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NEURAL CONTROL OF HEART BEAT IN THE AFRICAN GIANT SNAIL, *ACHATINA FULICA* FÉRUSSAC

II. INTERCONNECTIONS AMONG THE HEART REGULATORY NEURONES

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SUMMARY

The synaptic connections between identified heart regulatory neurones were examined in the central nervous system of the African giant snail, *Achatina fulica* Férussac. Two cerebral ganglion cells, the dorsal right and left cerebral distinct neurones (d-RCDN and d-LCDN), were found to have excitatory connections with several neurones in the suboesophageal ganglia (the periodically oscillating neurone, PON, the tonically autoactive neurones, TAN, TAN-2 and TAN-3, and the visceral intermittent firing neurone, VIN) and the connections are probably monosynaptic. VIN had a weak electrical coupling with PON. VIN inhibited TAN, TAN-2 and TAN-3, and the connections were considered to be monosynaptic. At the same time, TAN, TAN-2, TAN-3 and the visceral ganglion neurone (VG1) inhibited PON and VIN although the connections are unlikely to be monosynaptic. Another neurone in the pedal ganglia, the dorsal left pedal large neurone (d-LPeLN), was found to excite PON, VIN, TAN, TAN-2 and TAN-3. These connections were not monosynaptic. These results are interpreted in relation to heart regulation in *Achatina*.

INTRODUCTION

In the heart regulatory network of *Aplysia*, there are no direct interactions between heart regulatory motoneurones: their coordinated firing patterns result from the action of interneurones (Mayeri *et al.* 1974). This organization is also found in *Helix* (S.-Rózsa, 1979*a*) but is not present in the African giant snail, *Achatina fulica* Férussac, in which three tonic heart excitors (TAN, TAN-2 and TAN-3) are known to be interconnected by weak electrical synapses (Furukawa & Kobayashi, 1987).

In the preceding paper, seven neurones (PON, TAN, TAN-2, TAN-3, d-RCDN, d-LCDN and VG1) were identified as heart regulatory neurones in the central nervous system of *Achatina* (Furukawa & Kobayashi, 1987). In this paper, synaptic interconnections among these neurones are examined to clarify the structure and function of the heart regulatory network of *Achatina*. Identification of heart regulatory interneurones is also carried out and their relationships to the previously

Key words: identified neurone, neural connection, molluscan neurone.

identified heart regulatory neurones are examined. Finally, a diagram illustrating the heart regulatory network is presented.

MATERIALS AND METHODS

The preparation and the recording methods were essentially as described in the preceding paper (Furukawa & Kobayashi, 1987). In a few cases, the isolated ganglia preparation was used. In some experiments, the neurone was loaded with Cs^+ by using a recording electrode filled with $2\,mol\,l^{-1}$ CsCl. When testing for monosynapticity, the ganglia were perfused with high-Ca^{2+} and high-Mg^{2+} solution which contained three times more divalent ions than the normal physiological solution as described by Cohen, Weiss & Kupfermann (1978). The osmolarity was partially compensated by reducing NaCl. The composition of this solution was as follows (in $mmol\,l^{-1}$): NaCl, 25; KCl, 3·3; $CaCl_2$, 32·1; $MgCl_2$, 39; glucose, 5; Hepes, 10 (pH adjusted to 7·5 by titration with NaOH). All experiments were performed at room temperature (20–25 °C).

RESULTS

Synaptic connections between the heart regulatory neurones described in the preceding paper (Furukawa & Kobayashi, 1987) were examined by recording simultaneously from two or three cells in various combinations. The heart regulatory network is complex and its features are briefly described here before the presentation of evidence (see also Fig. 9).

Four heart excitatory motoneurones (PON, TAN, TAN-2 and TAN-3) and an interneurone (VIN) receive excitatory inputs from two cerebral neurones (d-RCDN and d-LCDN) and a pedal neurone (d-LPeLN). There are no connections from the heart excitatory motoneurones or VIN back onto the cerebral neurones and d-LPeLN.

VIN, electrically coupled with PON, inhibits TAN, TAN-2 and TAN-3. At the same time, TAN, TAN-2 and TAN-3 inhibit PON and VIN.

Interneurone VG1 inhibits PON and VIN. There are no connections among d-RCDN, d-LCDN, d-LPeLN and VG1.

The excitatory action of d-RCDN and d-LCDN on TAN, TAN-2 and TAN-3

Evoked activity of two cerebral neurones, d-RCDN and d-LCDN, was found to increase the firing frequencies of TAN, TAN-2 and TAN-3. Fig. 1A shows an example in the case of TAN-3. In Fig. 1B, underlying synaptic inputs in TAN-3 are shown by hyperpolarizing TAN-3 and making d-LCDN fire at 2 Hz. Although it was rather difficult to analyse the synaptic responses (the amplitude of an individual EPSP was usually less than 1 mV), close inspection revealed a one-to-one relationship between spikes and EPSPs. These EPSPs were not blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution, which would be expected to block polysynaptic pathways by raising the spike threshold of any interneurone. Under this condition, each EPSP was more easily discernible, probably because of the effect of raised $[Ca^{2+}]$ on synaptic transmission.

