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Excitation and Inhibition of the Heart of the Snail, *Lymnaea*, by Non-FMRFamidergic Motoneurons

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SUMMARY AND CONCLUSIONS

1. The present paper extends the model of neuronal control of the *Lymnaea* heart by the use of intracellular recording techniques to identify further types of cardioactive neurons in the CNS that, like the previously described E heart excitor (E_{he}) cells, influence the myogenic heartbeat.

2. Four new types of neuron that act on the heart are described. These are excitatory H_{he} and S_{he} cells (H and S heart excitors) and the inhibitory K_{hi} cell (K heart inhibitor). The fourth class, tonus pericardium excitor (T_{pe}), modulates the heart by action on pericardial tissue.

3. Pharmacologic, electrophysiological, and anatomic evidence is presented that shows that these cells are motoneurons, innervating heart muscle fibers directly: blocking central chemical synapses failed to prevent the actions of the neurons on the heart; simultaneous intracellular recordings showed unitary EJPs in heart muscle after 1:1 and with constant delay from evoked neuronal action potentials; intracellular injection of the dye Lucifer yellow showed all cells had axonal branches entering the intestinal nerve (which innervates the heart).

4. The use of selective antagonists to 5-hydroxytryptamine (5-HT) (cinanserin), dopamine (ergonovine), and acetylcholine (α-bungarotoxin) provided evidence that the actions of S_{he} and H_{he} cells are mediated by 5-HT, whereas those of the K_{hi} cell are mediated by acetylcholine.

5. A cyclically active network of three interneuronal inputs acting on the heart motoneurons is described.

6. One of these, input 3, is responsible for periodic excitation of the heart via its effects on the H_{he} cells.

INTRODUCTION

The molluscan heart is, like that of vertebrates, myogenic and will continue to beat when all nervous innervation is removed. However, heart activity is modulated by central neurons. Early studies demonstrated that the heart responded to nervous stimulation and described the effects of putative classical transmitters (Hill and Welsh 1966; Jones 1983; Koester et al. 1974). Several cardioactive peptides have also been identified (Walker 1986). This groundwork has made the molluscan heart a suitable model for asking questions about the relative roles of peptides and classical transmitters in controlling a single target organ. To do this effectively, individual cardioactive neurons containing classical and peptide transmitters need to be located. However, in only a few examples has a study of the central motoneuron innervation of the heart been made. The most comprehensive of these studies were those of Mayeri et al. (1974), Koester et al. (1974), and Leibeswar et al. (1975), who reported the identification of central cardioactive motoneurons and command interneurons in *Aplysia* and provided good evidence for the roles of 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) as authentic transmitters of heart motoneurons. More recently, the cardioregulatory neurons R3–R14 of *Aplysia* have been shown to produce mRNA coding for a peptide, although the role of this peptide is not yet clear (Nambu et al. 1983).

In *Lymnaea*, we examined the actions of the cardioexcitatory peptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) (Price and Greenberg 1977a,b) and related peptides (Ebberink et al. 1987) as well as monoamines and ACh on the heart (Buckett et al. 1990a,b). We also provided evidence for a pair of heart motoneurons whose actions are probably mediated by FMRFamide. The present study reports on further details of this model system by describing new types of motoneurons involved in regulating heart activity.

Two excitatory and one inhibitory classes of neuron are described, as well as another type that altered heart output by causing contractions of pericardial tissue. A pharmacologic examination of the likely transmitter substances suggested that 5-HT and ACh were the excitatory and inhibitory compounds utilized by these motoneurons. Interneurons forming part of a central network responsible for regulating the heart were also investigated.

MATERIALS AND METHODS

All the experiments were carried out on the semi-intact heart-brain preparation described in the previous paper (Buckett et al. 1990b). Methods for recording and stimulating the heart and central neurons were also the same. To obtain evidence for putative transmitters used by the motoneurons, the heart was perfused with selective antagonists previously identified from experiments on the isolated heart (Buckett et al. 1990a). The antagonist drugs were perfused through the heart during motoneuronal stimulation. Blocking of responses in the heart suggested that specific neurotransmitters were being released.

RESULTS

Identification of motoneurons

Four cell types were identified that had effects on the heart. These and the previously described FMRFamide-containing E heart excitor (E_{he}) cells (Buckett et al. 1990b) were all located on the dorsal surface of the visceral ganglion (Fig. 1). Three of the cell types formed parts of neuronal clusters identified in previous studies, the H cells, K

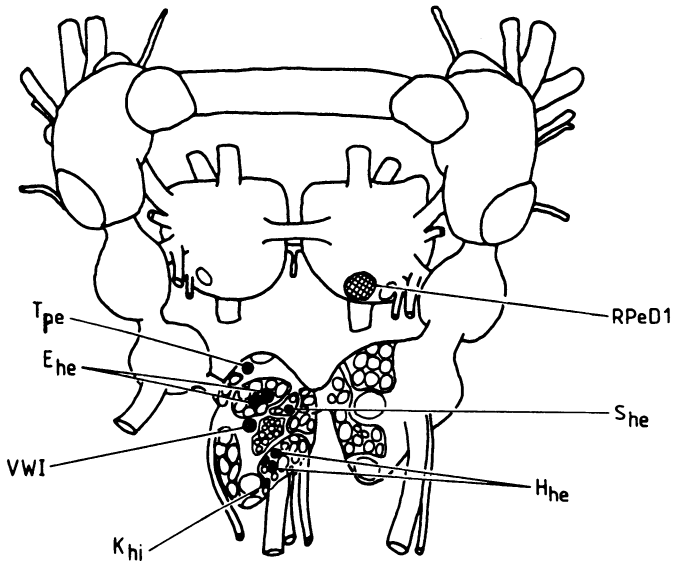

FIG. 1. Schematic representation of the dorsal surface of the *Lymnaea* brain showing location of identified heart motoneurons (●) and other noncardioactive cells, which have been previously identified (○). Cardioactive motoneurons of the E_{he} and S_{he} types occur as members of 2 larger groups of cell (the E group and S group). The H_{he} and K_{hi} cells are members of a more heterogenous cluster (the H I J K cells), and the T_{pe} is an isolated cell. Interneurons involved in cardioregulation (◉) are the visceral white interneuron (VWI) and the right pedal dopamine-containing cell (RPeD1).

cells, and E group; but two others, the S and T cells, were completely new cell types.

S heart excitor (S_{he}) motoneuron

The S group was a cluster of three neurons of similar color, electrophysiological properties (firing pattern, spontaneous synaptic input, and spike shape), and size (20- to 40-μm soma diameter). They, therefore, could not be separated until their effects on the heart were tested by electrical activation. Only one of these cells was found to be cardioactive, causing an excitatory response of the heart. The S group was located on the dorsal surface of the visceral ganglion, between the E group and the Yellow Cells (1), (Fig. 1) and commonly lies buried beneath the soma of the Yellow Cells. Often, some of the adjacent Yellow Cells had to be removed before the S group could be located and recorded from. The S_{he} cells had resting membrane potentials of about −60 mV and were usually silent. Spontaneous activity was seen in a few preparations, but this was intermittent, with firing interspersed between long periods of silence.

Current-evoked spike bursts in S_{he} cells often resulted in positive chronotropic and inotropic responses from the heart that far outlasted the spike burst itself (Fig. 2*D*). Alternatively, in a smaller number of preparations (~30%), a much weaker response was seen, with bursts of similar strength causing only single extra beats (Fig. 3). Single spikes in S_{he} cells could never initiate heartbeats, and bursts of 4 spikes were the minimum number that could reliably induce single contractions (Fig. 2*C*). The ability of S_{he} cells to excite the heart suggested that they were heart motoneurons. Further evidence for this was obtained from electrophysiological and anatomic data. S_{he} cells, injected with the fluorescent dye Lucifer yellow (Stewart 1978, 1981), had a simple geometry with a single axonal branch entering the intestinal nerve, the nerve innervating the heart (Fig. 2*A* and 2 other preparations). Spikes in the S_{he} cells elicited 1:1 excitatory junction potentials (EJPs), recorded intracellularly in auricular muscle fibers. The latency for response was constant in the same preparation (Fig. 2*B*) but varied between 100 and 140 ms in different preparations, probably reflecting differences in the distance from CNS to heart. It remained possible that cells within the CNS interposed between the S_{he} cells and the heart were responsible for the effects seen. However, when the CNS was bathed in saline containing Co^{2+}, the response of the heart persisted (Fig. 3). This treatment had previously been shown to completely block central chemical synapses (Buckett et al. 1990b). This suggested that S_{he} cells were heart motoneurons, acting directly on the heart, though the possibility of interposed electrotonically coupled neurons cannot be discounted.

