display of IPSPs is shown for the record in Fig. 5.11. IPSP onset was within one minute of 5-HT application, and lasted for 4 minutes. In contrast to its normal chronotropic effects, 5-HT evoked only small increases in burst rate in these preparations.

F2 was unique in that it was the only hormone tested that caused desensitization when applied repeatedly to the same cell. As shown in Figure 5.12, the first puff of F2 caused a 47% increase in burst rate, while the second application after a long wash period (17 minutes) produced a smaller response (24% increase). Desensitization was observed in 5 of 16 cells that received two successive F2 treatments.

Figure 5.7 Summary of isolated ganglionic neuron responses to hormones. The open bars are control values, and each of the hormone treatments is specified below its corresponding bar. During control and hormone treatments, each neuron was maintained at a steady hyperpolarized level by current injection via the recording electrode. The effect of this hyperpolarization was that the values for control and hormone treatments shown here were lower than the resting values reported in Table 5.1. Each bar represents from 15–20 cells and data are shown as mean ± S.E. Significant differences from control ($p < 0.05$) are indicated by asterisks (*).


control
Burst amplitude (mV)
15
10
5
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)
control
Burst rate ( / minute)
100
75
50
25
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)

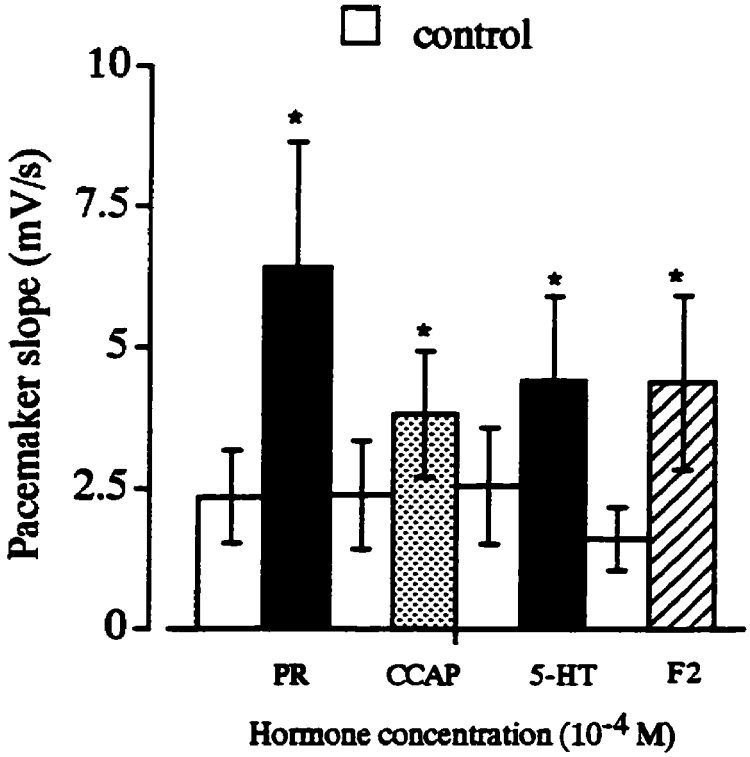
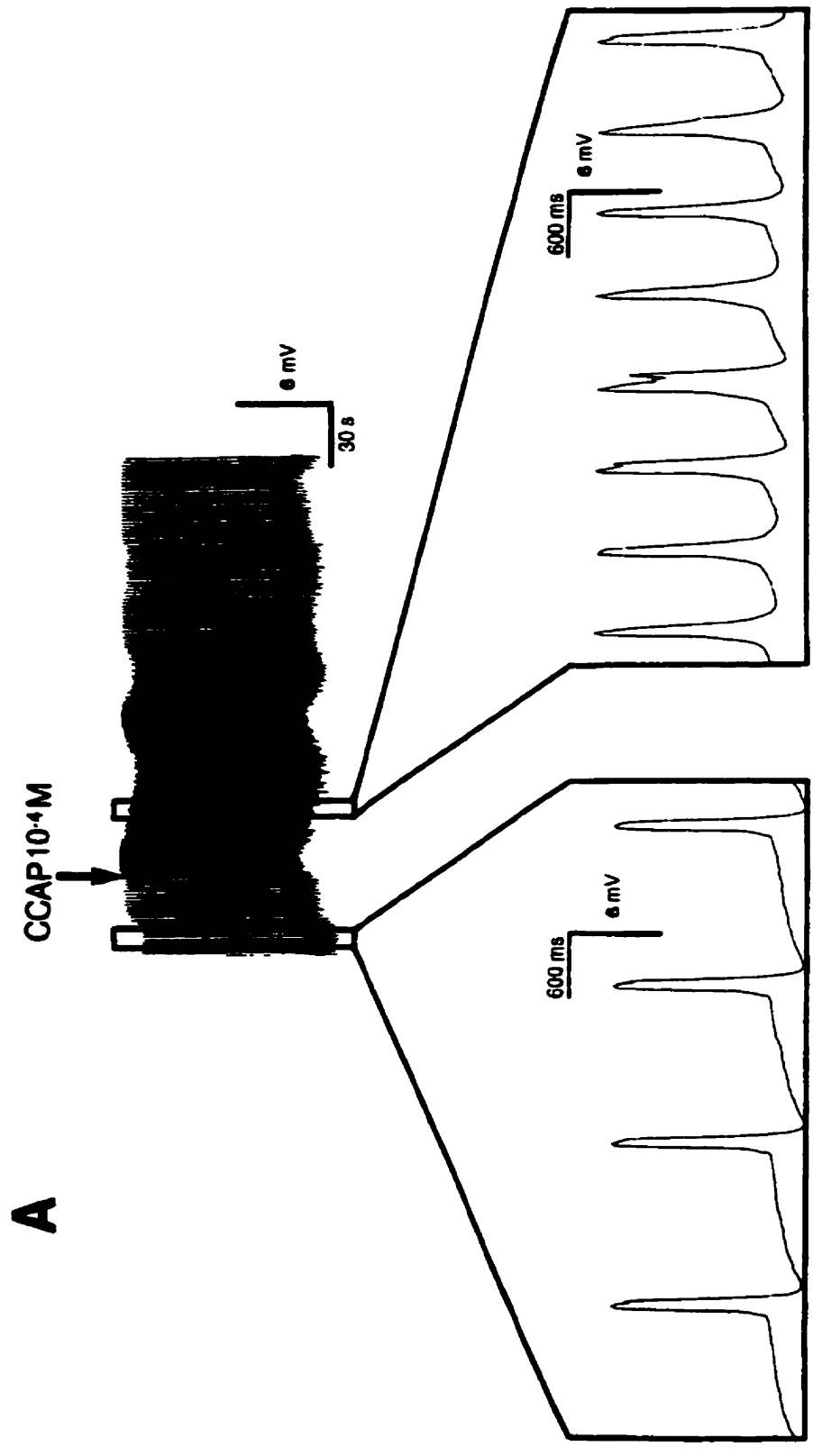
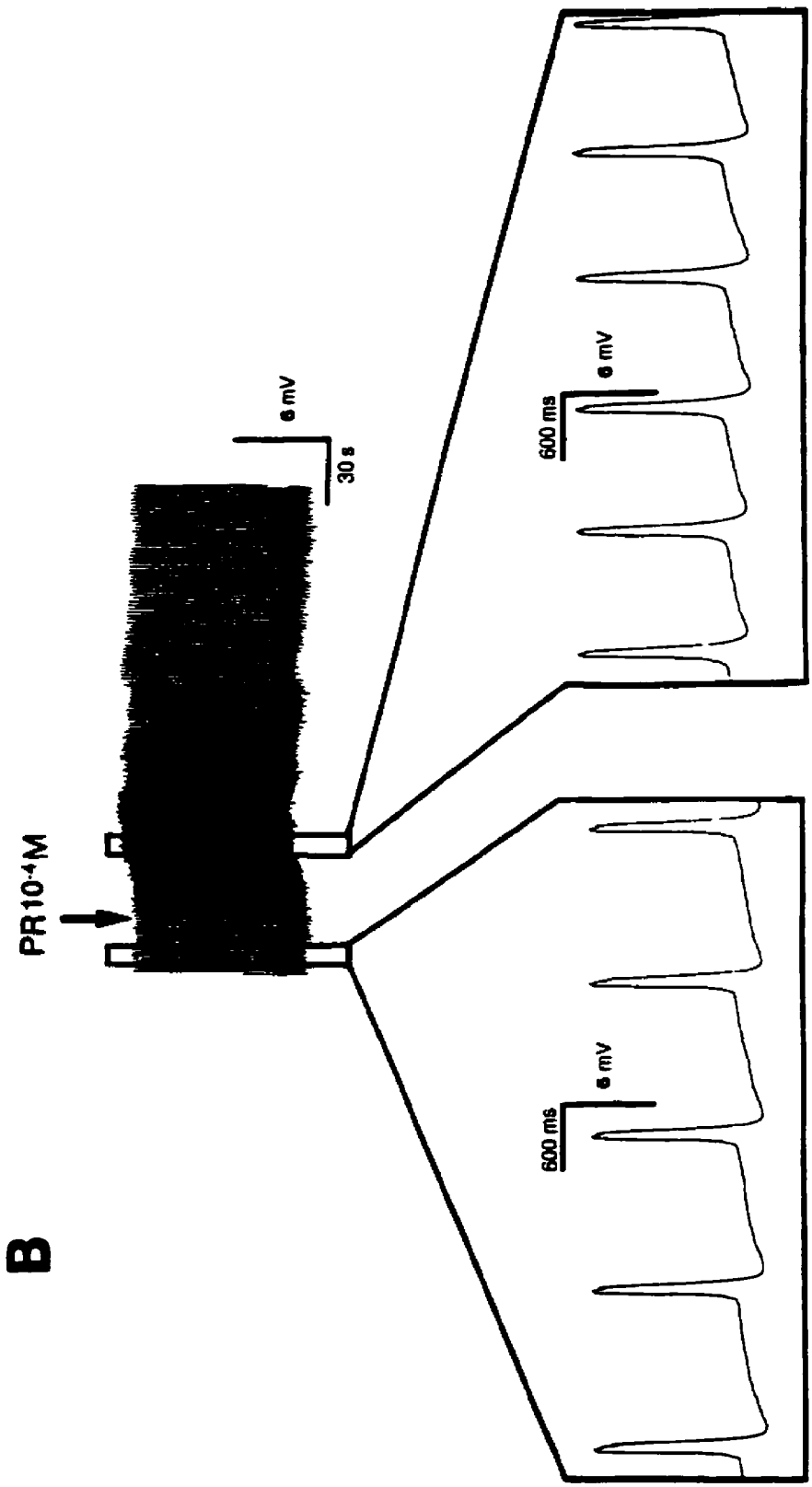
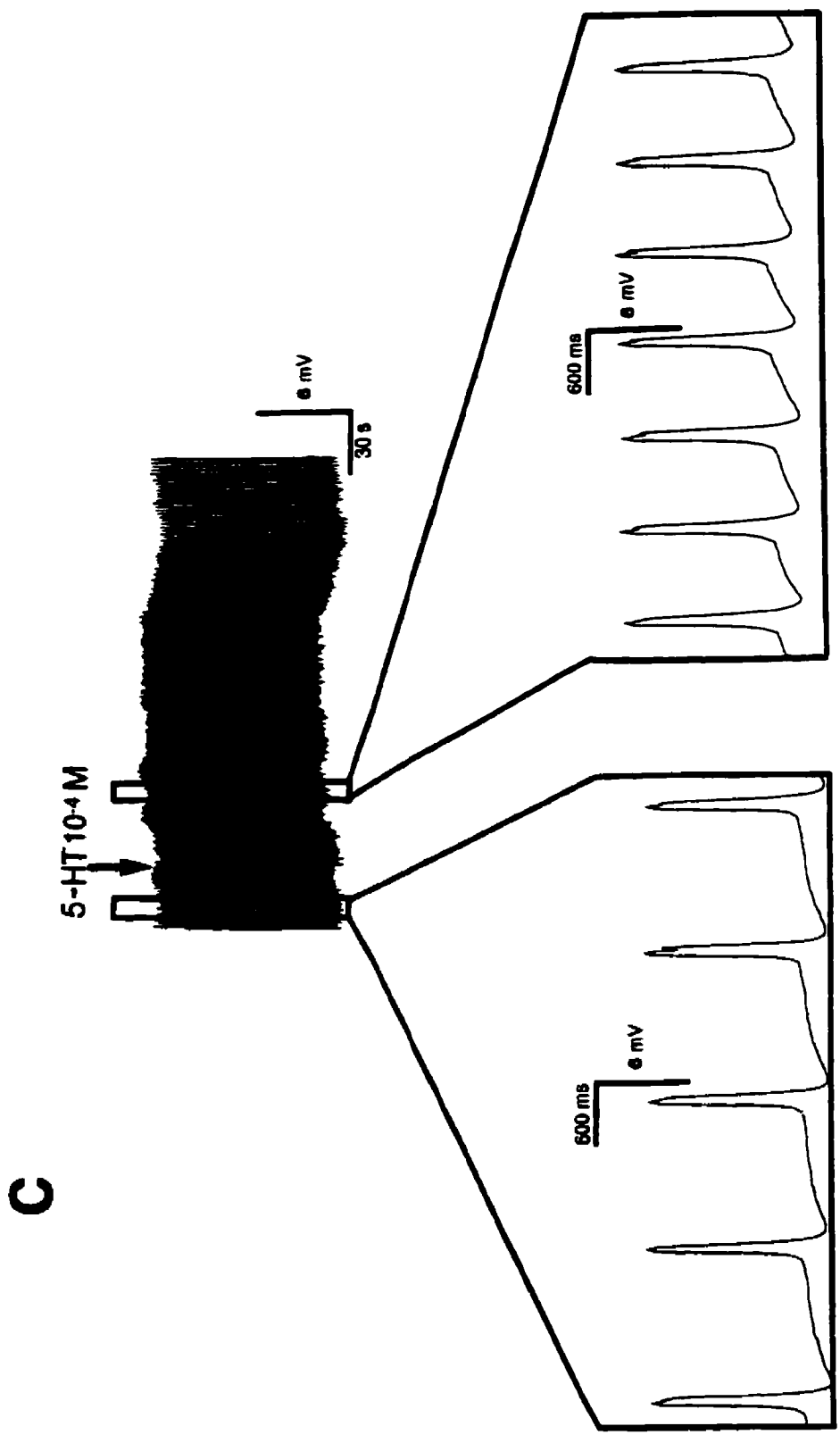

control
Pacemaker slope (mV/s)
10
7.5
5
2.5
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)

Figure 5.8 Responses of one neuron in a single ganglion to applications of each of the four hormones. All hormones were applied by pressure ejection (indicated by the arrow) to neuron LC5. Each trace shows an expanded view of individual waveforms in the control and hormone periods. The order of the traces follows the sequence of hormone application. (A) Response to CCAP $10^{-4}$ M application. (B) Response to PR $10^{-4}$ M is preceded by a slight (< 10 second) delay. (C) Effects of 5-HT $10^{-4}$ M. Note the delay in onset of effects that is typical of 5-HT application; this delay was typically 10–30 seconds, and was longer than that seen for PR in B. (D) Trace showing the effects of a puff of $10^{-4}$ M F2.


