Neural Control Mechanisms of the Heart Beat in the African Giant Snail, *Achatina fulica* Fèrussac

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CHAPTER I

INTRODUCTION

The purpose of this work is to understand the mechanisms of the neural control of the heart in the African giant snail, *Achatina fulica* Fèrussac, (gastropoda, pulmonata) on the cellular level. In this chapter, the background of this work is described as well as the usefulness of molluscs in the studies of neurobiology.

Molluscan nerve cells in neurobiology

In the field of neurobiology, molluscs provide the most useful preparations for the study of neuronal signaling and neural network. Some molluscan animals (pulmonata and opisobranchia) have the largest nerve cells in the animal kingdom and they are situated at the surface of the ganglia. In large specimens of a sea hare, *Aplysia*, and a sea slug, *Tritonia*, the diameter of the largest cells reaches to 1 mm (Dorsett, 1986). These conditions are quite opposite to those in the vertebrate brain where most neurones are situated deep in the brain and small in size. Needless to say, the number of neurones is far less in the molluscan ganglia; i.e. the number of cells in the pulmonate ganglia is estimated to be in the order of 10,000 (Dorsett, 1986). Thus, in some molluscan ganglia, many neurones can be identified convincingly in different specimens and easily studied by the intracellular microelectrodes. These identifiable giant neurones promoted the detailed investigations about biophysical properties of several ion channels (Meech, 1986) and actions of neurotransmitters and

other chemicals (Walker, 1986). Many discoveries in molluscan nerve cells (e.g. several ion channels) have later been found in the vertebrate central neurones. Thus, the molluscan nerve cells are suitable to study the mechanisms of neuronal signaling.

It seems to be evident that one of the ultimate goals of neurobiology is to understand the behaviors of animals including man on the cellular and molecular levels. Although we must be cautious to deduce the neuronal mechanisms in the higher vertebrates from the results obtained in the invertebrate preparations, the molluscs provide nonetheless useful model systems to analyze the cellular basis of behavior because of their identifiable neurones and the small number of neurones. Many behaviors of the molluscs are now extensively studied on the cellular level; e.g. "swimming" of *Tritonia* (Hume *et al.*, 1982; Hume & Getting, 1982a,b), "locomotion" of *Aplysia* (Fredman & Jahan-Parwar, 1983), and "feeding" of several gastropods (Bulloch & Dorsett, 1979a,b; Benjamin & Rose, 1979; Benjamin *et al.*, 1979; Rose & Benjamin, 1979; Gillette *et al.*, 1982; Cohen *et al.*, 1978). Probably, most exciting examples are the sensitization of gill-withdrawal reflex in *Aplysia* and the associative learning of a sea slug, *Hermissenda* (Kandel & Schwartz, 1982; Alkon, 1984), where some important molecular aspects underlying the behavioral changes have been shown.

Considering the usefulness of molluscan central nervous systems for the cellular analysis of the somatic functions, it is a rather wonder that the studies of the vegetative functions are much less. This may be derived from the subtleness of vegetative functions compared to the somatic

functions which can be clearly seen as the *behavior* in the intact animals. However, the vegetative functions are undoubtedly important for survival of animals, and are considered to have correlations with the somatic functions. Thus, the investigation of the neural regulations of vegetative functions has importance as well as that of somatic functions in neurobiology of molluscs.

Neural regulation of the molluscan heart

One interesting vegetative function in mollusc is the circulation. The molluscan heart is myogenic, i.e. its beating originates from the pace maker activity of muscle itself. However, the heart receives the dual innervations by the central nervous system and its beating is regulated by the neuronal activities (see review, Hill & Welsh, 1966). On the cellular level, the neural regulation of the heart activity has been studied mainly in two species (*Aplysia californica* and an edible snail, *Helix pomatia*). In *Aplysia*, two heart excitors, two heart inhibitors and higher order interneurones have been identified (Mayeri *et al.*, 1974; Koester *et al.*, 1974; see also review, Koester & Koch, 1987). In the heart regulatory network of *Aplysia*, there are no direct interactions between heart regulatory motoneurones and their coordinated firing patterns result from the action of interneurones. In *Helix*, two heart inhibitors, four heart excitors and one interneurone have been found as well as many neurones which receive synaptic inputs from the cardio-renal system (S.-Rôzsa & Salânki, 1973; S.-Rôzsa, 1979a; S.-Rôzsa, 1981). The heart regulatory network of *Helix* is considered to be similar to that of *Aplysia*.

The African giant snail

The African giant snail, *Achatina fulica* Fèrussac, is the large land snail (Fig. 1.1) and is distributed in the subtropical and tropical zones. In Japan, this snail inhabits in Okinawa and Ogasawara, and is more suitable to obtain as the experimental animals in large quantities than other land snails. The identifiable giant neurones of *Achatina* have been extensively used for the studies of actions of several neurotransmitter candidates and their analogues by Takeuchi and his colleagues (see Takeuchi *et al.*, 1984). However, the functions of such identifiable neurones are not yet known. The heart regulatory network of this snail has been studied by S.-Rôzsa (1979b), but the heart excitatory motoneurones have not been identified.

In the present work, heart regulatory neurones in the central ganglia of *Achatina* were identified and synaptic interconnections among these neurones were examined to clarify the structure and function of the heart regulatory network of *Achatina*. Also, certain synaptic connections among the heart regulatory network were studied in detail.

Fig. 1.1. African giant snail, *Achatina fulica* Fèrussac.

CHAPTER II

INNERVATION OF THE HEART

In this chapter the anatomy of the central nervous system and the heart is described as the introductory section for grasping the innervation of the heart in the African giant snail, *Achatina*. Experimental results indicating the dual innervations (inhibitory and excitatory) of the heart are also presented.

Central nervous system

The central nervous system of *Achatina* is composed of three ganglia; the buccal, the cerebral and the suboesophageal ganglia (Goto *et al.*, 1986). Fig. 2.1 shows the schematic drawing of the central nervous system of this snail. The buccal ganglia are connected to the cerebral ganglia by the cerebro-buccal connectives. The suboesophageal ganglia are connected to the cerebral ganglia by the cerebro-pedal and the cerebro-pleural connectives. The suboesophageal ganglia are divided into seven components; the right and left pedal, the right and left pleural, the right and left parietal and the visceral ganglia.

There are many nerve bundles going to the periphery, and one of them, the intestinal nerve, goes to the heart.

Fig. 2.2 is a picture showing the visceral and right parietal ganglia. The connective capsule and the inner sheath covering the dorsal surface of two ganglia were removed to expose nerve cells. Under this condition, many giant neurones are clearly seen.

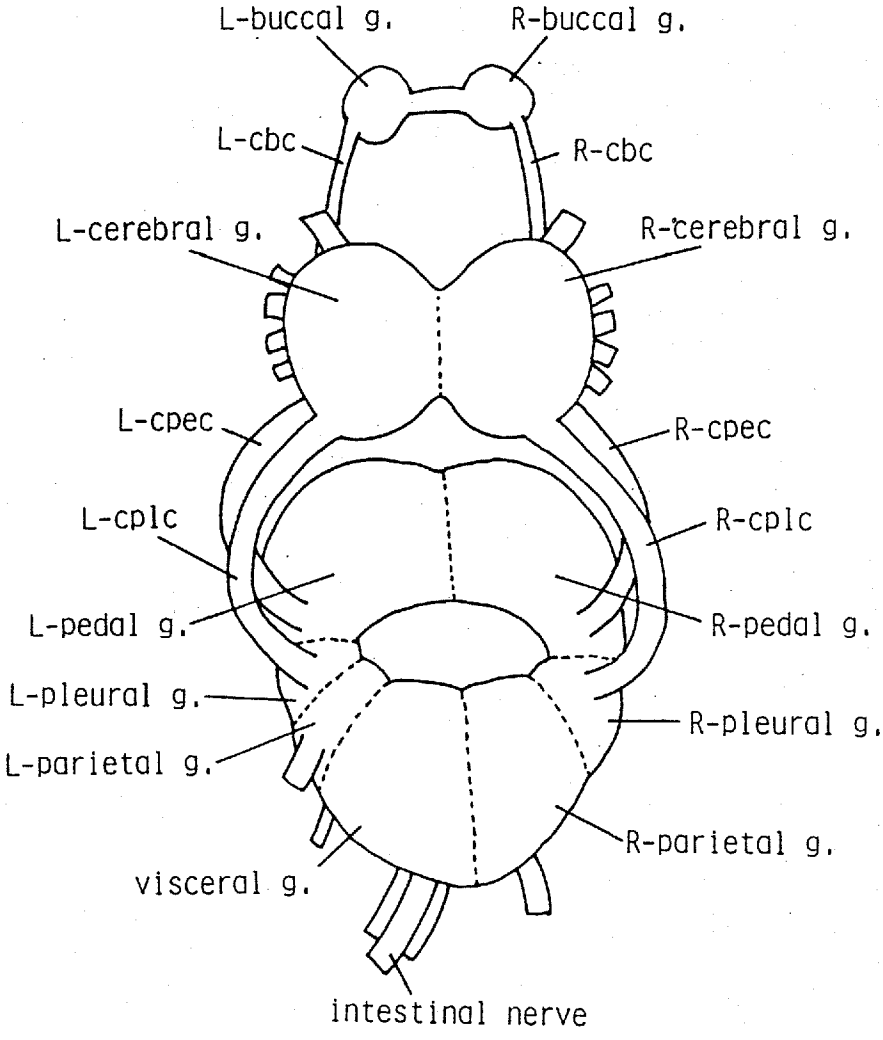

Fig. 2.1. Schematic drawing of the central nervous system of *Achatina*. L-buccal g (left buccal ganglion), R-buccal g (right buccal ganglion), L-cbc (left cerebro-buccal connective), R-cbc (right cerebro-buccal connective), L-cerebral g (left cerebral ganglion), R-cerebral g (right cerebral ganglion), L-cpec (left cerebro-pedal connective), R-cpec (right cerebro-pedal connective), L-cplc (left cerebro-pleural connective), R-cplc (right cerebro-pleural connective), L-pedal g (left pedal ganglion), R-pedal g (right pedal ganglion), L-pleural g (left pleural ganglion), R-pleural g (right pleural ganglion), L-parietal g (left parietal ganglion), R-parietal g (right parietal ganglion), visceral g (visceral ganglion). All nerve bundles arising from the buccal and pedal ganglia as well as small bundles from other ganglia are omitted in this figure.

