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This study describes the anatomy of cardiac innervation and the physiological actions of identifiable motorneurons in the nudibranch mollusc Archidoris montereyensis. Standard techniques of electrophysiological recording and intracellular staining were used to identify excitatory and inhibitory cardiac motorneurons in the right pleural and visceral ganglia of this animal. These neurons innervate the heart via the arterial and ventricular branches of the pericardial nerve emanating from the visceral ganglion. Stimulation of the pericardial nerve or its fine terminal branches in the wall of the heart evoked multiphasic inhibitory and

excitatory junction potentials within cardiac muscle cells.

The pleural ganglion contained an excitatory heart motorneuron (Pl_{HE}) and an inhibitory motorneuron (Pl_{HI}) which were particularly effective in regulating cardiac activity. Low levels of spontaneous activity (<1 Hz) in Pl_{HE} were strongly correlated with ventricular contractions of increased amplitude which were reversibly eliminated when Pl_{HE} was hyperpolarized below its firing threshold. Stimulation of activity in Pl_{HE} above this spontaneous level caused heart contractions of greater frequency, amplitude, and baseline tonus. Spontaneous activity in the inhibitory motorneuron, Pl_{HI}, was strongly correlated with decreased rate of heart contractions, and this inhibition was reversibly eliminated when Pl_{HI} was hyperpolarized. Tonic stimulation of this cell caused sustained inhibition of the heart. The visceral ganglion contained at least one heart excitatory motorneuron, V_{HE}, and two inhibitory motorneurons, $V_{HI1 \text{ and } 2}$, that affected the heart in a similar manner the pleural ganglion cells although not as powerfully.

Lucifer Yellow injections of the excitor and inhibitor motorneurons in the pleural ganglion reveal that the dendritic arbor of Pl_{HE} extends throughout the pleural ganglion while that of Pl_{HI} is more localized in the region of its cell body. The axons of both neurons pass

through the visceral ganglion where they branch before entering the pericardial nerve en route to the heart.

Thus, the heart of Archidoris appears to be regulated by a few, relatively powerful motorneurons located in a restricted region of the central nervous system. Pl_{HE} and Pl_{HI} are especially effective in regulating amplitude and rate of heart contractions, respectively, suggesting that these two cells may comprise the most important pathway for cardiac regulation. It is noteworthy that the actions of excitatory and inhibitory motorneurons in Archidoris are similar to those of sympathetic and parasympathetic nerves to the heart in vertebrates. In both systems inhibitory input to the heart appears to primarily affect the rate of cardiac contraction, while excitatory input is more generalized and affects both the rate and strength of contraction.

Anatomical and Physiological Characterization of Cardiac Innervation in the Nudibranch, *Archidoris montereyensis*

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INTRODUCTION

Behavioral neurobiologists seek to understand how nervous systems regulate animal behavior. Studies of the neuronal basis of behavior are founded on the assumption that any animal's behavior can ultimately be defined in terms of the functions of individual nerve cells within the central nervous system and the properties of interactions between these neurons. Thus, studies in behavioral physiology attempt to understand how single neurons regulate muscular and glandular responses, how circuits of neurons coordinate the activities of whole organs, and ultimately, how the entire nervous system initiates and integrates the activities of several organ systems during a complex change in behavior. Among the numerous taxa of animals used in cellular-level studies of nervous system function, invertebrates have long been recognized as advantageous preparations since the brains of these animals contain relatively few neurons. Molluscan nervous systems are particularly attractive

preparations because a significant proportion of the neurons contained in the brain are "giant" cells that can be individually identified by their morphological and physiological properties. By mapping the locations of these neurons it is possible to study their interactions with other identifiable neurons, and thus to define neural circuits that regulate particular components of a behavior (Kandel, 1976). Studies of behavioral physiology at the cellular level often suffer, however, from over simplification; as the numbers of neurons analyzed is reduced to render a neural circuit tractable for neurophysiological studies, the relevant behavior can become trivial or unquantifiable. The challenge to behavioral physiologists, therefore, is to identify simple circuits of neurons that regulate significant and quantifiable components of behavior.

The neuronal circuitry controlling the cardiovascular organs of gastropod molluscs is likely to provide a good system to study for several reasons. First, optimal functioning of an animal is likely to depend on adequate circulation of blood to active tissues, therefore, circulation should be appropriately regulated during any change in behavioral state. Secondly, hearts generally maintain a simple behavior in isolation, a uniform and rhythmic contractile activity that is easily monitored and

quantified in terms of its frequency and amplitude. Third, gastropod cardiovascular organs and relevant parts of the nervous system controlling them can be easily exposed in semi-intact or isolated preparations of the animal. This allows investigators to analyze an intact and functioning regulatory system. Finally, the number of nerve cells required to regulate the heart is small, in theory requiring only one excitatory and one inhibitory motorneuron. Thus, the neural signals that modulate heart activity during a transformation in behavior are likely to pass through this small population of cells which can be monitored by standard electrophysiological procedures. Given these circumstances it is surprising that the neuronal mechanisms regulating the cardiovascular system in molluscs are not better understood. Neural regulation of the heart has been superficially examined in several molluscs, but details of the circuit have only been well described in one mollusc, the gastropod Aplysia californica. With these considerations in mind I began this study of cardiovascular regulation in the marine gastropod, Archidoris montereyensis, a large and readily available nudibranch off the Oregon Coast. The goal of these studies was to define the properties of motorneurons that regulate the heart of Archidoris, and to compare the

organization and functioning of these cells to their counterparts in other molluscs.

STRUCTURE AND FUNCTION OF MOLLUSCAN CARDIOVASCULAR SYSTEMS

Hearts of gastropod molluscs are usually two chambered (atrial/ventricular) pumps. The heart pumps hemolymph through one or more major arteries to tissues where it percolates into the hemocoel and eventually returns to the heart through the gill and kidney. The pericardial cavity surrounding the heart is partitioned from the hemocoel by a sheet of muscle and connective tissue, the pericardial membrane, which contracts periodically and forces fluid from the pericardial cavity into the kidney. Molluscan hearts can beat continuously or irregularly (Bourne, 1983), and contractions of the atrium and ventricle may occur independently or in a coupled sequence. Contraction of the thin-walled atrium forces hemolymph into the ventricle and proximal arteries, while contraction of the muscular ventricle forcefully propels hemolymph into the entire arterial system (Sanger, 1979; Jones, 1983; Furgal and Brownell, 1987). A pair of semilunar valves between the atrium and ventricle, and a single semilunar valve between the ventricle and aorta, prevent backflow of

hemolymph into the heart during the contraction cycle (Krijgsman and Divaris, 1954).

Molluscan hearts, like those of mammals, are myogenic, although the location of the pacemaker and the mode of neural regulation are not as well described. Several lines of evidence suggest the atrio-ventricular valve is the pacemaker region in gastropod hearts. The highest densities of nerve terminals occur in the region of the atrio-ventricular valve (Jones, 1983), and Kuwasawa (1979) has shown that muscle cells of the atrio-ventricular valve of opisthobranchs contain more diffuse myofibrils and less differentiated sarcomeres. A similar cellular differentiation is found in the pacemaker cells of the mammalian sinoatrial node which receives a high density of nerve terminations (Cormack, 1984). Most importantly, Kuwasawa (1979) has shown that action potentials in the molluscan heart usually appear first in cells of the atrio-ventricular valve and subsequently invade other regions of the heart.

In molluscs, the central nervous system regulates the rate and force of heart contraction through both excitatory and inhibitory inputs. Carlson (1905b) was among the first to study cardio-regulatory innervation in molluscs using gross electrical shocks to stimulate "cardiac" nerves. He found that nerve stimulation had

variable effects on cardiac activity including inhibition in cephalopods, excitation in prosobranchs and opisthobranchs, and a mixture of inhibitory and excitatory effects in pulmonates. More recently Mackay and Gelperin (1972) and Silvey (1968) used higher resolution techniques for extracellular electrode stimulation to show that discrete regions of gastropod and bivalve ganglia contain neurons that either excite or inhibit heart activity. These studies suggest that inhibitory cardiac motorneurons may be concentrated in the visceral ganglion whereas excitatory motorneurons are predominantly located in cerebral and pleural ganglia.

Studies of individual motorneurons regulating cardiac activity are few in number. The most comprehensive investigations have been conducted on the opisthobranchs Aplysia californica (Mayeri et al., 1974; Koester et al. 1974) and A. depilans (S.-Rozsa et al., 1980), and the pulmonates Helix pomatia (S.-Rozsa, 1979a) and Achatina fulica (S.-Rozsa, 1979b). Generally, the hearts of these molluscs appear to be regulated by a small number of fast-acting motorneurons which either increase or decrease the frequency and/or force of heart contractions. The neuronal circuit controlling the heart in Aplysia has been particularly well studied, and the principles of neural regulation of the cardiovascular system in this animal are

usually taken to be representative of other gastropod molluscs.

NEURAL REGULATION OF THE CARDIOVASCULAR SYSTEM IN APLYSIA CALIFORNICA

All seven of the motorneurons that have been shown to regulate cardiovascular activity in Aplysia are located in the abdominal (parietovisceral) ganglion (Mayeri et al.,1974; Koester et al.,1974). Two excitatory and two inhibitory heart motorneurons have been identified. Axons of these cells project to the heart through the pericardial nerve which divides into two branches, one that enters the heart along the arteries and another that passes along the ventral surface of the pericardial membrane and enters the atrium of the heart through the renal-pericardial duct. The auricular branch divides further and terminates within the atrio-ventricular valve and the ventricle.

The two heart excitor motorneurons, cells RB_{HE} and LD_{HE}, affect the heart differently. RB_{HE} is a serotonergic neuron (Liebeswar et al., 1975) and discharges tonically at about 1 Hz. Mayeri and co-workers (1974) believe that RB_{HE} has a tonic excitatory effect on

heart rate since selective and reversible removal of this cell from the heart circuit by injection of hyperpolarizing current causes an 18 percent decrease in heart rate. However, Feinstein et al. (1977) recorded no change in heart rate after removing the abdominal ganglia from Aplysia, and Dieringer et al. (1978) recorded only a 5 percent decrease in heart rate upon cutting the pericardial nerve in Aplysia. The other heart excitor motorneuron, LD_{HE}, appears to affect the heart transiently. It is usually a silent cell in dissected preparations and only briefly excites the heart when stimulated even for prolonged periods. The two heart inhibitor motorneurons, LD_{HI1} and LD_{HI2}, are cholinergic neurons which have similar inhibitory actions on the heart (Mayeri et al., 1974; Liebeswar et al., 1975). They are normally silent neurons in dissected preparations but occasionally discharging in strong bursts of impulses driven by interneurons in the ganglion.