A
CCAP10⁻⁴M
6 mV
30 s
600 ms
6 mV
600 ms
6 mV


B
PR10⁻⁴M
6 mV
30 s
600 ms
6 mV
800 ms
6 mV


C
5-HT10-4M
6 mV
30 s
6 mV
600 ms
6 mV
600 ms

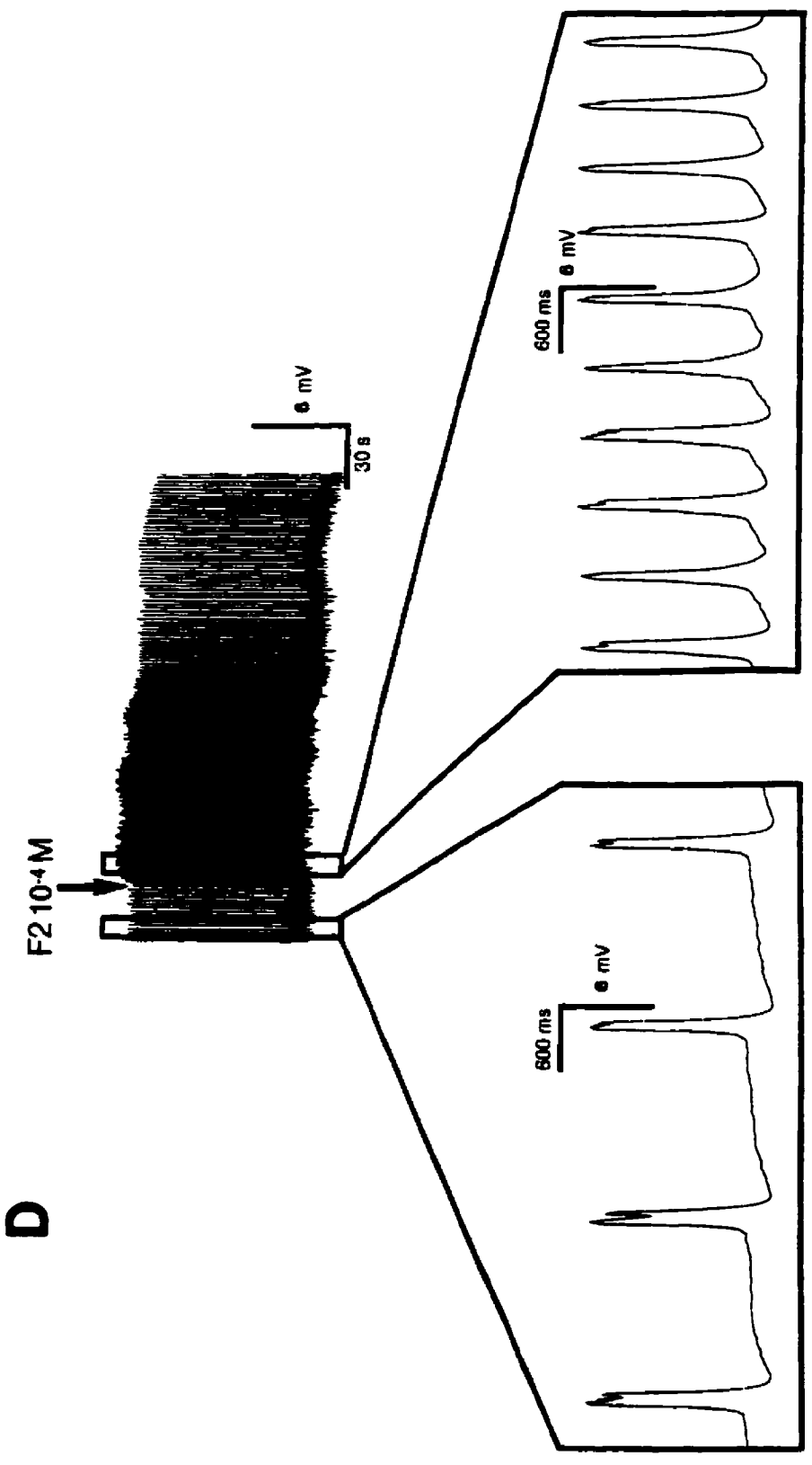
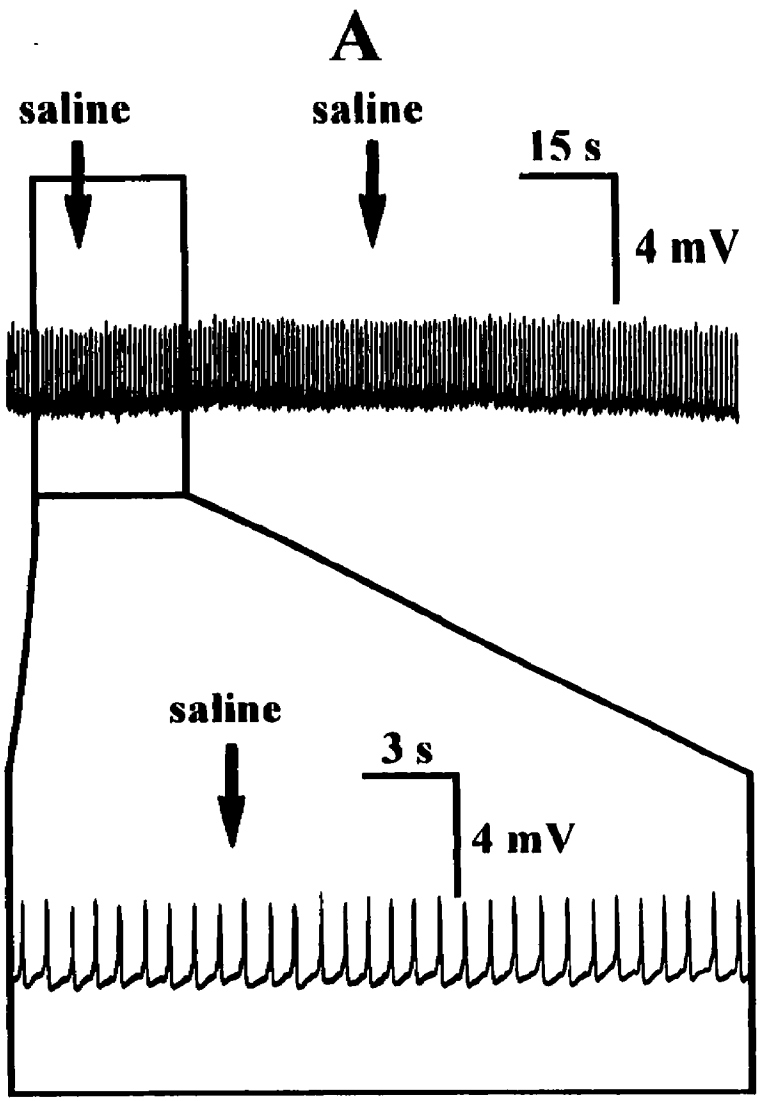

D
F2 10⁻⁴ M
6 mV
30 s
600 ms
6 mV
600 ms
6 mV

Figure 5.9 Control pressure applications of saline did not elicit responses in cultured neurons. (A) Two saline applications (arrows) to LC5 in an isolated ganglion preparation had no effect on burst characteristics. Inset shows an expanded trace of bursts before and after saline application. (B) Saline application (straight arrow) to cultured LC3 had no effect. Curved arrows indicate that hyperpolarizing current was either shut off (HYP off) or turned on (HYP on). Expanded trace shows bursts after saline pressure application.


A
saline
saline
15 s
4 mV
saline
3 s
4 mV

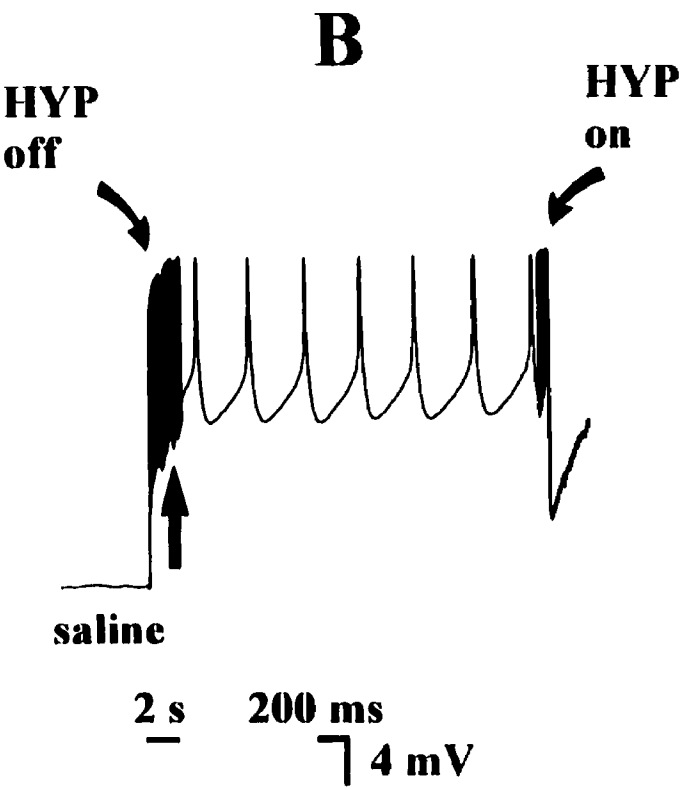
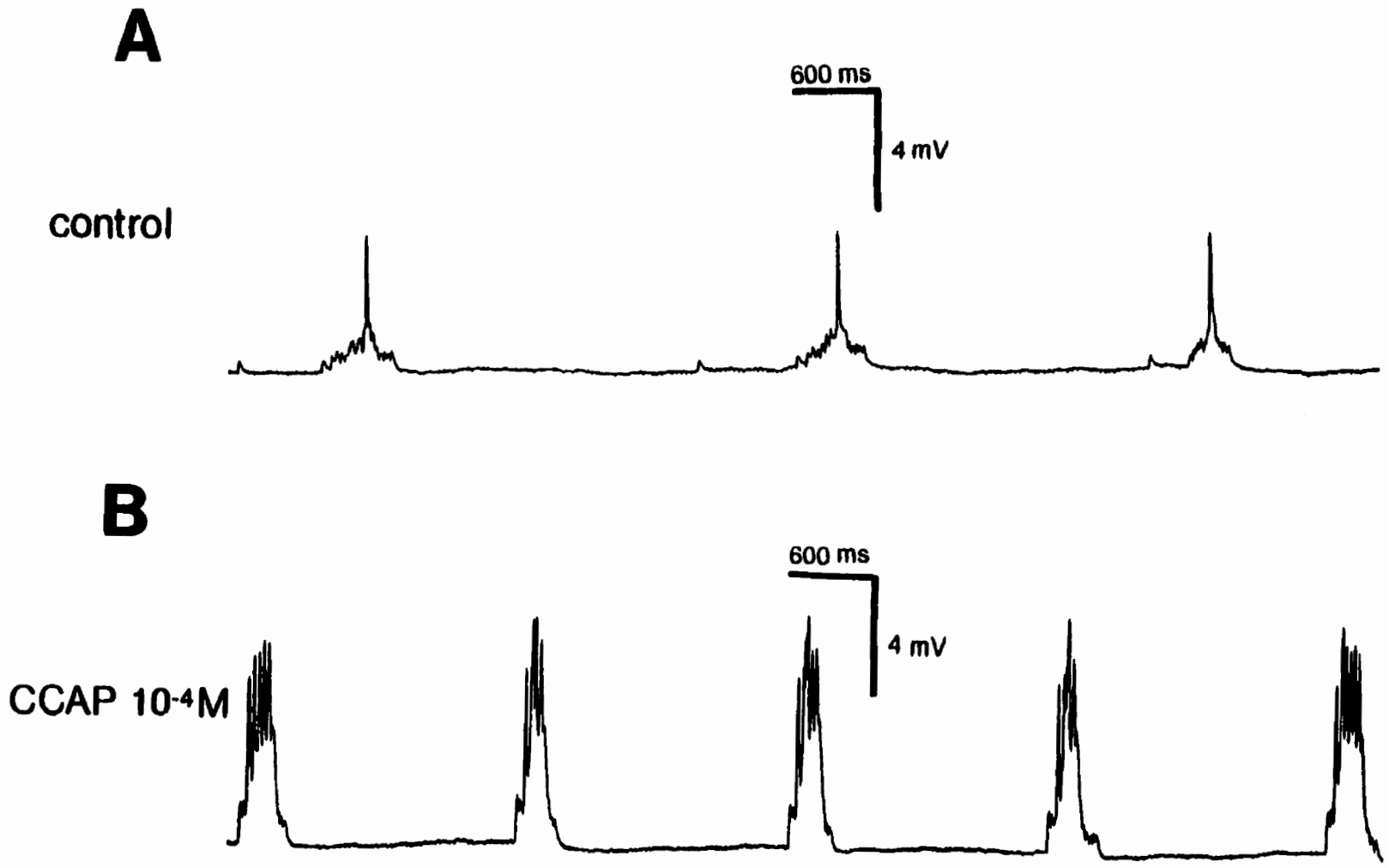

B
HYP
off
HYP
on
saline
2 s
200 ms
4 mV

Figure 5.10 Hormonal modulation of electrical activity. Each of the hormones tested on isolated ganglia preparations altered burst rate and appearance. Two examples of this regularizing and organizing effect are shown for CCAP treatments. (A) Control LC3 recording from a slowly bursting ganglion. (B) At 1 minute after CCAP $10^{-4}$ M application, burst rate increased, and bursting became more regular. Additionally, there were more spikes per burst, and each burst was condensed. (C) Control LC3 recorded from a different isolated ganglion. Bursts were of different amplitudes and some were doublets. (D) At 1 minute following CCAP $10^{-4}$ M application, bursts were all one amplitude and the spikes riding the DPs were more compact.