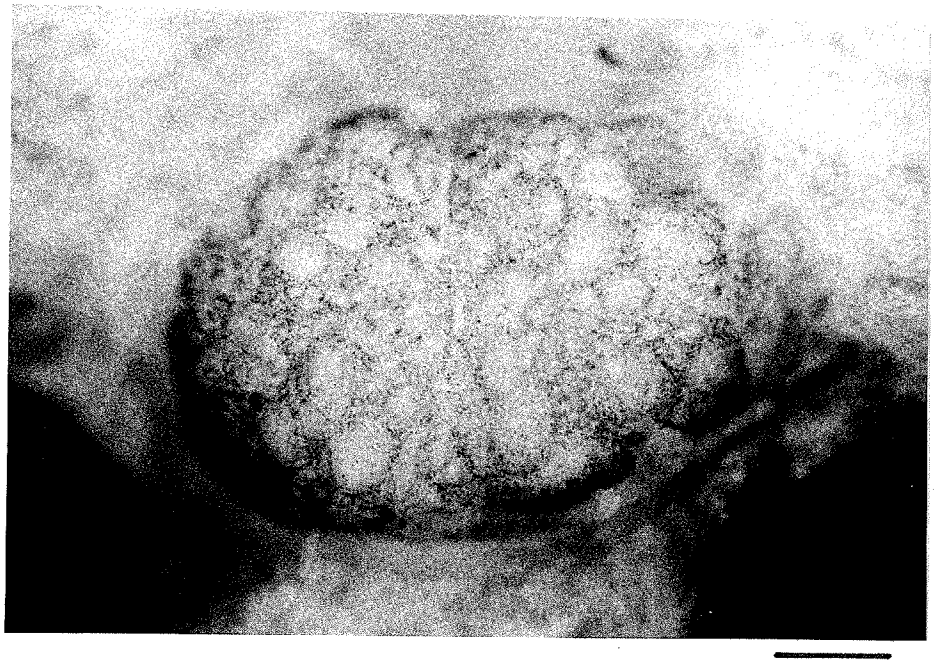

Fig. 2.2. A part of suboesophageal ganglia. The connective capsule and the thin inner sheath covering the dorsal surface of the visceral and right parietal ganglia were removed, and the preparation was lightly stained with 0.1 % methyleneblue. Several large cell bodies can be clearly seen. Scale: 200 μm.

Innervation of the heart

The circulation of this snail is the open circulation like most other molluscs (see Hill & Welsh, 1966). The heart consists of one auricle and one ventricle. There are two valves in this heart; one is between the auricle and the ventricle, the other between the ventricle and the aorta. These valves inhibit the back-flow of body fluid. The body fluid is collected to the auricle through the pulmonary vein and pumped out through the aorta.

Fig. 2.3 illustrates the heart and its innervation in *Achatina*. The heart is innervated by the intestinal nerve. The intestinal nerve bifurcates to two similar sized nerve bundles near the genital organs and one of them goes to the heart. This bundle further branches off at some points near the heart and most of small branches go to the kidney. The largest branch goes to the auricle along the pericardium. The heart is also innervated by the small branch going to the aorta.

Modulation of the heart beat

The heart of *Achatina* is myogenic like that of other molluscs, i.e. isolated heart can continue to beat by the pacemaker activity of the heart muscle itself. The example of myogenic beating of the heart and its modulation by the stimuli of the intestinal nerve is shown in Fig. 2.4. The preparation was the isolated ventricle with the intestinal nerve, and the central ganglia were cut away. The muscle contraction was monitored by the strain gauge.

Myogenic beating of the heart was stopped by the electrical stimuli of the intestinal nerve given at low frequency and the slight increase in peak tension was also

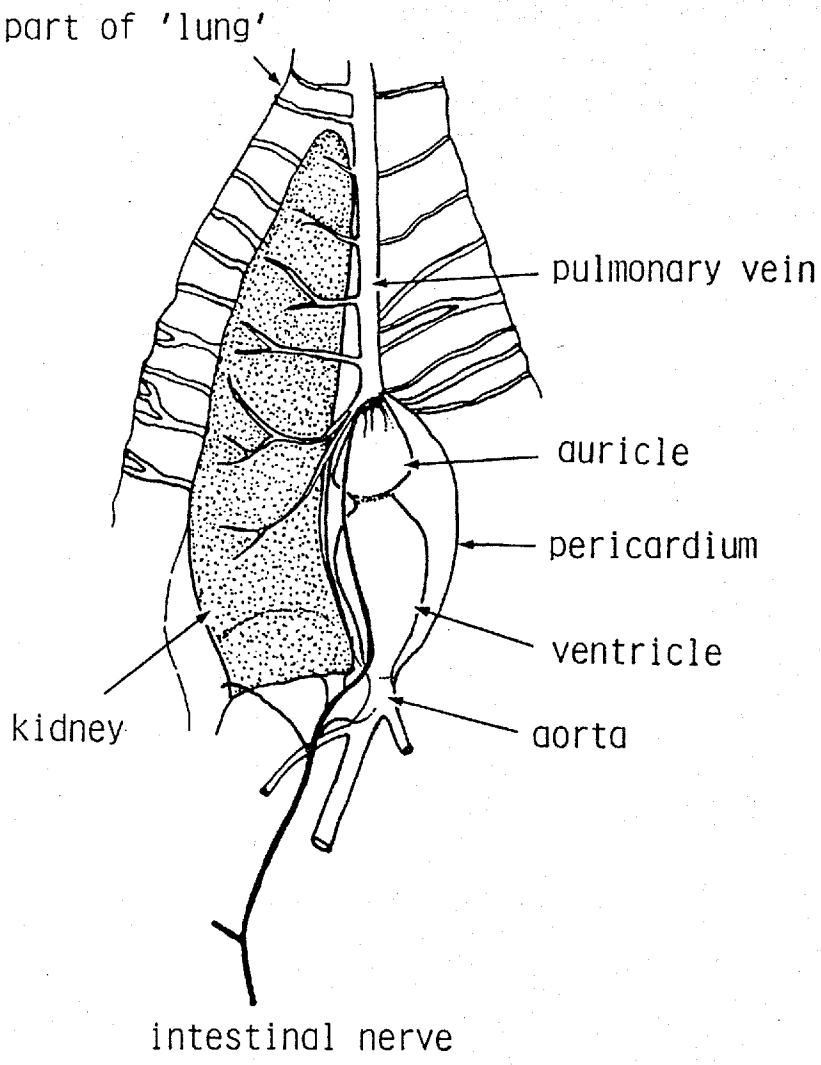

Fig. 2.3. The *Achatina* heart and its innervation by the intestinal nerve. The heart is enclosed by the pericardium and the branch of the intestinal nerve going to the auricle is attached on the pericardium.

seen after the inhibition (Fig. 2.4A). The stimuli at high frequency produced more pronounced excitatory action (Fig. 2.4B). Although there was a variability in the necessary strength of the stimuli for producing such modulation, in a given preparation weaker stimuli tended to produce the inhibition mainly and stronger stimuli produced the inhibition followed by the excitation. These results indicate that the heart of *Achatina* receives the dual innervations (inhibitory and excitatory) through the intestinal nerve.

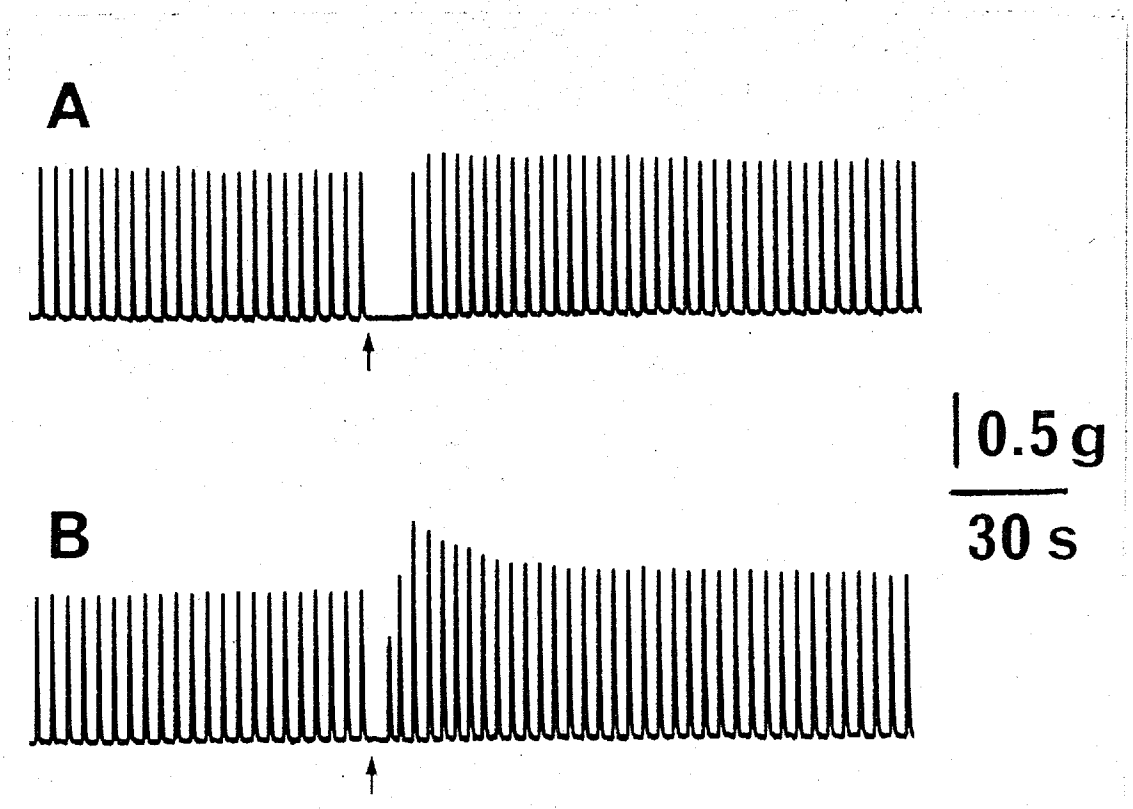

Fig. 2.4. Myogenic beating of the isolated ventricle of *Achatina* and its modulation by stimulation of the intestinal nerve (arrow). The intestinal nerve was stimulated by brief pulses (0.2 ms, 8 V), at the frequency of 2 Hz in A and 10 Hz in B. The number of stimulus was 20 in both cases.