In Aplysia the pattern of impulse activity in these excitatory and inhibitory heart motorneurons is largely determined by two interneurons with antagonistic actions: Interneuron I (L10; Koester et al., 1974), which excites the heart and inhibits the gill, and interneuron II (L25 cells; Byrne, 1983; Byrne and Koester, 1978; Koester et al., 1974), which inhibits the heart and causes strong

contractions of the gill and siphon. These interneurons coordinate activity of the heart and respiratory organs by affecting the firing patterns of heart and gill motorneurons through antagonistic synaptic actions on these motorneurons. For example, L10 excites RB_{HE} and simultaneously inhibits both of the LD_{HI} cells; Interneuron II excites the LD_{HI} cells and inhibits RB_{HE}. Additionally, the interneurons form mutual inhibitory synaptic connections with each other, and each shows different modes of spontaneous firing which result from the intrinsic pacemaker capabilities of the cells. Thus, the activities of these cells result in stereotyped motor responses involving changes in the activity of the heart. L10 activity causes an increase in heart rate and a decrease in vasomotor tone which raises cardiac output, and interneuron II stimulates respiratory pumping during which contractions of the siphon and gill increase circulation of seawater through the mantle cavity and hemolymph through the heart.

The cardiovascular system in Aplysia may also be regulated by neurons that secrete peptide neurotransmitters. The peptidergic bag cell neurons of the abdominal ganglion synthesize and secrete neuropeptides that trigger egg-laying behavior through their action on the central nervous system and peripheral

tissues (Kupfermann and Kandel, 1970). Among their several actions, transmitters from bag cells cause a biphasic response in L10 - inhibition lasting 5-10 minutes followed by prolonged excitation lasting 1-2 hours (Mayeri et al., 1979). Long-term excitation of L10 leads to the facilitation of synaptic potentials in excitatory heart motorneurons innervated by L10, therefore, activity of the heart would presumably be increased via the excitatory effects of L10 on the heart excitor RB_{HE} (Mayeri et al., 1979). Ligman and Brownell (1985) have shown that bag cell peptides may also act directly on the vasculature via hormonal mechanisms. Additionally, Rittenhouse and Price (1986) have found axonal terminations of peptidergic cells from the abdominal ganglion innervating a variety of cardiovascular tissues. The functions of these "white" cells have yet to be determined, but the distribution of their axonal processes suggest that they have a neurohormonal role in regulating cardiovascular functions.

In summary, the cardiovascular system in Aplysia appears to be regulated by a small number of neurons in the abdominal ganglion. The circuitry includes fast-acting excitatory and inhibitory motorneurons coordinated by the actions of interneurons, and peptidergic neurons that act both synaptically and hormonally on the heart or neurons in the heart regulatory circuit.

PREVIOUS STUDIES OF THE NERVOUS SYSTEM IN ARCHIDORIS

Archidoris has been used for a few cellular neurophysiological studies but very little is known about the basic anatomy and physiology of its nervous system. Rose (1971) described the nerves which innervate the buccal mass of A. pseudoargus, and Blackshaw and Dorsett (1976) and Dickinson (1979) identified neurons in the pleural and cerebral ganglia of A. pseudoargus and A. wellingtonesis, respectively, which control gill movements. The only studies regarding neural regulation of the heart in Archidoris were done by Carlson (1905b) who identified a nerve that excited the heart when stimulated by gross extracelluar electrodes. This "cardiac" nerve, which is probably homologous to the pericardial nerve of other molluscs, originates from the right side of the central ganglionic ring and projects posteriorly along the dorsal surface of the hemocoel. The cardiac nerve branches at the base of the cerebral artery, near the junction of the aorta and the ventricle, and again near the renal-pericardial duct. There is no visible branch into the atrium, although stimulation of the whole cardiac nerve accelerates the rate of auricular contraction even when the atrium was severed from the ventricle (Carlson, 1906). Blackshaw (1976) also found

this nerve in A. pseudoargus where it originates from the non-symmetrical visceral ganglion and terminates in the cardiac region. However, she did not conduct physiological studies to observe its effects on cardiac activity.

The study presented in this thesis describes the anatomical and physiological characteristics of cardiac innervation in Archidoris montereyensis. First, I have re-examined cardiovascular anatomy and described the neural pathways affecting the heart in greater detail than previous investigators. Secondly, I have identified several motorneurons in the central nervous system which have excitatory or inhibitory actions of the heart. These studies show that Archidoris is a relatively simple and tractable preparation for further study of cardiovascular neural regulation.

MATERIALS AND METHODS

I. ANIMALS

Archidoris montereyensis, ranging from 3 to 8 centimeters in length and 3 to 40 grams in weight, were collected year-round from two rocky intertidal areas (Strawberry Hill and Yaquina Head) on the Oregon coast. They were kept in a re-circulating, natural seawater aquarium (13°C; 16L/8D photoperiod) and fed their native food, the sponge *Halicondria* sp. Approximately 80 animals, which fed regularly and responded normally to tactile stimulation, were used for these experiments.

II. PHYSIOLOGICAL RECORDINGS

Physiological recordings of cardiac and neuronal activity were obtained from dissected preparations of *Archidoris* in which the cardiovascular organs were exposed. All innervation to the cardiovascular and other organ systems was maintained. In preparation for dissection animals were anesthetized by injecting an isotonic $MgCl_2$ solution (approximately 25% of body weight) into the hemocoel through the foot. The dorsal body wall

anterior to the gill was dissected away to expose the heart and central ganglia, and the anterior portion of the foot and the buccal mass were also removed so that neurons in the ganglia could be visualized with substage illumination. The dissected animal was pinned to a clear Sylgard (Dow Chemical) foundation in a plexiglass recording chamber and perfused with aerated, buffered (5 mM Hepes, pH 7.8) artificial seawater enriched with 0.2% glucose, Eagles Minimum Medium essential (0.2 X) and non-essential (0.5X) amino acid, and vitamin solutions (0.5 X) (Gibco, Grand Is., N.Y.) (ASW recipe modified after Koch et al., 1984). All experiments were conducted at room temperature (20^{o}C) after a thorough (>1 hr.) wash of the dissected preparation with several volumes of perfusion medium.

Contractile activity of the heart was recorded by attaching the ventricle to a tension transducer (Cambridge Technology Model 400) using 7-0 suture thread and a stainless steel hook. In a dissected animal the heart was often inactive until attached to the tension transducer used to record its activity. This slight tension caused the heart to beat for hours at a rate ranging from 30 to 60 beats per minute (bpm). Normal spontaneous activity of the heart ranged between 5 and 35 bpm as observed through a small incision in the body wall of an otherwise intact

animal. Thus, the apparatus used to record heart activity caused an artifactual increase in baseline heart rate.

The cut ends of nerve trunks and fine terminal branches in the heart were stimulated using a glass suction electrode filled with perfusion saline. Glass microelectrodes (10-40 MΩ) filled with 2 M potassium acetate were used to record intracellularly from neurons and cardiac muscle cells. All experimental data was recorded on chart (Gould 2400s) and FM tape (Hewlett Packard 3968A) recorders.

III. HISTOLOGY

The anatomical arrangement of nerves innervating the myocardium was determined using vital Methylene Blue staining (1% for 12 hours at 4°C) of intact tissues (Alexandrowicz, 1933) and Mallory's Triple staining of sectioned tissues (Humason, 1979). Prior to Methylene Blue staining, the heart and anterior aorta were pinned in a Sylgard lined petri dish and cut open longitudinally along the dorsal wall to facilitate entry of the dye into the lumen of the heart.

IV. IDENTIFICATION OF NEURONS INNERVATING THE HEART

Nerves containing axons of cardiac motorneurons were identified by stimulating each nerve trunk exiting the head ganglia while monitoring changes in cardiac activity. Those nerves that affected activity of the heart were backfilled with cobalt chloride ($CoCl_2$) to label the somata of all neurons projecting into the nerve. To backfill nerves with $CoCl_2$, the cut end of the nerve was pulled into a vaseline well which was filled with distilled water. A fresh, diagonal cut was made in the end of the nerve, and after two minutes the distilled water in the well was replaced with a 400 mM solution of $CoCl_2$. The preparation was then incubated at $4^{o}C$ for 3 days. Backfilled ganglia were rinsed in saline and submerged in a 1 - 2% solution of ammonium sulfide to precipitate cobalt and to visualize the labeled neuronal somata in the central nervous system. The tissue was then dehydrated, cleared, and observed with a dissecting microscope to localize positions of filled cells in the ganglia.

Cardiac motorneurons were identified by their effect on the heart when stimulated to fire by intracellular injection of depolarizing current. The morphology of select cardiac motorneurons was determined by iontophoretic injection (hyperpolarizing current pulses of 45-100 nA, 1 sec duration, 0.5 Hz, 60-120 min) of a 3%

solution of Lucifer Yellow in 0.1% $LiCl_2$ (Stewart, 1981). The ganglia were then fixed in 4% paraformaldehyde for 12 hours, dehydrated, and cleared in methyl salicilate. Filled neurons were viewed through an epifluorescence microscope (Zeiss Standard) equipped with excitation and barrier filters appropriate for Lucifer Yellow.

V. NOMENCLATURE

Following the convention of Blackshaw (1976), nerves projecting from the central ganglia are indicated by descriptive letters and numbered consecutively as they exit from the ganglia (clockwise on the right side of the circumesophageal ring and counterclockwise on the left side). To facilitate discussion, however, the visceral nerve, VN1, which contains axons innervating the heart and kidney, is referred to here as the "pericardial nerve." This is consistent with the nomenclature used in Aplysia (Mayeri et al., 1974). The nomenclature used to identify cardiac motorneurons is also consistent with that used in Aplysia (Mayeri et al., 1974). The neurons are identified by letters appropriate to their ganglionic location (normal script) and function (subscript) (i.e., Pl_{HE} = pleural ganglion cell that excites heart).