In molluscan neurones, cell bodies usually lack synapses and synaptic contacts may be rather far from the soma where the recording electrode is inserted. Synaptic potentials recorded in the soma are therefore generally small in amplitude. To

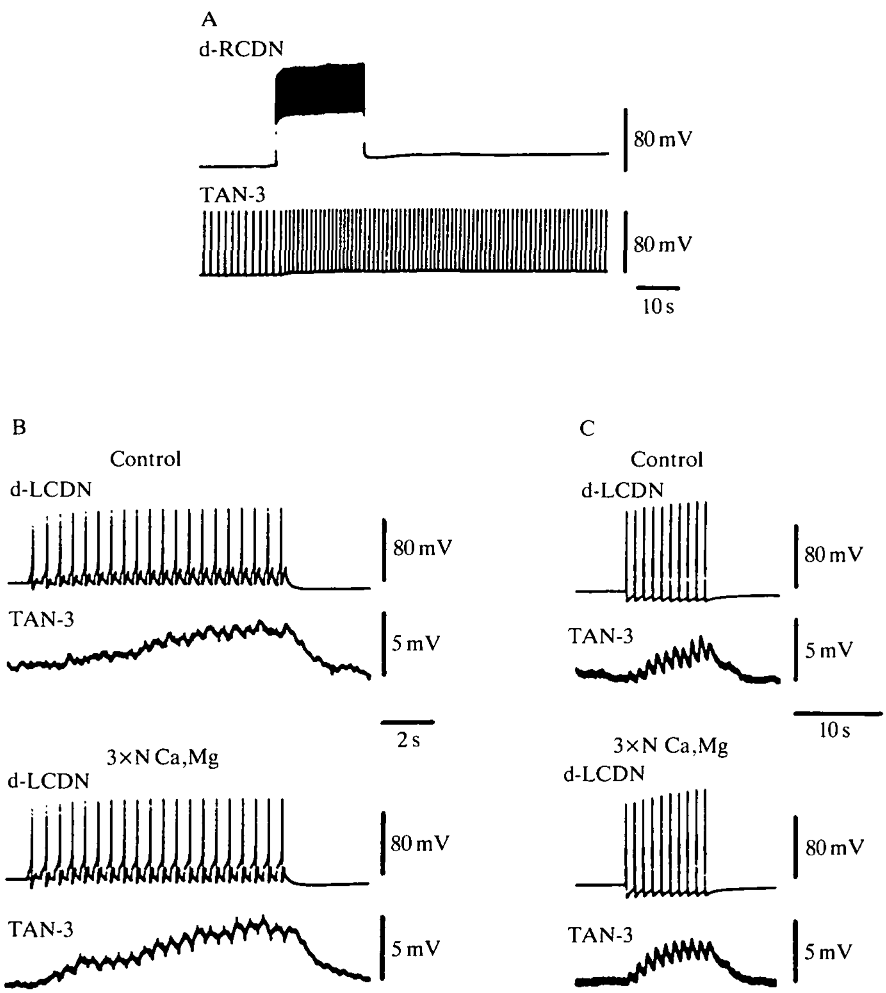

Fig. 1. (A) Effect of a burst of spikes in d-RCDN on the spontaneous activity of TAN-3. d-RCDN was driven to fire by current injection (20 s, 16 nA). (B) EPSPs of TAN-3 produced by the evoked spikes in d-LCDN and the effect of high-Ca^{2+} and high-Mg^{2+} solution (3×N Ca,Mg) on these EPSPs. d-LCDN was driven to fire by current injection at 2 Hz. The membrane potential of TAN-3 was set at −120 mV. The recording electrode for TAN-3 was filled with potassium acetate. (C) EPSPs of Cs^+-loaded TAN-3 produced by evoked spikes in d-LCDN and the effect of high-Ca^{2+} and high-Mg^{2+} solution on these EPSPs. d-LCDN was driven to fire by current injection at 1 Hz. The membrane potential of TAN-3 was set at −80 mV. The recording electrode for TAN-3 was filled with CsCl. Note the discrete EPSPs under these conditions. Latency of EPSP in this figure was 100 ms.