CHAPTER Ⅲ

EXPERIMENTAL PREPARATIONS AND METHODS

The African giant snail, *Achatina fulica* Fėrussac, captured in Okinawa and transported by air to Hiroshima, was bred in our laboratory at 24 ℃ .

< Preparations >

Heart-ganglia preparation

This preparation consisted of the cerebral ganglia, suboesophageal ganglia, intestinal nerve and the heart. Usually, the pericardium was cut around the base of the ventricle and the heart was exposed to record the heart beat efficiently. This treatment involved transection of the smaller nerve branch going to the aorta (see Fig. 2.3 in Chap. Ⅱ). Thus, the innervation of the heart muscle by this branch is neglected. In a few experiments, the smaller branch going to the aorta was left intact and the pericardium was cut from the auricle side. As this treatment destroyed the main innervation to the auricle, the preparation was used to investigate the innervation of the ventricle by the smaller nerve branch going to the aorta.

The preparation was pinned to the bottom of a chamber covered with a silicone resin (Fig. 3.1). The recording chamber was separated into two compartments by a partition having a slit through which the intestinal nerve was led. The slit was sealed by silicone grease, which enabled perfusion of both compartments (ganglia compartment and heart compartment) independently. The perfusion rate was

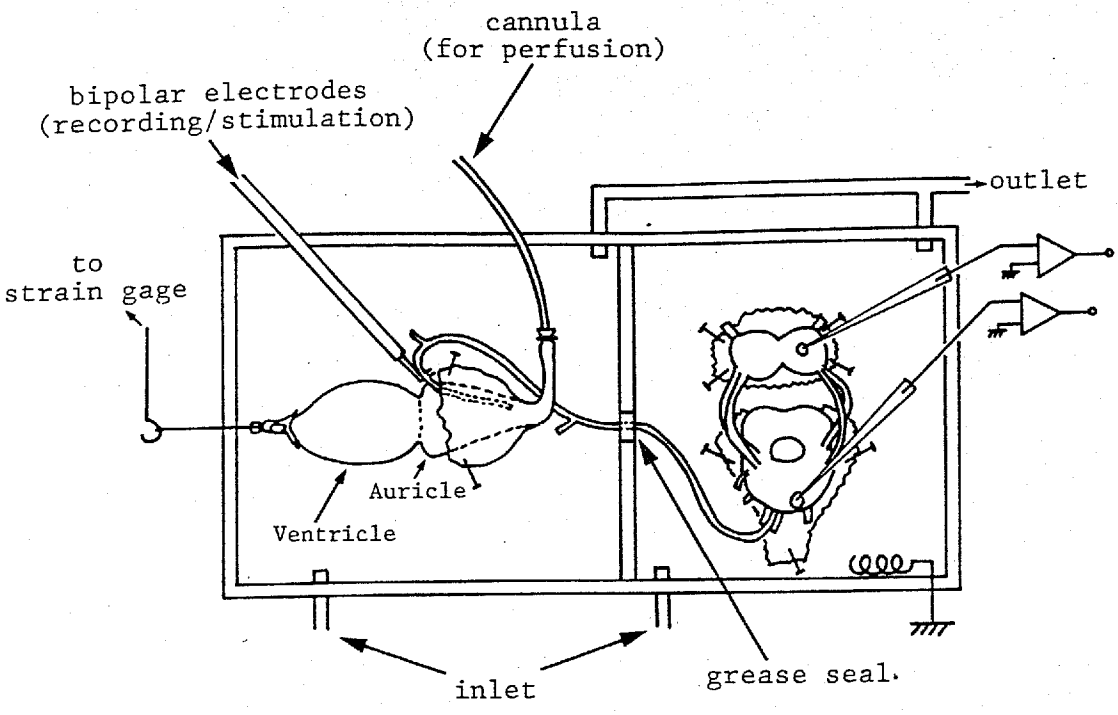

Fig. 3.1. Schematic diagram of the experimental set-up for simultaneous recording of the heart and neuronal activities.

2-5 ml/s. The heart was also perfused internally with the normal physiological solution (see Table 3.1) by means of a cannula inserted into the vein, unless otherwise noted in the following chapter. Usually, the outer thick connective capsule but not the inner thin sheath covering the ganglia was removed by dissection. In some cases, the inner thin sheath was also carefully removed to expose the nerve cells.

This preparation was used in the experiments described in chapters IV & V .

Isolated-ganglia preparation

This preparation consisted of the suboesophageal ganglia and the cerebral ganglia. The thin inner sheath covering the ganglia was always removed with the thick connective capsule. Effective volume of the chamber was reduced to 0.5 ml for this preparation.

This preparation was used mainly in the experiments described in chapter VI .

Isolated cell-body preparation

In some experiments described in chapter VI , the nerve cell-body of the neurone PON (see Fig. 4.1 & 4.2) was isolated by the axotomy. The axotomy of PON was done by cutting the septum between the visceral and the right-parietal ganglia where the axon of this cell is running (Chap. IV ; see also Goto *et al.*, 1986). No synaptic input was seen after this operation. The preparation was kept in the perfused normal physiological solution for more than 30 min before the experiment, and those cells, which had the resting potential of more than -40 mV and showed the all-or-none action potential when a depolarizing current was

injected, were used.

< Recording procedures >

Simultaneous recording of the heart beat and the neuronal activity

Intracellular recording and stimulation of neurones were carried out using microelectrodes filled with 3 M KCH_3COO, of 5-10 MΩ resistance. In some cases, a second microelectrode was inserted into a cell for current injection. Heart beat was monitored using a strain gauge connected to the aorta by a fine thread. The aorta was cut open between the ligating point and the ventricle to allow exudation of the perfusate. In a few cases, an extracellular recording of the intestinal nerve activity was made at the point just before entering the pericardium using an Ag-AgCl bipolar electrode. This electrode was also used for stimulation.

Voltage-clamp

Neurone was voltage-clamped by the two microelectrodes method (Furukawa & Kobayashi, 1986). Both recording and current-passing electrodes were silver-painted to within 2 mm of the tip, insulated with nail polish and filled with the mixture of 3 M KCH_3COO and 0.1 M KCl. The resistance of voltage-recording electrode was about 5 MΩ and that of current-passing electrode was 2-3 MΩ . The silver screen of each electrode was driven by positive feedback from a unity gain in the head stage, which reduced the stray capacitance of electrodes and improved the frequency response of the system. A grounded shield was placed between two electrodes to minimize coupling between electrodes. The gain of loop

was about 4000. Under these conditions, the rise time of a square voltage pulse was less than 200 μs. Membrane currents were read as a voltage drop across a 1 MΩ resistor interposed in the feed-back loop or were measured by a virtual ground circuit. The indifferent electrode was the 3 M KCl-Agar bridge with Ag-AgCl electrode which was connected to ground or the input of a virtual ground circuit. Series resistance compensation was carried out by subtracting appropriate fraction of current signal from the summing point of voltage-clamp circuit so as to give the fastest capacitive transient.

In some experiments, Cs^+ or EGTA was injected into the neurone ionophoretically by using a separate electrode which contained 2 M CsCl or 0.5 M EGTA. The injection was carried out under voltage-clamped condition at the intensity of 0.5 μA for 10 min.

Patch-clamp

The activity of a single ion channel of the nerve cell-body was recorded by the improved patch-clamp method (Hamill, *et al.*, 1981). The feedback resistor of current-voltage converter was 10 GΩ. Usually, the resting potential of the neurone was recorded simultaneously by the microelectrode. The bath was connected to the earth via the indifferent electrode which was the same as that described above. The patch-pipette had the outer tip diameter of 1-3 μm and the tip was fire-polished. The pipette was filled with suitable solution for the recording of interested ion channel. When the pipette solution was different from the bath solution, a liquid-junction potential was developed. The measured potential was corrected for such potential

after the experiment. Exposed nerve cell was treated by 0.5 % trypsin (2000 u/g, WAKO PURE CHEMICAL INDUSTRIES) for 15-20 min for the cleaning of cell surface, which was essential for the formation of "giga-seal" (see below). After the pipette was pressed against the membrane of cell-body, gentle suction was applied. This produced the high resistance seal (10-100 GΩ) between the pipette-rim and the membrane in most cases. Such seal is usually called a giga-seal. After a giga-seal was made, single channel activity could be recorded by varying the potential inside the patch-pipette. This recording condition is called the "cell-attached" configuration, because the pipette was attached on the cell. In some experiments, single channel recording was made in the "inside-out" configuration (Hamill *et al.*, 1981). The patch of membrane attached to the pipette-tip can be isolated from the cell by pulling the pipette away from the cell, without destroying the membrane patch containing ion channels. The isolated patch is called the inside-out patch because the intracellular surface of the patch is exposed to the external solution. Further details for the conditions of single channel recording will be given in the appropriate position in this paper.

< Data analysis >

The data were displayed on an oscilloscope (NIHON KOHDEN, VC-10) and stored on an FM tape recorder (SONY, A-85) for later analysis. The permanent records were produced using a pen-recorder (NIHON KOHDEN, PMP-3000) or a X-Y recorder (YOKOGAWA, type 3077) with the aid of a transient memory (8 bits, 1024 bytes, clock rate 10 ms-5 μs) equipped to VC-10. For the subtraction of capacitive and linear leak

currents, currents elicited by identical hyperpolarizing and depolarizing command pulses were summed by the signal averager (NIHON KOHDEN, DAT-1100). In some cases, digitized data by the A/D converter of VC-10 were stored on floppy disks for later treatments with a computer (NEC, PC-9801 VM2). Single channel data were filtered at 300 Hz or 1 kHz by the low-pass filter equipped to VC-10. All electrical signals in the figures of this paper were displayed in the conventional way; e.g. outward current across the membrane was described as a positive sign and upward.

< Intracellular staining of the neurones >

Intracellular staining was made by pressure injection of 5 % Lucifer Yellow CH (Sigma Chemical Co.) based on the standard method of Stewart (1978). The ganglia were fixed in 4 % formaldehyde for 12 h at 4 ℃, then they were dehydrated, cleared in methylbenzoate, and viewed in wholemount using a fluorescence microscope. The stained neurone was photographed at several depths and reconstructed.

< Experimental solutions and drugs>

The composition of experimental solutions is shown in Table 3.1. To block the chemical synapses in the ganglia, the ganglia compartment was perfused with 3Mg, Ca-free solution. When testing for monosynapticity, the ganglia were perfused with 3Ca, 3Mg solution which contained three times higher divalent ions than the normal physiological solution as described by Cohen *et al.* (1978).