RESULTS

I. PATTERN OF CARDIAC INNERVATION

The major components of the cardiovascular and nervous systems of Archidoris are shown in Figure 1. Injection of dyes into the ventricle of Archidoris revealed three large arteries that convey blood from the heart: a single anterior aorta that supplies blood to cephalic organs and the body wall, and two lateral arteries that supply blood to digestive and reproductive organs. The kidney is located ventral to the heart and major arteries and receives fluids from the pericardial cavity via the renal-pericardial duct. All of the major ganglia in Archidoris are contained within the circumesophageal nerve ring (Carlson, 1905a,b; Blackshaw, 1976). Inspection of dissected tissues revealed that all of the cardiovascular and pericardial organs appear to be innervated predominately by the pericardial nerve.

The distribution of nerve terminals in the heart and their relationship to the pericardial nerve was examined in stained whole mounts and sectioned tissues. As shown in Figure 1, the arterial and ventricular branches of the pericardial nerve enter the ventricle near the origin of

the arteries and pass superficially along the inner ventral surface of the heart. Many of these branches appear to terminate in the region of the atrio-ventricular valve, but at least two branches cross into the atrium and project along trabecular muscles there. I also observed fine nerve processes in the atrium that do not appear to originate from the ventricular nerves. These processes may originate from the arterial division of the pericardial nerve which passes ventral to the heart and possibly innervates arteries there. Methylene Blue also stained 4 to 8 neuron-like somata embedded in the myocardium of the atrium. Cells of this type are commonly found in molluscan visceral tissues and may be interconnected with local nerve nets.

Individual nerves exiting the central ganglia were severed and stimulated distally with a suction electrode to determine which ones influenced cardiac activity. Stimulation of the pericardial nerve had the greatest effect, characterized by an immediate increase in the beat frequency and tonus of the heart (Fig. 1, 1). Stimulation of pleural nerve RPlN4 also affected tonus of the heart, but the response was delayed by a second or more from the time of stimulation and the amplitude of response was much reduced. Thus, innervation of the heart through RPlN4 appears to be indirect. This conclusion is supported by

the observation that RPlN4 terminates in the body wall near the base of the gill and does not have obvious branches into the atrium or ventricle.

The branches of the pericardial nerve containing heart regulatory axons were identified by independent stimulation of each branch while monitoring changes in activity of the heart. As shown in Figure 1, stimulation of the arterial (location 2) and ventricular (location 3) branches resulted in an immediate and transient increase in heart rate and tonus which were similar to the response evoked by stimulation of the entire nerve trunk. The response lasted approximately 5 seconds and was sometimes followed by a period of reduced activity lasting several seconds. Stimulation of the pericardial nerve posterior to the ventricular branch (location 4) had no excitatory effect on the heart and probably no inhibitory action (note: in this example what appears to be inhibition is merely a continuation of ongoing activity in that the heart was alternating between periods of increased and decreased contractile amplitude). These observations suggest that axons of cardio-acceleratory motorneurons are contained only in the arterial and ventricular branches of the pericardial nerve.

To characterize effects of nerve stimulation on electrical properties of the heart, I monitored intracellular electrical activity in heart muscle cells tactile stimulating the whole pericardial nerve or its terminal processes embedded in the wall of the heart. The average resting membrane potential of cardiac muscle cells was -54 mV (the 10 largest potentials recorded ranged from 50 to 65 mV). As shown in Figure 2, stimulation of the pericardial nerve or its terminal processes evoked complex junction potentials of graded amplitude. Three phases of synaptic action could be identified. At low intensities of stimulation an inhibitory junction potential of graded amplitude was observed; as stimulation intensity increased a biphasic excitatory potential of graded amplitude was recruited into the response. The excitatory potentials were most evident during stimulation of terminal branches where a fast excitatory potential was coupled to a delayed and longer lasting potential. When the amplitudes of fast and slow excitatory potentials were plotted against stimulus intensity (Fig. 2.B_2), there was a clear difference in the threshold of response and the rate at which the two potentials increased with stimulus intensity. This may indicate that the fast and slow potentials are mediated by different neurotransmitters released from the same excitatory nerve terminals, or that

the slow potential is a voltage activated process induced by the fast depolarizing potential preceding it. These results also show that excitatory and inhibitory axons project to the heart through the same nerves and terminal processes and most likely terminate on the same muscle cells in the region of the atrio-ventricular valve.

II. IDENTIFICATION OF CARDIAC MOTORNEURONS

Infusion of $CoCl_2$ into the cut end of the pericardial nerve revealed neuronal somata that project axons into the pericardial nerve from the cerebral, pleural, and visceral ganglia. As shown in Figure 3, at least nine neurons were labeled by cobalt within the central ganglia, although not all of the cells were filled each time (n=11). The filled cells included the two readily identifiable medial giant neurons in the cerebral ganglia (Blackshaw, 1976), seven unidentified cells in the right and left pleural ganglia, and most of the neurons in the visceral ganglia. The connective between the pleural ganglia also contained a large process that backfilled, but the cells of origin for this structure were not visible.

To identify which of these neurons affected cardiac activity, I penetrated neurons in regions of the backfilled cells and stimulated each one individually by

current injection. Two cardiac motorneurons were found on the dorsal surface of the right pleural ganglion in the same location as three of the cobalt labeled cells, and at least three cardiac motorneurons were found in the visceral ganglion. Figure 8 is a summary diagram illustrating the positions of these cardiac motorneurons relative to readily visible "landmark" neurons in the circumesophageal ganglia. The two cerebral giant neurons, LC1 and RC1, and several other small cells with axons projecting into the pericardial nerve had no affect on cardiac activity when stimulated.

A. Cardiac Motorneurons in the Pleural Ganglion

One excitatory heart motorneuron, referred to here as Pl_{HE}, was located in the right pleural ganglion (Fig. 8). As shown in Figure 4, the heart was very sensitive to endogenous and stimulated fluctuations in Pl_{HE} activity (n=28). This neuron was spontaneously active (average spike rate 20-30 spikes per minute) in approximately 50 percent of the experiments in which it was identified, and fluctuations in Pl_{HE} activity were always positively correlated with increases in the amplitude of heart contractions. These large amplitude contractions were associated with strong contractions of the ventricle which usually did not occur when Pl_{HE} was silent. When

spontaneous impulse activity of Pl_{HE} was reversibly eliminated by injection of hyperpolarizing current, the stronger contractions of the ventricle were eliminated for the duration of the hyperpolarization (Fig. 4.A). These results suggest that Pl_{HE} has an unusually powerful influence on ventricular contraction and may be uniquely important in the excitatory neural pathway regulating the heart.

When Pl_{HE} was forced to fire at higher rates than normal by injection of depolarizing current, the frequency, amplitude, and baseline tonus of heart contractions increased (Fig. $4.B_1$). The cardiac response gradually accommodated if high levels of activity (> 5 Hz) were sustained for several seconds, but there was generally a good correlation between peak response and intensity of Pl_{HE} firing. This relationship was most easily quantified by comparing maximum baseline tonus of heart contractions to the spike rate of Pl_{HE} (Fig. $4.B_2$). Visual observation of the heart revealed that most of this tonic tension increase is attributable to sustained contraction of the ventricle.

The potency of Pl_{HE} control over ventricular contraction is also indicated by its effect on activity of hearts that were very near the threshold for strong ventricular contraction. As shown in Figure 4.C, single

action potentials from Pl_{HE} caused a sustained period (15-30 sec) of increased ventricular contraction in these hearts. This observation suggests that in addition to its immediate actions on the ventricle, transmitters from Pl_{HE} may also have slow and more sustained excitatory effects on the heart.

The pleural ganglion also contained a neuron that strongly inhibited the heart. This inhibitory motorneuron, hereafter referred to as Pl_{HI}, was located near Pl_{HE}. Like Pl_{HE}, fluctuations in activity of Pl_{HI} were closely correlated with spontaneous changes in activity of the heart, but in a reciprocal manner (n=7). In the example shown in Figure 5.A_1, each period of increased spike activity in Pl_{HI} was clearly associated with a period of reduced frequency and amplitude of cardiac contraction and reduced baseline tonus of the heart muscle.

The correlation between increases in Pl_{HI} spike activity and reduction in heartbeat frequency was best observed when spontaneous activity of the neuron was relatively high (> 2 Hz). At these levels of activity Pl_{HI} appeared to be the primary regulator of heart rate. This was supported by the observation that reversible hyperpolarization of Pl_{HI} increased the average heartbeat frequency and completely eliminated spontaneous bouts of

heart inhibition (Fig. 5.A_1). Moreover, the action of Pl_{HI} on the heart did not accommodate during prolonged periods of Pl_{HI} stimulation (Fig. 5.B). Such strong and sustained effects on the heart would be expected for neurons involved in chronic regulation of cardiac activity.

While Pl_{HI} seemed to have an especially important role in regulation of the heart it was not the only central neuron capable of inhibiting the heart. In Figure 5.C, for example, spontaneous excitatory synaptic input to Pl_{HI} evoked action potentials in this neuron which were synchronous with inhibition of the heart. Occasionally these synaptic inputs failed to generate action potentials in Pl_{HI} but inhibition of the heart was still evident. The simplest explanation of this observation is that sensory neurons or interneurons that excite Pl_{HI} also excite other heart inhibitory motorneurons. The identity and location of these cells was not determined but it is possible that they were heart inhibitor motorneurons located in the visceral ganglion (see below).

Pl_{HI} also contributes to inhibition of cardiac activity induced by mechanical stimulation of the mantle (dorsal surface) of <u>Archidoris</u>. Both Pl_{HI} and Pl_{HE} appear to receive direct excitatory synaptic input from mechano-receptors in the mantle, but the inhibitory neuron

dominated this reflex and inhibited the heart when the mantle was touched. Tactile stimulation of the heart stimulated mechano-receptor input to Pl_{HE} and excited the heart.