A
600 ms
4 mV
control
B
600 ms
4 mV
CCAP 10-4M

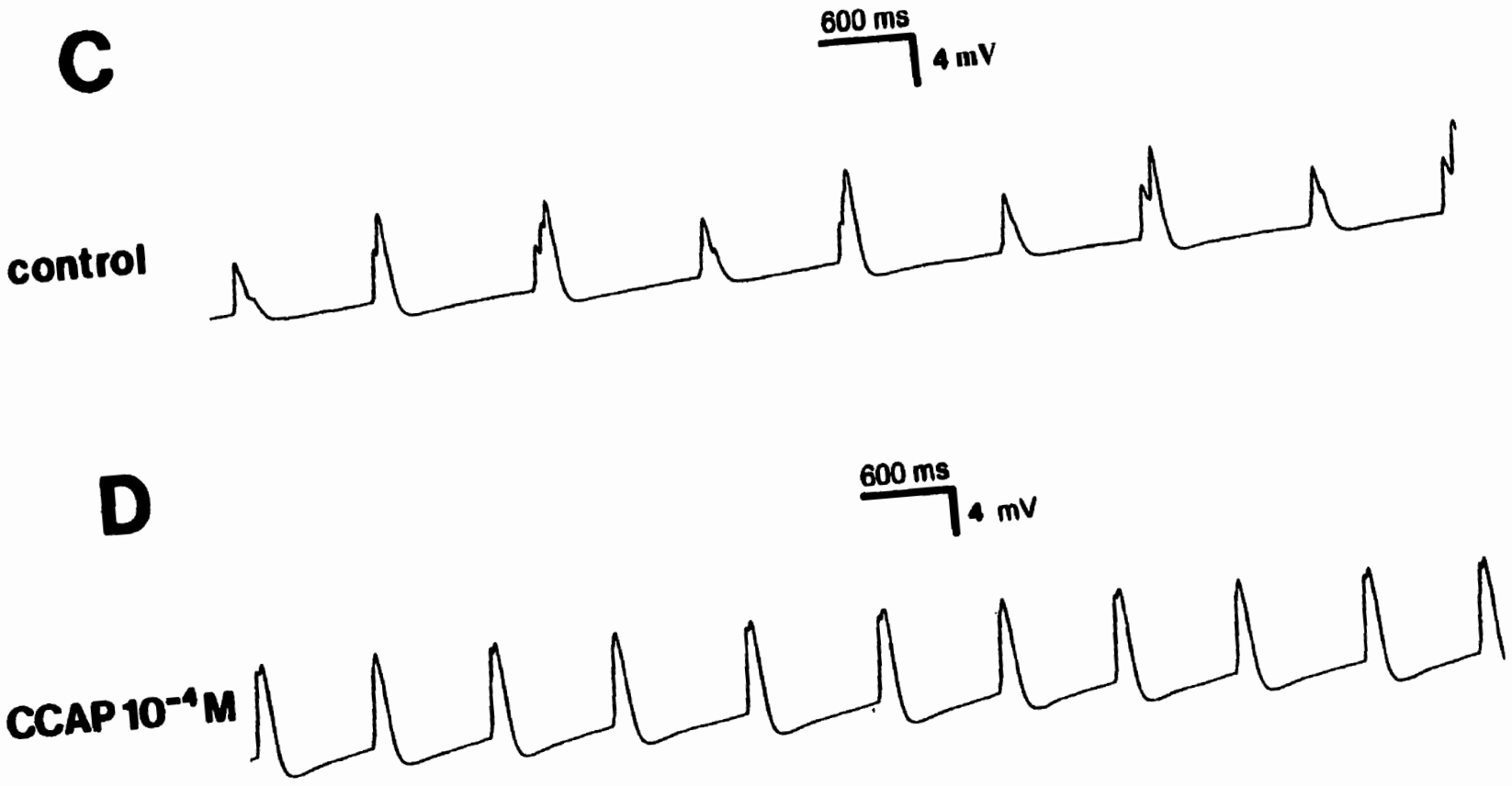

C
600 ms
4 mV
control
D
600 ms
4 mV
CCAP 10⁻⁴ M

Figure 5.11 A rare display of what appears to be inhibitory post-synaptic potentials (IPSPs) in LC 3 following 5-HT treatment in an isolated ganglion. (A) An illustration of control bursting, including an expanded trace of individual waveforms. (B) IPSPs started nearly 1 minute after 5-HT ($10^{-4}$ M) was applied to LC3. This trace was taken at 1 minute 45 seconds following 5-HT application.

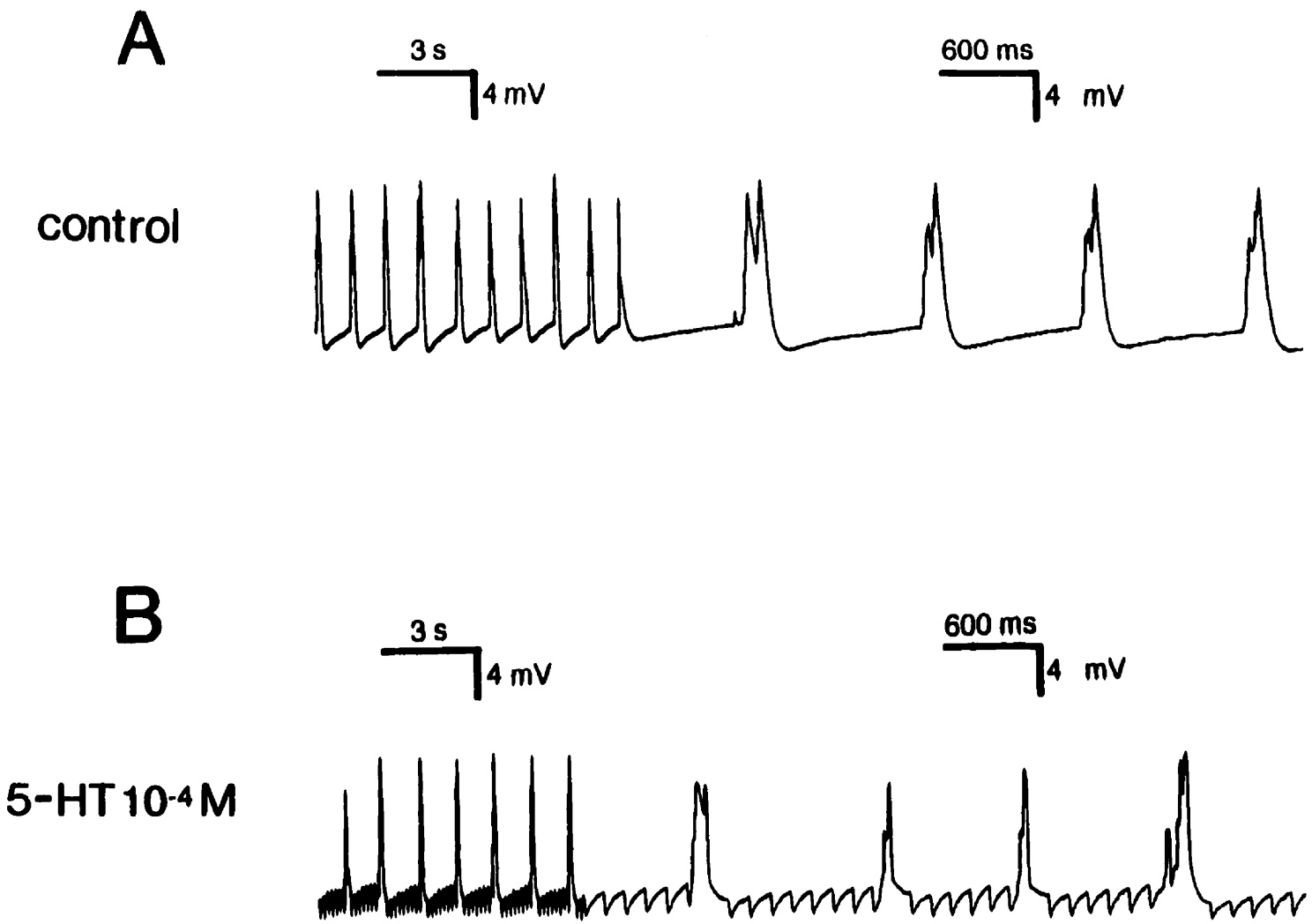
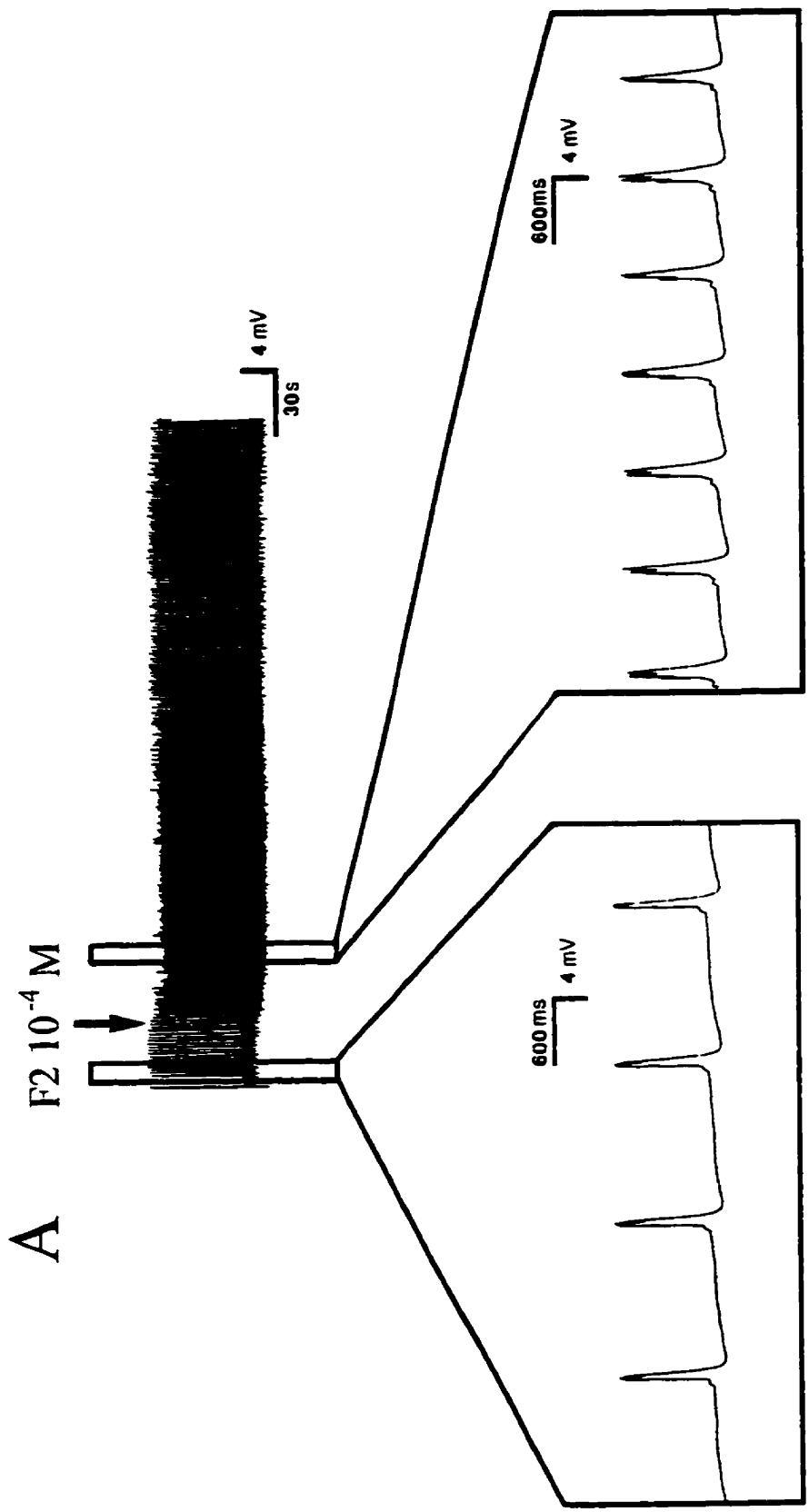
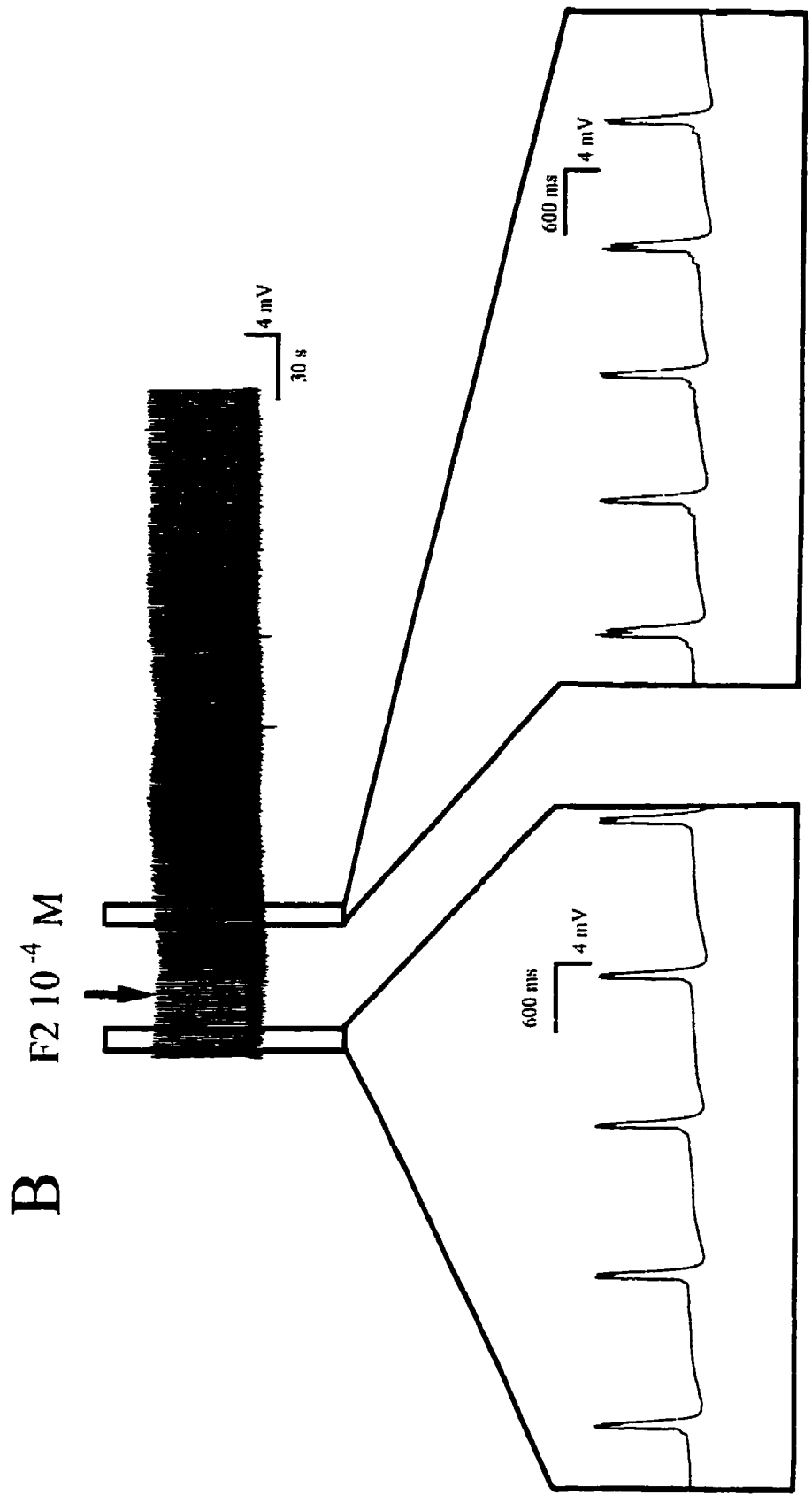

A
3 s
4 mV
600 ms
4 mV
control
B
3 s
4 mV
600 ms
4 mV
5-HT 10-4 M

Figure 5.12 Multiple applications of F2 to the same neuron produced desensitization. (A) The first application of F2 $10^{-4}$ M to neuron LC5 in an isolated ganglion preparation caused nearly a two-fold increase in burst rate. Shown in the insets are waveforms from the control period prior to F2 application, and waveforms at 30 s after F2 application. (B) After 17 minutes of saline wash, the second pressure application of F2 $10^{-4}$ M to the same neuron in the same preparation elicited a smaller response. Individual waveforms from control and 30 s after F2 treatment are shown, inset.


A
F2 10-4 M
4 mV
30s
600ms
4 mV
600 ms
4 mV


B
F2 10$^{-4}$ M
4 mV
30 s
600 ms
4 mV
600 ms
4 mV

The above data were obtained from LCs that were isolated from surrounding cardiac muscle, regulatory nerves (cardioinhibitor and cardioaccelerators), and endogenous hormones (pericardial organ secretions). There are, however, strong electrotonic and chemical synaptic connections between LCs and SCs (reviewed in Hartline, 1979) within the ganglion. It was not possible, therefore, to ensure that these hormone-induced effects were *direct* effects on a particular cell due to the possibility of synaptic interactions. In order to determine intrinsic and hormone-modulated properties of cells isolated from synaptic input, recordings were also made from cells isolated in culture.