5-Hydroxytryptamine creatinine sulfate (5-HT, Sigma) was dissolved in the experimental solution and applied by

bath perfusion. Duration of the application was usually less than 5 min and at least 20 min interval was allowed between the 5-HT applications for reproducible responses. The 5-HT antagonist, methysergide-hydrogenmaleinate (methysergide, Sandoz) was also applied by bath perfusion. These drugs were freshly dissolved before experiments as 1 mM stock solution, and stored in the refrigerator for later use.

Most experiments were done at room temperature (20-25 ℃). In the experiments described in Chap. Ⅵ, the temperature of the perfusate was maintained at 24 ℃ by a thermo-electric device.

TABLE 3.1. Composition of experimental solutions (mM)

solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	$BaCl_2$	TrisCl	TEACl	$CoCl_2$	Glu[2]	HEPES
NPS[1]	61	3.3	10.7	13	–	–	–	–	5	10
3Mg, Ca-free	38	3.3	–	39	–	–	–	–	5	10
3Mg, 3Ca	25	3.3	32.1	39	–	–	–	–	5	10
3K	54.4	9.9	10.7	13	–	–	–	–	5	10
Na-, K- ,Ca-free	–	–	–	13	–	64.3	–	10.7	5	10
Na-, Ca-free, 10K	–	33	–	13	–	31.3	–	10.7	5	10
Ca-free	61	3.3	–	13	–	–	–	10.7	5	10
TEA, Ba[3]	–	3.3	–	13	10.7	–	61	–	5	10
Tris	–	3.3	–	23.7	–	61	–	–	5	10
85mM K	–	85	–	13	–	–	–	–	–	10
70mM Ba	–	–	–	–	70	–	–	–	–	10
High-K, Ca-free[4]	–	100	–	–	–	–	–	–	5	10

pH adjusted to 7.5 by titration with HCl or NaOH.

1) NPS, normal physiological solution.

2) Glu, glucose.

3) 5 mM 4-AP (4-aminopyridine) was also added in some cases.

4) 5 mM EGTA was added and pH was adjusted to 7.5 by adding 30 mM KOH. Thus, final K concentration of this solution was 130 mM.

CHAPTER IV

IDENTIFICATION OF THE HEART REGULATORY NEURONES

Location of the heart regulatory neurones in the ganglia

The cell bodies of neurones which were identified as members of the heart regulatory network were typically located in the ganglia as shown in Fig. 4.1. All neurones except VG1 have been described previously (Takeuchi *et al.*, 1975; Ku & Takeuchi, 1983; Ku *et al.*, 1985; Boyles & Takeuchi, 1985; Matsuoka *et al.*, 1986). Each neurone except VG1 could be identified easily by its cell size, location and colour. The location of VG1 is rather variable from preparation to preparation and there are two other similar sized cells near VG1. Of these three cells, however, VG1 is the only one which has its axon in the intestinal nerve. Thus, VG1 could be identified by the demonstration of antidromic action potentials in response to stimulation of the intestinal nerve.

The heart excitatory action of PON

Recent morphological investigations (Goto *et al.*, 1986; Matsuoka *et al.*, 1986) show that PON, TAN, TAN-2 and TAN-3, but not VIN, have axons in the intestinal nerve, and PON has multiple axons only in this nerve. Fig. 4.2 shows the morphology of PON revealed by the injection of Lucifer Yellow. The primary axon of this cell is relatively thick and goes to the visceral ganglion where it branches off to several axons. These results confirmed the previous observation. One clear feature of this cell is that all

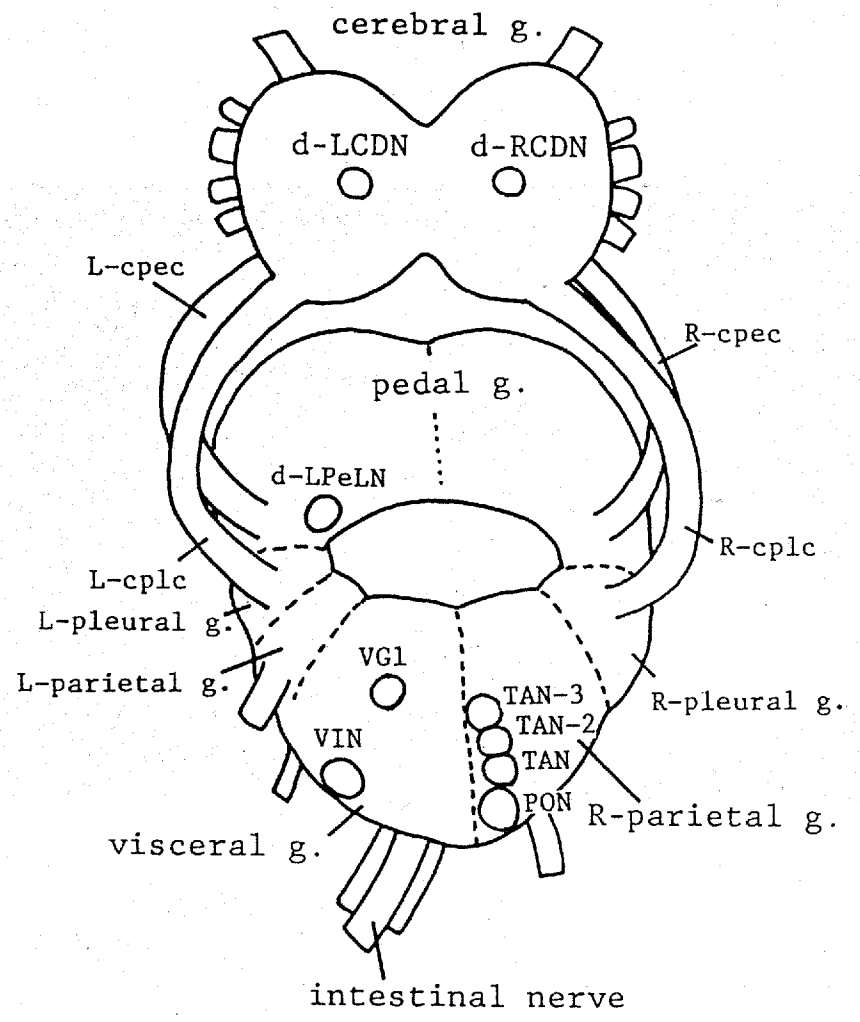

Fig. 4.1. Schematic drawing of the cerebral and suboesophageal ganglia showing the positions of neurones which were examined in this paper. d-LCDN (dorsal left cerebral distinct neurone), d-RCDN (dorsal right cerebral distinct neurone), d-LPeLN (dorsal left pedal large neurone), VIN (visceral intermittent firing neurone), TAN (tonically autoactive neurone), PON periodically oscillating neurone), L-cpec (left cerebro-pedal connective), R-cpec (right cerebro-pedal connective), L-cplc (left cerebro-pleural connective), R-cplc (right cerebro-pleural connective).

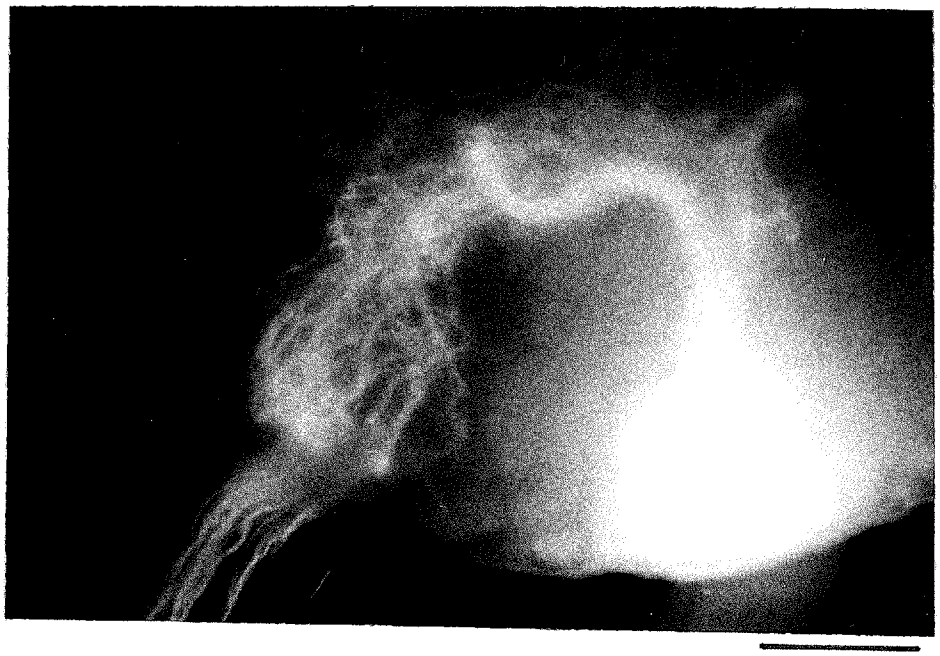

Fig. 4.2. Morphology of PON stained by the injection of Lucifer Yellow. Scale: 200 μm.

branched axons are going to the periphery through the intestinal nerve and the functional significance of such morphology will be discussed in chapter VI . Unfortunately, however, the terminal of this and other neurones could not be stained, because the distance from the suboesophageal ganglia to the heart is rather long (3 to 4 cm).

PON can be identified as a bursting neurone in the isolated ganglia preparation (Takeuchi *et al.*, 1975). However, in the present, more intact, preparation, PON usually showed irregular firing and received numerous inhibitory inputs (Fig. 4.3A). When the intestinal nerve was cut and the ganglia were isolated from the heart, PON began to show periodical bursting (Fig. 4.3B). This result is considered to suggest that the bursting activity of PON is usually depressed by inhibitory inputs originating from the periphery.

The spontaneous activity of PON was correlated with an increase in peak tension and in beating frequency of the heart. When PON was inhibited, the heart beat was also depressed (Fig. 4.4A). If PON was driven to fire by depolarizing current injection, the heart rate and beating amplitude increased dramatically (Fig. 4.4B). The heart excitatory action of PON was so strong that only 5 to 6 action potentials at a frequency of 1 Hz were enough to produce positive inotropic and chronotropic actions. Usually, 10 to 20 spikes at a frequency of 1-2 Hz produced 50-70 % increase in beat amplitude and 30-70 % increase in heart rate. These results suggest that PON is one of the heart excitatory neurones.