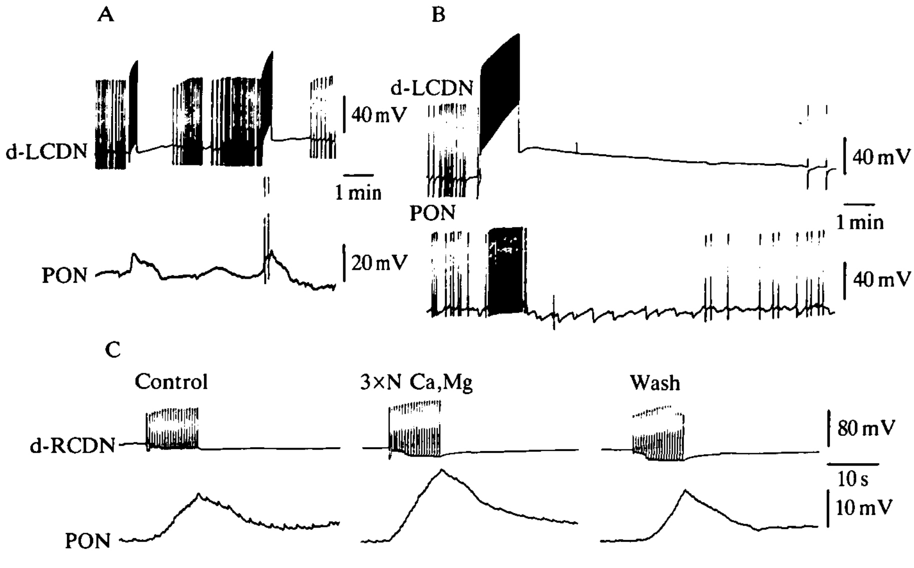

Fig. 2. The excitatory action of d-RCDN and d-LCDN on PON. (A) Slow depolarizing response in PON induced by a burst of spikes in d-LCDN. (B) Effects of longer-duration burst in d-LCDN on PON. (C) Effect of high-Ca^{2+} and high-Mg^{2+} solution (3×N Ca,Mg) on the slow depolarizing response in PON induced by d-RCDN. d-RCDN was driven to fire at 2 Hz. The membrane potential of PON was set at −100 mV. The recording electrode for PON was filled with CsCl.

overcome this problem, the postsynaptic cell was penetrated by a CsCl-filled electrode to load Cs^+ into the cell. Cs^+ is known to block K^+ channels (Akaike, Lee & Brown, 1978) and to raise the membrane input resistance, thus producing an increase in PSP amplitude. Fig. 1C shows results in TAN-3 recorded using a CsCl-filled electrode. As predicted, the action potentials of d-LCDN produced discrete EPSPs which showed facilitation and summation in TAN-3, and they were not blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution. The latency between the cerebral cell spike and the beginning of the EPSP was fairly constant, being about 100 ms. Although this seems to be rather long, comparable values have been reported in other molluscan ganglia (Berry & Cottrell, 1975).

The excitatory action of d-RCDN and d-LCDN on PON

A moderate burst of spikes induced in d-LCDN produced a slow depolarization of PON (Fig. 2A). A longer-duration burst in d-LCDN drove PON into activity and this was followed by delayed inhibitory synaptic inputs (Fig. 2B). These inhibitory inputs to PON were presumably due to activation of interneurone(s) driven by the burst of spikes in d-LCDN. Fig. 2C illustrates synaptic inputs produced by driven spikes in d-RCDN at a frequency of 2 Hz. In this experiment, the recording electrode penetrating PON was filled with CsCl and the membrane potential of PON was set at −100 mV. The spikes in d-RCDN produced a slow depolarization in PON which was not blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution, but increased in amplitude.

The excitatory action of d-RCDN and d-LCDN on VIN

VIN is a periodically firing neurone in the isolated ganglia preparation and has no output axons to the periphery (Ku & Takeuchi, 1983; Goto, Ku & Takeuchi, 1986). However, in the present preparation that included the heart, the spontaneous activity of VIN was greatly reduced to the level seen in PON (Furukawa & Kobayashi, 1987). When VIN and PON were recorded simultaneously, many common inhibitory inputs were seen and the active phase of both neurones was correlated (Fig. 3A). These two neurones were weakly electrically coupled (Fig. 3B). However, in contrast to PON, the evoked activity in VIN did not produce heart excitation.

VIN also received excitatory inputs from d-RCDN and d-LCDN. The effect of spikes in d-RCDN on VIN is illustrated in Fig. 3C. In this experiment, VIN was penetrated by a CsCl-filled electrode and the membrane potential of VIN was set at