H-cell heart excitor (H_{he}) motoneurons

The H cells are a dispersed group of neurons sharing similar patterns of electrical activity and spontaneous synaptic input (Benjamin and Winlow 1981) located in the posterior dorsal region of the visceral ganglion, around the roots of the intestinal, anal, and genital nerves (Fig. 1). As many as four of these cells have been found to have an

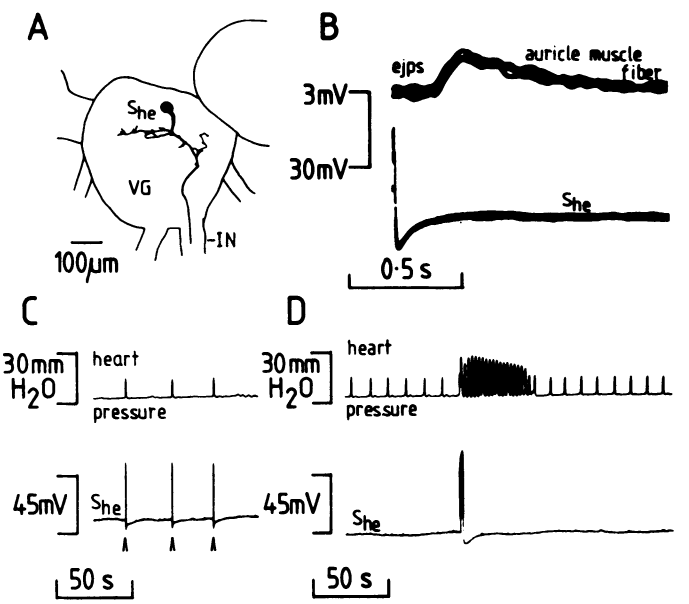

FIG. 2. The S_{he} motoneuron. *A*: reconstruction of a Lucifer-yellow–injected cell in the visceral ganglion (VG) of the CNS drawn from photomicrographs of a whole-mount preparation showing the typical S_{he} neuronal geometry, with a single axonal projection that enters the intestinal nerve (IN). *B*: simultaneous intracellular recordings from an S_{he} cell (*bottom*) and an auricle muscle fiber (*top*). Four superimposed sweeps triggered by spikes in the S_{he} cell show constant latency 1:1 EJPs in the muscle fiber. *C*: current-induced spike bursts (arrowed) in the S_{he} produced single beat responses (monitored as blood pressure) in a quiescent heart preparation. *D*: response of a weakly beating heart to a brief evoked S_{he} spike burst consists of an increase in beat amplitude and rate, which lasts for almost 1 min.

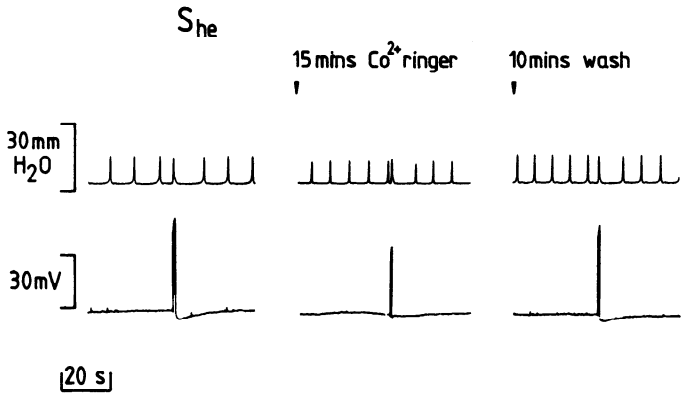

FIG. 3. Excitatory effects on heart beat of S_{he} activation were maintained before (*left*), during (*middle*), and after (*right*) cobalt saline block of central synaptic transmission. Normal saline was perfused through the heart chamber throughout the experiment.

effect on the heart in a single preparation, although it was more usual to find only two or three. The cells were normally spontaneously active, having firing frequencies from 0.2 to 2 Hz, with occasional higher frequency bursts because of synaptic input (Fig. 4*B*; see also Benjamin and Winlow 1981). Long-duration–evoked bursts were required to produce excitatory responses in the heart. This resulted in a series of high-frequency arrhythmic contractions with an increase in underlying tonus accompanied by a striking reduction in the amplitude of individual beats (Fig. 4*B*). Unlike the S_{he} cell, the duration of the response did not outlast the stimulus.

H_{he} anatomy was consistent with a heart motoneuron function. Each Lucifer-yellow–filled cell showed a large peripheral projection entering the intestinal nerve (Fig. 4*A*), with finer additional processes in the same nerve. A smaller collateral projection entering the genital nerve was also

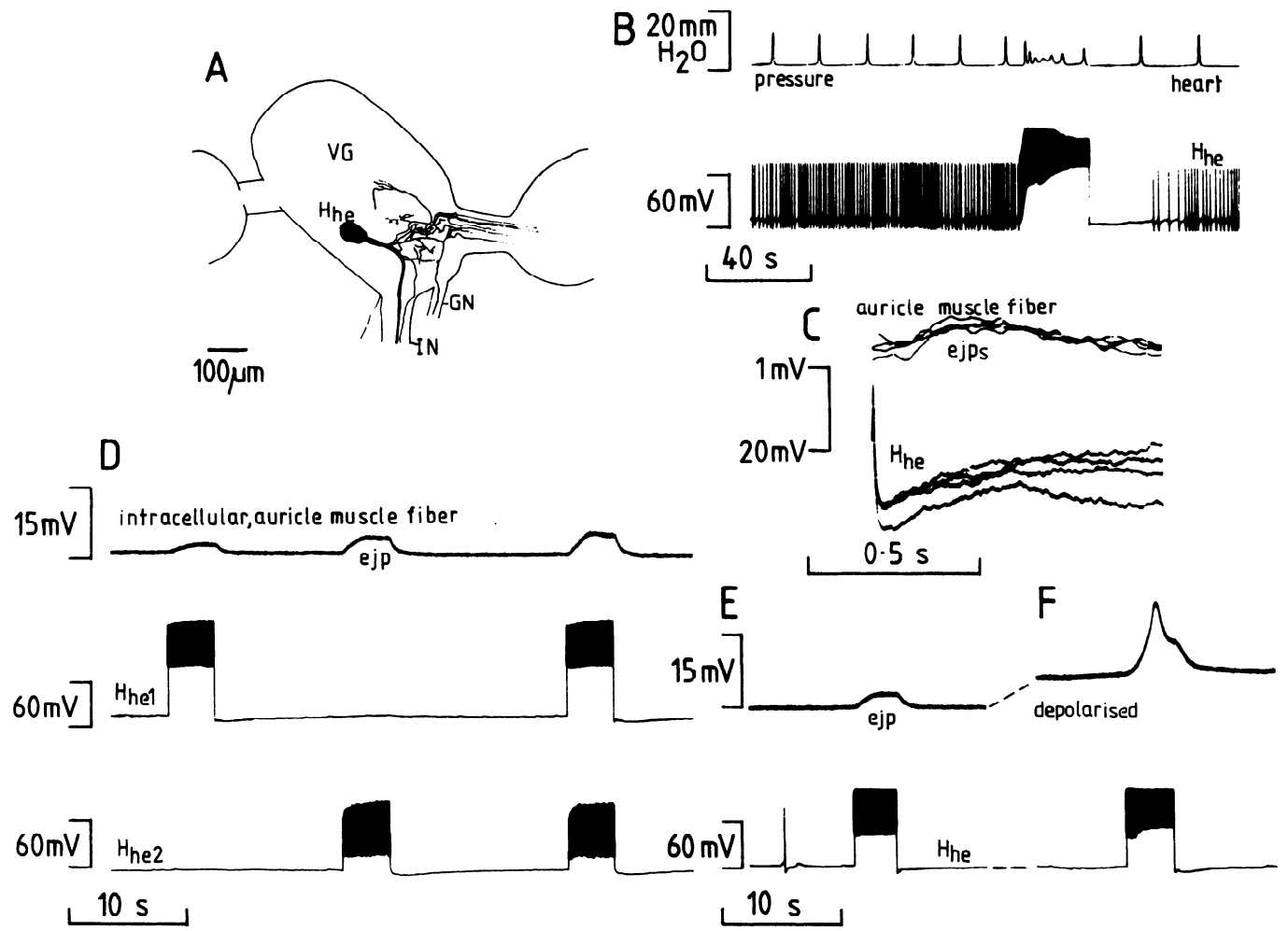

FIG. 4. The H_{he} motoneurons. *A*: reconstruction of a Lucifer-yellow filled cell from the visceral ganglion (VG) made from a series of photomicrographs showing the major axonal projection entering the intestinal nerve (IN). Also present is neuropile arborization around the visceral-left parietal connective and 2 minor neuritic branches that enter the intestinal and genital nerves (GN). *B*: pressure response of the heart to H_{he} cell stimulation. Arrhythmic, low-amplitude contractions result from a long-duration evoked spike burst. *C, D, E,* and *F*: simultaneous intracellular recordings from H_{he} cells (*bottom traces*) and auricle muscle fibers (*top traces*). *C*: constant latency 1:1 EJPs in an auricle muscle fiber follow from 4 superimposed H_{he} cell spikes. *D*: separate bursts of spikes in 2 different H_{he} cells (H_{he1} and H_{he2}) each produced slow summated excitatory postsynaptic potentials (EPSPs) in the same auricular muscle fiber (3 and 5 mV amplitudes). There is no synaptic connection between the neurons. When the 2 cells are activated together, a larger summed response was produced (8 mV amplitude). *E*: Summated EJPs from a single H_{he} cell only produce an action-potential–like event in the muscle fiber when the muscle membrane was first depolarized extracellularly (*F*). This event evoked a visually observed muscle contraction.

present in all four preparations filled. The cells also had characteristic fine neuritic branching in the neuropile of the visceral and right-parietal ganglia.