Because the intracellular staining of terminals of neurones was not successful as described above,

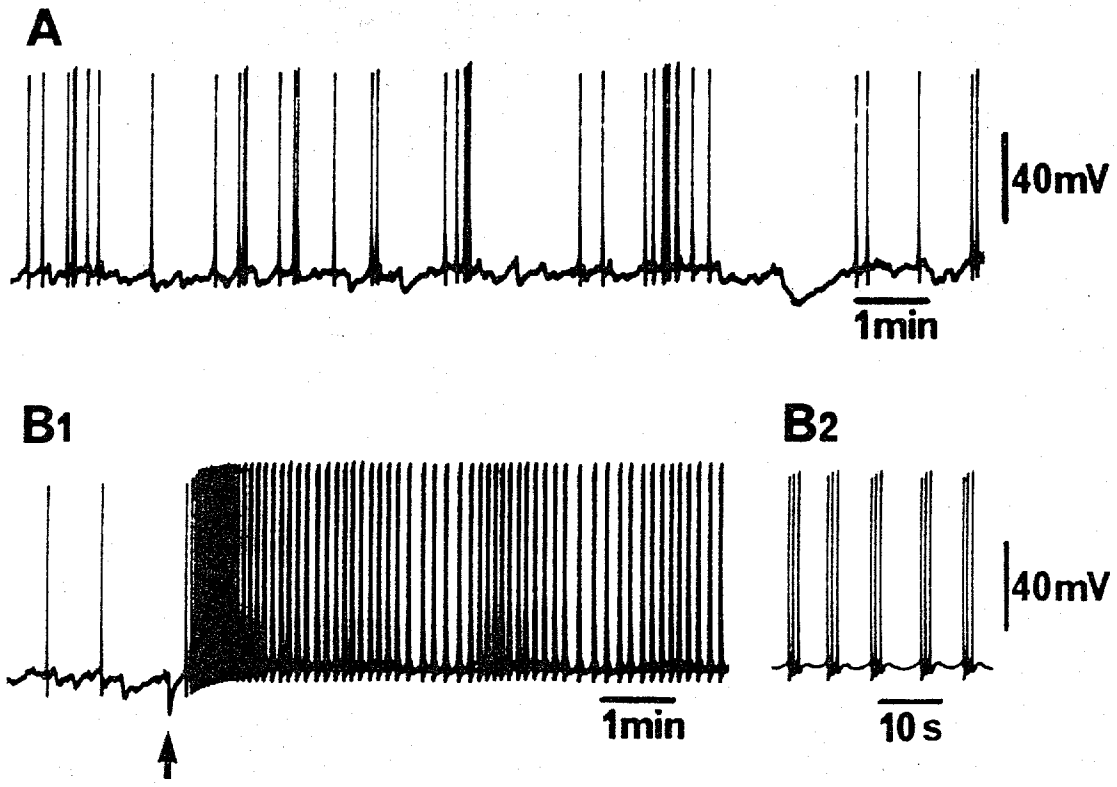

Fig. 4.3. A:Activity of PON. B1:Effect of cutting the intestinal nerve (arrow) on the activity of PON. B2:High speed recording of the burst-like activity in B1 following cutting of the intestinal nerve. A and B are records from the same preparation.

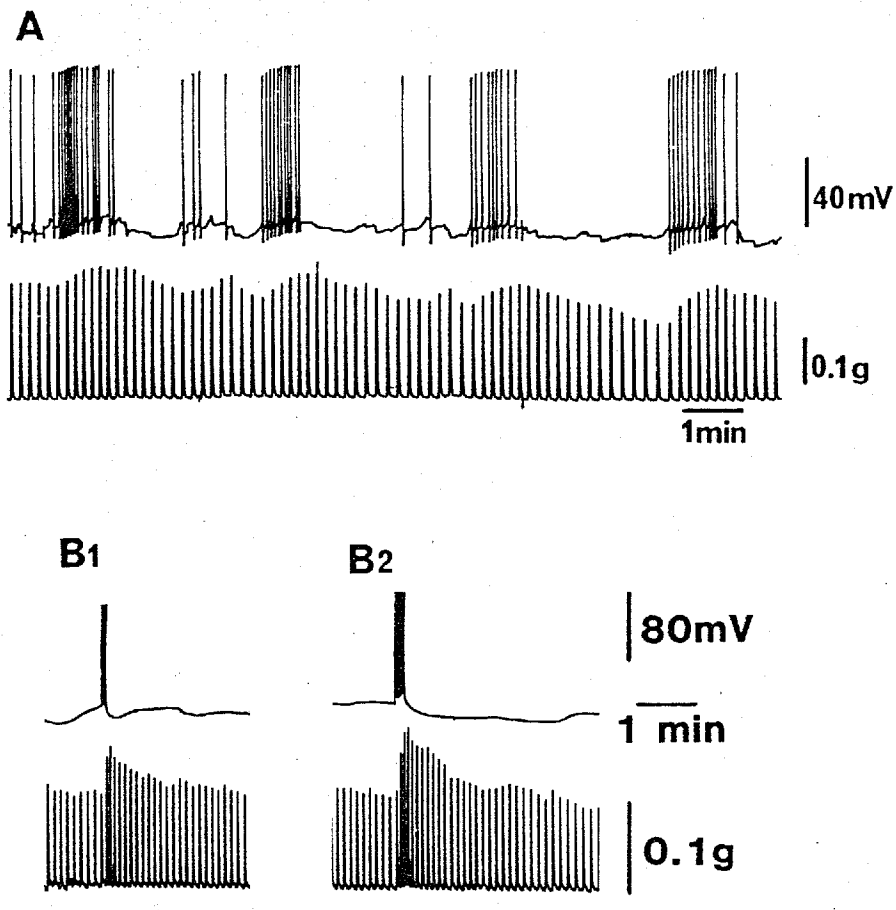

Fig. 4.4. A:Simultaneous recording from a spontaneously firing PON and the heart. B:Effect of the spikes of PON induced by current injection on the heart activity. The firing frequency was 2 Hz and the number of spikes was 10 (B1) and 20 (B2).

electrophysiological techniques were used to examine whether the axons in the intestinal nerve go to the heart. When the intestinal nerve was stimulated at the point just before entering the pericardium (see Fig. 3.1 in Chap. Ⅲ), an antidromic action potential was recorded at the soma of PON (Fig. 4.5A1). If the somatic membrane was hyperpolarized, separation of the axonal spike from the somatic spike was seen as a hump on the rising phase of the antidromic action potential (Fig. 4.5A2). The somatic spike was inhibited by further hyperpolarization (Fig. 4.5A3) and the invading axonal spike was also depressed (Fig. 4.5A4). Excitatory post-synaptic potentials (EPSPs) were not seen. Fig. 4.5B shows the simultaneous measurement of PON membrane potential and extracellularly recorded spikes of the intestinal nerve at the point just before entering the pericardium. Action potentials of PON were correlated with the largest spikes in the intestinal nerve (see Fig. 4.5B2). The conduction velocity of PON action potential was about 25 cm/s. These results indicate that the axon of PON extends to the heart.

To investigate whether the heart excitatory actions of PON were produced by the actions of PON upon another neurone, the excitation was examined before and after blockage of chemical synapses by perfusion of the ganglia with 3Mg, Ca-free solution. This treatment was seen to block synapses, because the large inhibitory input received by PON when cerebro-pleural connective was stimulated (Fig. 4.6A2) was almost completely absent in 3Mg, Ca-free solution (Fig. 4.6B2). However, the heart excitatory action of PON was not blocked by this treatment (Fig. 4.6B1). Consequently, PON was considered to be a heart excitatory motoneurone.

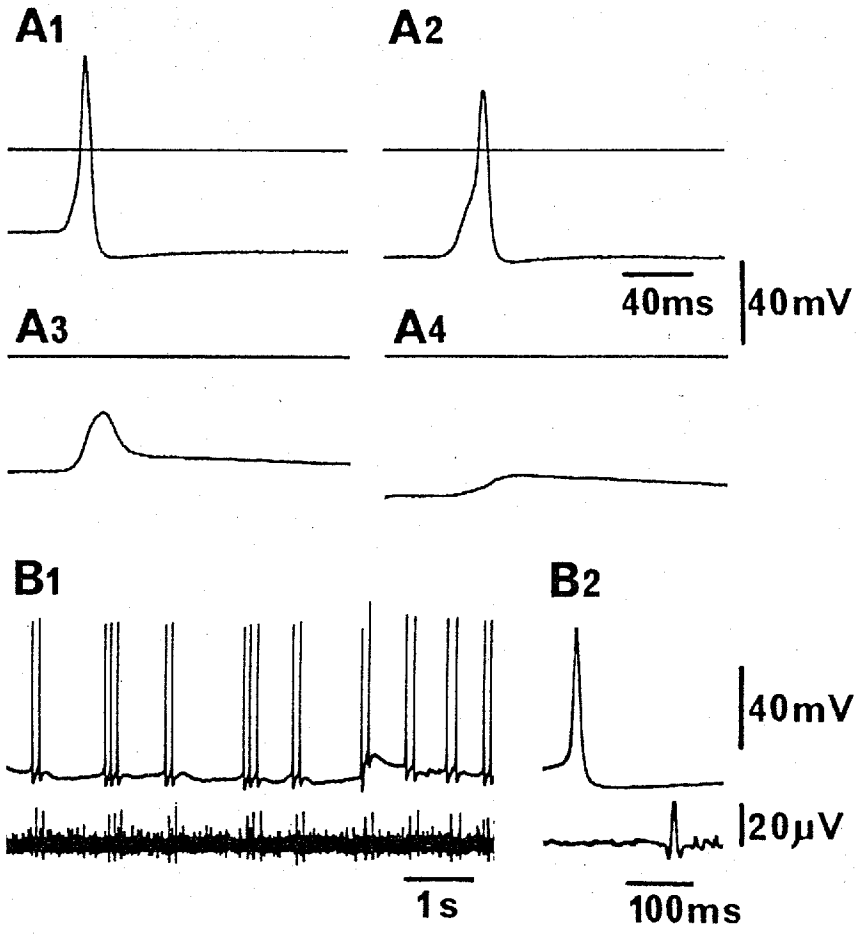

Fig. 4.5. A:Antidromic action potential of PON elicited by stimulation of the intestinal nerve. Straight line shows 0 mV level. The membrane of PON was hyperpolarized gradually from A1 to A4. The stimulus points are off the records. B1:Simultaneous recording from PON and the intestinal nerve. B2:High speed recording of one action potential from B1. The records in A and B were taken from different preparations. The conduction velocity of the spike was 23 cm/s in A and 27 cm/s in B.