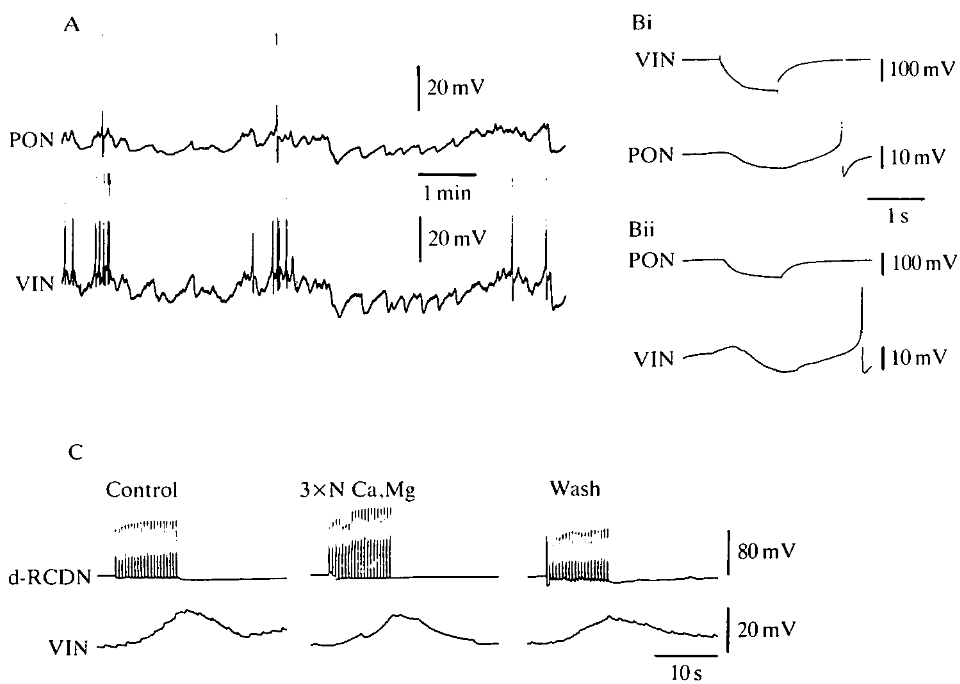

Fig. 3. (A) Simultaneous recording from PON and VIN. Note the numerous common inhibitory inputs. (B) Weak electrical coupling between PON and VIN. Hyperpolarizing current was injected into VIN (Bi) or PON (Bii). (C) Slow depolarizing response in VIN induced by d-RCDN and the effect of high-Ca^{2+} and high-Mg^{2+} solution (3×N Ca,Mg) on this response. d-RCDN was driven to fire at 2 Hz. The membrane potential of VIN was set at −100 mV. The recording electrode for VIN was filled with CsCl.

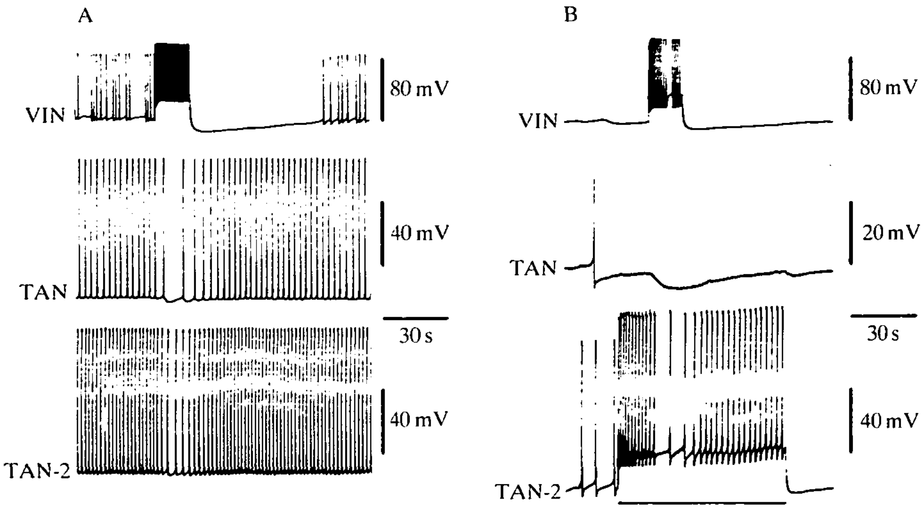

Fig. 4. Inhibitory action of VIN on TAN and TAN-2. (A) Inhibition of the activity in TAN and TAN-2 produced by the burst of spikes in VIN. (B) Effect of high-Ca^{2+} and high-Mg^{2+} solution on the inhibitory response. TAN-2 was depolarized by current injection during the period indicated by the bar. VIN was driven to fire by current injection (15 s, 6 nA).

−100 mV. The spikes of the cerebral neurones produced a slow depolarization in VIN and this effect was not blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution (Fig. 3C).

The inhibitory action of VIN on TAN, TAN-2 and TAN-3

A burst of spikes in VIN depressed the spontaneous activity of TAN, TAN-2 and TAN-3 (Fig. 4). In the experiment shown in Fig. 4B, the ganglia were perfused with high-Ca^{2+} and high-Mg^{2+} solution. In this state, spontaneous activity of TAN almost ceased and a clear hyperpolarizing potential was seen in response to the burst of spikes in VIN. At the same time, TAN-2 was depolarized to fire in order to show the inhibition clearly; the driven activity of TAN-2 was also depressed by the burst of spikes in VIN. A similar result was also obtained in TAN-3 (not shown). The burst of spikes in VIN had no effect on the other heart regulatory neurones described in the preceding paper.

The inhibitory action of TAN, TAN-2 and TAN-3 on PON and VIN

Evoked activity in TAN, TAN-2 and TAN-3 produced an inhibition of PON and VIN (Fig. 5Ai,Bi). However, if the heart was dissected away by cutting the intestinal nerve, this inhibition was not seen (Fig. 5Aii,Bii). Thus, the inhibitory action of TAN, TAN-2 and TAN-3 is considered to be an indirect one, and appears to be dependent on the heart activity. TAN, TAN-2 and TAN-3 had no effects on the other identified heart regulatory neurones.