### 5.3.3 Cultured cells

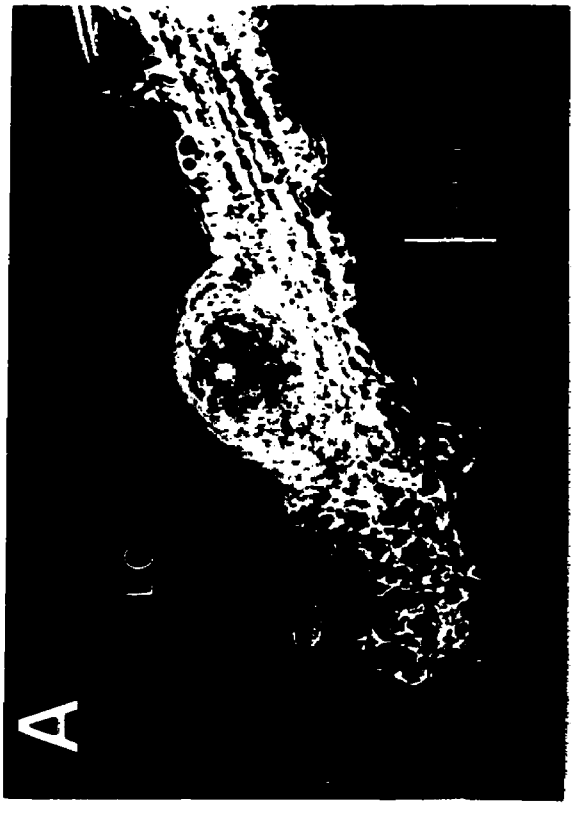
#### 5.3.3.1 General characteristics of neurons

Since this is the first report of cultured cardiac ganglion neuronal activity, this section describes the burst characteristics of cultured cells and also compares these properties to the corresponding activity recorded from LCs in isolated ganglia. Electrophysiological recordings were made from 15 isolated LCs in culture; data were obtained one day following the culture procedure. This represents 33% of cells isolated in culture, the remainder did not possess resting potentials or show spontaneous electrical activity (oscillations or bursts). Cells devoid of axon stumps did not possess resting potentials and did not show spontaneous activity. Membrane potentials of cultured neurons were similar to those recorded from neurons in the isolated ganglion (range: -10 to -56 mV, average -34.2 ± 5.6 mV). Typical cultured LC spontaneous activity, including pacemaker depolarizations and driver potentials with superimposed spikes, is illustrated in Fig. 5.4B. 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, while oscillations were only observed in 5 of 30 isolated CG preparations (Fig. 5.4). Table 5.1 shows the average resting burst rate, burst amplitude, pacemaker slope, and membrane oscillation amplitude recorded from cultured cells.

Three SCs were examined in culture, however, it was not possible to maintain a stable membrane potential for any of these cells throughout the duration of a hormone application. None of the SCs exhibited membrane potential oscillations. The cellular appearance and burst characteristics of SCs were very different from LCs (Figs. 5.13 & 5.14). All SCs fired tonically at a fast rate (3.0 ± 0.3 Hz), and spikes were of greater amplitude than those recorded from LCs. Resting pacemaker slope was 14.92 ± 3.26

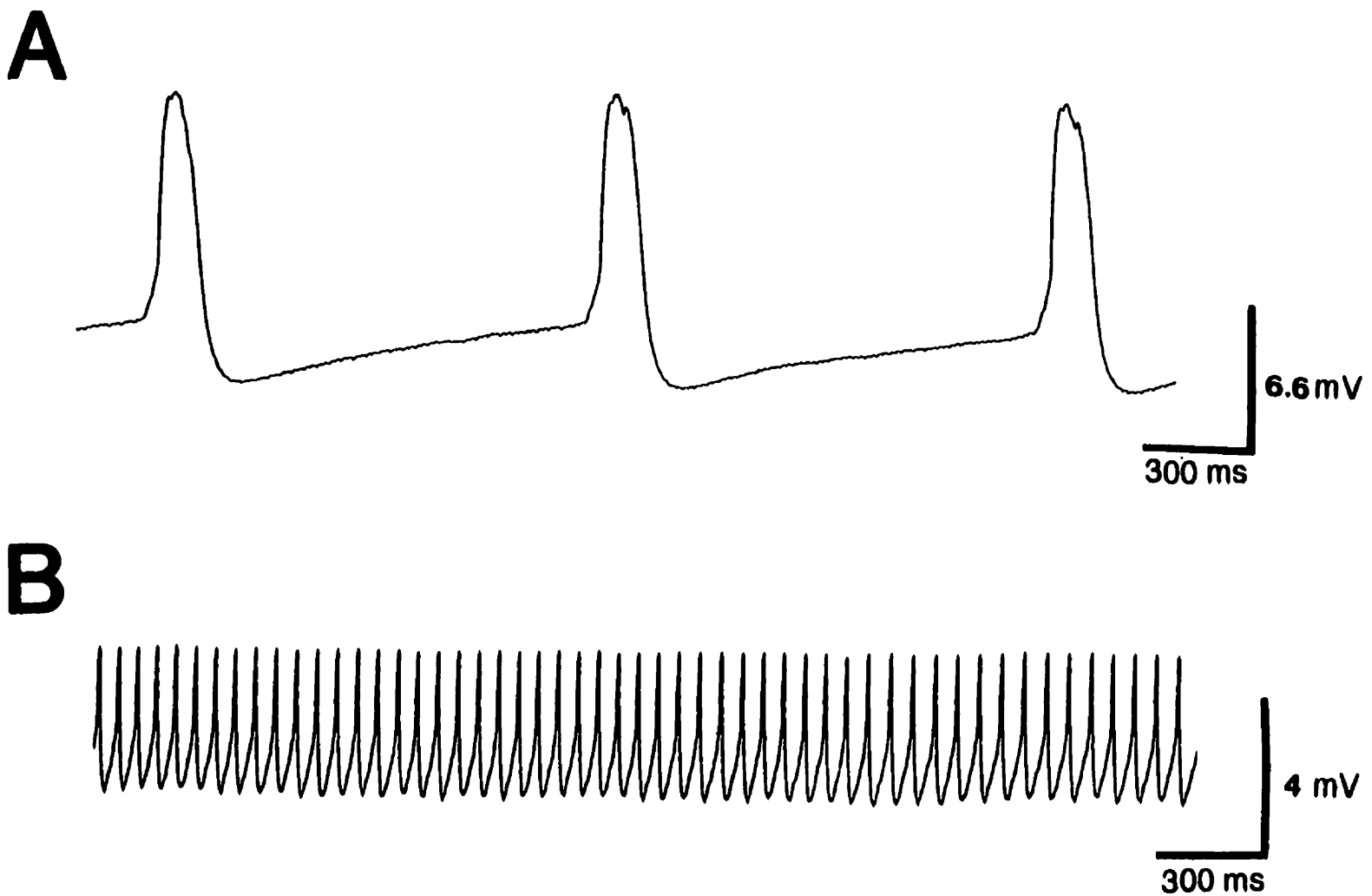
Figure 5.13 Photographs of LC and SC somata illustrating that while LCs are covered with thick connective tissue, SCs are devoid of this covering. (A) LC soma close-up (arrow). (B) SCs (arrows) shown at the same magnification as the LC in A. (C) Another SC (arrow), more clearly illustrating that the soma is bare of any connective tissue or glial covering. All scale bars are 50 μm.


B


A


C

Figure 5.14 Comparison of cultured LC and SC burst characteristics. (A) A record of LC1 bursting behaviour recorded from a cultured cell. Spikes riding the DP plateau are attenuated. (B) Firing behavior of a cultured SC neuron. Note the absence of bursting, the larger amplitude of spikes, and the faster rate of firing for the SCs in comparison to the LC. Time scale is the same in A and B to show the faster rate of SC firing.


A
6.6 mV
300 ms
B
4 mV
300 ms

mV/s ($n$ = 2). These values for SC pacemaker slope are slightly greater than the slopes calculated for LCs.

### 5.3.3.2 Hormone effects on cultured LCs

It was of interest to test the same hormones on cultured LCs to determine if there were any differences between these effects in culture and when the cells were connected in a ganglion, and whether these differences could be attributed to loss of synaptic inputs. As with the isolated ganglion preparation, all data for hormonal effects on cultured neurons were obtained from LCs; hormones were applied to 11 of the 15 cultured LCs studied. The time course of the onset and recovery of each hormone's effects were similar to those observed in isolated ganglia. More of the cultured neurons were quiescent prior to hormone treatment in comparison to neurons in the isolated ganglion preparation (3 of 30). In general, hormone application induced bursting in quiescent cells (Fig. 5.15), and increased burst rate in spontaneously active cells. These changes in burst rate were significant (p = 0.001–0.008, Fig. 5.16). Control saline applications had no effect on the burst characteristics of cultured LCs (Fig. 5.9B).

On three different occasions, all four hormones were tested on a single neuron (Fig. 5.17 shows an example of one such experiment). For oscillating cells, CCAP was the only hormone to significantly increase oscillation amplitude (67%, p = 0.0072). In fact, CCAP was unique in its effects as it significantly increased all measured variables (rate: p = 0.001, amplitude: p = 0.017, slope: p = 0.004, Figs. 5.16–5.17). PR, CCAP and 5-HT significantly increased pacemaker slope (p = 0.002–0.004, Fig. 5.16). Desensitization was observed in 2 of 4 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. 5.16).

In comparing Figures 5.7 and 5.16, 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 all measured parameters. However, greater increases in burst rate, amplitude and pacemaker slope were recorded in response to these same hormones in cultured cells. Cultured cells appeared to be more responsive to hormones, as indicated by a greater number of significant changes in these values for cultured cells than for isolated ganglion preparations.

Figure 5.15 PR application induced a quiescent cell to begin bursting. The cell was maintained at a hyperpolarizing potential via current injection throughout this trial. Cultured neuron LC 4/5 was silent at rest, and induced to burst with a pressure pulse of PR $10^{-4}$ M. Singlet, individual waveforms at 30 s after PR ejection are shown in the expanded trace.

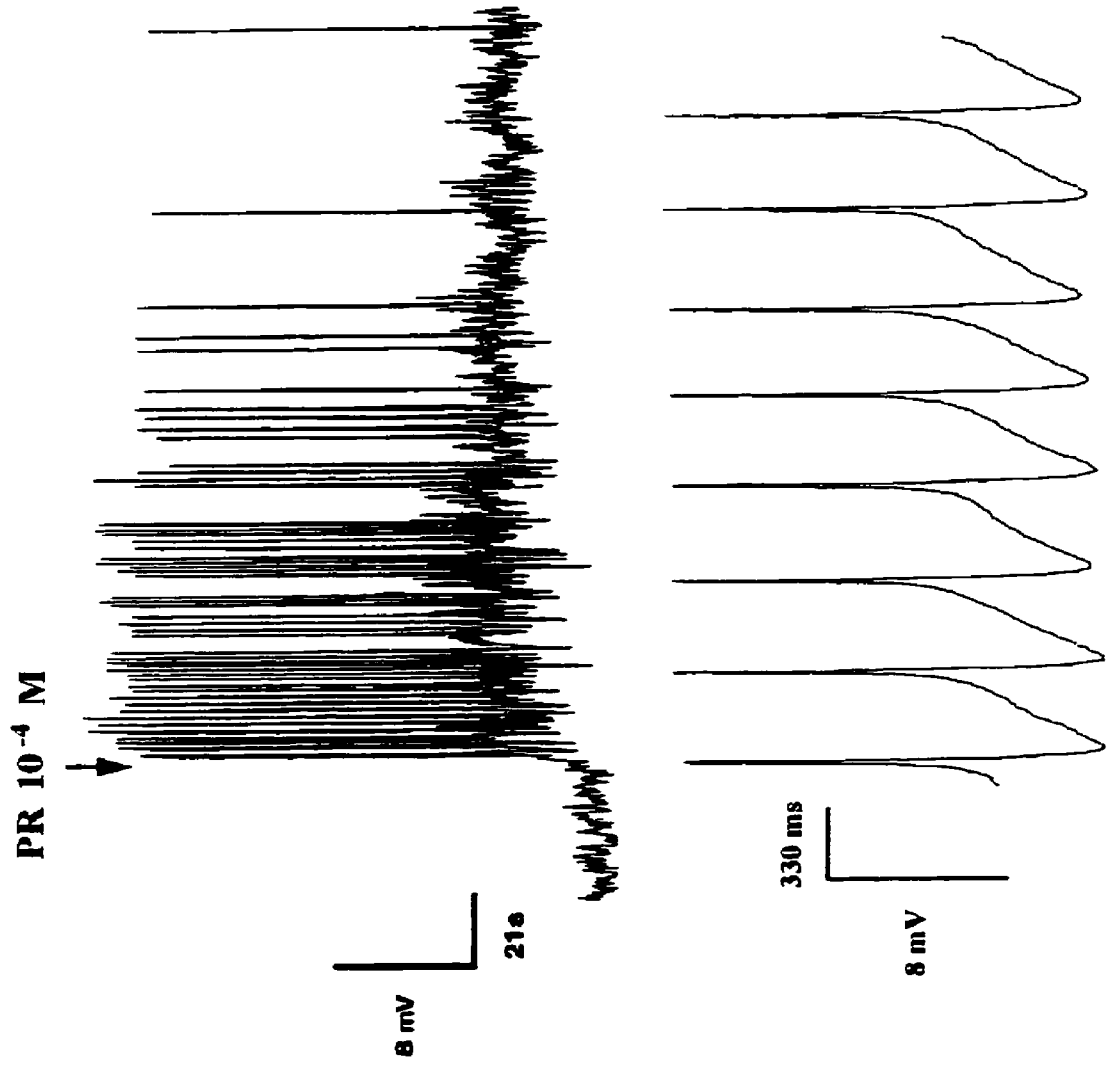

PR $10^{-4}$ M
8 mV
21s
330 ms
8 mV

Figure 5.16 Summary of cultured neuron responses to hormones. The open bars are control values, and each of the hormone treatments is specified below its corresponding bar. Data were collected from neurons that were maintained at a steady hyperpolarized level during control and hormone treatments. The effect of this hyperpolarization was that the values for control and hormone treatments shown here are lower than the resting values reported in Table 5.1. Each bar represents N = 5–9 and data are mean ± S.E. Significant differences from control ($p < 0.05$) are indicated by asterisks (*).