Simultaneous intracellular recording from H_{he} cells and heart muscle fibers provided further evidence for a motoneuronal function for this cell type. Spikes induced 1:1 EJPs with a constant latency of ~100 ms (Fig. 4*C*). The unitary EJPs from a particular H_{he} cell were small, <1 mV in amplitude, accounting for the failure of single spikes in H_{he} cells to evoke heart contractions. However, a single auricular muscle fiber could be innervated by two H_{he} cells (Fig. 4*D*), and so summation of EJPs from synchronously active motoneurons was possible. In fact, very strong activation of even two H_{he} cells rarely evoked strong contraction in the heart, and only when the muscle was depolarized by extracellular current did a single H_{he} cell induce a "spikelike" response in a muscle fiber (Fig. 4, *E* and *F*) with an accompanying contraction of the heart. However, despite the weak effects of individual H_{he} cells, strong synaptic activation of the H_{he} population as a whole occurred spontaneously, and this had a strong influence on heart beat. This will be described in a later section.

Co^{2+} saline applied to the CNS did not prevent H_{he} effects on the heart (Fig. 5), and it was concluded that these cells are another class of excitatory heart motoneurons.

5-HT was the putative transmitter for S_{he} and H_{he} cells

Application of 5×10^{-5} M cinanserin to the heart, with normal saline perfusing the CNS, blocked the excitatory effects of both S_{he} and H_{he} cells. This provided direct evidence that 5-HT was the likely transmitter used by these cells as this concentration was previously shown to be an effective and selective antagonist to 5-HT on the *Lymnaea* heart (Buckett et al. 1990b). In the experiment shown in Fig. 6*A*, both S_{he} and H_{he} cells were recorded at the same time. Either cell could evoke a heart contraction (pressure recording) in this quiescent preparation. Twenty minutes' perfusion with cinanserin blocked both cells' ability to induce heartbeats. The response was reversible, showing that the heart was still capable of responding to neuronal stimulation. The other strong candidate for transmitter status in the *Lymnaea* heart was dopamine (Buckett et al. 1990a). However, perfusing with the selective dopamine antagonist, ergonovine, did not prevent either cell type from exciting the heart (Fig. 6*B*).

Tonus pericardium excitor (T_{pe}) motoneuron

The T_{pe} cell was a single cardioactive cell located on the extreme anterior edge of the visceral ganglion (Fig. 1). It had strikingly different effects on the heart to either the S_{he} or H_{he} cells. Evoked bursts of spikes produced a reduction in heartbeat amplitude with a concomitant elevation in diastolic tonus (Fig. 7*B*). This was the usual response (in 8 preparations), but on one occasion the heartbeat was completely abolished by activation of this cell (Fig. 7*C*). However, because the predominant response was tonotropic, the cell was given the designation T_{pe} (tonus pericardium excitor).

The T_{pe} cell exhibited irregular spontaneous firing patterns with periods of silence lasting tens of seconds. The spontaneous bursts were never seen to result in changes in heart activity, and only when strong sustained firing was induced by depolarizing current injection (↑ and ↓, Fig. 7, *B* and *C*) did the heart show the tonotropic response.

The anatomy of the T_{pe} cell (2 cells of this type were filled with Lucifer yellow) was similar to that described for other heart motoneurons in that there was a single axonal projection that entered the intestinal nerve (Fig. 7*A*). Also, when central chemical synapses were blocked by perfusion of Co^{2+} saline into the CNS chamber, the response of the heart was maintained (Fig. 7*D*). These data all suggested a motoneuronal function for the T_{pe} cell.

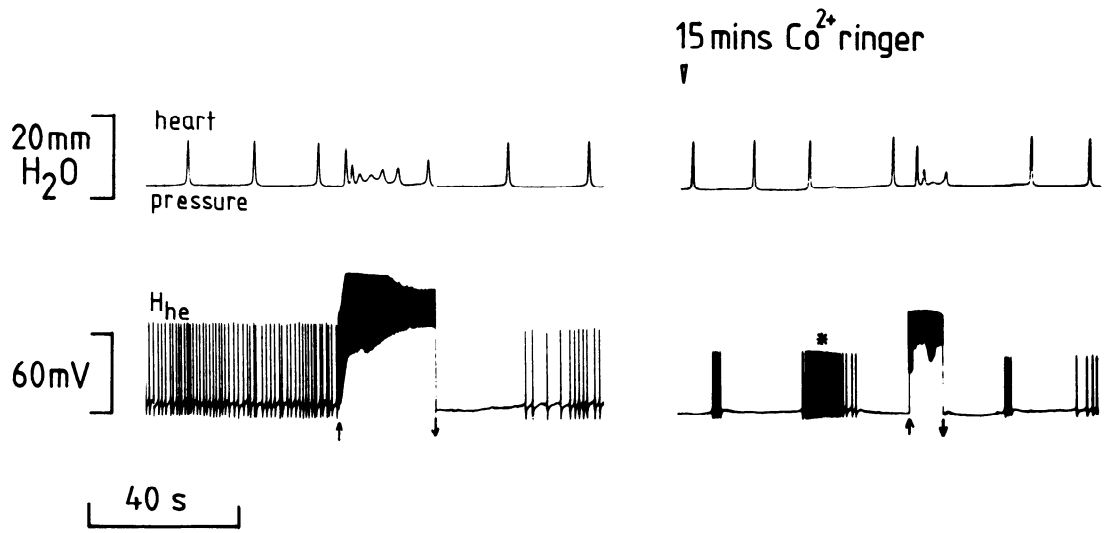

FIG. 5. Pressure response of the heart (*top traces*) to H_{he} cell stimulation persists in the presence of cobalt saline perfused over the CNS. Normal saline was continuously perfused through the heart. A strong spontaneous burst (*) has only a small effect on heart pressure, but a higher frequency evoked burst (↑ and ↓) produces a response similar to that found in normal saline. Note the induced bursting activity in the neuron when calcium entry is inhibited. The recording is from the same cells as in Fig. 4*B*.

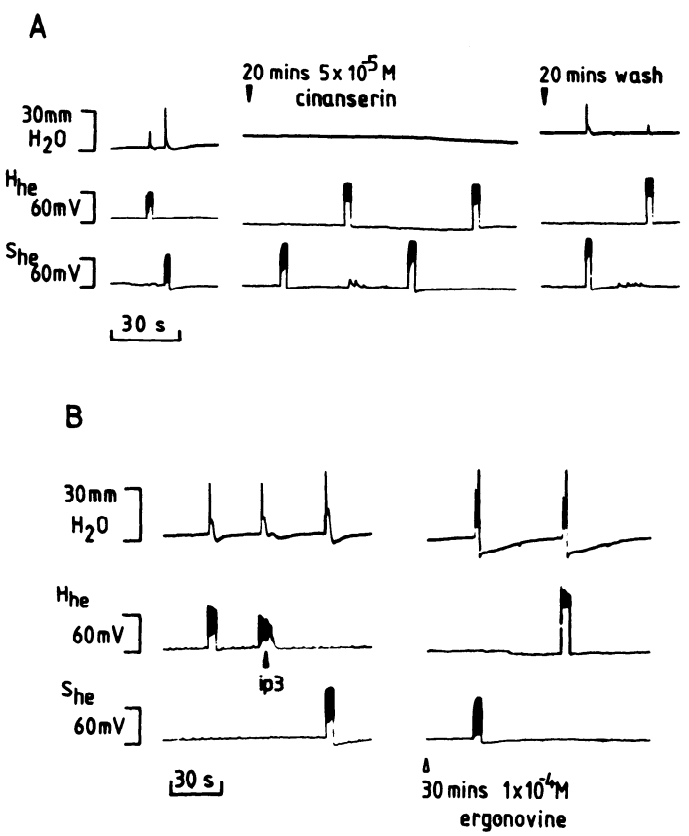

FIG. 6. Effects of the 5-HT antagonist, cinanserin, and the dopamine antagonist, ergonovine, on S_{he} and H_{he} cell excitation of the heart. Joint recordings of S_{he} and H_{he} cells were made from 2 preparations (*A* and *B*). In both preparations heart activity has been reduced by adjustments to perfusion pressure head heights so that effects of motoneuronal stimulation on heartbeat could be seen more clearly. *A*: evoked spike bursts in H_{he} (*middle*) and S_{he} (*bottom*) cells produce single heart contractions prior to cinanserin perfusion (*left*). These responses are abolished in cinanserin (*center*) and return on wash out (*right*). *B*: in another preparation ergonovine (*right*) has no effect on the excitatory action on the heart of either cell type (*left*). Note that in the *left middle trace,* the H_{he} cell is spontaneously activated by the interneuronal input, input 3. This produces heart excitation similar to that induced by evoked spikes in the H cell. These results suggest that 5-HT, and not dopamine, is the transmitter responsible for both S_{he} and H_{he} excitatory effects on the heart.