The inhibitory action of VG1 on PON and VIN

The activity of VG1 was found to inhibit PON and VIN. The strength of this inhibitory action was somewhat variable from preparation to preparation (Fig. 6). In a few preparations, one action potential in VG1 was enough to produce the hyperpolarizing responses in PON and VIN (Fig. 6Ai,C). In most cases, a burst of spikes was needed to inhibit these postsynaptic cells (Fig. 6Bi,D). In either case, PON and VIN appeared to receive common inhibitory input (Fig. 6C,D). This inhibitory action of VG1 was also lost if the heart was dissected away (Fig. 6Bii), as for the effect of TAN, TAN-2 and TAN-3 on PON and VIN. There was no clear action of VG1 on the other identified heart regulatory neurones.

The excitatory action of d-LPeLN on PON, VIN, TAN, TAN-2 and TAN-3

Boyles & Takeuchi (1985) described pharmacological characteristics of three giant neurones in the pedal ganglia of *Achatina*. In the present study, one of these neurones, d-LPeLN, was found to excite PON, VIN, TAN, TAN-2 and TAN-3.

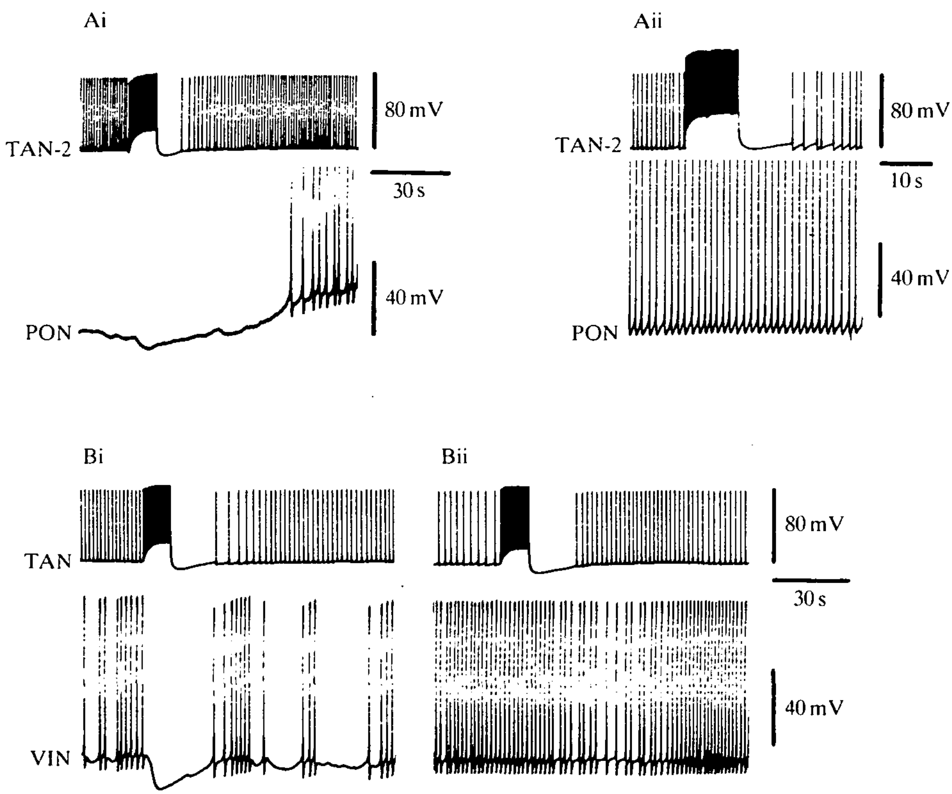

Fig. 5. (A) Inhibitory action of TAN-2 on PON (Ai) and the effect of cutting the intestinal nerve on this response (Aii). The firing frequency of TAN-2 was increased by current injection (10 s, 6 nA). (B) Inhibitory action of TAN on VIN (Bi) and the effect of cutting the intestinal nerve on this response (Bii). The firing frequency of TAN was increased by current injection (10 s, 6 nA).