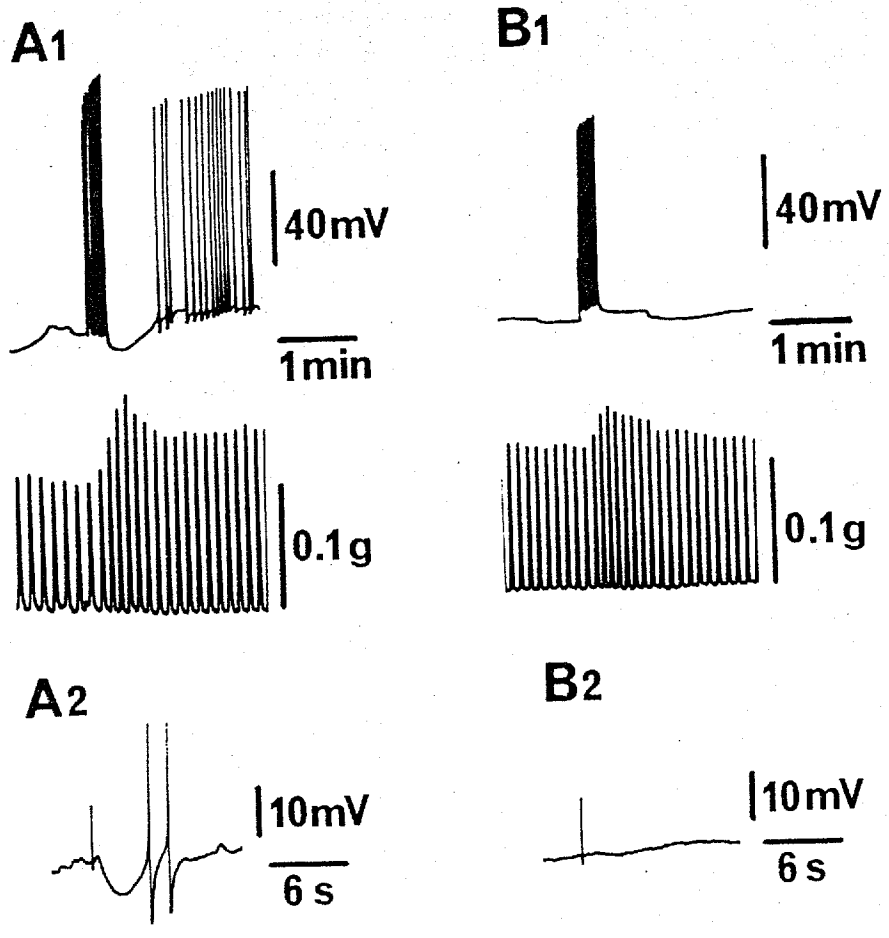

Fig. 4.6. Effect of perfusion of the ganglia with 3Mg, Ca-free solution on the heart excitatory action of PON. A:Control B:Records in 3Mg, Ca-free solution. PON was driven to fire at 1 Hz and the number of spikes was 14 in both A1 and B1. The heart excitatory action of PON is not blocked by this treatment (B1). In comparison, the response of PON to the single stimulation of the right cerebro-pleural connective (1 ms, 10 V) is shown (A2 and B2). The small vertical deflections in these figures are stimulus artifacts. The hyperpolarizing response of PON is completely blocked.

The heart excitatory action of TAN, TAN-2 and TAN-3

TAN, TAN-2 and TAN-3 fire tonically at a frequency of 0.5 to 2 Hz. They have a similar sensitivity to several neurotransmitter candidates, and have similar morphology (Matsuoka *et al.*, 1986). These neurones have axons in several nerve bundles, including the intestinal nerve (Fig. 4.7), and were found to have an excitatory effect upon the heart. The effect was less pronounced than that of PON. When the spontaneous activity of these neurones was stopped by hyperpolarizing current injection, the heart beat was decreased to 90-70 % (Fig. 4.8A). When the firing rate was increased, the heart activity was increased (Fig. 4.8B); a burst of spikes (20 s) produced 20-30 % increase in beat amplitude and heart rate. In a given preparation, one of these neurones (usually TAN) generally showed a stronger action than the other two.

Stimulation of the branch of the intestinal nerve that entered the pericardium produced antidromic action potentials in TAN, TAN-2 and TAN-3 (Fig. 4.8C), indicating that these neurones had axons in this branch. The conduction velocity of these spikes was less than that of PON; i.e., about 15 cm/s.

Fig. 4.8D shows that the heart excitatory action of TAN was not blocked by perfusion of the ganglia with 3Mg, Ca-free solution.

Although many features of these three neurones were quite similar, their spontaneous activity was not usually coordinated. To examine whether any coupling exists among these cells, two cells were impaled simultaneously and the effect of current injection in one cell on the other was examined. Although not very strong, there were electrical

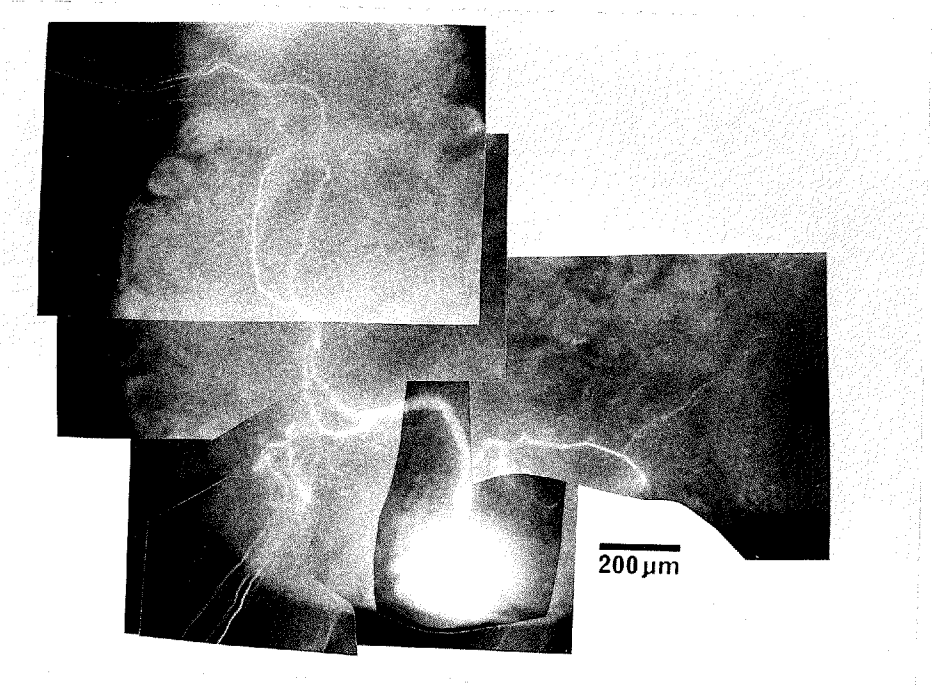

Fig. 4.7. Morphology of TAN stained by the injection of Lucifer Yellow.

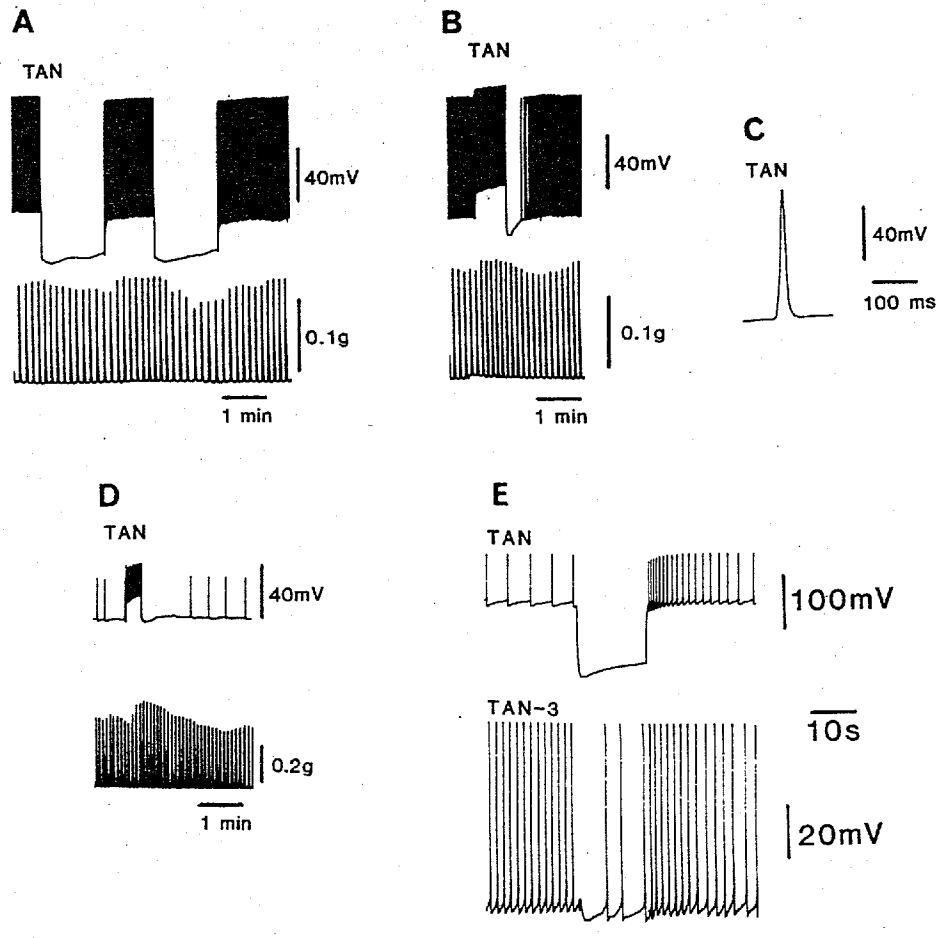

Fig. 4.8. A:Effect of stopping the spontaneous activity of TAN on the heart activity. B:Effect of increasing the firing frequency of TAN by current injection (40 s, 3 nA). C:Antidromic action potential of TAN elicited by stimulation of the intestinal nerve (1 ms, 8 V). The conduction velocity of this spike was 15 cm/s. The stimulus point is off the record. D:Effect of perfusion of the ganglia with 3Mg, Ca-free solution on the heart excitatory action of TAN. TAN was driven to fire by current injection (20 s, 5 nA). E:Weak electrical coupling between TAN and TAN-3.

couplings among these neurones (e.g. Fig. 4.8E).