B. Cardiac Motorneurons in the Visceral Ganglion

$CoCl_2$ backfills of the pericardial nerve labeled most, possibly all, of the cells in the visceral ganglion. Therefore, I randomly surveyed all of the larger (>50 μm) visceral ganglion neurons to determine which ones directly influenced heart activity. As in the previous study of the pleural ganglion, I found cells that both excited and inhibited the heart. In 20 experiments where I could clearly visualize most of the larger cells in the ganglion, I found only one cell, V_{HE}, that excited the heart when depolarized (n=5). As shown in Figure 6.A, V_{HE} was normally inactive. High frequency discharge (3 Hz) of the cell increased the frequency and amplitude of heart contractions (Fig. $6.A_1$) but lower levels of firing (< 1 Hz) only mildly affected the spontaneous pattern of heart activity. In Figure $6.A_2$, for example, V_{HE} activity facilitated the amplitude of heart (ventricular) contraction but had little effect on beat frequency. Thus, V_{HE} appears to influence the heart in the same manner as Pl_{HE} but not as strongly.

Occasionally I found that strong bursts of spike activity caused by the initial penetration of cells in the visceral ganglion triggered long-term changes in the pattern of heart activity. One example of these prolonged excitatory actions is shown in Figure 6.B, where penetration of an unidentified neuron in the visceral ganglion initiated strong ventricular contractions which gradually increased in frequency. The absence of a close correlation between neuronal and cardiac activity suggests that this cell or others affected by it may excite the heart through slow-acting transmitters.

At least two heart inhibitor motorneurons, $V_{HI1 \text{ and } 2'}$ were found in the visceral ganglion (n=9). Activity in either of these cells appears to affect the heart in the same way as Pl_{HI} but not as strongly (Fig. 6.C). The V_{HI} cells were generally inactive, but strong excitation of either V_{HI} cell by direct depolarization greatly reduced heart contractions for the period of stimulation but did not completely inactivate beating. Additionally, the frequency of spiking required to inhibit heart contractions was much greater for V_{HI} than for Pl_{HI}. Thus, the V_{HI} cells appear to be less potent inhibitors of the heart than Pl_{HI}.

C. Morphology of Cardiac Motorneurons

The distribution of axonal and neuritic branches from the pleural heart excitor and inhibitor motorneurons were visualized by iontophoretic injection of Lucifer Yellow into their somata. As shown in Figure 7.A, the soma of a dye-injected Pl_{HE} is approximately 170 micrometers in diameter (n=2). The axon from this cell projects through the visceral ganglion where it bifurcates into two branches, one that enters the pericardial nerve and another that enters VN3. The pericardial branch of Pl_{HE} branches a second time into the arterial division of that nerve. The dendrites of this cell arborize extensively throughout the pleural ganglion. The dye-injected soma of Pl_{HI}, shown in Figure 7.B, is approximately 100 micrometers in diameter (n=2). The pattern of Pl_{HI} axonal branching is very similar to that of Pl_{HE}, with branches into the pericardial nerve and its arterial division. The dendritic arbor of this cell is much smaller than that of Pl_{HE} and localized nearer to its cell body. Additionally the axons of both Pl_{HE} and Pl_{HI} sprout fine processes in the visceral ganglion, raising the possibility of synaptic interaction between pleural and visceral pools of cardiac motorneurons.

III. OTHER VISCEROMOTOR NEURONS

Serial paraffin sections of the visceral ganglion show that it contains at least 25 cells most of which appear to project to their target organs through the three major visceral nerves. Intracellular stimulation of these cells showed that, in addition to the heart motorneurons described above, there are at least three visceral neurons which innervate the kidney sacs. One of these can be identified consistently because it is generally the largest cell in the visceral ganglion. Another neuron causes weak contraction of the gill when stimulated. Like the cardiac motorneurons, these neurons probably innervate their target organs via the pericardial nerve since both stimulation of the nerve and intracellular stimulation of the neurons causes identical effects on the kidney and gill. Therefore, it appears that the visceral ganglion of Archidoris is similar to that of other molluscs in that it contains motorneurons innervating several tissues other than the heart.

DISCUSSION

The heart of Archidoris montereyensis is regulated by a small number of powerful motorneurons. Two excitatory and three inhibitory motorneurons were identified in two ganglia - the right pleural ganglion and the visceral ganglion. All five motorneurons appear to innervate the heart via the pericardial nerve, which terminates diffusely throughout the heart but mainly in the region of the atrio-ventricular valve. Neurons regulating the kidney and gill were also found in the visceral ganglion, suggesting that this area of the central nervous system controls several visceromotor functions. The locations of these motorneurons and the organs they innervate are shown schematically in Figure 9.

The excitatory and inhibitory motorneurons contained in the pleural ganglion were particularly powerful cells. When these motorneurons were spontaneously active they accounted for much of the observed fluctuation in cardiac behavior. Likewise, when they were reversibly inactivated by hyperpolarization, cardiac activity was strongly altered. It is unusual to find single neurons with such strong effects on the heart. In Aplysia, for example, the excitatory heart motorneurons, RB_{HE} and LD_{HE}, must discharge at relatively high rates to excite the heart

(Mayeri et al., 1974). Additionally, strong stimulation of the command interneuron L10 often produces only mild cardio-acceleration (Koester et al., 1974). The extent of the dendritic processes of the pleural motorneurons in Archidoris is consistent with their importance in regulation of the heart. The dendritic arbor of Pl_{HE} is particularly extensive indicating that this cell receives input from a number of neurons in the central nervous system and may be involved in many of the animal's behavioral responses.

Excitatory and inhibitory cardiac motorneurons in the visceral ganglion of Archidoris affected the heart much like the pleural motorneurons, however, the actions of the visceral motorneurons were not as strong or long-lasting. The excitor, V_{HE}, acts in a similar manner to Pl_{HE} in that low levels of activity in the cell predominantly affected the amplitude of heart contractions, whereas higher levels of activity affected the rate, tonus, and amplitude of contraction. The two inhibitor motorneurons, $V_{HI1 \text{ and } 2}$, largely affected the rate of contraction, as does Pl_{HI}, although the spike rate required to elicit complete inhibition of the heart is much greater for the V_{HI} neurons. The presence of cardiac motorneurons in both the pleural and visceral ganglia of Archidoris is noteworthy because motorneurons in other gastropods have only been

identified in the parietal and visceral ganglia (Mayeri et al., 1974; S. Rosza, 1979a,b; S. Rosza et al., 1980) It is possible, therefore, that more powerful cardio-regulatory neurons may be found in the pleural ganglia of these other molluscs.

The modes of action of the cardiac motorneurons in Archidoris are similar to those observed in Aplysia. In Aplysia, strong depolarization of the inhibitory LD_{HI} motorneurons decreases heart rate as does stimulation of the inhibitory motorneurons in Archidoris. However, the action of the LD_{HI} cells are more similar to the V_{HI} neurons in Archidoris in that the effects of these neurons on the heart are transient and often do not last the entire period of stimulation. The excitatory cardiac motorneurons in Aplysia, like those in Archidoris, increase heartbeat frequency when stimulated to fire at a high rate. However, low levels of activity in the excitatory motorneurons of the two animals appear to have different effects. In Aplysia, low levels of spontaneous activity in RB_{HE} may have a slight tonic effect on heart rate (Mayeri et al., 1974). In Archidoris spontaneous activity in Pl_{HE} did not noticeably affect heart rate, but it did have a large effect on the amplitude of ventricular contraction. It is possible that RB_{HE} also has an effect on the amplitude of contraction, however, this parameter

has not yet been measured in Aplysia. It would be of interest to determine if the cardiac excitatory motorneurons of other molluscs also have a dominant affect on the amplitude of heart contractions.

Neural regulation of the heart in Archidoris has noticeable similarities to the pattern observed in vertebrates. The parasympathetic nervous system in vertebrates has a tonic inhibitory effect on the heart via the vagus nerve (Ganong, 1983). The vagus terminates diffusely throughout the heart but mainly on the sinoatrial (S-A) and atrioventricular (A-V) nodes. Vagal stimulation has two major effects of the heart; it decreases the rate of S-A node discharge and decreases the excitability of the A-V junctional fibers (Guyton,1981). Thus, normal levels of vagal activity decrease heart rate, and strong stimulation can inhibit ventricular contraction. Nerves from cervical and thoracic sympathetic ganglia innervate the S-A and A-V nodes, however, unlike parasympathetic innervation, sympathetic fibers also innervate the ventricular myocardium. Thus, stimulation of the sympathetic nerves increases the rate as well as the force of ventricular contraction (Guyton, 1981). In this way, parasympathetic stimulation of the heart in vertebrates predominantly influences heart rate and sympathetic stimulation increases the rate and force

of heart contraction. Neural control of cardiac activity in Archidoris appears to be similar because physiologial levels of impulse discharge in the inhibitory and excitatory cardiac motorneurons predominantly affect heart rate and amplitude respectively. The pattern of cardiac innervation in Archidoris is also similar to that of vertebrates in that terminations of the pericardial nerve are concentrated in the ventricular myocardium and particularly in the region of the atrio-ventricular valve, which is thought to be the pacemaker region in gastropod molluscs.

In summary, Archidoris will be an advantageous preparation for use in future studies of neural regulation of the cardiovascular system. The motor component of the cardio-regulatory circuit appears to be the simplest yet described, consisting of a small number of very powerful motorneurons localized in a discrete region of the central nervous system. Future studies will address several questions that this work has raised. First, what transmitters are employed by these cardiac motorneurons, particularly the two powerful pleural motorneurons? The biphasic excitatory potentials recorded in cardiac muscle cells upon stimulation of the pericardial nerve suggest that both slow and fast excitatory transmitters may be employed by the excitatory motorneurons. Additionally,

the tonic actions of the heart motorneurons provide evidence that slow-acting, possibly peptidergic, transmitters are acting on the heart. Secondly, how are the actions of the heart inhibitors and excitors coordinated, and do mutually inhibitory synaptic interactions exist between these neurons? Based on the evidence that the heart motorneurons in Aplysia are coordinated by interneurons (Koester et al., 1974) one might expect the excitatory and inhibitory motorneurons in Archidoris to be independently acting neurons under the influence of command interneurons. However, observations that the axons of Pl_{HE} and Pl_{HI} pass through the visceral ganglion suggest that these neurons may not be acting entirely independently but instead may make synaptic connections with the visceral motorneurons. Thirdly, what are the sensory inputs into the system, and how are they integrated to determine the behavior of the heart? For example, preliminary observations suggest that Pl_{HE} receives synaptic input from mechano-receptors in the heart. It would be of interest to determine how this input helps to maintain proper cardiac functioning. Finally, the ultimate question raised by these studies is how are the key motorneurons, Pl_{HE} and Pl_{HI}, affected during more complex behaviors such as feeding and reproduction?