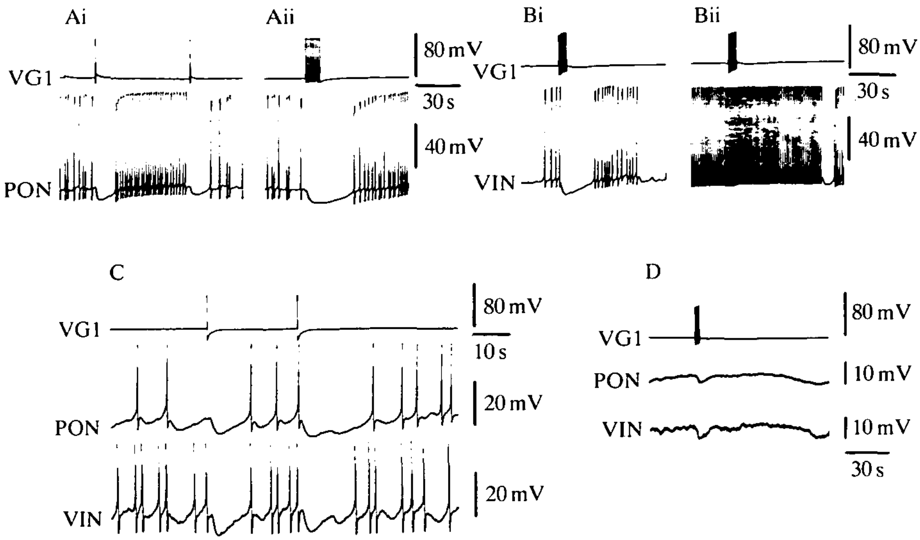

Fig. 6. Inhibitory action of VG1 on PON and VIN. (A) Inhibitory action of VG1 on PON. VG1 was driven to fire by current injection. Ai shows the effect of a single action potential, and Aii shows that of repetitive spikes (1 Hz, 10 spikes) on PON. Both records are taken from the same preparation. (B) Inhibitory action of VG1 on VIN (Bi) and the effect of cutting the intestinal nerve on this response (Bii). VG1 was driven to fire by current injection (5 s, 4 nA). (C) Simultaneous recording from VG1, PON and VIN showing spontaneous activity. (D) Simultaneous hyperpolarizations of PON and VIN induced by a burst of spikes in VG1. VG1 was driven to fire by current injection (3 s, 4 nA).

d-LPeLN is situated in the left pedal ganglion (Fig. 7), and has main axonal processes in both sides of the suboesophageal ganglia and dendritic arborizations in the pedal ganglia. The main axonal processes separate into several branches which go into the left anterior pallial accessory nerve 1, the left anterior pallial accessory nerve 2, and the intestinal nerve. The branches in the intestinal nerve are not considered to go to the heart, as antidromic action potentials were not recorded when the intestinal nerve entering the pericardium was stimulated.

In the experiments shown in Fig. 8, the isolated ganglia preparation was used so that PON and VIN showed periodic bursting activity. A burst of spikes in d-LPeLN induced by current injection clearly increased PON (Fig. 8Ai) and TAN (Fig. 8Aii) activity. The effect on VIN was not as striking because of its strong spontaneous activity (Fig. 8Aiii). In Fig. 8B, the underlying synaptic potential in PON is shown by hyperpolarizing PON. The excitatory action of d-LPeLN was completely blocked if the ganglia were perfused with high-Ca^{2+} and high-Mg^{2+} solution (Fig. 8B), suggesting that this connection is polysynaptic. Similar results were also obtained in the cases of TAN, TAN-2, TAN-3 and VIN (data not shown). d-LPeLN had no effect on the other identified heart regulatory neurones.

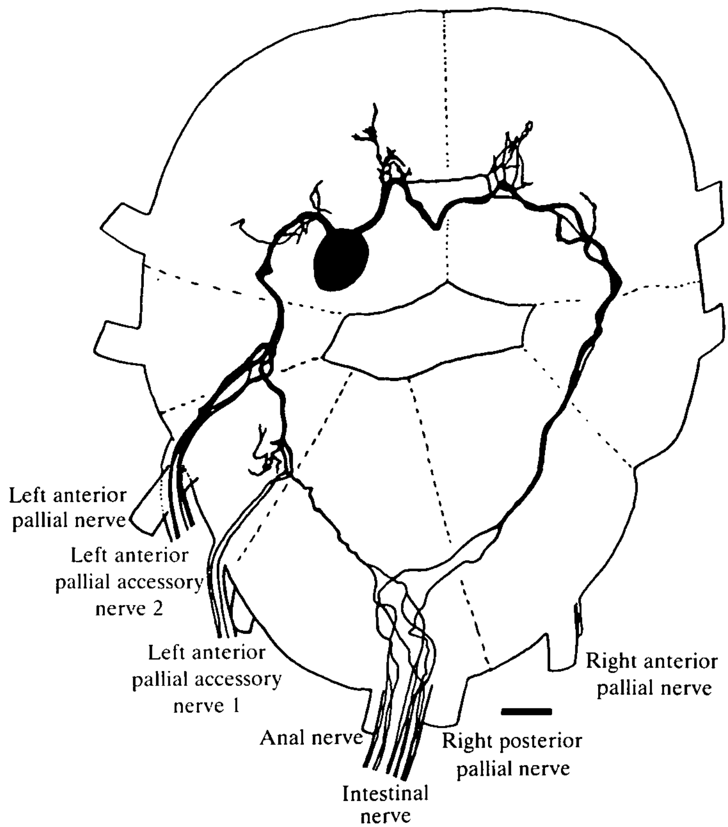

Fig. 7. Morphology of d-LPeLN stained by the injection of Lucifer Yellow. Scale bar, 200 μm.

DISCUSSION

In the present study, synaptic connections between the heart regulatory neurones of the African giant snail, *Achatina fulica* Férussac, are described, as summarized in Fig. 9. As there were no different properties among TAN, TAN-2 and TAN-3, these neurones will be described simply as TAN in the following section.