The heart excitatory action of d-RCDN and d-LCDN

Two cerebral ganglion cells, d-RCDN and d-LCDN, are symmetrically situated on the dorsal surface of the ganglia (Ku *et al.* 1985; see Fig. 4.1). As shown in Fig. 4.9, these neurones send their axons to the contralateral ganglion, where they bifurcate near the origin of the cerebro-pleural connectives. The two branches go into the contralateral cerebro-pleural connectives. No axons of these cerebral cells could be traced in the suboesophageal ganglia. The absence of these processes might be due to the failure of the dye to penetrate the more distal processes of the neurones.

These neurones were usually silent or firing at low frequencies (<0.3 Hz). A burst of spikes (less than 20 s) in these cells produced a 20-40 % increase in beat amplitude and heart rate. As shown in Fig. 4.10 for d-RCDN, the excitation of the heart produced when these neurones were made to fire was not blocked when the ganglia were perfused with 3Mg, Ca-free solution (Fig. 4.10A).

Stimulation of the intestinal nerve entering the pericardium produced antidromic action potentials in d-RCDN and d-LCDN (Fig. 4.10B). EPSP-like potentials were not seen following stimulation of the intestinal nerve when the cell membrane was hyperpolarized. The conduction velocity of the antidromic action potentials was comparable to that of TAN. There is some doubt about the axonal projections of these cerebral neurones. Out of more than 40 d-RCDN and d-LCDN examined, 40 % of these neurones did not show antidromic action potentials following stimulation of the intestinal

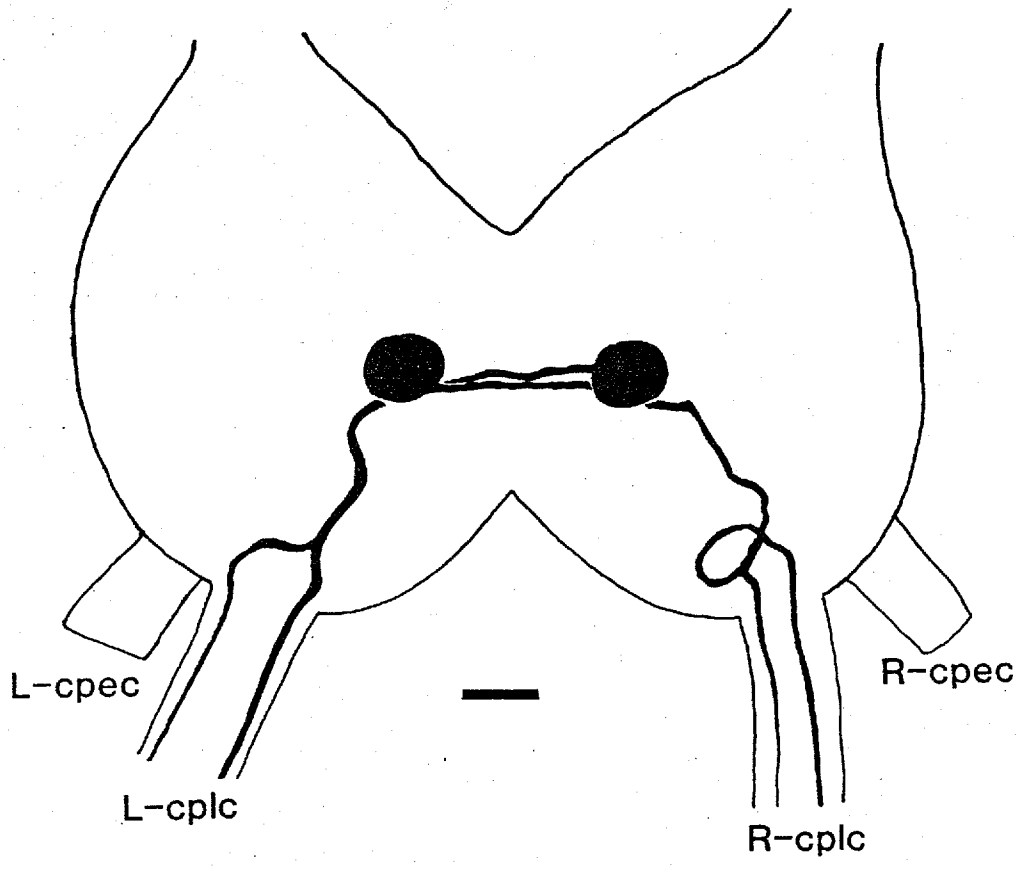

Fig. 4.9. Morphology of d-RCDN and d-LCDN stained by the injection of Lucifer Yellow. R-cpec (right cerebro-pedal connective), L-cpec (left cerebro-pedal connective), R-cplc (right cerebro-pleural connective), L-cplc (left cerebro-pleural connective). Scale: 200 μm

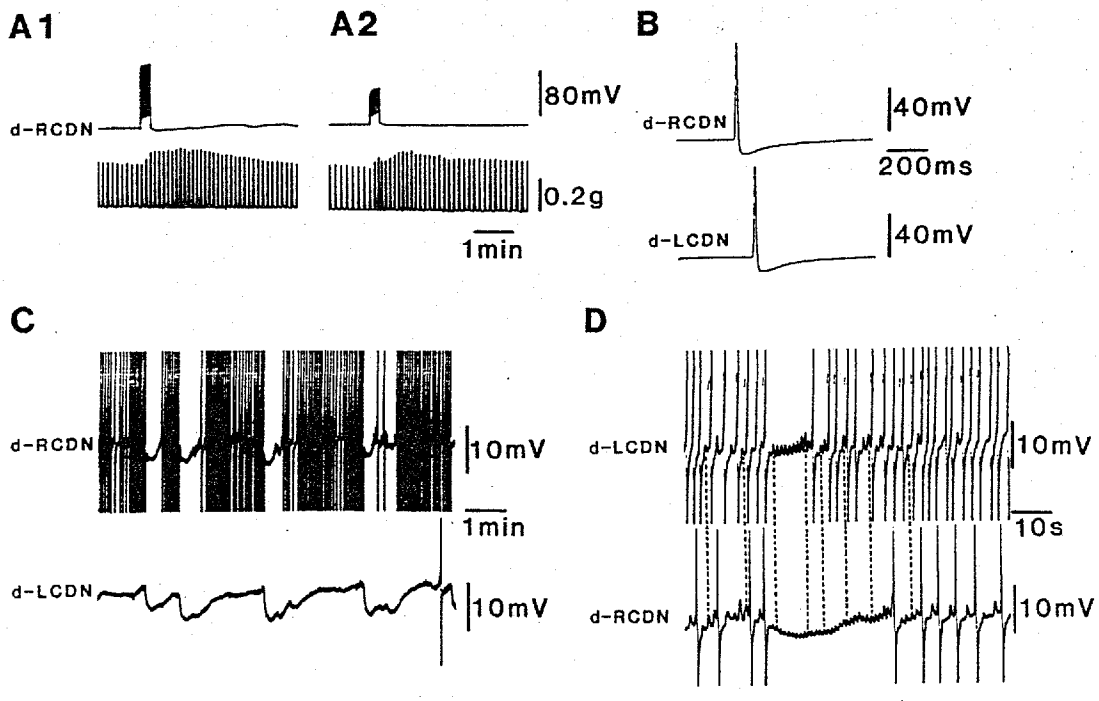

Fig. 4.10. A:The heart excitatory action of d-RCDN. A1:Control A2:Record in 3Mg, Ca-free solution. d-RCDN was driven to fire by current injection (15 s, 8 nA in A1 and 15 s, 6 nA in A2). B:Antidromic action potential of d-RCDN and d-LCDN elicited by stimulation of the intestinal nerve (1 ms, 10 V). The conduction velocities of these spikes were 16 cm/s in d-RCDN and 12 cm/s in d-LCDN. The stimulus point is the beginning of sweep in each record. C:Simultaneous recording from d-RCDN and d-LCDN which shows the common inhibitory inputs. D:Simultaneous recording from d-LCDN and d-RCDN which shows the common excitatory inputs. Some common inputs are connected by the broken line.

nerve. This does not seem to be the result of the poor state of the preparations, as in a given preparation the loss of antidromic action potentials was seen in d-RCDN but not in d-LCDN, and *vice versa.* Also, in some cases, the antidromic action potential was recorded following stimulation of the proximal position of the intestinal nerve, but not recorded following stimulation of the branch going to the heart (see Fig. 2.3).

Although these bilaterally symmetrical neurones did not necessarily show coordinated firing patterns, it was found that they received common inhibitory (Fig. 4.10C) and excitatory (Fig. 4.10D) inputs. At present, the source of these inputs is not known, but they originate centrally as they were also seen in the isolated brain preparation. There was no electrical coupling between these neurones.

The actions of VG1 on the heart activity

VG1 has not previously been described. As shown in Fig. 4.11, the soma lies near the middle of the dorsal surface of the visceral ganglion. This neurone has two main axons and some thin collaterals which go into the intestinal nerve. There are also other thin collaterals going to the right posterior pallial nerve. Whether all axons in the intestinal nerve go to the heart is unknown at present.