FIGURE 1. Diagram of the cardiovascular system, circumesophageal ganglia, and cardiac innervation in Archidoris (dorsal view). Chart recordings at right (1-4) show responses of the heart to brief (50 msec pulse at arrow) suction electrode stimulation of the cut pericardial nerve (PN) at points 1-4 in the diagram. The cardio-excitatory response elicited by stimulation at points 1-3 indicate that the major proportion of axons innervating the heart pass through the arterial (2) and ventricular (3) branches of this nerve but not through branches innervating the posterior viscera (4). The heart walls are pulled away from a dorsal longitudinal cut to reveal innervation patterns along the lumenal surface of the ventricle (Vt) and atrium (At). The circumesophageal ganglia are not drawn to scale. AA, anterior artery; Ao, aortic valve; Av, atrio-ventricular valve; C, cerebral ganglion; CA, cerebral artery; HG, hind gut; K, kidney; Pd, pedal ganglion; Pl, pleural ganglion; R, rhinophore ganglion; RP, renal-pericardial duct; St, statocyst; V, visceral ganglion.

FIGURE 1

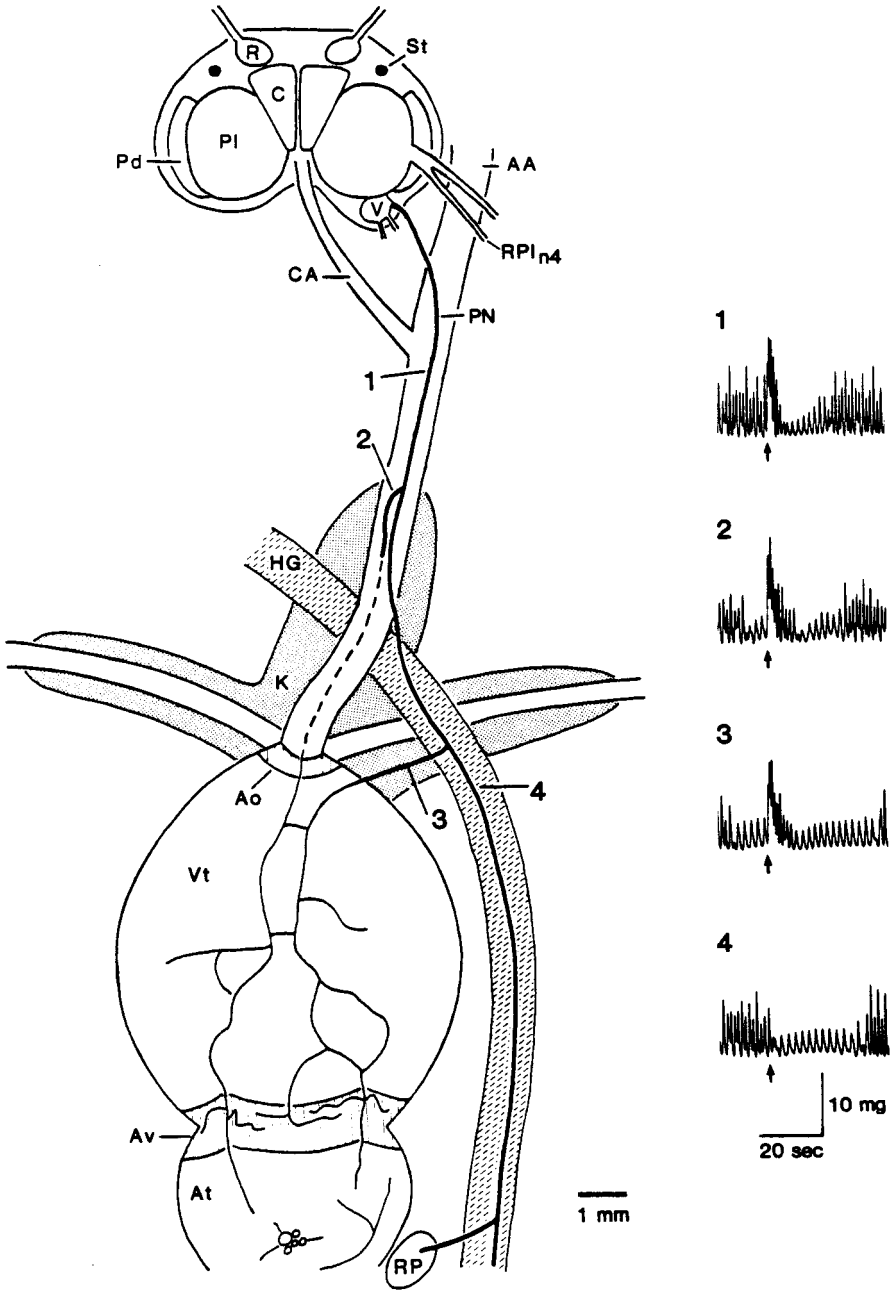

R
St
C
Pl
Pd
AA
V
RPl n4
CA
PN
1
2
HG
K
Ao
3
4
Vt
Av
At
RP
1 mm
1
2
3
4
10 mg
20 sec

FIGURE 2. Synaptic potentials in cardiac muscle cells. Stimulation of the pericardial nerve (A) and its terminal branches (B1) evoked multiphasic junction potentials in muscle cells near the atrio-ventricular valve, indicating that the heart receives direct excitatory and inhibitory inputs from the central nervous system. A. Stimulation (arrows) of the pericardial nerve (at level 1 in Fig.1) evoked a unitary inhibitory potential at the threshold of stimulation. Fast (▼) and slow (▽) excitatory potentials of graded amplitude were recruited into the response as stimulus intensity was gradually increased. B1. Stimulation (arrow) of terminal branches of the pericardial nerve in the wall of the ventricle evoked similar inhibitory and excitatory potentials in muscle cells. Excitatory potentials were biphasic with fast (peak;▼) and slow (shoulder;▽) components. B2. A plot of synaptic potential amplitude as a function of stimulus intensity (arbitrary units) for the inhibitory junction potential and the fast and slow phases of the excitatory potential recorded in B1. Each potential type can be distinguished by stimulus threshold and the rate at which they increase with stimulus intensity.

FIGURE 2

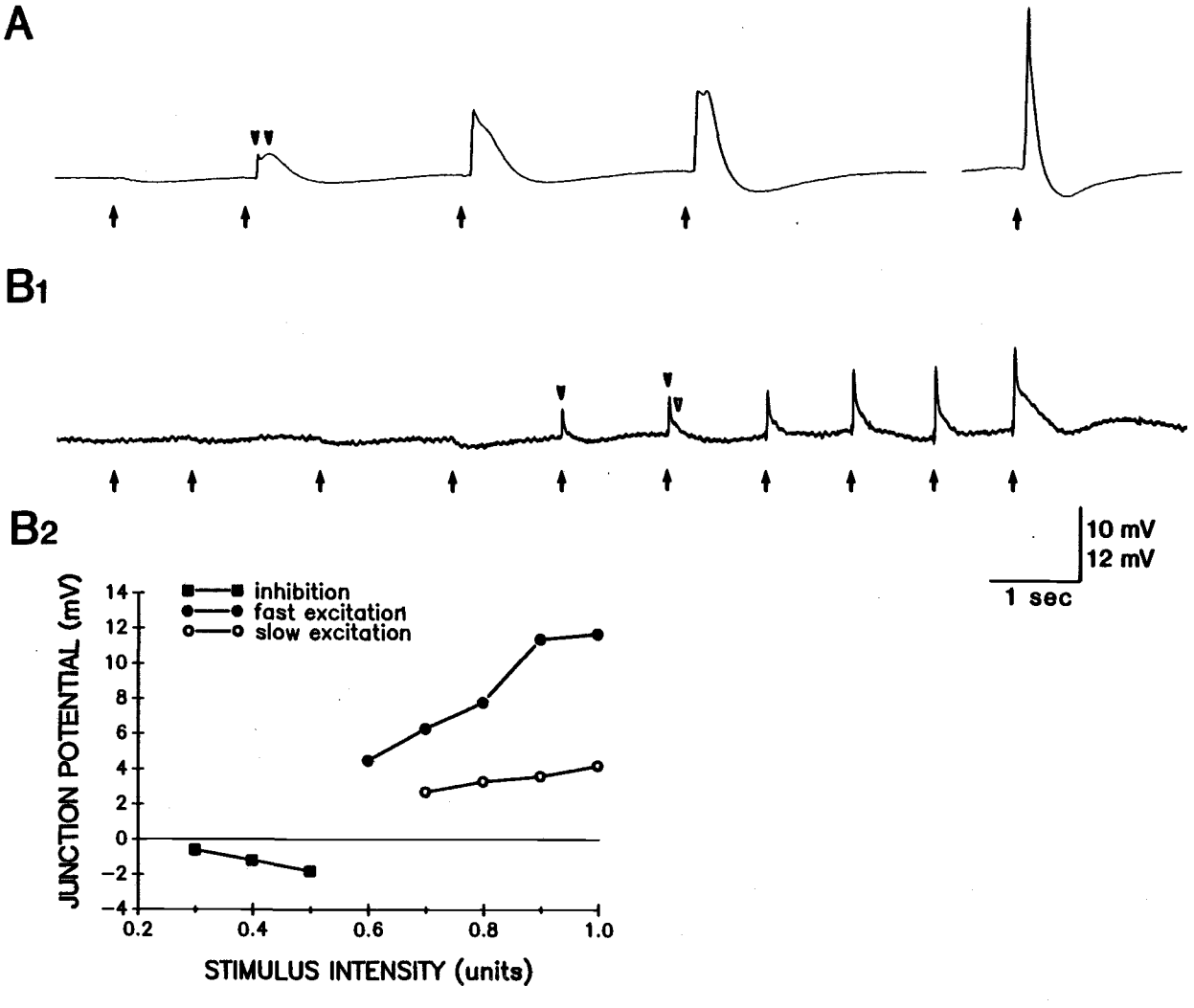

A
B1
B2
10 mV
12 mV
1 sec
inhibition
fast excitation
slow excitation
JUNCTION POTENTIAL (mV)
14
12
10
8
6
4
2
0
−2
−4
0.2
0.4
0.6
0.8
1.0
STIMULUS INTENSITY (units)

FIGURE 3. Schematic diagram of the circumesophageal ganglia (dorsal view) showing positions of cell bodies that backfilled with $CoCl_2$ (stippled cells) through the pericardial nerve (PN). Most of the neurons in the visceral ganglion (V) were backfilled preventing the identification of individual filled cells. Seven cells in the pleural ganglia (Pl) and two cells in the cerebral ganglia (C) were also labeled. Stippling in the circumesophageal connective (CE) represents a large process that was labeled by this procedure. Pd, pedal ganglion; St, statocyst.