However, unlike the other heart motoneurons, the T_{pe} cell did not appear to act directly on the heart itself, but on the contractile pericardium that surrounds it. This conclusion was based on results comparing heart responses with the use of a mechanotransducer hooked into the ventricle (pericardium slit) with pressure recordings made from the whole heart (pericardium intact). T_{pe} effects could never be recorded by direct mechanical monitoring of the heart muscle but only by pressure recording in the whole structure (Fig. 8*A*). Thus only when the pericardium was intact could responses be recorded. Immediately after the pericardium was cut, the increase in tonus was lost (Fig. 8*B*). The pericardial tissue either side of the slit, however, was still seen to contract when the cell was stimulated, suggesting that the changes in heart activity during T_{pe} activation were because of contraction of the pericardium squeezing the heart. Because of this indirect action of the T_{pe} cell on the heart, no pharmacologic experiments to identify its transmitter molecule were performed.

K-cell heart inhibitor (K_{hi}) motoneuron

Up to four K cells occurred in the posterior region of the visceral ganglion, scattered among a large group of cells called the HIJK cells (Fig. 1). The K cells were normally silent and received several types of spontaneous inhibitory synaptic input (Benjamin and Winlow 1981). In any given preparation, only one cell could influence heartbeat, and this was called the K_{hi} cell. This was the only cell among the identified *Lymnaea* heart motoneurons that was capable of completely inhibiting heartbeat (Fig. 9*B*). Evidence that

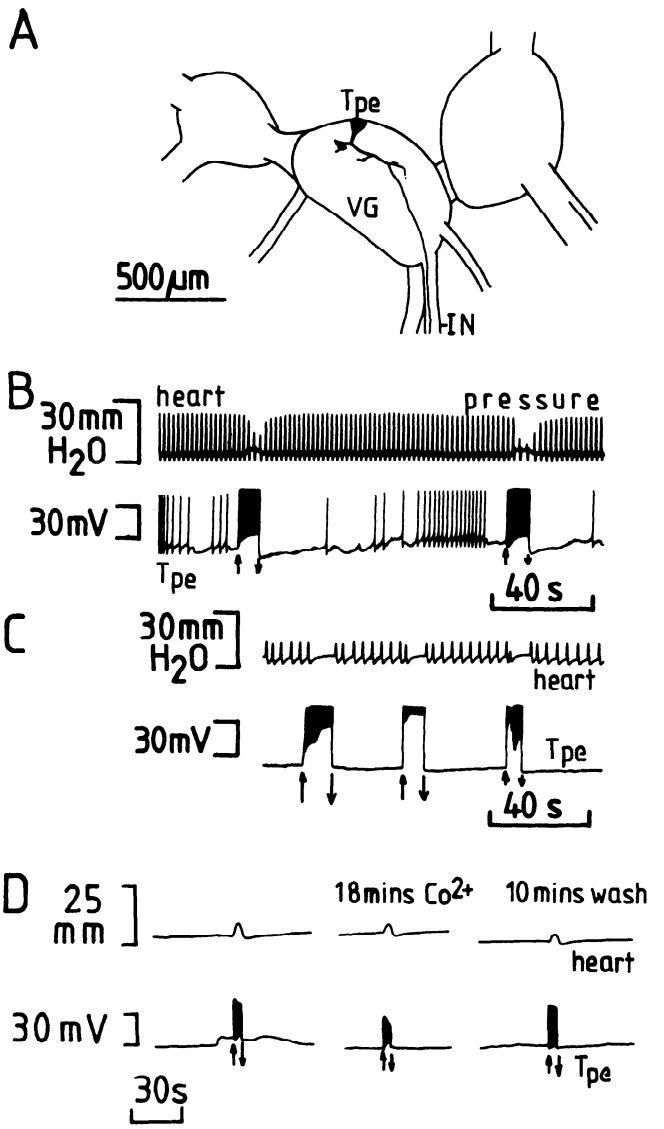

FIG. 7. The T_{pe} motoneuron. *A*: reconstruction of a Lucifer-yellow-filled cell in the visceral ganglion (VG) made from a series of photomicrographs of a whole-mount preparation showing a single axonal projection that enters the intestinal nerve (IN). *B–D*: pressure responses of the heart to current-evoked bursts of spikes (↑ and ↓) in the T_{pe} cell in 3 different preparations. *B*: example of the most typical type of response, where tonus is increased and beat amplitude decreased. Complete abolition of the heartbeat shown in *C* was seen only on 1 occasion. *D*: lack of effect of centrally applied cobalt saline on the heart response to T_{pe} cell stimulation. Here, heart activity has been suppressed by reducing the input pressure to the heart to show clearly the continued presence of the tonus response in cobalt saline.

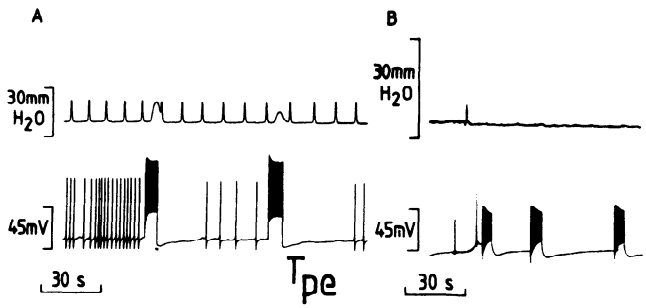

FIG. 8. Pericardial innervation by the T_{pe} cell. Increase in heart tonus due to evoked bursts of spikes in the T_{pe} cell (*A*). These are abolished when the pericardium is slit (*B*).

the K_{hi} was a motoneuron was that action potentials were followed by 1:1 in action potentials in auricular muscle fibers (Fig. 9*C*), the axon of the cell was restricted to the intestinal nerve (Fig. 9*A*), and that responses persisted when the CNS was bathed in Co^{2+} saline (Fig. 10).

The cardiac response to the K_{hi} cell was more complex than other heart motoneurons so far described, for as well as inhibiting the heartbeat (Fig. 9*B*), it could also cause an initial brief excitation followed by a much longer lasting inhibition. This was best seen with shorter duration bursts of evoked spikes in the K_{hi} cell. In Fig. 11, a burst of 12 spikes caused a sudden increase in the instantaneous heart rate, whereas a longer burst of 18 spikes caused an initial increase followed by a sudden decrease, indicating the bi-