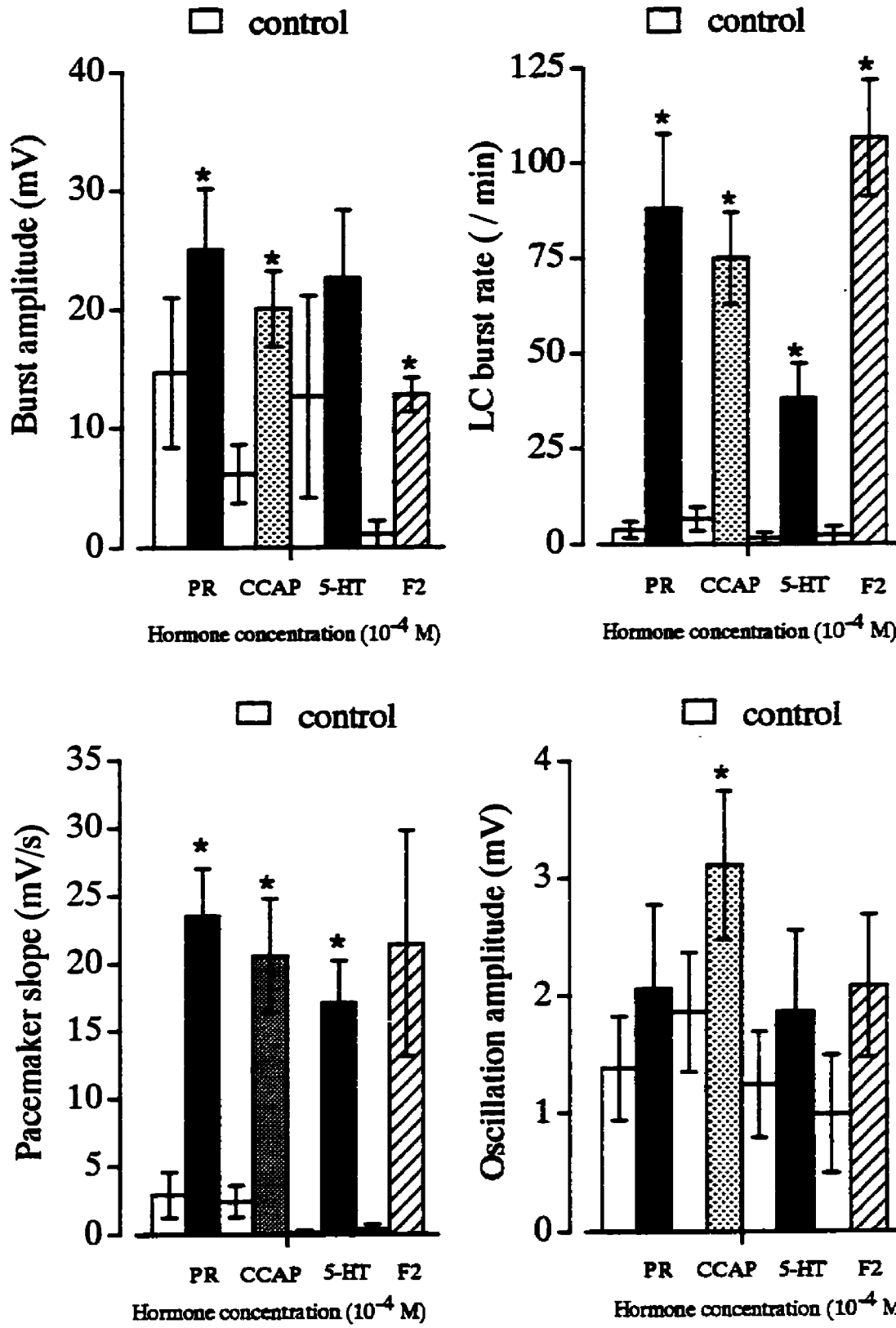
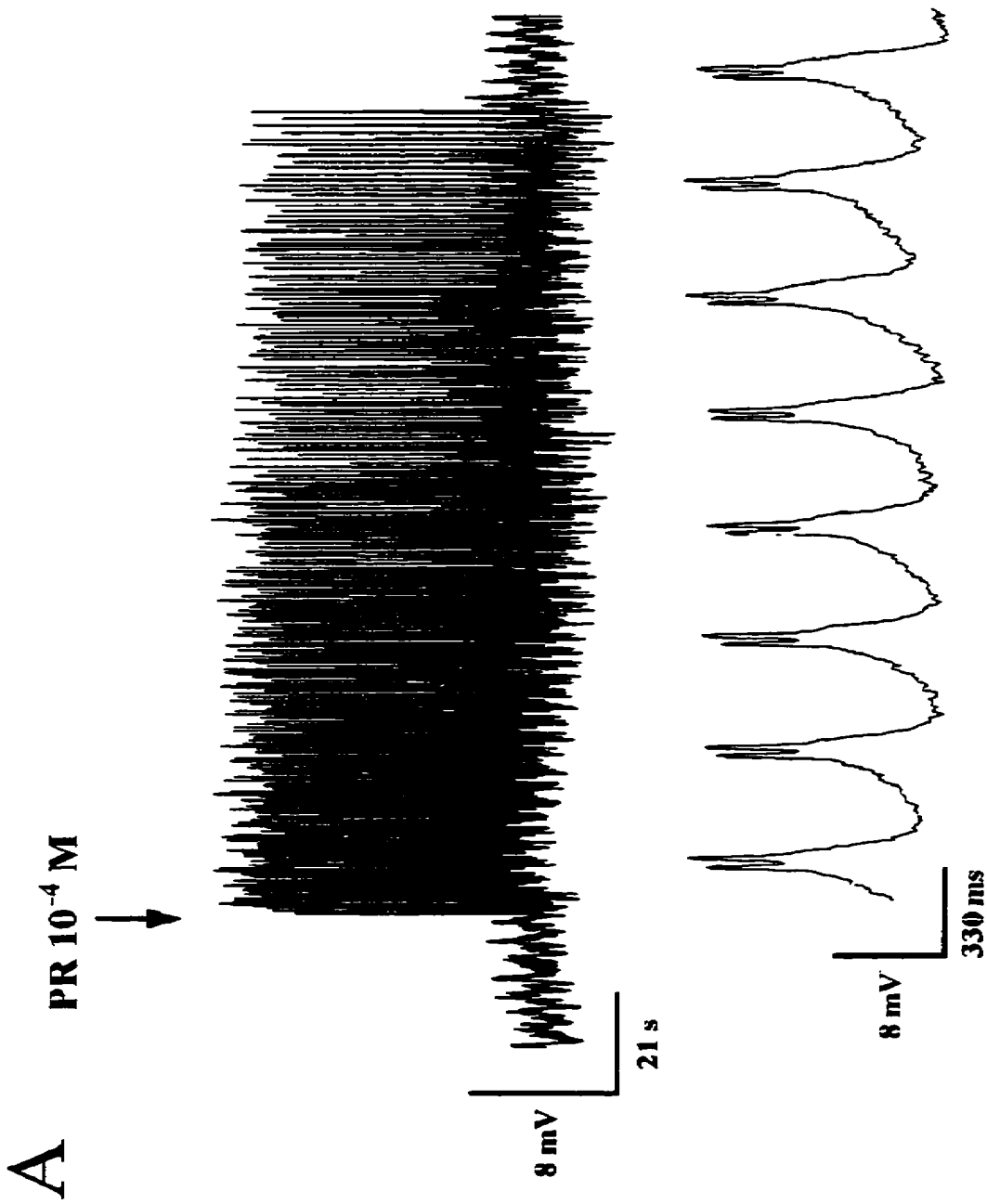
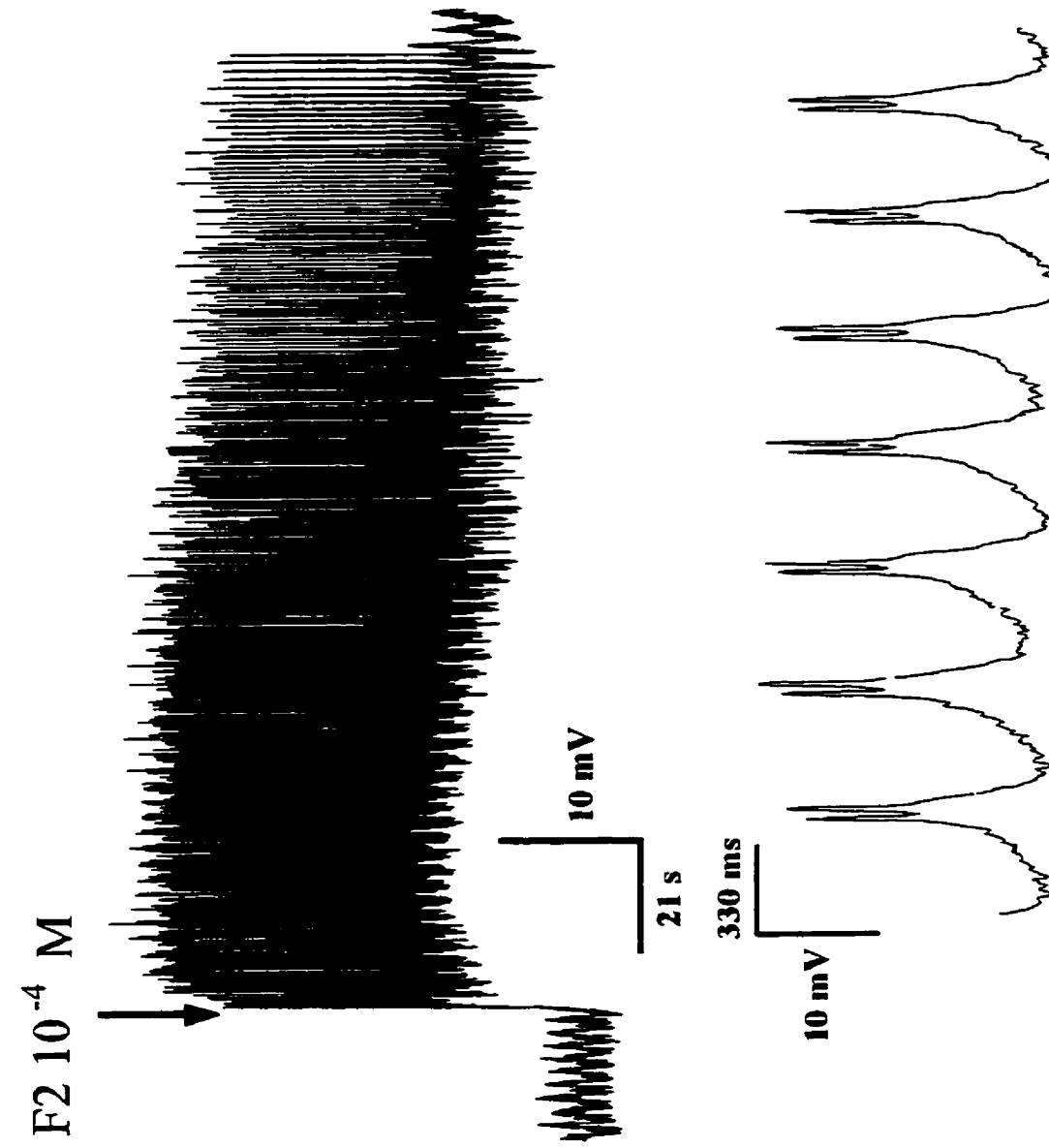
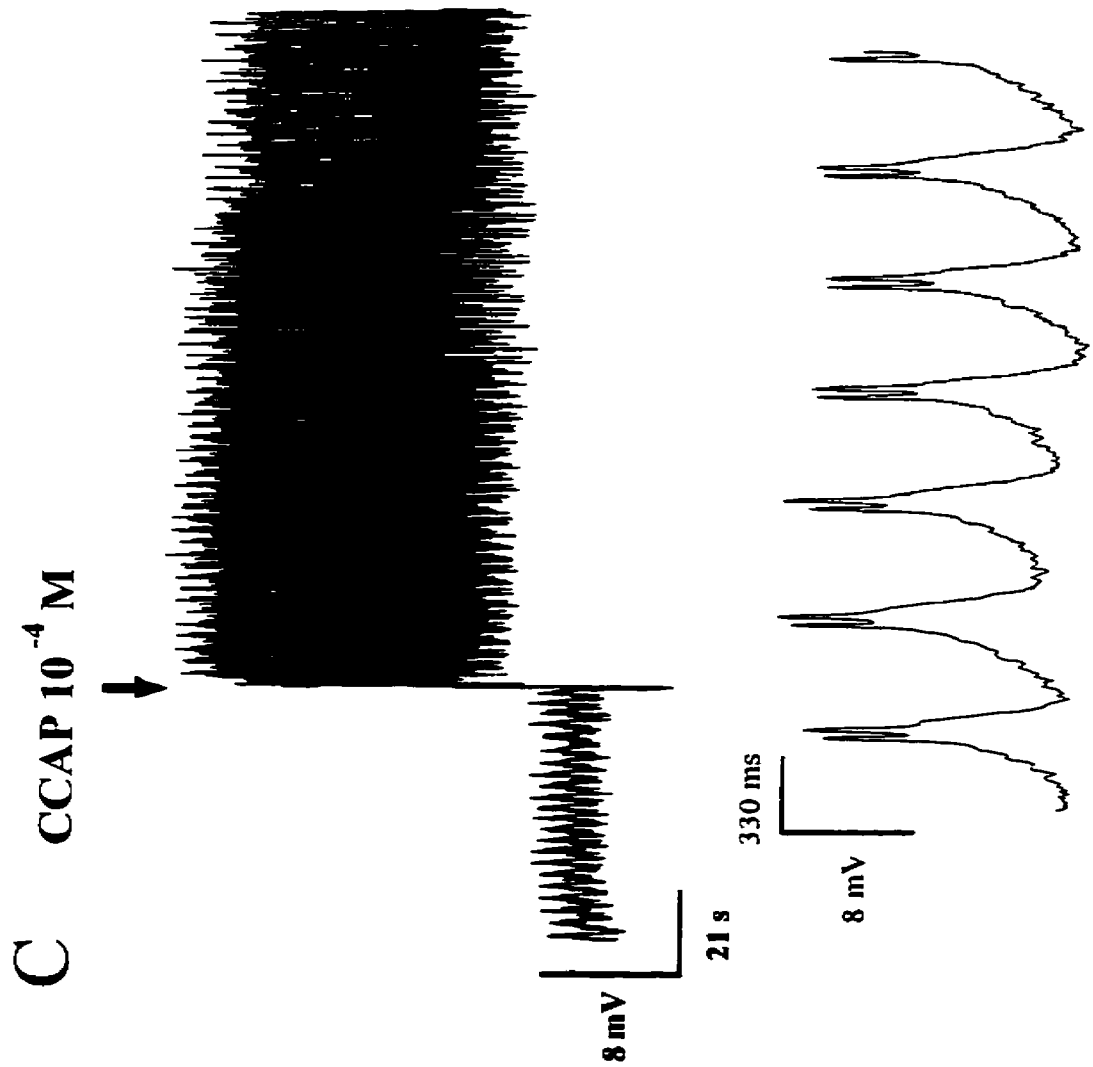
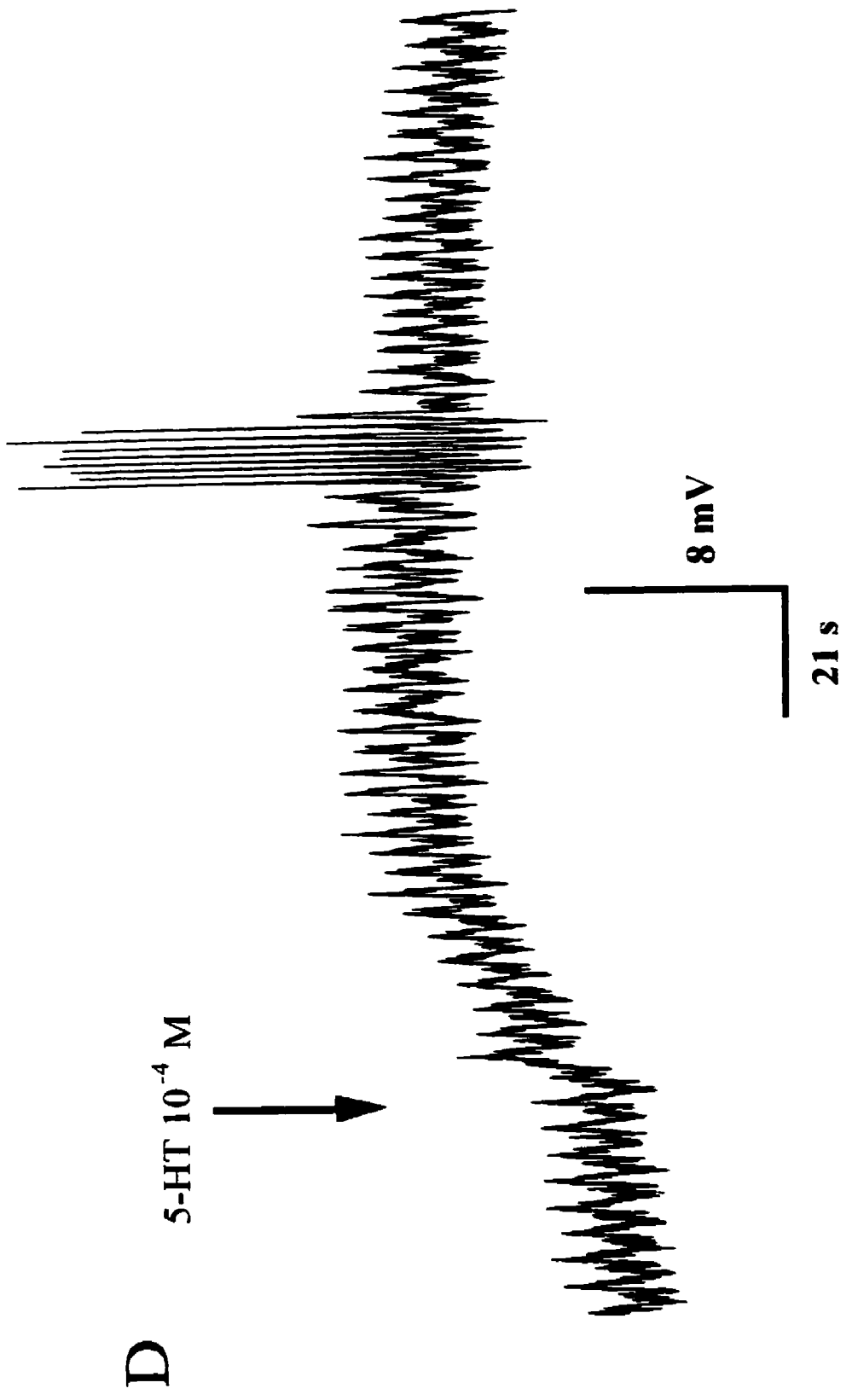