Two cerebral neurones, d-RCDN and d-LCDN, were found to be higher-order neurones in the heart regulatory network, in addition to their role in direct excitation of the heart, described in the preceding paper. The activity of these cells increased the firing frequency of TAN and produced a slow depolarization in PON and VIN. Each spike in the two cerebral cells produced a one-to-one EPSP in TAN with constant latency, and these EPSPs showed facilitation and summation. One-to-one relationships were not certain in PON and VIN but summated slow depolarization was produced by evoked activity in the two cerebral neurones. Neither type of response was blocked when the ganglia were perfused with high-Ca^{2+} and high-Mg^{2+} solution. These results, although not conclusive, suggest that the connections are monosynaptic, as a high concentration of Ca^{2+} would be expected to block interneuronal connections by raising the spike threshold (Berry & Pentreath, 1976).

These cerebral neurones also seemed to drive the inhibitory pathways in the heart regulatory network, as rather strong bursts of spikes in these cells produced delayed inhibition of PON (see Fig. 2B). According to the results mentioned above, d-RCDN and d-LCDN may function as 'command' elements in the heart regulatory network. Although comparable neurones to these cells have not been reported as far as we are aware, their function may have some similarity to that of MCC in *Aplysia*, which has several follower cells in the buccal ganglia and also has modulatory actions on the buccal muscles (Kandel, 1976; Weiss, Cohen & Kupfermann, 1978). The homologous neurones to *Aplysia* MCC have been reported in other molluscan species (Cottrell & Macon, 1974; Gillette & Davis, 1977; McCrohan & Benjamin, 1980).

VIN is an interneurone whose axon bifurcates. The branches go to the left and right pedal ganglion, respectively (Goto *et al.* 1986). This neurone had a weak electrical coupling with PON, and received common inhibitory inputs with PON. The activity of this neurone was also correlated to that of PON. Moreover, the actions of other known presynaptic neurones on VIN and PON were also similar. However, a burst of spikes in VIN did not produce heart excitation but inhibited TAN, in contrast to PON spikes which excited the heart but did not inhibit TAN.

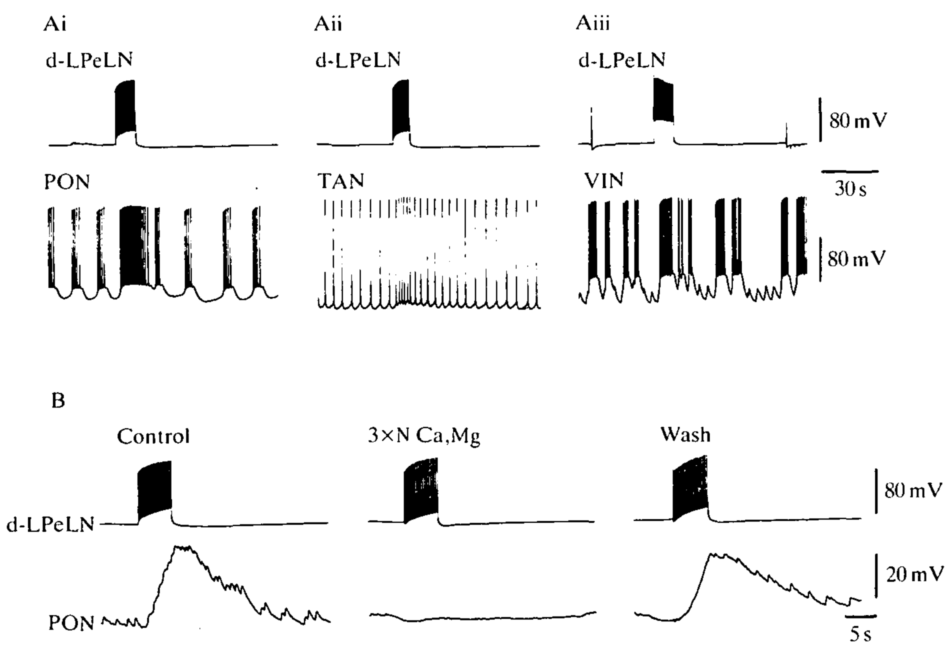

Fig. 8. Excitatory action of d-LPeLN on PON, TAN and VIN. (A) Effects of a burst of spikes in d-LPeLN on the spontaneous activity of PON (Ai), TAN (Aii) and VIN (Aiii). d-LPeLN was driven to fire by current injection (10 s, 10 nA). (B) Slow depolarizing response in PON induced by d-LPeLN and the effect of high-Ca^{2+} and high-Mg^{2+} solution (3×N Ca,Mg) on this response. The membrane potential of PON was set at −90 mV. The recording electrode for PON was filled with CsCl. d-LPeLN was driven to fire by current injection (5 s, 20 nA).