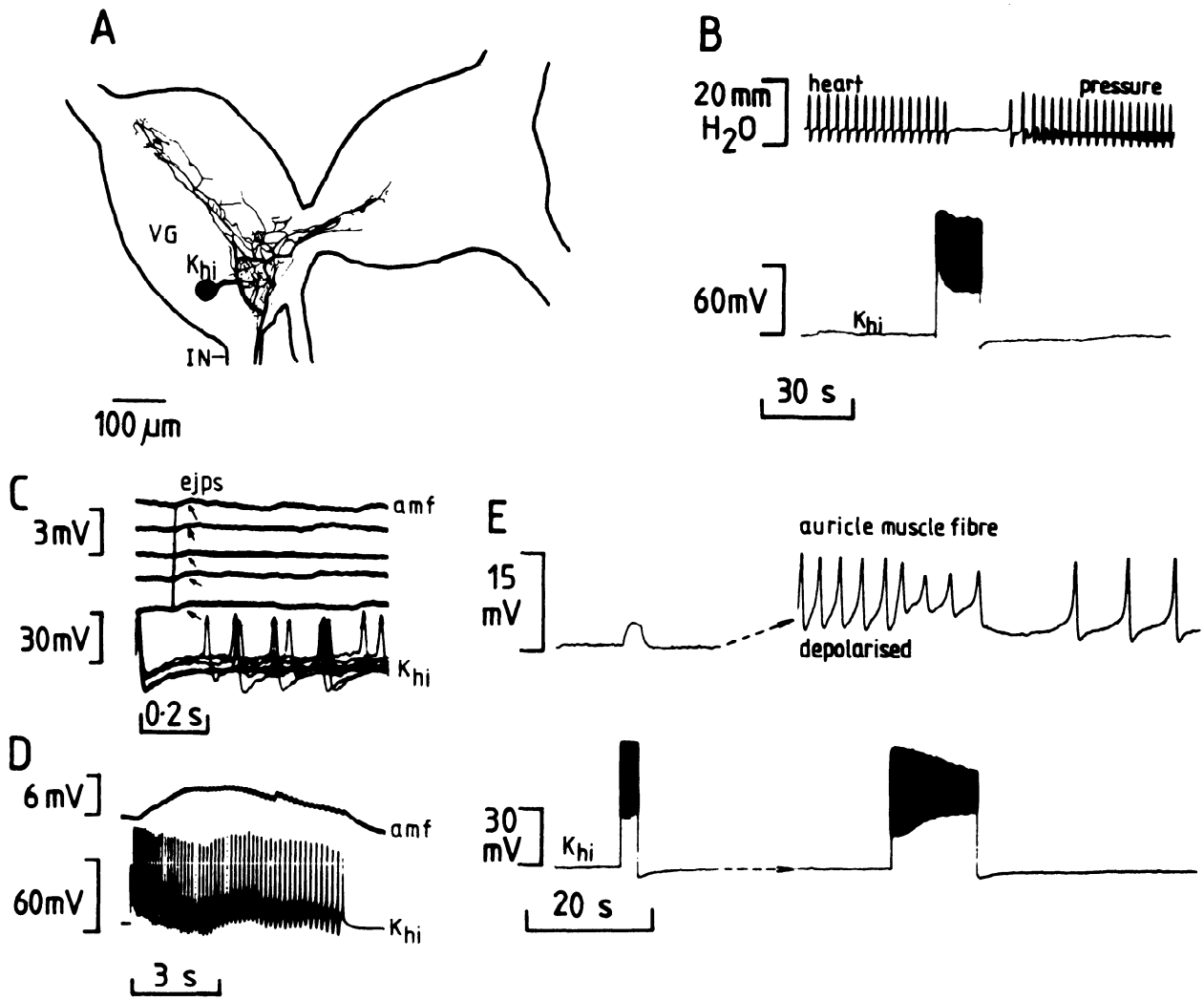

FIG. 9. The K_{hi} motoneuron. *A*: reconstruction from a series of photomicrographs of a Lucifer-yellow–filled K_{hi} cell in the visceral ganglion (VG) showing a single axonal projection entering the intestinal nerve (IN). *B*: pressure response of the heart to K_{hi} cell electrical stimulation. The evoked spike burst results in the total abolition of the heartbeat. *C*, *D*, and *E*: simultaneous intracellular recordings from K_{hi} and auricle muscle cells. *C*: multiple sweeps from an oscilloscope triggered by K_{hi} spikes showing small EJPs in an auricular muscle fiber following 1:1 (consecutive sweeps have been offset). *D*: oscilloscope recording showing summating EJPs from a K_{hi} cell spike burst. In both *C* and *D* there are additional EJPs recorded in the auricular cell originating from neuron(s) other than the recorded K_{hi} cell. *E*: slower time-base recordings showing the depolarizing membrane response of a muscle fiber at (*left*) resting membrane potential (−65 mV) and (*right*) after depolarization beyond threshold for spike initiation. Depolarizing current was applied via the extracellular suction pipette used to hold the auricle. The K_{hi} cell spike burst, at first, depolarizes the muscle membrane and then inhibits action potential formation for nearly 20 s.

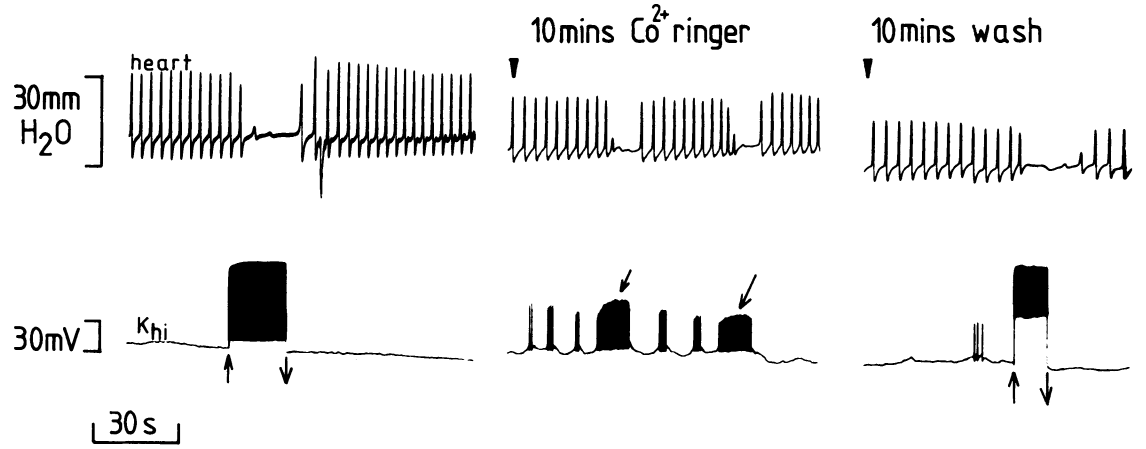

FIG. 10. Failure of centrally applied cobalt to block inhibition of the heartbeat by evoked bursts of spikes (↑ and ↓) in the K_{hi} cell. In Co^{2+}, K_{hi} spike height is attenuated, and the normally silent cell fires in cyclical bursts, which on 2 occasions are of sufficient strength (↙) to cause heart inhibition. The bursting activity ceases after wash with normal saline (*right*).

phasic e-i (excitation followed by inhibition) nature of the response.

Given the effects of K_{hi} activation, it might have been expected that biphasic junctional potentials, depolarization followed by hyperpolarization, would have been recorded in heart muscle fibers. However, when the heart was recorded intracellularly, only depolarizing junction potentials (JPs) were seen to follow evoked K_{hi} spikes (Fig. 9*C*). These small unitary JPs followed 1:1 from neuronal spikes and had a constant latency of ~120 ms. The JPs from a burst of spikes summated to produce a smooth depolarizing potential (Fig. 9*D*). When the heart muscle was depolarized by extracellular stimulation to a level where it would beat (indicated by muscle cell action potentials), an evoked K_{hi} burst caused an extra depolarization. This was eventually followed by a cessation of muscle spikes (Fig. 9*E*) during which the membrane potential hyperpolarized.

One possible explanation for a lack of a more striking hyperpolarizing synaptic potential during inhibition of spike activity was that chloride defusing from the (KCl-filled) electrode tip reversed or reduced the size of a chloride-mediated inhibitory postsynaptic potential (IPSP).

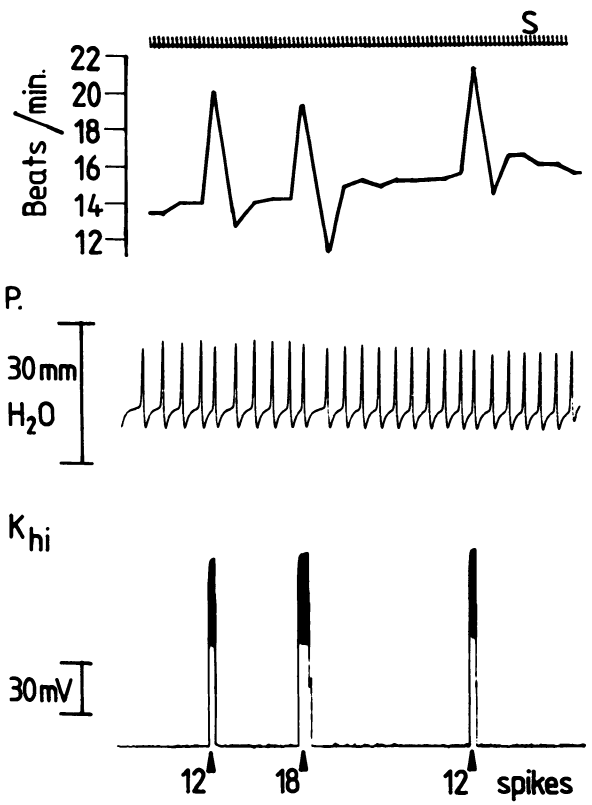

FIG. 11. The differential effects of K_{hi} bursts of different duration on heartbeat. Twelve spike bursts elicit an advancement of the next heartbeat. When the burst is increased by 50% to 18 spikes, the response becomes biphasic, with the first beat advanced and the next delayed. This is seen clearly in the instantaneous heart rate record (*top*; beats/min). *Middle*: blood pressure (P) showing individual heartbeats.