control
Burst amplitude (mV)
40
30
20
10
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)
control
LC burst rate ( / min)
125
100
75
50
25
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)
control
Pacemaker slope (mV/s)
35
30
25
20
15
10
5
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)
control
Oscillation amplitude (mV)
4
3
2
1
0
PR
CCAP
5-HT
F2
Hormone concentration ($10^{-4}$ M)

Figure 5.17 Cultured CG neuron (LC1) responded to direct pressure ejection of each of the four hormones. The order of the traces followed the sequence of hormone application, and an expanded trace showing maximum hormone-induced burst rate is included below each full trial. (A) PR $10^{-4}$ M. (B) F2 $10^{-4}$ M. (C) CCAP $10^{-4}$ M. The top trace of the CCAP response was truncated before the firing rate had returned to baseline, which occurred more than 3.5 minutes after the pressure pulse of CCAP. (D) 5-HT $10^{-4}$ M. Note the delay in response to 5-HT, which was typical of this amine's effects. Three of the other seven cells treated with 5-HT also responded poorly in this manner.


A
PR $10^{-4}$ M
8 mV
21 s
8 mV
330 ms


B
F2 10-4 M
10 mV
21 s
330 ms
10 mV


C
CCAP $10^{-4}$ M
8 mV
21 s
330 ms
8 mV


D
5-HT $10^{-4}$ M
8 mV
21 s

## 5.4 Discussion

### 5.4.1 The *in vitro* cell culture system

Isolated CG preparations have been used extensively in the past to study network and intrinsic ganglionic neuronal properties in several crustacean species (Tazaki and Cooke, 1979a-c, 1983b, 1986; Sullivan and Miller, 1984; Berlind, 1985, 1989). Ligation of the ganglion or use of two-pool isolation studies allowed these investigators to 'isolate' neurons or groups of neurons. Using these types of preparations, CG network connections have been well mapped out (Hartline, 1979 review) and more recently, the conductances underlying DP generation were examined in detail (Tazaki and Cooke, 1986). However, Sullivan and Miller (1984) suggested that CG ligation may disrupt the physiological and pharmacological characteristics of the neurons.

Attempts to examine the basic electrophysiological characteristics of the *C. maenas* CG network were hampered by the inability to visualize (and hence isolate) the ganglion from these small crabs. Therefore, in the present study, techniques were developed first to enzymatically separate the ganglion from surrounding heart musculature, and then to culture the individual CG neurons. The aim was not to study cellular growth, regeneration, or synapse formation in culture, but to establish an *in vitro* cell culture system that produced viable, healthy cells for electrophysiological study. A second aim was to validate the utility of the *in vitro* preparation for further studies directed at the mode of action of cardioregulatory hormones. This is the first study to accomplish these goals using the CG neural network.

Cell culture techniques were developed originally by researchers utilizing various molluscan species. The complexity of the mammalian brain precludes *in vivo* studies of neuronal interactions such as synaptogenesis; hence, investigators have turned to simpler models, such as molluscs. Utilizing cell culture techniques, various aspects of neuronal interactions in isolation can be directly explored (Feng et al., in press). Some of these culture techniques were described for molluscan neurons as early as the 1970s and 1980s (Kaczmarek et al., 1979; Haydon et al., 1984; see Bulloch and Syed, 1992). Not only have molluscan neurons been shown to survive in culture and retain excitability, but many exhibit neurite outgrowth and make synaptic connections *in vitro* (Wong et al., 1981; Syed et al., 1990; Bulloch and Syed, 1992). Several other invertebrate cell or tissue culture systems have been established, using leech (soma-soma synapses; Fuchs et al., 1990) and insect neurons (cockroach, locust, fruit fly, and grasshopper, reviewed in Thomas et al., 1987) as models. Generally, cell culture models provide powerful systems

in which mechanisms underlying learning, memory, growth cone behavior, synaptic plasticity, regeneration, and synapse formation can be examined (Bulloch and Syed, 1992).

The crustacean field lags behind molluscan research in terms of cell culture techniques. However, cardiac ganglion neurons can now be added to the short list of crustacean neurons that have been cultured. Only crab (*Cardisoma carnifex*) and lobster (*Panulirus marginatus*) eyestalk X-organ peptidergic neurons (Cooke et al., 1989; Grau and Cooke, 1992), and lobster STG neurons (*P. interruptus*, Panchin et al., 1993; Turrigiano and Marder, 1993) have been examined morphologically and electrophysiologically in 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 were also encountered in the present culture system due to the glial sheath covering LC neurons. In this study the mechanisms underlying neurite outgrowth from CG neurons were not investigated, as this was not the goal. Neurite outgrowth requires growth factors, protein synthesis, and substrate adhesion molecules (Bulloch and Syed, 1992). In the future, neurite outgrowth of CG neurons could be examined using appropriate conditioned media and trophic factors.

In the present study, cultured CG neuron recordings were made after one day in culture. At this time cells exhibited bursting behavior similar to that recorded from isolated ganglia. This contrasts with studies on the lobster STG in which neurons were initially inactive but regained tonic firing and subsequent bursting ability after a few days in culture (Panchin et al., 1993; Turrigiano and Marder, 1993; Turrigiano et al., 1994, 1995). Another finding from this study that contrasted previous literature was the burst patterns of LC neurons. Previously published studies showed that either chemically or electrotonically mediated inputs from the SCs or LCs were necessary for LC burst generation (Tazaki and Cooke, 1983c; reviewed in Cooke, 1988). In the present study, however, both SCs and LCs isolated from the above synaptic inputs showed spontaneous bursting patterns suggesting that they exhibit intrinsic membrane conductances necessary for burst generation. When SCs and LCs are connected in a ganglion, synaptic connections serve to coordinate these conductances in order for all five neurons to fire together as a unit.

Enzyme treatments are standard protocols in cell culture studies and such enzyme treatments could affect ion influx, neuronal excitability, and synaptic transmission,

although some of these effects may be reversible (Hermann et al., in press; P. Hermann, personal communication). Additionally, enzymatic action could possibly break down extracellular components of hormone receptors. In the present study, both the morphological appearance of cells and their excitability indicated that cells were healthy after enzyme treatment. In terms of excitability, cell membrane potentials recorded in the present study (range: -12 to -65 mV, isolated ganglia vs. -10 to -56 mV for cultured cells) were within the range of those recorded from lobster and crab isolated CGs that were mechanically isolated, i.e., in the absence of enzyme. Values were reported to range from -42 to -60 mV in *H. americanus* lobsters (Miller and Sullivan, 1981) and were documented as -54 mV in portunid crabs (Tazaki and Cooke, 1979a). Another measure of excitability, the bursting activity of the LCs, was similar in the present study to that of previously recorded studies (Benson, 1980; Miller and Sullivan, 1981; Tazaki and Cooke, 1983b; Berlind, 1985). Finally, as detailed below, cell responses to hormone application matched previous studies (Cooke and Hartline, 1975; Lemos and Berlind, 1981; Miller and Sullivan, 1981; Freschi, 1989). Whether or not the hormonal responses of the *in vitro* CG neurons replicated those of intact *C. maenas* neurons is unknown. To test this possibility, it would be necessary to mechanically isolate the CG from the *C. maenas* heart and compare its responses with those of a ganglion receiving enzyme treatment. These experiments have not been performed. All in all, the characteristics of isolated cells were similar to those in the intact ganglion and give confidence in utilizing the *in vitro* cell culture system.

Burst characteristics of *in situ* and *in vitro* isolated neurons were similar, yet there were some qualitative differences in hormone responses (Figs. 5.7 and 5.16). These differences were attributed to the cellular, synaptic or network properties of the ganglion. All five motoneurons are coordinated to fire together in the intact ganglion. Hormone application increased the coordination between these neurons, as indicated by more regular and organized bursts recorded during hormone application in this study. This entrainment likely occurred either by strengthening electrotonic and/or synaptic connections between the cells. The overall effect of more coordinated ganglionic neuronal firing would be increased EJP amplitude and burst frequency, which ultimately results in increased rate and force of heart contraction. Several lines of evidence indicated that hormones also altered individual neuronal excitability. Cultured cells lack the long axons, gap junctions and chemical synaptic connections to other neurons that exist in the intact ganglion. These features of cultured cells serve to decrease $R_{input}$ and enhance cell excitability. The *in*

*vitro* neurons isolated from such interactions showed more dramatic changes in bursting behaviour in response to direct hormone application than neurons in isolated ganglion preparations, which may suggest that hormones directly alter specific ion conductances. Additionally, the same level of hyperpolarizing current injected into either *in situ* or *in vitro* CG neurons resulted in only the *in vitro* neurons becoming quiescent. Activity of the *in situ* neurons, however, was only slightly suppressed. Thus not only did hormones alter synaptic properties, as indicated by more coordinated and regular bursts in the isolated ganglion, but these same hormones affected neuronal excitability.

### 5.4.2 Small cells

Most previous recordings from SCs reported in the literature were made indirectly *via* extracellular wire (Tazaki and Cooke, 1979b,c; Sullivan and Miller, 1984) or suction (Berlind, 1985) electrodes applied to the nerve trunks. To my knowledge, only 3 groups of investigators have made direct intracellular SC penetrations in nearly 50 years of studying this ganglion. These other direct SC recordings were made from ligatured crab (*P. sanguinolentus*) semi-isolated CGs (Tazaki and Cooke, 1979a), and lobster CGs (*P. interruptus,* Friesen, 1975; and *P. japonicus*; Tameyasu, 1976). Recordings from crab SCs showed slow, prolonged pacemaker-like depolarizations that were more dominant than LC pacemaker potentials (Tazaki and Cooke, 1979a). Prolonged depolarizations, driver potentials (DPs), with superimposed spike trains were recorded from SCs; the duration of DPs was longer in SCs than LCs (Tazaki and Cooke, 1979a; Tameyasu, 1976).

This study provides the first anatomical description of *C. maenas* SCs. All the SCs observed in this study were clustered below LCs 4 & 5, and were encased in a "pocket" of connective tissue burried deep within the CG. However, those SCs that were removed from the ganglion for study in culture showed no evidence of the thick glial sheath that covers each individual LC neuron. Rather, these cells were devoid of any covering (see Fig. 5.13). Cooke (1988) suggested that since SCs are ensheathed by glial and connective tissues, they are inappropriate for study by patch clamp techniques. On the contrary, once the SCs were removed from this connective tissue "pocket", they appeared to be excellent specimens for patch-clamping. However, since visualizing these SCs was difficult, better visualization procedures would still be required before this kind of study is attempted.

Although only 4 SC recordings were made from both preparations in the present study, some generalizations can be drawn about SC burst characteristics. In both preparations, SC bursting was very different from that of LCs. The pacemaker potentials were faster and steeper than those observed in other SCs (Tazaki and Cooke, 1979a; Tameyasu, 1976). SCs fired tonically and did not generate slow driver potential depolarizations; this is in dramatic contrast to the prolonged DPs with superimposed spikes recorded in other studies (Tazaki and Cooke, 1979a; Tameyasu, 1976). The only finding consistent with previous SC studies was the fast rate of the spike train. The firing rate of spikes riding the DP was twice or more than that of LC firing (Tazaki and Cooke, 1979a).

In the present isolated ganglion preparation, where SCs were still synaptically connected, an explanation for the tonic firing could simply be that network properties were different than was once thought. Alternatively, cultured SCs were removed from ganglionic synaptic and CNS regulatory inputs. This absence of extrinsic regulation could mean that the tonic firing pattern observed for SCs was an intrinsic property of this cell type, which is again different from previous suggestions. However, an explanation for these discrepancies in SC firing recorded in the literature and at present require further investigation because only a few recordings were obtained from SCs.

### 5.4.3 Large cells

#### 5.4.3.1 Proctolin effects

The peptide proctolin is present in shore crab POs (Stangier et al., 1986). A myotropic role for PR has been documented and effects on contractility were described elsewhere in this thesis (Chapters Three & Four). This section focuses on PR action on CG neurons.

PR increased heart rate, electromyogram amplitude, and intracellularly-recorded excitatory junction potentials in intact, open and isolated *C. maenas* heart preparations (Chapters Three & Four). These findings suggested that one site of action for PR is at the CG. In isolated lobster CGs, PR depolarizes LCs, increases their burst frequency and pacemaker potentials (Miller and Sullivan, 1981; Freschi, 1989). In TTX-silenced lobster ganglia, PR application evoked depolarization and repetitive DPs (Miller and Sullivan, 1981). Doublet bursts were often observed with PR treatment. A continued or prolonged discharge of the SCs during the period in which a LC recovers from its refractory period

can provide the depolarization for the second burst of a doublet burst. This opinion is shared by A. Berlind (personal communication).

PR effects recorded from isolated ganglia and cultured cells in this study correspond with other studies. PR increased all burst parameters, and induced immediate bursting activity in silent cells. The main difference between the present findings and those of previous studies was the time course of PR action. Immediate responses to PR were recorded here (within 0–10 seconds), whereas the other studies reported slow onset (60–90 seconds) and long-lasting effects (10–20 min) (Sullivan and Miller, 1984; Freschi, 1989). The different time courses can be attributed to different methods and amounts of peptide application. In this study, small amounts of PR (estimated to be 0.5–1 μL) were pressure applied ('puffed') directly onto the LC soma under investigation. Other authors bath applied PR, where peptide was continuously infused for 2–3 min., or applied small pulses (aliquots of 50–100 μL) 'upstream' of the preparation (Miller and Sullivan, 1981; Sullivan and Miller, 1984).