FIGURE 3

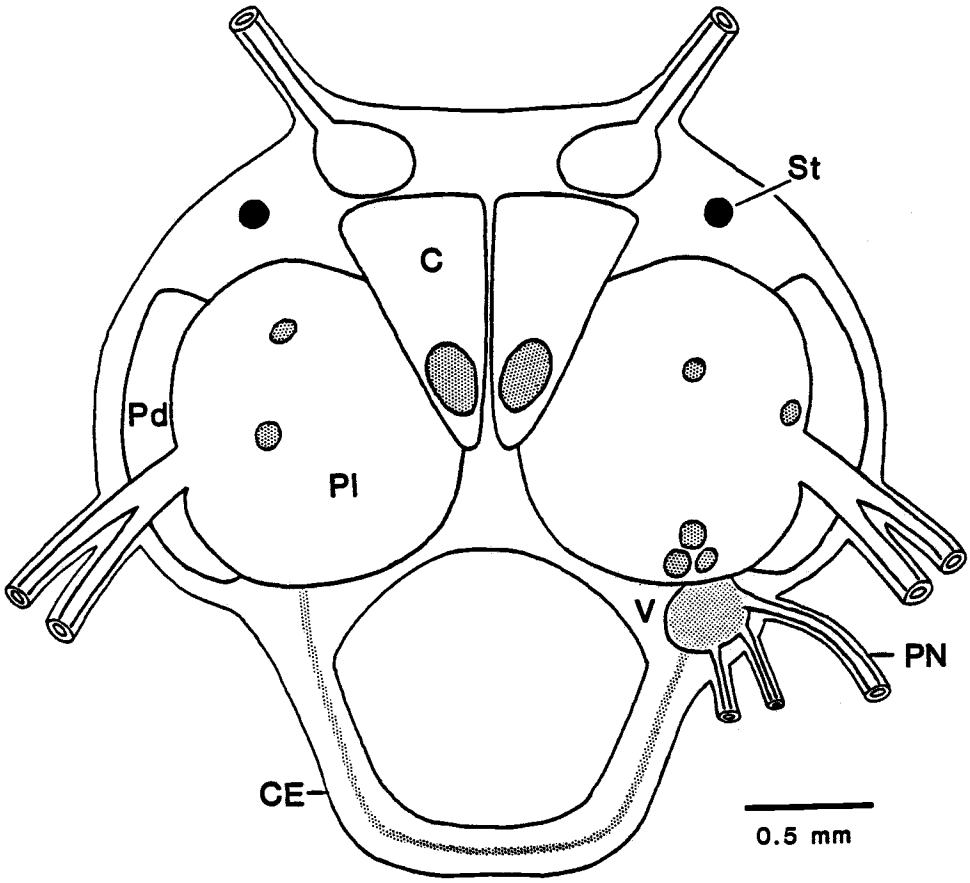


FIGURE 4. Cardio-excitatory actions of Pl_{HE}. A. Fluctuations in spontaneous activity of motorneuron Pl_{HE} (lower trace, average spike rate < 1 Hz) were positively correlated with changes in amplitude of ventricular contractions (upper trace). When spontaneous activity of Pl_{HE} was reversibly eliminated by injection of hyperpolarizing current into the cell (horizontal line) these stronger ventricular contractions ceased. B1. Depolarization of Pl_{HE} (bars beneath trace) increased spike activity of the cell (frequency indicated beneath bars) and stimulated contractile activity of the heart. B2. Stimulated spike activity in Pl_{HE} was positively correlated (r=.93) with the average increase in tension of the heart muscle. Symbols represent results obtained in four experiments. C. Single action potentials (arrow) from Pl_{HE} stimulated long-term increases in strength of ventricular contraction in preparations where the heart was near threshold for large amplitude ventricular contractions.

FIGURE 4

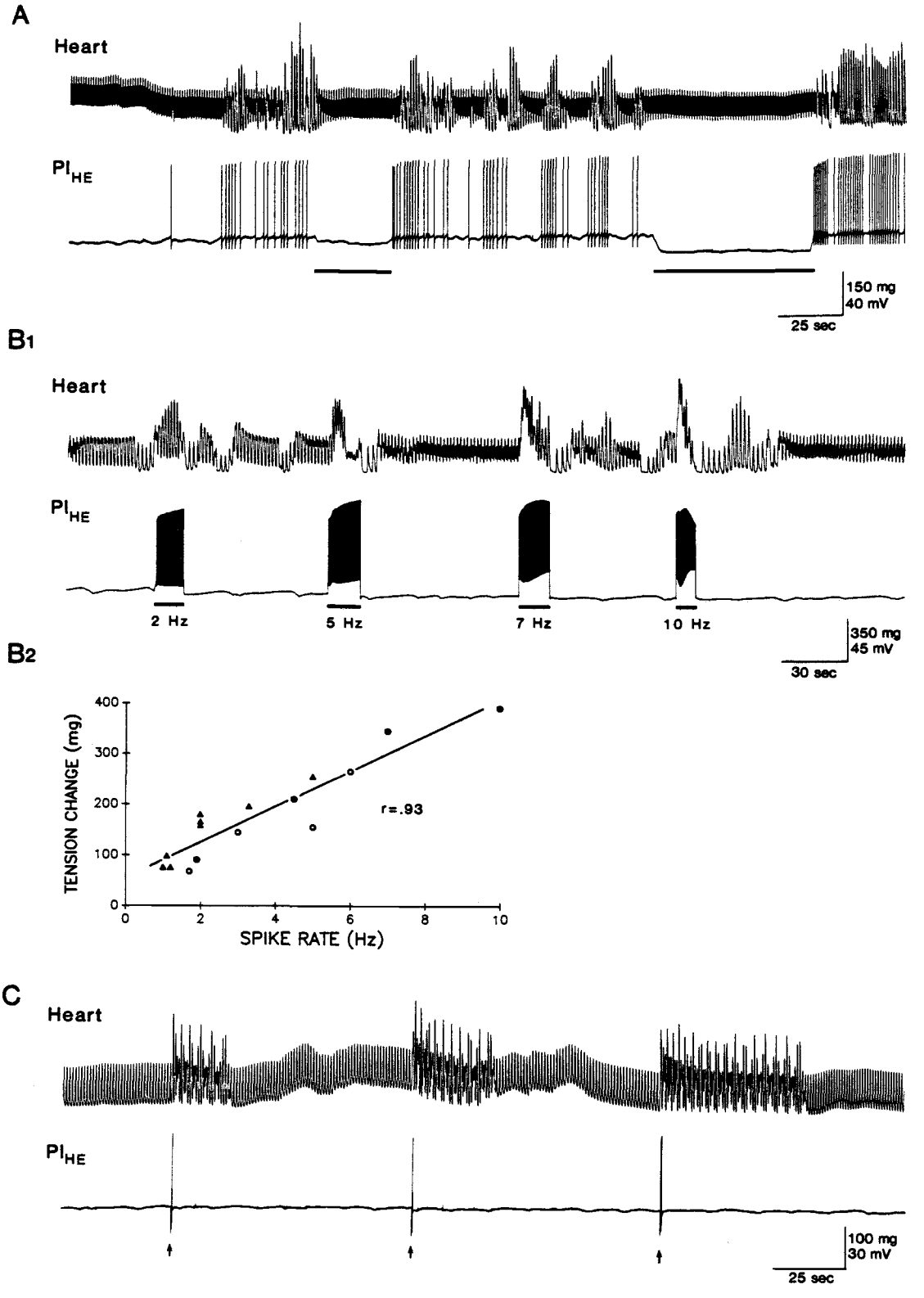

A
Heart
PI HE
150 mg
40 mV
25 sec
B1
Heart
PI HE
2 Hz
5 Hz
7 Hz
10 Hz
350 mg
45 mV
30 sec
B2
TENSION CHANGE (mg)
400
300
200
100
0
r=.93
0
2
4
6
8
10
SPIKE RATE (Hz)
C
Heart
PI HE
100 mg
30 mV
25 sec

FIGURE 5. Cardio-inhibitory actions of Pl_{HI}. A1. Spontaneous fluctuations in spike activity of the motorneuron Pl_{HI} were coincident with decreased activity of the heart. Hyperpolarization of Pl_{HI} by direct current injection (horizontal line) raised the average frequency of heartbeat and eliminated spontaneous periods of cardiac inhibition. A2. Heartbeat frequency showed a strong negative correlation with the spontaneous spike activity in Pl_{HI} (A1) ($r= -.96$). B. When Pl_{HI} was stimulated to fire at rates above 1 Hz activity of the heart was completely inhibited (average Pl_{HI} spike frequencies are indicated below each stimulus). C. Spontaneous bursts of excitatory synaptic potentials in Pl_{HI} were correlated with inhibition of the heart even though the excitatory synaptic activity did not always cause Pl_{HI} to fire.