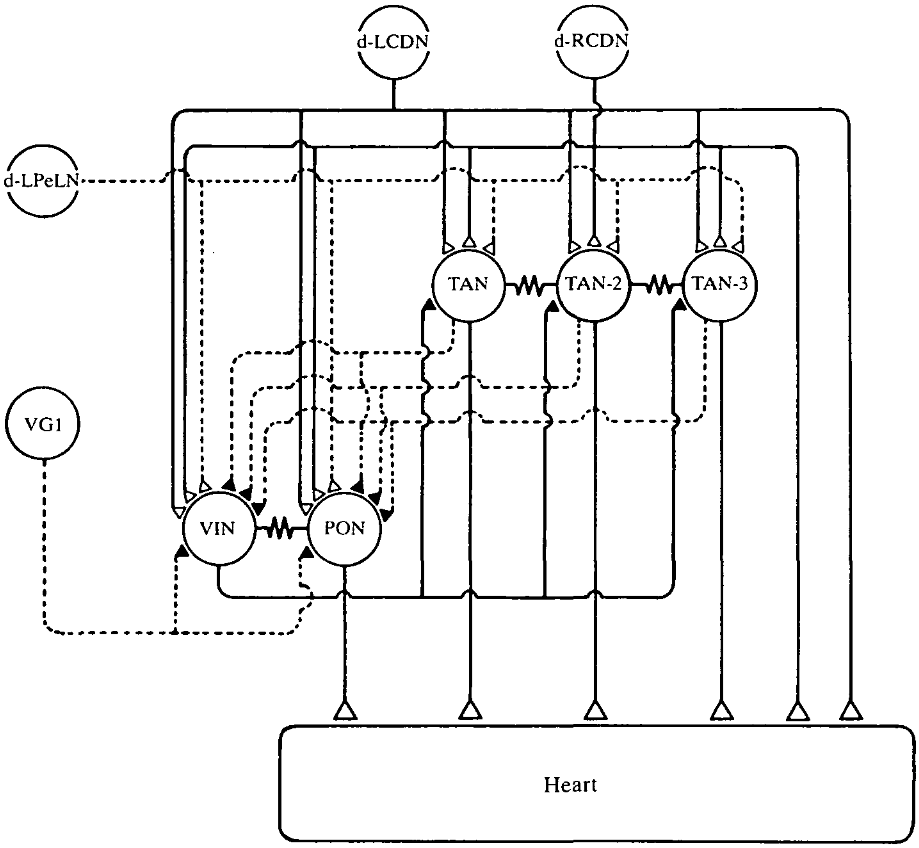

Fig. 9. Schematic diagram showing synaptic connections between the heart regulatory neurones. Open and closed triangles indicate excitatory and inhibitory synapses, respectively. Zigzag lines indicate weak electrical couplings. Broken lines indicate the involvement of the other neurones in the pathways.

The connection from VIN to TAN is considered to be monosynaptic as the response was not blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution. VIN may function as a negative feedback to the heart by depressing the activity of TAN (tonic heart excitor) when the activity of PON (the most effective heart excitor) is high. In contrast, increased activity of TAN produced inhibition of VIN and PON although the connections were not considered to be direct. Thus, some of the inhibitory inputs usually seen in PON and VIN are considered to originate from the activity of TAN. This inhibitory pathway from TAN to PON may be important for heart regulation as the heart excitatory action of PON is rather acute (Furukawa & Kobayashi, 1987).

VG1 is the only identified neurone whose activity produces heart inhibition and this inhibitory action is probably mediated by other neurones (Furukawa & Kobayashi, 1987). This neurone also inhibited PON and VIN indirectly. From these results, this cell may be considered as an interneurone in the heart regulatory network. The inhibitory pathways from VG1 to PON and VIN are rather complex as the inhibitory action was not seen when the heart was dissected away by cutting the intestinal nerve. This property applies also to the inhibitory pathways from TAN to PON and VIN. These results suggest the involvement of the peripheral systems in these inhibitory pathways. In *Helix pomatia*, several neurones receive inputs from the cardiorenal system (S.-Rózsa, 1979*a*, 1981). Similar results have also been reported in *Achatina* (S.-Rózsa, 1979*b*). The sensory elements were not examined in the present experiments but they may interpose in the inhibitory pathways described here. Further experiments will be needed to clarify this problem.

One of the pedal ganglion cells, d-LPeLN, identified by Boyles & Takeuchi (1985), was found to excite PON, VIN and TAN. The connections are not considered to be monosynaptic, as the responses were completely blocked by perfusion of the ganglia with high-Ca^{2+} and high-Mg^{2+} solution. d-LPeLN may be an interneurone of the heart regulatory network or form a link to other neural networks. Connections between some pedal neurones and heart excitatory motoneurones have also been reported in *Helix* (S.-Rózsa, 1981).

In *Aplysia* (Mayeri *et al.* 1974; Koester, Mayeri, Liebeswar & Kandel, 1974) and *Helix* (S.-Rózsa, 1979*a*), the heart regulatory motoneurones are not interconnected and higher-order interneurones can produce their actions on the heart by activating different motoneurones. This principle is not found in the heart regulatory network of *Achatina*, because some monosynaptic or polysynaptic connections between heart regulatory motoneurones are found in this snail. Thus, for example, any neurone which increases the activity of TAN should result in the inhibition of PON through the inhibitory pathways from TAN to PON, if that cell did not excite PON at the same time (see Fig. 9).

In the present and preceding papers, nine neurones involved in heart regulation of *Achatina* have been identified. They are giant neurones of more than 100 μm in diameter and can be easily identified. This makes them a promising system for future analysis of the neural control of heart regulation and the relationships between heart regulation and other behaviour in this mollusc.

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