ACh was the putative transmitter for K_{hi} cells

Our initial pharmacologic study (Buckett et al. 1990a) showed that ACh perfused into the *Lymnaea* heart produced inhibitory or excitatory effects on heartbeat similar to those produced by the K_{hi} cells. Both these responses could be blocked by the snake venom toxin α-bungarotoxin (α-Bgt) (Buckett et al. 1990a; Geraerts et al. 1981). Thus ACh became the prime transmitter candidate for K_{hi} effects on the heart. This was confirmed by perfusing the heart with α-Bgt while stimulating a K_{hi} cell (Fig. 12). Per-

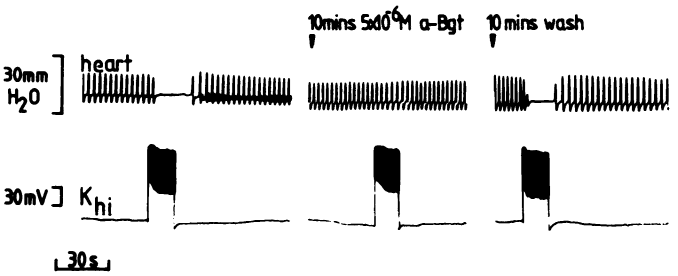

FIG. 12. Blocking effect of α-Bgt on the inhibitory response of the heart to K_{hi} cell activation. The response (*left*) is completely blocked by 5×10^{-6} M α-Bgt perfused into the heart for 10 min (*middle*). The block is reversed after 10-min wash in normal saline.

fusion of the heart for 10 min with 5 μM α-Bgt completely abolished the inhibitory response of the heart. This antagonistic effect was reversible, following wash out with normal saline for 10 min.

In vertebrate systems, ACh is known to activate both fast excitatory (nicotinic) and slow inhibitory (muscarinic) receptors on central neurons (cf. Horn and Dodd 1985). If these two types of receptor channel occurred on *Lymnaea* heart muscle cells, this could account for the initial excitation followed by the inhibitory response seen in Fig. 11. However, because the antagonistic action of α-Bgt was reversible (unlike at the vertebrate nicotinic receptor), the molluscan ACh receptors are unlikely to be exactly the same as those of vertebrates. This conclusion supported earlier work on ACh receptors in mollusks reported by Parmentier and Carpenter (see Walker 1986).

Synaptic inputs to heart motoneurons

The motoneurons identified in this study received a variety of synaptic inputs, some of which originated in the

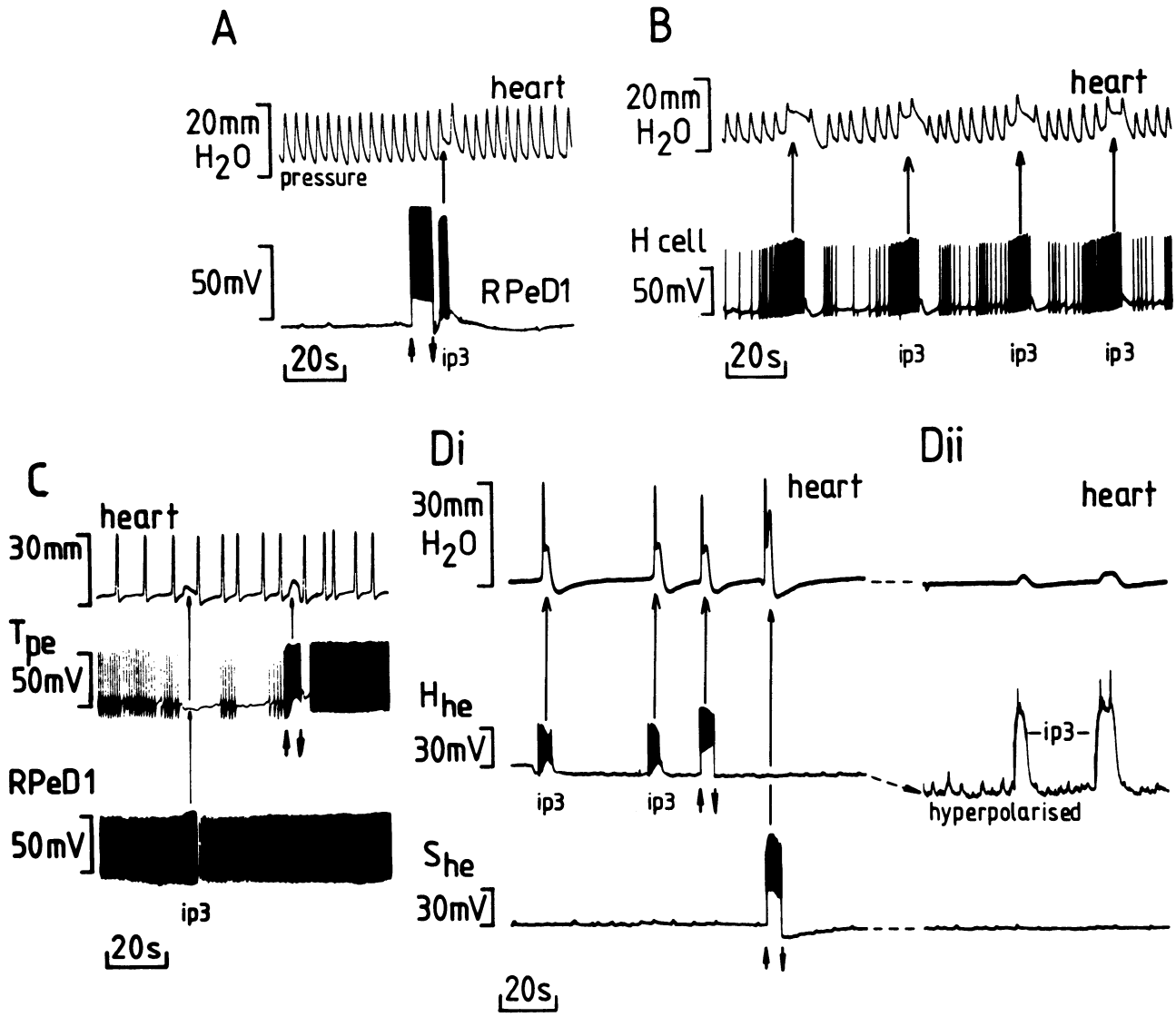

FIG. 13. Input 3 (ip3)—a central synaptic input to heart motoneurons. Its occurrence is correlated with heart excitation. *A*: a burst of spikes in RPeD1 triggers a delayed input 3, which excites RPeD1 itself and is correlated with an increase in heart tonus. Note that there is no direct effect of RPeD1 on the heartbeat. This cell is merely used to trigger input 3 by some unknown pathway and monitor the occurrence of the input on central neurons. The interneuron(s) responsible for input 3 have not been identified. *B*: input 3 causes regular bursting activity in an H_{he} cell and concurrent tonus excitation of the heart. *C*: input 3, monitored on RPeD1 as a sudden spontaneous increase in activity, is inhibitory on the T_{pe} cell. Therefore activity in the T_{pe} cell cannot be contributing directly to input 3s ability to excite the heart. A strong burst of spikes in the T_{pe} cell evoked by current injection causes an increase in heart tonus. *Di*: in a quiescent heart preparation input 3 excites an H_{he} cell but not an S_{he} cell. Input-3–induced bursts in the H_{he} cell (*middle*) are accompanied by increase in tonus and heartbeats. A current-evoked burst in either the H_{he} cell or a S_{he} cell has a similar effect ($\uparrow$ and $\downarrow$). *Dii*: the H_{he} cell has been hyperpolarized so that spontaneous input 3 elicits only a few spikes. As a consequence, the major component of the heart response is lost. Paired arrows beneath the traces in *A*, *C*, and *D* refer to injection of depolarizing current through the recording electrode.

CNS, whereas others could be initiated by sensory stimulation of the heart. No synaptic connections were found between the different types of motoneurons. Three previously described interneuronal sources give rise to synaptic input. These are input 3 [a wide-acting input originating from unidentified interneurons within the CNS (Benjamin and Winlow 1981)], the giant dopamine-containing cell RPeD1 (Winlow et al. 1981), and the visceral white interneuron (VWI) (Benjamin 1984). These form a rhythmically active central network (Benjamin 1984), at least part of whose function is to modulate heart activity.