FIGURE 5

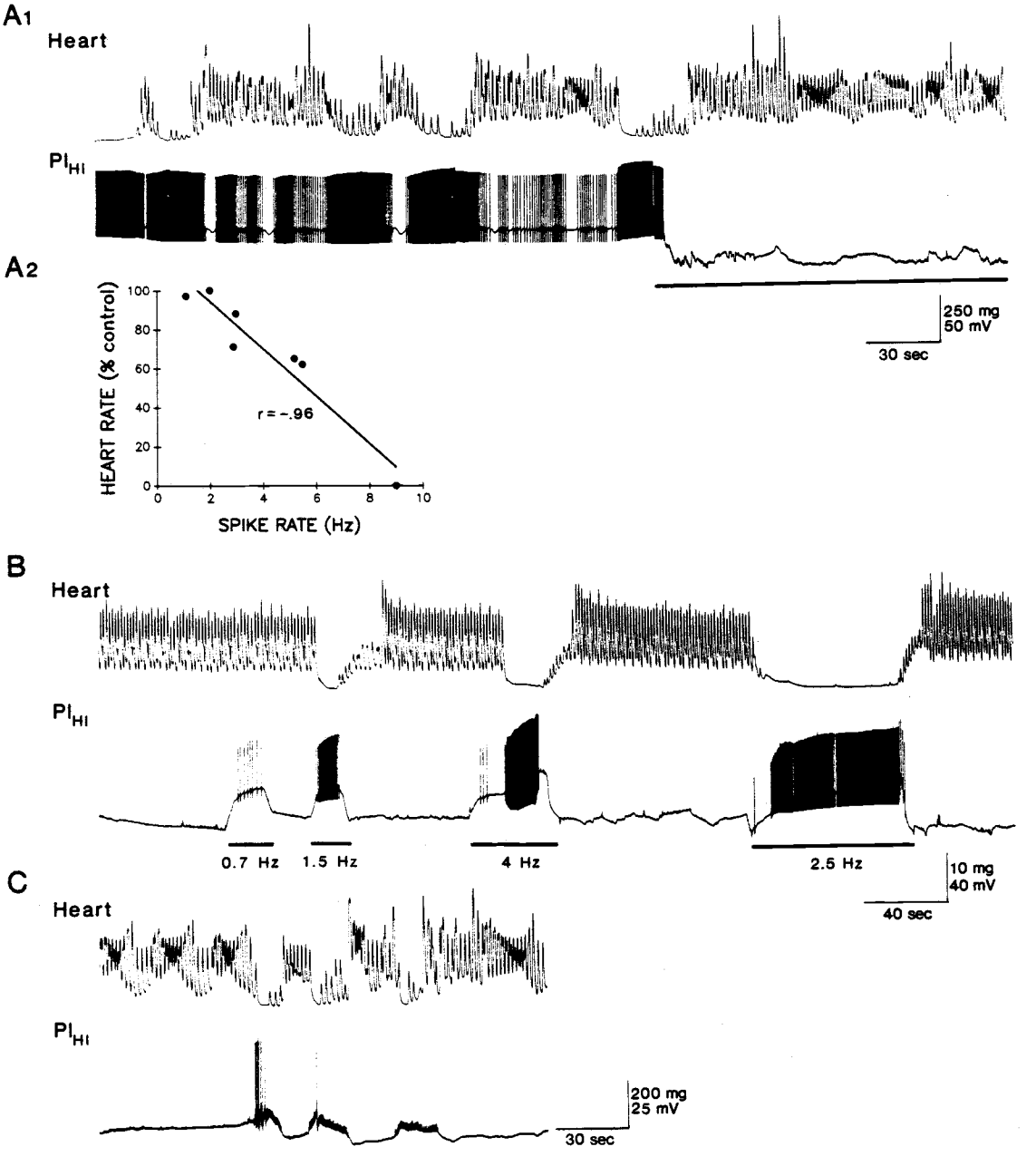

A1
Heart
PIHI
A2
HEART RATE (% control)
SPIKE RATE (Hz)
r = -.96
250 mg
50 mV
30 sec
B
Heart
PIHI
0.7 Hz
1.5 Hz
4 Hz
2.5 Hz
10 mg
40 mV
40 sec
C
Heart
PIHI
200 mg
25 mV
30 sec

FIGURE 6. Cardio-excitatory and inhibitory actions of visceral ganglion heart motorneurons. A. High rates of stimulated spike activity in V_{HE} increased the frequency and amplitude of cardiac contractions (A1); Mild stimulation of V_{HE} prolonged spontaneous bouts of increased amplitude cardiac contractions (A2). B. Sudden onset of a spike discharge in an unidentified visceral ganglion neuron (VN), caused by electrode penetration (arrow), triggers long-term changes in cardiac activity. C. Stimulated spike activity in a V_{HI} cell reduces the frequency of heart contractions and the amplitude of ventricular contraction.

FIGURE 6

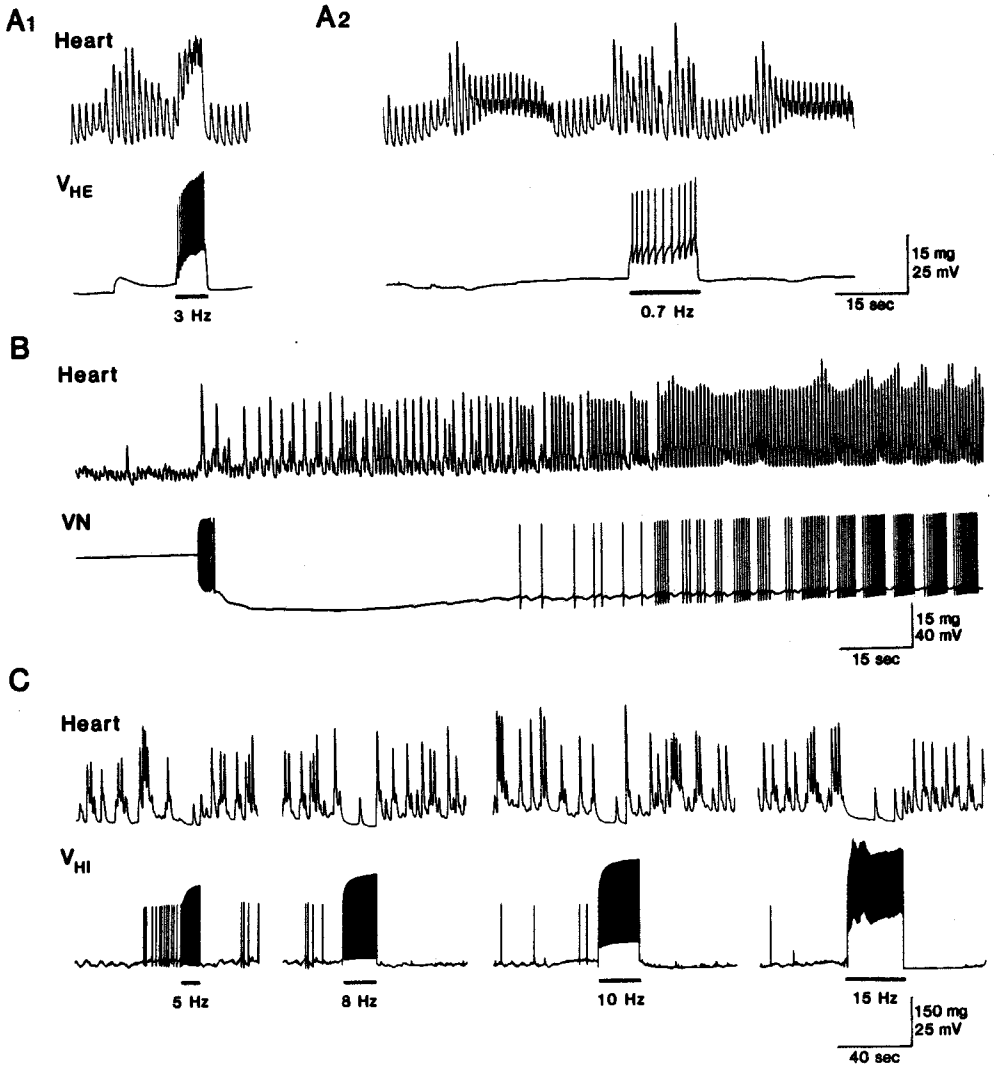

A1
Heart
VHE
3 Hz
A2
0.7 Hz
15 mg
25 mV
15 sec
B
Heart
VN
15 mg
40 mV
15 sec
C
Heart
VHI
5 Hz
8 Hz
10 Hz
15 Hz
150 mg
25 mV
40 sec

FIGURE 7. Fluorescence micrograph showing dendritic morphology and axonal projections of Pl_{HE} and Pl_{HI} injected with Lucifer Yellow. A. The axon of Pl_{HE} enters the visceral ganglion and divides into two branches, one which projects into the pericardial nerve (PN) and another which projects into the third visceral nerve. The dendritic arbor of this cell extends throughout the pleural ganglion with some dendrites extending into the cerebral ganglia (not shown). B. The axon of Pl_{HI} projects into the pericardial nerve and into another visceral ganglion nerve. The dendritic arbor of this cell is localized near its soma within the pleural ganglion. Scale bar is 50 micrometers.