The mechanism of PR action on the CG neurons was not examined. The PR mechanism of action on *H. americanus* LCs, however, was suggested to be due to decreased $K^+$ conductance (Sullivan and Miller, 1984). Alternatively, Freschi (1989) showed evidence for PR action in *H. americanus* LCs via activation of a voltage-dependent $Na^+$ current. In light of the significant increases in burst rate and pacemaker slope produced by PR treatment in cultured cells in this study, one suggestion is that PR accelerates the pacemaker depolarization; however, the exact mechanism of this pacemaker potential is unknown in CG neurons. If the pacemaker mechanism in this system involves an interplay of $I_h$ (mixed $Na^+$ and $K^+$ inward current) and $I_T$ (transient $Ca^{2+}$ current), as in other bursting neurons (Pape, 1996), then PR may facilitate ion channel opening to accelerate the pacemaker slope.

### 5.4.3.2 Effects of CCAP

This is the first report of the effects of CCAP on individual CG neurons. Several previous studies testing CCAP on intact or isolated *C. maenas* hearts have shown this peptide to produce chronotropic responses (Stangier, 1991; Wilkens and Mercier, 1993; Chapter Three). The chronotropic nature of CCAP's effects suggests that its site of action is exclusively at the CG. Curiously, other crustaceans such as the lobster *H. americanus* and the crayfish *P. clarkii*, show negligible heart rate responses to this nonapeptide (J.L. Wilkens, personal communication). This species-specific effect of

CCAP is attributed to the different concentration of CCAP in the POs of each species. *C. maenas* crab POs contain greater amounts of CCAP than other crustaceans (Stangier et al., 1987, 1991), which confirms the idea that these animals make use of CCAP and have receptors for it.

The chronotropic effects of CCAP on intact and open hearts are reflected in the increases in LC neuronal burst rate, pacemaker slope and burst amplitude observed in the present study. Data from this study allow speculation as to the CCAP mode of action on LCs. CCAP may enhance the inward calcium current ($I_{Ca}$, Tazaki and Cooke, 1986) responsible for DP generation. This could explain the increased burst rate and amplitude observed at present. Burst duration was not measured, but it is predicted to decrease due to the role of $Ca^{2+}$ in inactivating this $Ca^{2+}$ current, and in increasing $I_{K(Ca)}$, which aids repolarization. CCAP accentuated the pacemaker depolarization. If the pacemaker is generated by a combination of $I_h$ and $I_T$ currents in a manner similar to other bursting neurons (Pape, 1996), then it could be speculated that CCAP enhances these currents. However, mechanisms underlying the pacemaker potential are unknown in CG neurons.

CCAP proved to be unique in that it was the only hormone to significantly increase 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 (>$10^{-8}$ M) to pharmacologically isolated lateral pyloric neurons caused membrane potential oscillations. Although the concentrations differed between the study by Wiemann et al. (1992) and the present study, similar responses were observed in each system.

#### 5.4.3.3 Effects of 5-HT

The amine 5-HT has been tested previously on isolated CGs from the lobsters *H. americanus* (Cooke and Hartline, 1975; Lemos and Berlind, 1981) and *P. japonicus* (Kuramoto and Yamagishi, 1990). The general consensus of these studies is that 5-HT increases burst frequency. Table 5.2 attempts to summarize the 5-HT responses described in the literature in comparison with those observed in the present study. In this study, 5-HT application to LCs in isolated ganglia and in culture significantly increased burst rate and pacemaker slope. Most 5-HT applications produced large increases in burst rate that were maintained for 3-4 minutes. Burst duration and number of spikes per burst were not calculated in this investigation.

Table 5.2 Literature reports of some effects of 5-HT on isolated CG neurons, compared with data obtained from 5-HT responses in the present study.

| Study | burst frequency | burst duration | # spikes/burst | pacemaker slope |
|---|---|---|---|---|
| Cooke & Hartline 1975[1] | increased | decreased[4] | increased[5] | N/A |
| Lemos & Berlind 1981[2] | increased | decreased | decreased | decreased |
| Kuramoto & Yamagishi 1990[1] | increased[3] | N/A | N/A | N/A |
| present study | increased | N/A | N/A | increased |

1 = extracellular study

2 = intracellular and extracellular recordings

3 = burst frequency increased to a greater extent when pacemakers affected

4 = burst length was reported to decrease unless the pacemaker SC9 was affected

5 = # impulses/burst was multiplied by # bursts/second, so this value is actually the number of impulses/second

In the present study, all hormones were applied directly to the LC somata. Previously, Cooke and Hartline (1975) found that the soma is not responsive to 5-HT, but the area between the soma and trigger zone in the axon is the most sensitive to hormone. Perhaps this is the reason for the observed delay in the effects of 5-HT recorded in this study. A 10–30 second delay always preceded the effects of 5-HT in isolated ganglia and cultured cells, and recovery was slower than for the peptides' effects. An alternate explanation for this delay may be that it is the time required for activation of an intracellular second messenger system. Although cAMP is not likely the mediator of 5-HT action in lobster ganglia (Lemos and Berlind, 1981), this does not rule out the possibility than another second messenger may be involved. Delayed onset and recovery of responses were also found in response to 5-HT in intact, open, and isolated *C. maenas* heart preparations (Chapters Three & Four).

#### 5.4.3.4 Peptide F2 effects

This is the first report of F2 effects on isolated CGs or cultured cells. The actions of the family of FMRFamide-related peptides (FaRPs) have been examined in heart preparations from several different crustaceans (lobster, Trimmer et al., 1987; blue crabs, Krajniak, 1991; crayfish, Mercier and Russenes, 1992). In particular, the peptide F2 (SDRNFLRFamide) was first isolated and sequenced from lobster, where it is found in high concentrations in the pericardial organs (POs), and is shown to be cardioexcitatory (*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 preparations. This suggests that F2, like all the other hormones that increased burst rate, may increase $Ca^{2+}$ influx to speed DP initiation or increase $I_{K(Ca)}$ to speed DP hyperpolarization. Either mechanism on its own, or a combination of both possibilities could increase burst rate. Interestingly, F2 was the only neurohormone tested that had desensitizatizing effects on burst rate; this desensitization was observed in some cells in both preparations. Desensitization from F2 treatment has also been demonstrated for heart rate responses in lobsters and ostium muscles (J.L. Wilkens, personal communication).

## 5.5 Summary

One goal of this study was to develop an *in vitro* cell culture system to examine neuronal excitability in a simple CPG. Evidence is shown to indicate that cells survive in culture and maintain excitability. The intrinsic properties of cells in each preparation were described. Cells responded to hormone treatment: spontaneous burst rate increased, and silent preparations were induced to burst. Each hormone produced characteristic effects on the burst pattern of LCs in each preparation. Peptides produced immediate effects, while amine responses were preceded by a delay and were longer-lasting. Data obtained from isolated CG preparations treated with PR and 5-HT matched the observations from other studies. F2 was the only hormone that evoked desensitization. In cultured cells, CCAP increased membrane potential oscillations. There is no homology between the amine 5-HT and any of the peptides. This suggests that the LCs must contain a specific receptor for each of the four different hormones.

Although only a few SC recordings were obtained, SC burst characteristics were different from LCs, and from previously documented SC activity. Use of this novel *in vitro* cell culture system opens new possibilities for studying the intrinsic properties of this CPG that was first introduced to neurophysiology nearly 50 years ago (Welsh and Maynard 1951).

# CHAPTER SIX: GENERAL DISCUSSION AND CONCLUSIONS

## 6.1 *In situ* preparations

### 6.1.1 Stability of hearts

The *in situ* heart preparations were developed in order to maintain endogenous nervous regulation as close as possible to the condition present in the whole animal, while still allowing experimental manipulations. It was common for both intact and open hearts to continue contracting for 6–10 hours. These *Carcinus maenas* hearts were very robust; however, the stability tests showed that ventricular pressure ($P_{vent}$) and heart rate ($f_h$) declined steadily over time in intact hearts. After 10 hours, $P_{vent}$, but not $f_h$, was significantly lower than the starting level. There was no significant difference in the decline in $P_{vent}$ or $f_h$ in hearts receiving glucose-supplemented or glucose-free saline. Hearts were only "stable" for about 3–4 hours, at which time there was a large decline in $P_{vent}$. CNS activity also ceased at this time, thereby implying that loss of CNS regulation contributes to this decrease in $P_{vent}$. While heart performance declined steadily over 10 hours, the time frame of most of the experiments described below was only 3–6 hours. At the end of an experiment, even though $f_h$ and $P_{vent}$ were below starting values, the effectiveness of each hormone did not change from the start of the experiment. The conclusion is that these preparations are stable enough for the purpose of surveying hormone effects on *in situ* hearts.

### 6.1.2 Hormones and the whole animal

This study began with a survey of the effects of five pericardial organ (PO) hormones on *in situ* heart preparations. All hormones produced characteristic effects on heart rate, $P_{vent}$ or tension, and electromyogram (EMG) amplitude, indicating that each hormone has a specific role in the animal. It is suggested that each hormone is released from the POs in response to some perturbation or signal in order to produce responses in the whole animal appropriate for the condition (environmental stress, presence of a predator, digestion, burrowing, mating, etc.). I will attempt to describe how the effects of each hormone are related to the overall behaviour of the whole animal.

#### 6.1.2.1 Proctolin

All hormones increased heart rate, and were shown to affect cardiac ganglion (CG) bursting. In both intact and open hearts, proctolin (PR) had multiple sites of action. In

addition to the CG, PR had an additional, direct effect on the myocardium which served to increase contractility. Myocardial contractility is a major contributor to stroke volume (SV), which can be described as the volume of hemolymph pumped out of the heart for each contraction. The combined increases in $f_h$ and contractility induced by PR could lead to increased cardiac output (CO) in the intact animal since CO, the amount of blood pumped by the heart each minute, is the product of $f_h$ and SV (Berne and Levy, 1992).

The CNS was another possible site of action for PR in the *in situ* hearts. Data suggested that the CNS regulates contractility, and the biphasic heart rate responses observed in some hearts treated with PR suggested that the CNS also has a role in modifying heart rate. It was thought that the lag period and subsequent cardiac arrest observed in several hearts treated with PR were due to circulation of the hormone to the CNS and action of the CNS cardioinhibitory nerves on the heart, respectively.

PR treatment also led to contraction of the cardioarterial valves. In the present study, PR-induced valve contracture was indicated by larger increases in force measured in intact than open hearts. Other investigators have shown that PR induces contraction in the anterior and posterior cardioarterial valves in Japanese lobsters (*Panulirus japonicus*, Kuramoto and Ebara, 1984), and decreases outflow through the sternal artery in Maine lobsters (*Homarus americanus*, Wilkens et al., 1996). The combined actions of PR on CO and valve closure may serve to divert hemolymph, in large volumes, to specialized body regions, and also to prevent backflow of hemolymph into the heart.

#### 6.1.2.2 Other PO neurohormones

The effects of the other hormones tested were attributed solely to action on the CG burst pattern. The amines dopamine (DA), 5-hydroxytryptamine (5-HT) and the peptide crustacean cardioactive peptide (CCAP) all strongly increased heart rate and EMG amplitude. For these hormones, increased heart rate alone could increase hemolymph delivery (CO) but not as effectively as the combination of increased $f_h$ and contractility (SV), as observed with PR. However, there is growing evidence that several hormones can be released at once from the POs and interact to produce different responses than just one hormone acting alone. For instance, in the present study OA did not significantly increase any of the measured variables. Other investigators have found that simultaneous application of OA and PR enhance responses more than application of either hormone alone. In crayfish skeletal muscle, OA alone does not activate $Ca^{2+}$ channels, but when co-applied with PR, it enhances $Ca^{2+}$ channel activity and muscle tension (Bishop et al.,

1991). Similarly, OA and PR applied together enhanced $P_{vent}$ and $f_h$ in lobster hearts more than PR alone (Wilkens et al., 1996). Perhaps OA is released from the POs together with PR in the whole animal to produce synergistic effects on heart rate and contractility. Although no combinations of hormones were tested in this study, it is suggested that the effects of any of the chronotropic hormones (DA, 5-HT, and CCAP) together with PR can lead to greater increases in CO in *C. maenas* hearts than that produced by the release of PR alone.

### 6.1.2.3 Summary of *in situ* findings

The purpose of examining *in situ* hearts was to determine the sites of action of several PO neurohormones on hearts from one species. *In situ* preparations were used in the introductory phase of this research to obtain a general overview of where each hormone acted. One aim in particular was to determine the relationship between CG electrical output and myocardial force in these *in situ* hearts. From data showing that all hormones increased heart rate and EMG amplitude, the conclusion was made that all hormones altered CG burst activity. Besides action on the CG, PR also affected myocardial contractility, cardioarterial valve closure, and CNS regulatory activity of heart rate. CG electrical output and myocardial force increased in parallel in response to application of hormones, with the exception of PR. PR increased force disproportionately to CG output in both intact and open hearts indicated by significant $P_{vent}$/EMG and tension/EMG ratios, respectively. These findings provided evidence for action of this peptide on the myocardium and cardioarterial valves. From these preliminary findings, the next obvious study was to use isolated hearts to reduce the scope of hormone action to only 3 possible sites.

## 6.2 Isolated heart preparations

The purpose of using isolated hearts was to remove the action of the CNS and the pericardial hormones on the heart. In essence, this would narrow the possible hormone sites of action to either the CG, the neuromuscular junctions between CG neurons and the myocardium, or the myocardium itself. The aim of this study was to determine the hormonal site(s) of action in the isolated heart. The hormones PR and 5-HT were chosen based on their contrasting effects on *in situ* hearts. 5-HT, which was mainly chronotropic in intact and open hearts, was expected to act at the CG. PR, however, was expected to alter both myocardial force and CG electrical output. Hormonal action on the

CG was indirectly determined by changes in intracellularly recorded myocyte excitatory junction potentials (EJPs), while action on the myocardium was indicated by disproportionate changes in tension and tension/EJP ratio. Possible mechanisms of hormonal action at the level of the myocyte were also investigated. In particular, intracellular electrodes were used to examine hormone-induced changes in myocyte membrane potential ($V_m$) and membrane input resistance ($R_{input}$). The question of whether hormone-induced changes in these myocyte membrane properties could account for changes in cardiac contractility was asked.

As in the *in situ* hearts, PR significantly increased myocardial force; this increase was greater than the corresponding increase in EJP amplitude. In other words, in isolated hearts PR induced increases in myocyte contractility that surpassed the CG electrical input. Neither PR or 5-HT significantly changed $V_m$ or $R_{input}$ of myocytes, which indicated that changes in these particular membrane properties did not alter cardiac contractility. This was not a surprising finding for 5-HT, which has consistently been found to exert chronotropic actions in intact and open hearts and appears to act solely at the CG neurons. However, this suggests that a mechanism other than altered influx or efflux of ions across the myocyte membrane must be responsible for the inotropic action of PR on myocytes.