INPUT 3 CAUSES PERIODIC EXCITATION OF THE HEART. Input 3 is a spontaneous synaptic input that occurred as predominantly inhibitory or excitatory compound potentials on many central neurons of the visceral and parietal ganglia of *Lymnaea* (Benjamin and Winlow 1981). The sign of the potential depended on the particular identified neuron. It often occurred periodically on neurons and could be seen to be accompanied by a strong increase in the tonus of the heart recorded at the same time (Fig. 13*B*). As the H_{he} heart motoneurons were both excited by input 3 (Fig. 13, *B* and *Di*) and were known to cause similar direct effects on the heart, they were considered to be the prime candidates for causing the increase in heart tonus during input 3. That they were at least partly responsible is shown in Fig. 13*D*. In a quiescent heart preparation, input 3 caused occasional bursts of spikes in the H_{he} cell, and this was accompanied by the expected increase in tonus in the heart (Fig. 13*Di*). Artificially induced bursts of spikes in the H_{he} cell caused similar responses in the heart showing that neuron was definitely capable of exciting the heart in the required way (Fig. 13*Di*). Suppressing input-3–induced bursts on the H_{he} cell by hyperpolarization (Fig. 13*Dii*) reduced the increase in tonus due to input 3 showing directly that the H_{he} cell was actually contributing to heart excitation during the spontaneous input. We supposed that H_{he} cells in the same preparation might be responsible for the remaining increase in tonus during spontaneous occurrences of input 3. No connections have been found between H cells either here or in previous work (Benjamin and Winlow 1981).

Could other motoneuron types be contributing to the periodic excitation of the heart during input 3? Neither the S_{he} or E_{he} cells are excited by input 3, so they cannot be important. This is shown for the S_{he} cells in Fig. 13*Di* where, although artificially evoked bursts of S_{he} spikes can cause heart excitation similar to the H_{he} cell recorded at the same time, no input 3 could be seen on the S_{he} cell, unlike the H_{he}. The other candidate heart excitor would be the T_{pe} cell, but these were, in fact, inhibited by input 3 (Fig. 13*C*). Input 3 also inhibits the only heart inhibiting neuron, the K_{hi} cell (Fig. 14*C*), and so removal of inhibition from this cell could have theoretically contributed to the periodic excitation of the heart—but this has not been tested directly.

RPeD1. As well as spontaneous input arising from unidentified sources, the giant dopamine-containing interneuron, RPeD1, also had widespread synaptic effects on central neurons in *Lymnaea* (Benjamin and Winlow 1981; Winlow et al. 1981) including some of the heart motoneurons. It was confirmed that RPeD1 had weak excitatory effects on H and K cells, but no responses on T_{pe}, S_{he}, or E_{he} cells, or on the heart itself were found. In the type of preparation used here or in the isolated preparations used previously (Benjamin and Winlow 1981), the weak excitatory effects RPeD1 had on the H and K cells did not significantly change their spike activity, so its importance is unclear. Of more importance may be the indirect effects of RPeD1 activation on input 3. An artificially induced burst of spikes in RPeD1 caused a delayed input-3 excitation on itself (Fig. 13*A*, arrows), and this was accompanied by the increase in tonus in the heart described in the previous section. This is presumably because of input 3 exciting the H_{he} cells at the same time (Fig. 13*Di*) and cannot be explained by any direct action of RPeD1 on the heart. The current-evoked burst of spikes in RPeDI caused no change in heart activity (Fig. 13*A*).

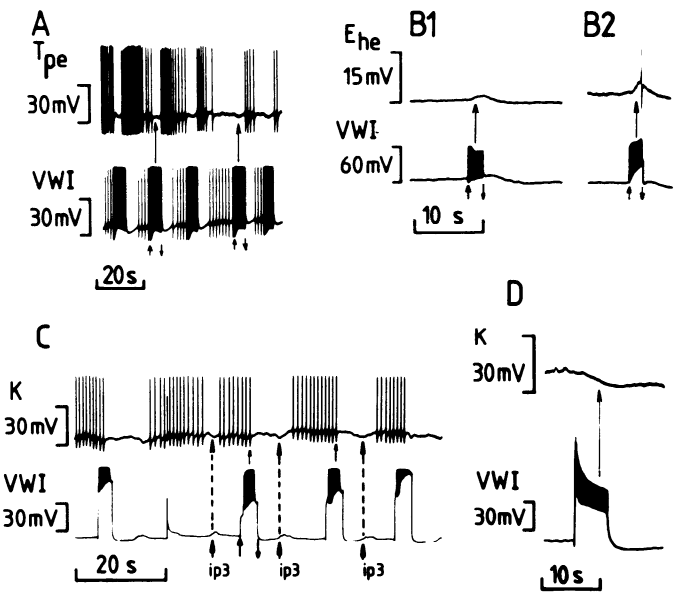

FIG. 14. Postsynaptic responses of heart motoneurons due to activation of the visceral white interneuron (VWI). *A*: bursts of spikes in VWI inhibit ongoing spiking in the T_{pe} cell. *B1*: a burst of VWI spikes depolarizes an E_{he} FMRFamide-containing heart motoneuron and leads to spiking if the E_{he} cell is steadily depolarized with weak DC current (*B2*). *C*: bursts of VWI spikes stop firing in a continuously depolarized K cell. Inhibition of firing is also because of the spontaneous occurrence of hyperpolarizing input 3 (arrows with dashed line) acting on the K cell. Input 3 also occurs as a depolarizing wave on the VWI. *D*: the VWI-evoked IPSP on the same K is seen more clearly at resting membrane potential. Paired arrows below VWI traces indicate bursts of spikes evoked by depolarizing current injection.

VWI. Like RPeD1, the VWI is a wide-acting interneuron with many central follower cells in the CNS (Benjamin 1984). It also had specific effects on heart motoneurons. Bursts of spikes in the VWI inhibited the K cells (Fig. 14*C*) via a compound IPSP, shown at a faster time base in Fig. 14*D*. The VWI was also shown to excite E_{he} cells (Fig. 14*B*) and to inhibit T_{pe} cells (Fig. 14*A*). It had no effect on S_{he} cells. Whether the responses of the motoneurons were because of monosynaptic connections is not known as in no

case have clear spikes leading to 1:1 postsynaptic potentials been recorded.

HEART SENSORY INPUT TO THE MOTONEURONS. Cardioactive motoneurons in the related snail, *Helix*, received chronosensory "feedback" from the heart, which allowed fine tuning of its activity (S.-Rozsa and Zhuravlev 1981). We looked for evidence of similar mechanoreceptor input to motoneurons in *Lymnaea* by applying pressure pulses to the heart or touching the heart with forceps. The S_{he} and H_{he} cells showed no responses when the heart was stimulated by either increased pressure applied at the perfusion heads or by direct touch. This was also true for E_{he} cells, described in the previous paper (Buckett et al. 1990b). However, both the T_{pe} and K_{hi} cells responded to heart stimulation by increasing their firing frequencies.

The response of the K_{hi} cell (frequency of spikes and duration of spike burst) increased as greater pressure was applied (Fig. 15*A*). Whether this feedback pathway is direct from (presumably) sensory cells in the heart or involves intermediate central interneuronal pathways has not been studied. However, two of the known interneuronal inputs to the K_{hi} cell (RPeD1 and input 3) played no apparent role. This was monitored in Fig. 15*A* by recording RPeD1 at the same time as K_{hi} during application of pressure. No activation of RPeD1 occurred either directly or because of input 3, which it normally received (e.g., Fig. 13*A*).

The T_{pe} cells were also excited when the heart was stimulated by sensory stimuli. Figure 15*B* shows excitatory responses resulting in spike bursts of a T_{pe} cell evoked by both pressure and tactile stimulation of the heart in the same preparation. In this example high-frequency spike bursts artificially evoked from the T_{pe} by current injection (↑↓) had no effect on the heart because the pericardium had been slit.