FIGURE 7

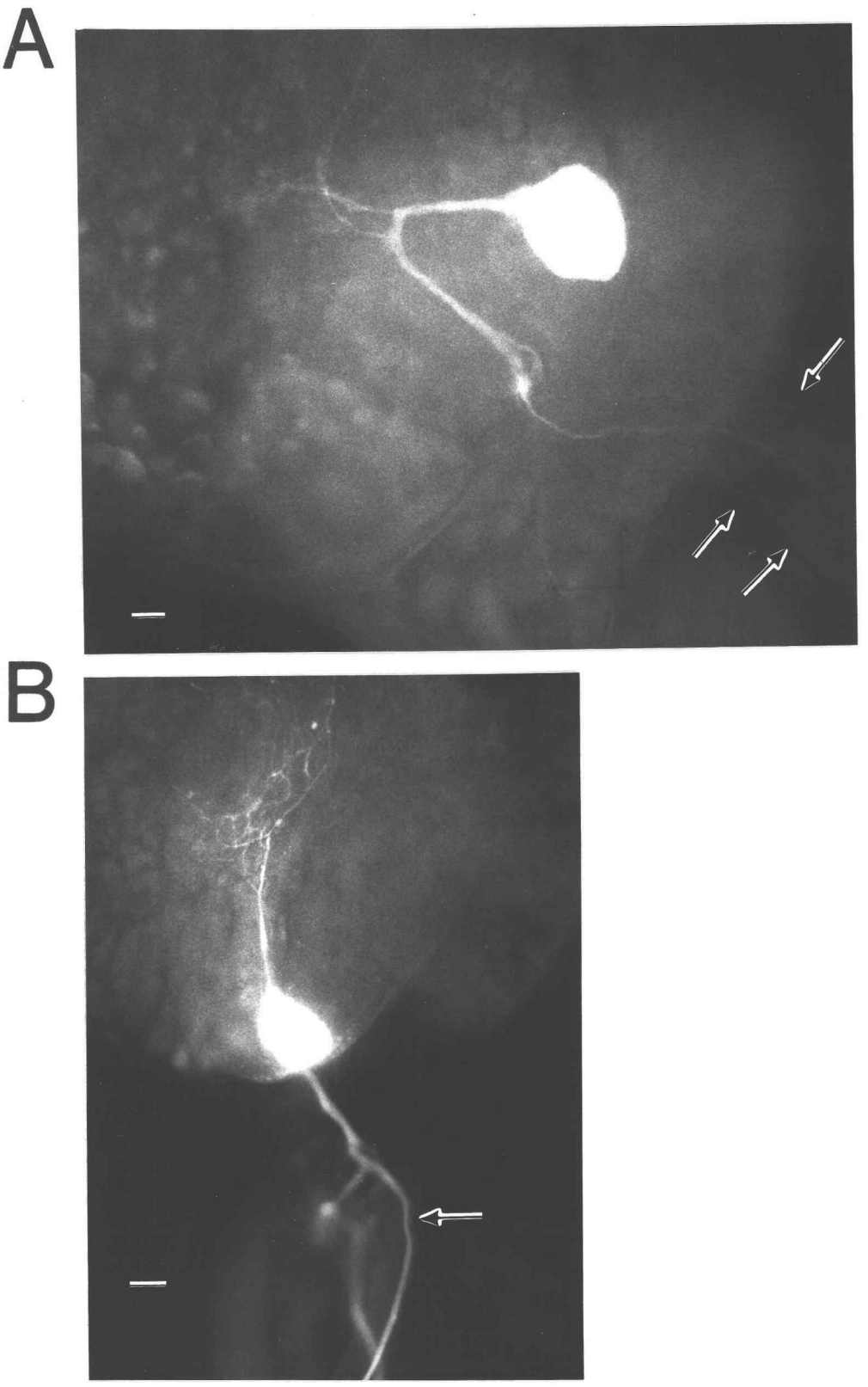


FIGURE 8. Schematic diagram of the circumesophageal ganglia (dorsal view) showing the positions of excitatory and inhibitory cardiac motorneurons identified in this study. Unmarked cells adjacent to the motorneurons are landmarks for orientation, and the cell medial to the pleural cardiac motorneurons is a white cell.

FIGURE 8

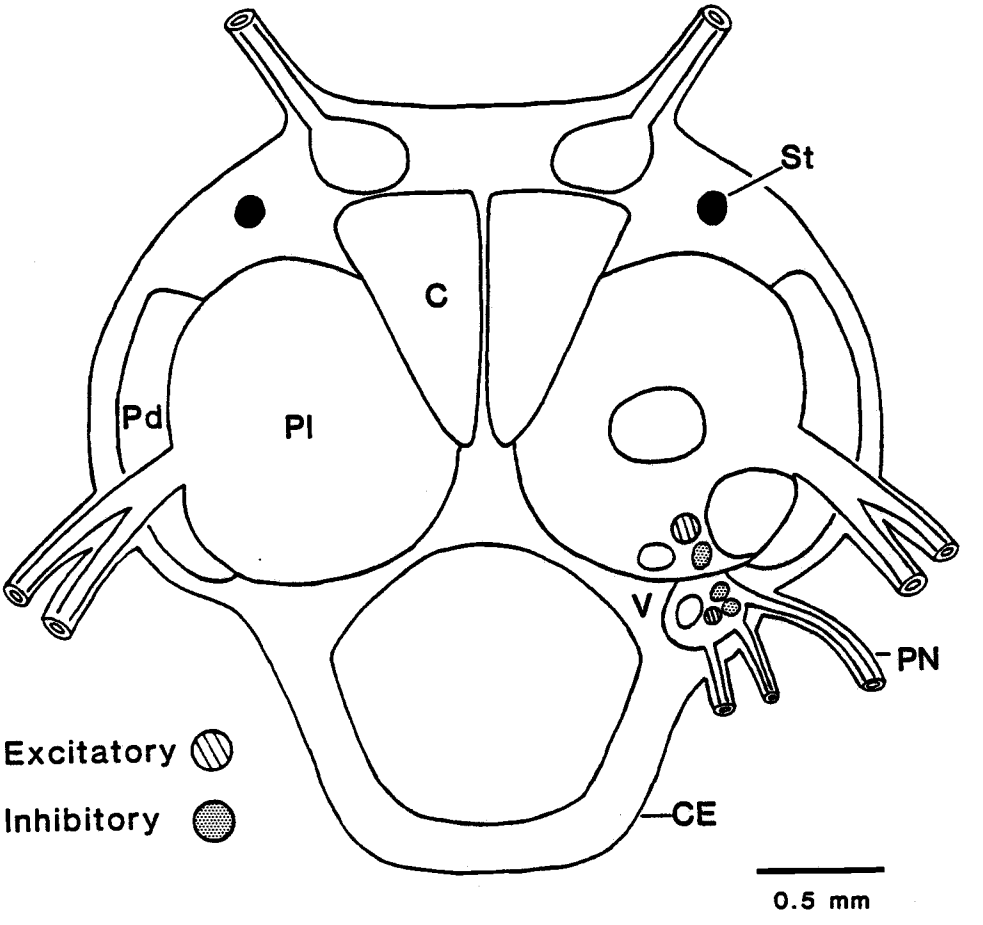
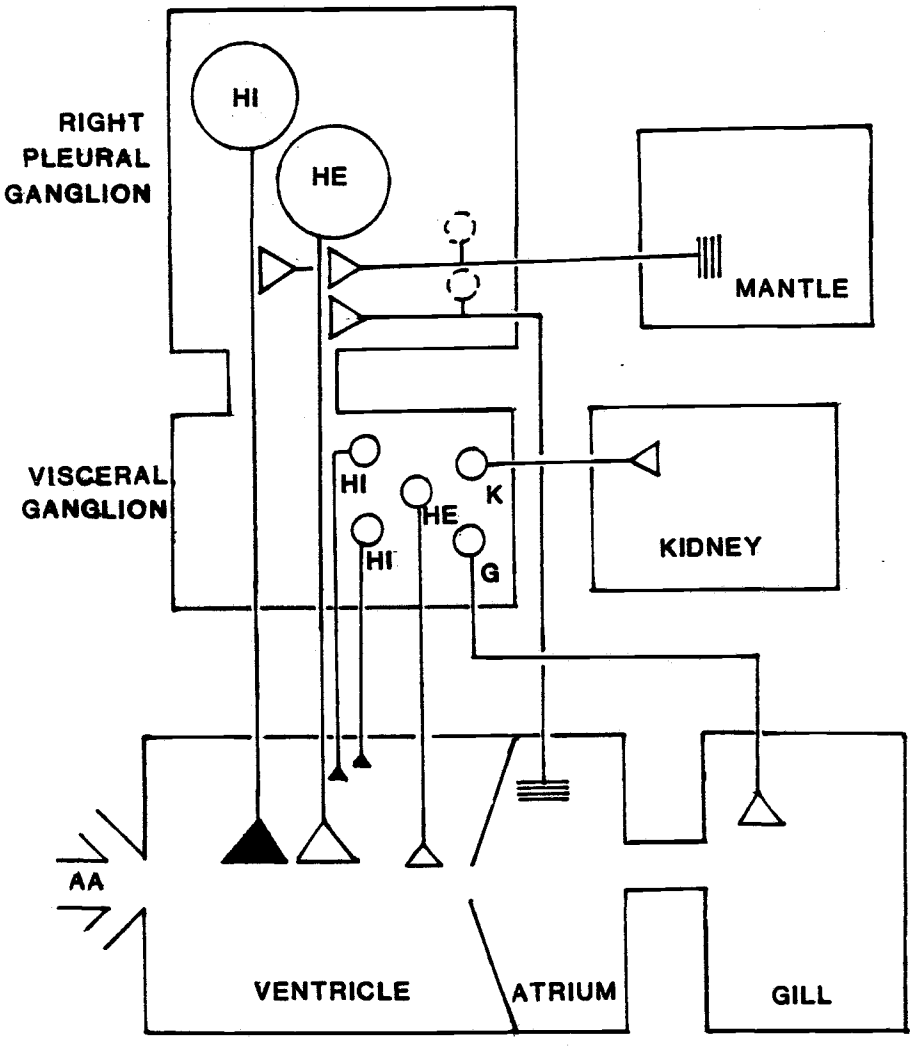


FIGURE 9. Schematic diagram of excitatory and inhibitory motorneurons innervating the heart of Archidoris. Representative visceral ganglion motorneurons innervating the kidney and gill are also shown. Putative sensory inputs to the pleural cardiac motorneurons from the mantle and heart are indicated by dashed cell bodies. Filled triangles represent inhibitory synapses and empty triangles represent excitatory synapses. AA, anterior aorta; G, gill motorneuron; HE, heart excitatory motorneuron; HI, heart inhibitory motorneuron; K, kidney motorneuron.

FIGURE 9


RIGHT
PLEURAL
GANGLION
HI
HE
MANTLE
VISCERAL
GANGLION
HI
HE
K
HI
G
KIDNEY
AA
VENTRICLE
ATRIUM
GILL

LITERATURE CITED

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