The next logical step to determine the mechanism of PR action would be to study isolated, individual myocytes. However, this approach was not taken since preliminary attempts revealed that heart myocytes were very long and profusely branched (data not shown). The "huge" size of myocytes was also documented in *C. magister* crab hearts (Meyerhöfer, 1993). Unfortunately, the task of enzymatically or mechanically isolating myocytes from *C. maenas* hearts proved to be impractical. Additionally, the size and extensive branches of these myocytes would have made patch clamp or voltage clamp studies unfeasible. Instead, the focus was shifted to study the direct actions of hormones on CG neurons.

### 6.3 Isolated cardiac ganglia and cultured neurons

The major goal of this portion of my research was to establish whether neuronal excitability and hormone responses recorded from cultured cells were similar to those in isolated cardiac ganglia and in previously reported literature. To answer these questions, an *in vitro* cell culture system was established to isolate single, viable and healthy CG neurons. First, cardiac ganglia were enzymatically dissociated from whole hearts, and then individual neurons were removed and maintained in culture. This is the first study to

describe a method for culturing CG neurons. In the following section, evidence is provided to indicate that cells were indeed viable, excitable, and responded to hormones in a similar manner either *in situ* or *in vitro*. Hormonal responses of *in situ* neurons were also similar to those previously documented in the literature.

### 6.3.1 Large cell excitability and responses to hormones

Ganglionic large cells (LCs) survived enzyme treatment and culture protocols. Microelectrode penetrations of these cells, however, often proved difficult because of the thick glial sheath surrounding the soma. Neurons were nevertheless found to be viable and membrane potentials of LCs in isolated ganglia and in culture were similar. Burst appearance was similar not only between isolated ganglia and individual neurons, but to that of previously published descriptions of LC activity (Tazaki and Cooke, 1979a,b,c, 1983a,b,c; Sullivan and Miller, 1984; Berlind, 1985, 1989). Although there was variability in the shape and rate of bursts from day to day, the events underlying bursts were similar. A slow pacemaker potential always preceded the driver potential (DP) depolarization. A spike train was superimposed on the driver potential, and an afterhyperpolarizing potential (AHP) followed each burst. In culture, a majority of the LCs showed membrane potential oscillations.

All hormones increased activity in spontaneously bursting neurons, and induced bursting in quiescent cells. Hormone effects were similar in isolated ganglia and cultured cells, however, greater increases in all variables were observed in cultured neurons. Both PR and 5-HT have previously been tested in isolated cardiac ganglia, but this study is the first where the effets of CCAP and F2 were examined. All of the cultured cell findings were novel, and there are no literature comparisons for these data.

PR increased burst rate and pacemaker slope in isolated ganglia and isolated neurons, effects that are consistent with the mild chronotropic action of this hormone in intact, open, and isolated hearts and also consistent with literature (Miller and Sullivan, 1981; Sullivan and Miller, 1984). The immediate effects of this peptide suggest direct action on soma receptors, however, the ion currents involved in mediating these effects are not known.

CCAP significantly increased all aspects of cultured cell bursting and was the only hormone to increase oscillation amplitude. The mechanism underlying membrane potential oscillations in these isolated neurons is unknown. The chronotropic action of CCAP

observed in isolated and *in situ* hearts can be explained at the single ganglionic cell level by these increases in burst rate and pacemaker slope.

Only 5-HT produced delayed effects in both isolated ganglia and cultured cells. It was suggested that this delay is required to activate a second messenger system. Or perhaps, as other authors suggest, it is the small cells (SCs) that are more responsive to 5-HT (Kuramoto and Yamagishi, 1990); therefore, in culture where LCs are isolated from SC synaptic inputs, the effects of this hormone on LCs are attenuated. All phases of this study have pointed to the chronotropic role of this amine, but in several trials only weak chronotropic responses were recorded from LCs in isolated ganglia and in culture. This also may be due to the possibility that SCs are more responsive to 5-HT than LCs. All previous studies of 5-HT effects on isolated ganglia also showed that this amine produces increased burst rate (Cooke and Hartline, 1975; Lemos and Berlind, 1981; Kuramoto and Yamagishi, 1990).

F2 was the only hormone to cause desensitization in isolated ganglia and cells. The physiological relevance of this desensitization is unclear. Like PR and CCAP, the immediate effects of this peptide suggest that receptors are located on the soma itself. Perhaps F2 is released from the POs in emergency situations that require short-lived, immediate, one-time only increases in heart rate, and hence increased mobilization of hemolymph as a result of the enhanced cardiac output.

### 6.3.2 Model of CG neurons

An interesting finding in this study was that when LCs were cultured, those soma plated without axons were not excitable and were physiologically inactive (i.e., dead). These soma had a granular appearance, and did not possess membrane potentials. These observations lead to the suggestion that the soma is required for generating action potentials, as was noted by Tazaki and Cooke (1983a) who showed that an impulse-initiating zone located more distally down the axon is the site of spike formation rather than the soma itself. Data from the present study supported the idea that there is regional specialization between the soma and the axon.

Findings from this study also indicated that CG neurons are endogenous bursters. Previously, it was thought that depolarizing input, whether from synaptic interactions or when injected as current from a microelectrode, was required to initiate the LC burst (Tazaki and Cooke, 1983c; Berlind, 1985, reviewed in Cooke, 1988). However, in culture, where the LCs were isolated from the synaptic inputs that usually provide some of

the depolarizing current for driver potential and burst initiation, LCs displayed spontaneous activity. Isolated SCs *in vitro* also demonstrated spontaneous activity. This is the first evidence for the endogenous bursting ability of these CG neurons.

In the present study, all hormones excited LC bursting, confirming the expectation that chronotropic actions observed in intact, open and isolated hearts could be explained as direct effects on CG burst output. Now that one site of action is confirmed to be at the CG neurons, the mechanism of each hormone's action can be determined.

Possible mechanisms were not tested in this study, but some speculations can be made based on knowledge of the inward and outward currents responsible for each portion of the DP, as outlined by Tazaki and Cooke (1986, 1990). All hormones increased burst rate, which leads to the suggestion that hormones enhance the inward $Ca^{2+}$ current required to initiate the upstroke of the DP, and perhaps also the outward $Ca^{2+}$-dependent $K^{+}$ current to accelerate termination of the DP. A combination of these events would produce the increases in burst rate and more regular bursting pattern that were observed with hormone treatment. All hormones also increased the depolarizing pacemaker slope, but the events underlying this potential have not been described in the literature and I will not attempt to speculate about this phenomenon.

A model of LC ganglionic neurons was presented in Fig. 1.3 in Chapter One. This model depicted the events involved in an LC burst, and showed the typical events that would be recorded from the soma and the axon. For instance, a microelectrode placed in the soma would record a small singlet burst, and at the axonal spike initiation zone, this would correspond to an action potential (AP). A somatic driver potential with superimposed spikes would elicit a barrage of APs in the axon (see Fig. 1.3). Sometimes microelectrode recordings from LCs in isolated ganglion preparations showed small interburst events (see Fig. 5.5). These small events were of an inhibitory nature, as they re-set the pacemaker slope, thereby indicating that they were not excitatory post-synaptic potentials (EPSPs). These small events likely did not provide enough depolarization for the spike initiation zone of axon membrane to reach AP threshold.

### 6.3.3 Small cells

In nearly 50 years of studying CG physiology, only 3 groups of researchers have reported SC data. In the present study, data was obtained from one SC connected in a ganglion, and from three isolated, cultured SCs. The anatomy of SCs *in situ* and *in vitro* and their bursting pattern were contrary to previous findings. All other decapod crabs and

lobsters have nine neurons in the CG, but *C. maenas* appears to be missing one SC. The bursting pattern of SCs did not consist of bursts at all, but rather these cells fired tonic singlet spikes at a high frequency (about 3 Hz). In addition to the absence of bursting recorded from SCs, the pacemaker slope was steeper and the amplitude of each spike was greater than the respective events in the LCs and compared to previously documented SC activity.

### 6.4 Overall summary and future directions

This study examined hormone effects on *C. maenas* hearts beginning with a systems level evaluation of several hormones and proceeding down to the effects of these hormones on single cells. In the whole animal, hormones increased heart rate and myocardial contractility which may serve to direct hemolymph to various body regions. At the isolated heart level, it was discovered that increases in myocardial contractility were not due to hormone effects on myocyte membrane potential or input resistance. Increases in heart rate observed in intact, open, and isolated hearts were attributed to hormone action on CG electrical output, and this was confirmed by using an *in vitro* cell culture system to examine direct hormone effects on individual neurons.

Although I have suggested how the hormone-induced changes in heart rate, force and valve activity contribute to whole animal behaviour, to get a full picture of the changes in hemolymph delivery, each hormone needs to be tested on each of the seven cardioarterial valves. It has been shown that hormones can contract specific cardioarterial valves, thereby altering the path of hemolymph flow to one region of the body or another (Kuramoto and Ebara, 1984; Wilkens et al., 1996). Additionally, it is of interest to test combinations of hormones on heart performance. OA and PR together would be expected to produce synergistic effects, as evidenced in the literature (Bishop et al., 1991; Wilkens et al., 1996). A combination of F2 and PR could produce different effects than each peptide on its own, as indicated by trials in cultured cells and isolated CGs in which application of PR following F2 led to a prolonged PR response. Seasonal variability of PR effects has been documented in crayfish skeletal muscle (Bishop et al., 1991), and perhaps this may be an explanation for the lack of response in some hearts treated with PR in this study. Thus, any further experiments on PR should be performed in the spring and summer months, where PR is the most effective (Bishop et al., 1991). In isolated hearts, neither PR nor 5-HT altered the myocyte membrane properties, $V_m$ and $R_{input}$. It was suggested that the PR-induced increase in myocardial contractility may be modulated by a

second messenger system. To determine the role of second messengers, a simple commercially available bioassay test could be used with isolated hearts.

The cell culture system provides the most exciting research opportunities for studying CG neuronal intrinsic properties. Although I have shown that all hormones tested exert direct action on LCs, the exact nature of these effects is unknown. Possible mechanisms include direct changes in membrane ion currents or activation of intracellular second messenger systems that alter membrane currents indirectly. To fully examine direct hormone effects on ion currents, bath ion substitutions combined with patch clamp, or two-electrode voltage clamp experiments are necessary. Patch clamping of LCs will be difficult, and perhaps unfeasible, unless some method is developed to remove the thick glial sheath surrounding each cell that is otherwise a barrier to formation of patch seals. The events underlying the pacemaker potential have never been studied, but these could be easily investigated using the present cell culture system, appropriate bath solutions and voltage clamp electrodes.

Nothing is known about cultured SC responses to hormones. Although it is a difficult task to locate SCs, let alone record from them, they would be excellent specimens for patch clamping studies since they are devoid of the thick glial/ connective tissue sheath found to cover the LCs. Some evidence from previous studies indicates that hormones differentially affect SC and LC burst rates, although the SCs were not recorded from directly (PR, Sullivan and Miller, 1984; 5-HT, Kuramoto and Yamagishi, 1990). Thus, it would be useful to record directly from each cell type both in isolation and when connected in the ganglion, to understand any differences in hormone responses between these neurons. More importantly, additional studies need to be performed on *C. maenas* intact ganglionic and cultured SCs to verify data from this investigation and to provide a fuller picture of SC activity.

Cellular growth and formation of synaptic connections *in vitro* were not a focus of this study, but these could be investigated in the future now that the cell culture system has been established. It would be interesting to determine whether LCs form synapses in culture, and whether these synapses retain similar function and excitability as in the isolated CG. In addition to pairing LCs in culture, it would be useful to form SC->LC synapses to determine the relative importance of electrotonic versus synaptic connections in the different cell pairings. Whatever studies are undertaken in the future, the *in vitro* cell culture system will be an important tool for understanding the intrinsic and network properties of this simple central pattern generator, the cardiac ganglion.

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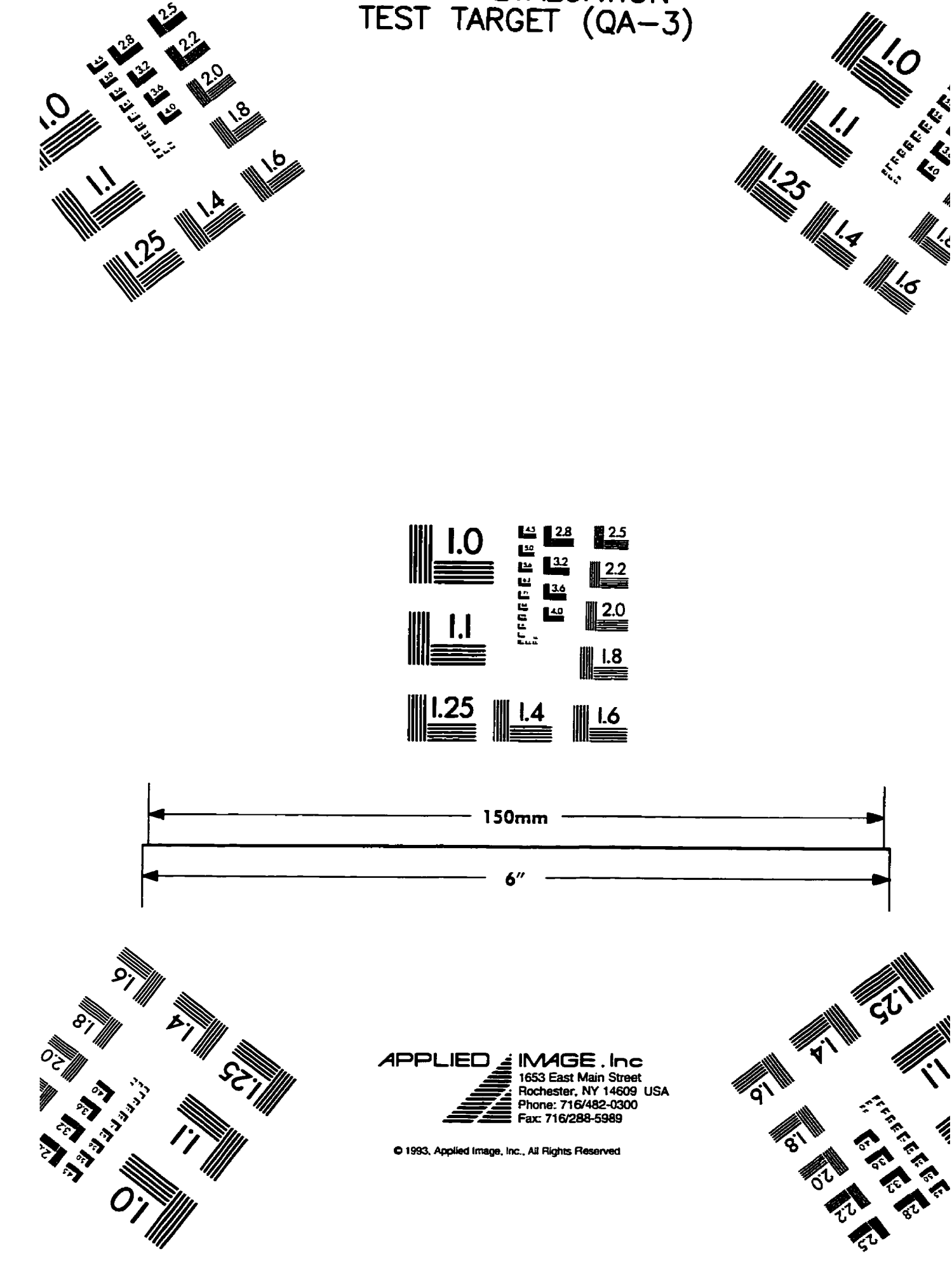


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