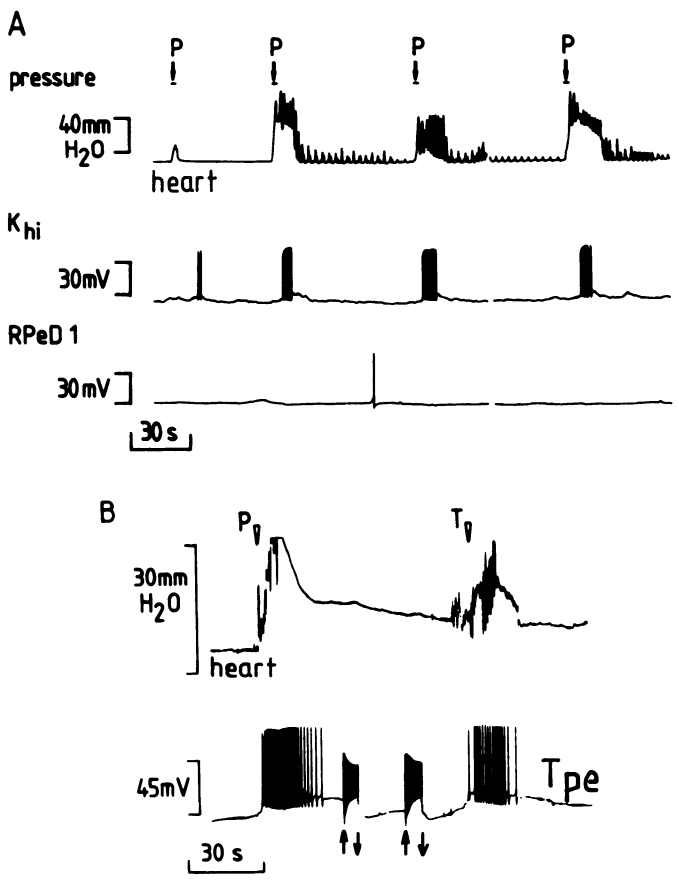

FIG. 15. Responses of the K_{he} and T_{pe} cells to mechanical stimulation of the heart. *A*: short-duration pressure pulses (P, *top*) applied via the perfusion reservoir cause K_{hi} spike bursts (*middle*) but have no effect on RPeD1 (*bottom*). Which neuron(s) are responsible for this K_{hi} excitation is unclear. It cannot be because of input 3 or VWI, which are both inhibitory on the K_{hi} (Fig. 14). *B*: increased pressure (P) applied to the heart via the perfusion reservoir results in a burst of T_{pe} cell spikes. Similar responses are evoked by touching the heart with forceps (T). Spike bursts evoked from the T_{pe} have no effect on heart pressure in this experiment because in the preparation used the pericardium (the target for the T_{pe} effects) has been slit.

DISCUSSION

This paper, together with the two preceding ones (Buckett et al. 1990a,b), examined the roles of specific motoneurons and putative transmitters in the control of the *Lymnaea* heart. The first identified likely transmitter substances by pharmacologic and biochemical methods (Buckett et al. 1990a). These were 5-HT, dopamine, and FMRFamide-like peptides (excitatory on the heart) and ACh (inhibitory). The second described a pair of cardioexcitatory motoneurons (E_{he} cells) and presented evidence that their effects were mediated by FMRFamide-like peptide (Buckett et al. 1990b). The present paper reported the discovery of other heart motoneurons and showed that their responses are likely to be mediated by classical transmitter substances. It also described three interneuronal inputs to these cells originating from central neurons and gave initial information on the role of sensory inputs to two classes of motoneuron.

Motoneurons

In the present paper, we described four new types of central neuron that acted on the heart to change its pattern of activity. These were the S_{he} cell, which, generally, had long-lasting excitatory effects on heart rate and beat amplitude, the H_{he} cells (up to 4), which increased rate but reduced amplitude, and the K_{hi} cell, whose main effect was to reduce amplitude and inhibit rate but which could also cause an initial transient excitation.

Evidence was presented that the S_{he}, H_{he}, and K_{hi} cells, were motoneurons, acting directly on the heart. This was based on their morphologies, all having major axons in the nerve that innervates the heart; on the occurrence of 1:1 JPs in heart muscle fibers following from spikes in their cell bodies; and on their continued ability to elicit responses from the heart in the presence of centrally applied Co^{2+} saline.

Transmitters

Pharmacologic evidence was presented suggesting that the effects on the heart of the S_{he} and H_{he} cells were mediated by 5-HT. Their excitatory actions were prevented by the 5-HT–selective antagonist, cinanserin. However, these

cells produced different effects on the heart. Responses due to S_{he} activation were long-lasting excitation of beat rate and amplitude, whereas those due to H_{he} cells were increases in tonus and rate but reduction in amplitude. Why they had such qualitatively different effects when evidence suggested they shared the same transmitter is not known. Perhaps this is because of release of a co-transmitter by one of the motoneurons. Another possibility might be the involvement of a second messenger in the case of the heart response to S_{he} activity.

One inhibitory motoneuron (K_{hi}) was identified. The likely transmitter of this cell was ACh. ACh mimicked the inhibitory effect on the heart of K_{hi} activation (Buckett et al. 1990a), and neuronal and chemical inhibition of heart were both blocked by preperfusion with α-Bgt. The blocking effect of α-Bgt on the initial transitory excitatory effect of normal stimulation was not tested.

The fourth cell type (T_{pe}) altered heart activity indirectly by causing contraction of the pericardial tissue surrounding the heart. As the drugs used in our experiments were normally applied to the inside of the heart, it was not possible to carry out tests to examine the transmitter used by this cell type.

Interneurons

Several types of interneurons within the CNS provided synaptic input to the motoneurons. Of these the so-called input 3 (arising from unidentified interneurons within the CNS) (3) was the most significant. This provided bursts of excitation to the serotonergic H_{he} cells, which excited the heart in a cyclical manner (see Fig. 13*B*). At the same time, input 3 inhibited the cholinergic K_{hi} cells, which were the only type of inhibitory motoneuronal type innervating the heart. These reciprocal effects reduce K_{hi} inhibitory effects on the heart in favor of the H_{he} cell excitation.

Synaptic inputs to heart motoneurons also came from the single identified interneuron called the VWI. This cell excited the E_{he} cells and inhibited the K_{hi} cells. This would, theoretically, allow the excitatory FMRFamide-mediated effect of the E_{he} cells on the heart to occur at the expense of K_{hi} cholinergic inhibition. However, as the VWI was normally silent in the heart-brain preparations used here, the precise circumstances under which the VWI could carry out this function were unknown.

Another potentially interesting interaction came from the previously reported mutual inhibiting connections between the VWI and input-3 interneuron (Benjamin 1984). Operationally, this would allow either serotonergic excitation of the heart via the H_{he} cells or FMRFamide excitation of the heart via the E_{he} cells depending on whether the VWI or input 3 was active. Again, the circumstances under which either of these situations might arise was unknown.

Finally, another identified interneuron, the dopamine-containing right-pedal giant cell (RPeD1) had excitatory synaptic connections with the H_{he} cells and inhibitory connections with the K_{hi} cells. Neither of these inputs was very strong but must be taken into account when a final model for interneuronal control of the heart is produced.

We conclude that the control of the *Lymnaea* heart is complex with both interneuronal-motoneuronal and interneuronal-interneuronal synaptic connections involved.

Sensory input from heart

Only the cells capable of inhibiting heart beating, the T_{pe} and K_{hi} cells, showed responses from mechanical stimulation of the heart. Both these cells were excited by increasing pressure in the heart. The direct inhibition of the heart by the K_{hi} cell may be part of the normal regulatory apparatus, acting as negative feedback to slow the heart after or during periods of increased heart activity (feeding or sex?). The T_{pe} cell does not innervate the heart itself but is involved in controlling the volume and pressure of the pericardial fluid. Because this cell often elicited a strong pressure increase response by apparently causing the heart to be compressed, it is possible that it plays a role in behaviors requiring rapid redistribution or removal of blood, such as whole-body withdrawal.

Similarities with heart control in Aplysia

Results reported in the present study can be compared to those of *Aplysia*, the other molluscan species where cardioactive motoneurons, interneurons and transmitters, have been described. In these animals, two excitatory (RB_{HE} and LD_{HE}) and a pair of inhibitory motoneurons ($LD_{HI\text{-}1}$ and $LD_{HI\text{-}2}$) were found that acted on the heart (Mayeri et al. 1974). As in *Lymnaea*, no direct connections between motoneurons were found, but they were activated or inhibited by a network of three interneuronal inputs (Koester et al. 1974).

The RB_{HE} motoneuron produced similar long-duration chronotropic responses to the S_{he}, although negatively inotropic responses were also recorded (Mayeri et al. 1974). Like the S_{he} cell, this excitatory neuron was blocked by cinanserin, suggesting that its effects were mediated by 5-HT (Leibeswar et al. 1975). The inhibitory *Aplysia* motoneurons, $LD_{HI\text{-}1}$ and $LD_{HI\text{-}2}$, had very similar effects to the *Lymnaea* K_{hi} cell, and evidence that ACh was its transmitter was also presented (Leibeswar et al. 1975). The transmitter of LD_{HE} in *Aplysia* was unknown, and no cells similar in action to the E_{he} cells of *Lymnaea* were identified.

Similarities in interneuronal control of heart motoneurons were suggested by comparing data for *cell L10* of *Aplysia* (Koester et al. 1974) with that of input 3 of *Lymnaea*. Like input 3, *L10* fired in spontaneous bursts with a period of ~1 s, during which it excited a serotonergic heart excitor and inhibited a cholinergic heart inhibitor. Similar motoneuronal responses occurred in *Lymnaea* (input 3 excited the serotonergic H_{he} cells and inhibited the cholinergic K_{hi} cells) suggesting an analogous function for *L10* and input 3.

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