The action of this neurone on the heart activity was somewhat variable. A burst of spikes produced in this cell by current injection usually induced a slight increase in peak tension of the heart beat but not an increase in heart rate (Fig. 4.12A1). When the firing frequency of this neurone was increased by larger current injection, the heart rate was decreased (Fig. 4.12A2 and A3). In a few

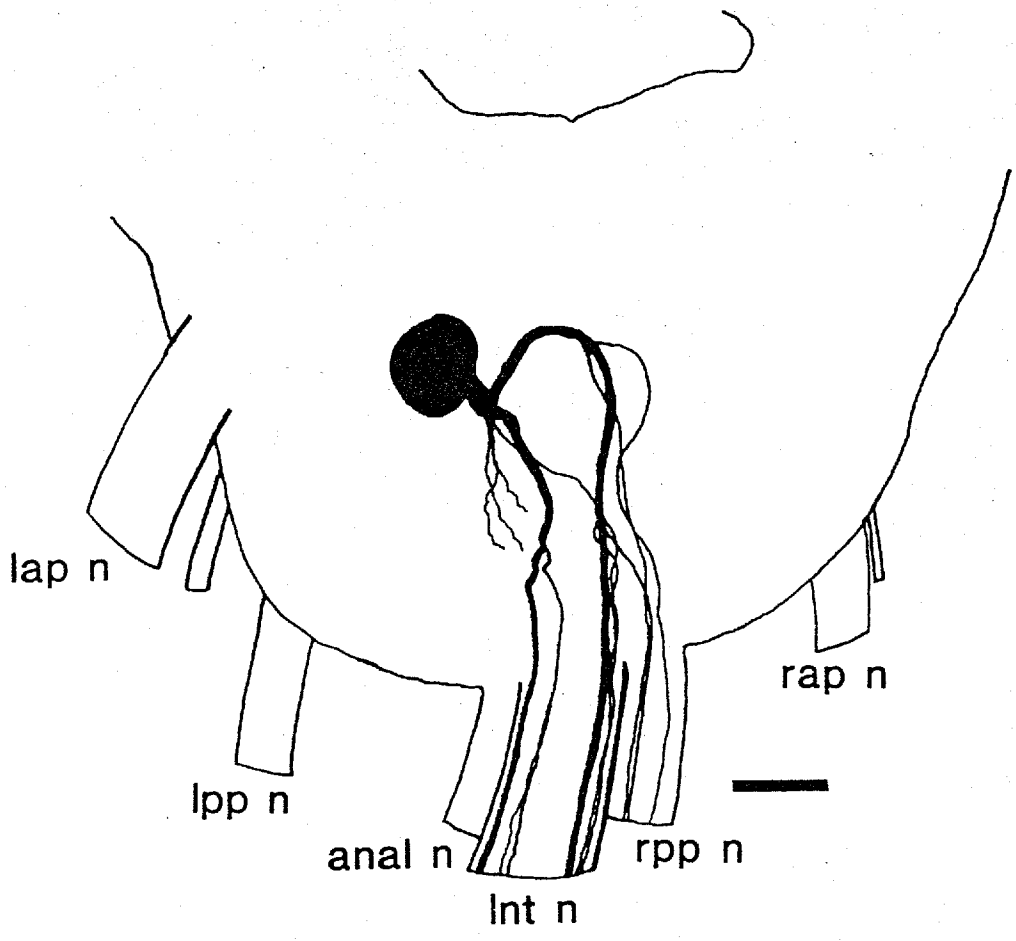

Fig. 4.11. Morphology of VG1 stained by the injection of Lucifer Yellow. lap n (left anterior pallial nerve), lpp n (left posterior pallial nerve), anal n (anal nerve), int n (intestinal nerve), rpp n (right posterior pallial nerve), rap n (right anterior pallial nerve). Scale: 200 μm

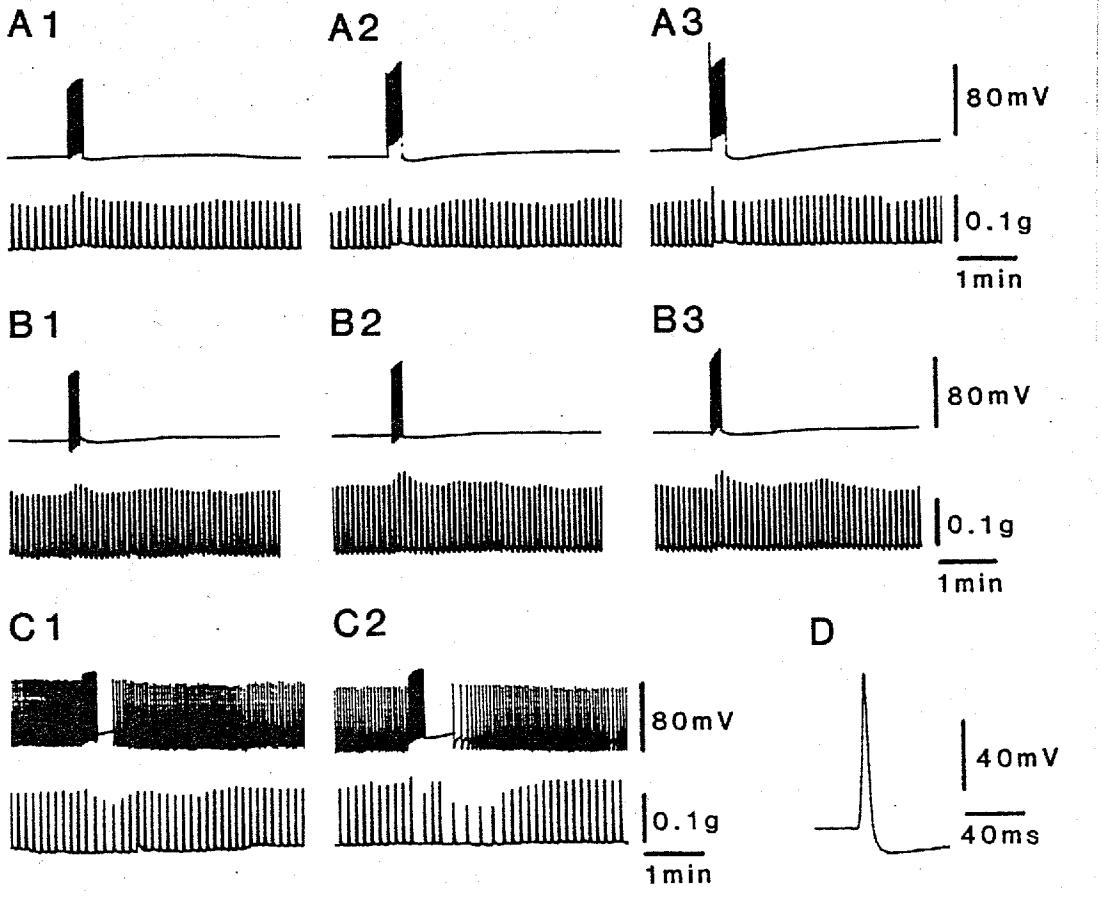

Fig. 4.12. Several examples of the action of VG1 on the heart activity. Records in A, B and C were taken from different preparations. In these figures, the activities of VG1 are increased from left to right by increasing the injected current. The injected currents were as follows: A1 (15 s, 4 nA), A2 (15 s, 6 nA), A3 (15 s, 8 nA), B1 (10 s, 3 nA), B2 (10 s, 4 nA), B3 (10 s, 6 nA), C1 (15 s,2 nA), C2 (15 S, 4 nA). D:Antidromic action potential of VG1 elicited by the stimulation of the intestinal nerve (1 ms, 8 V). The conduction velocity of the spike was 39 cm/s. The stimulus point is off the record.

preparations, only an increase in beat amplitude (Fig. 4.12B) or inhibition of heart rate and decrease in beat amplitude (Fig. 4.12C) were recorded. This neurone had an axon in the branch of the intestinal nerve going to the pericardium, as demonstrated by the antidromic action potential (Fig. 4.12D). No EPSP-like potential was seen following stimulation of the intestinal nerve when the membrane of this cell was hyperpolarized. Conduction velocity of this spike was much faster than that of other neurones described, i.e., about 40 cm/s.

Discussion

In this chapter, the features of seven neurones (PON, TAN, TAN-2, TAN-3, d-RCDN, d-LCDN and VG1) which were identified as being concerned with regulation of the heart of *Achatina* were described. Among these neurones, six cells had an excitatory effect on the heart.

PON, previously described as RPal (S.-Rôzsa, 1979b), is a bursting neurone in the isolated ganglia preparation. From the position in the ganglia, and the bursting characteristics, it is considered to be a homologous neurone to R15 in *Aplysia*, F1 in *Helix aspersa*, RPal in *Helix pomatia* and Cell 11 in *Otala lactea* (Chase & Goodman, 1977; Rittenhouse & Price, 1985; Kai-kai & Kerkut, 1979; S.-Rôzsa, 1979a; Gainer, 1972). All these neurones have axons in the nerve bundle which goes to the heart, suggesting their similar role as PON of *Achatina*. *Helix* F1 contains a cardio-active peptide whose structure has been reported (Price *et al.*, 1985). Application of this peptide as well as the homogenate of isolated F1 show excitatory actions on the heart (Cottrell *et al.*, 1981; Price *et al.* 1985).

However, the effects of stimulation of *Helix* F1 on heart activity have not been reported, and negative results have been reported in the cases of *Aplysia* R15, *Helix* RPal, *Otala* cell 11 and *Achatina* RPal (Koester & Koch, 1987; S.-Rózsa *et al.*, 1983; Gainer, 1972; S.-Rózsa, 1979b). S.-Rózsa (1979b) reported that *Achatina* RPal (i.e. PON) received inputs from the heart but was not involved in heart regulation. Although the difference between the present results and those reported for other snails may reflect the difference in species, the inconsistency between the present results and those of S.-Rózsa is not easily reconciled because PON has the strongest excitatory action on the heart among the identified heart excitors in the present experiments. Indeed, its action was so strong that frequent activity of this cell following injury masked the excitatory action of the other heart excitors. One possible reason may be the difference of experimental conditions. In S.-Rózsa's experiments, the pericardium was not removed and the heart activity was monitored by a photo-optic method. In my experience, if the pericardium was left intact, the heart of *Achatina* did not show regular beating after it was dissected out from the animal. Under this condition, it was very difficult to examine the action of PON on the heart.

In the present experiments, PON showed irregular firing interposed by many inhibitory inputs. Although direct presynaptic inhibitory neurones were not identified, these inhibitory inputs were suggested to originate from the periphery. Further accounts on this phenomenon will be given in chapter V . The bursting activities of *Helix* RPal and *Aplysia* R15 are also reported to be influenced by synaptic inputs (Salánki *et al.*, 1979; Koester *et al.* 1974).

TAN, TAN-2 and TAN-3 are tonically firing neurones and have several output axons in many nerve bundles arising from the suboesophageal ganglia (Matsuoka *et al.* 1986). This suggests that they have multiple functions. One clear function is the modulation of heart beat. The tonic heart excitatory action of these neurones is comparable to that of RB_{HE} in *Aplysia* (Mayeri *et al.* 1974).

In *Aplysia*, identified heart regulatory motoneurones are located in a rather restricted region of the abdominal ganglion (Mayeri *et al.* 1974). However, in *Achatina*, two cerebral ganglion cells, d-RCDN and d-LCDN, were shown to have axons which go to the heart, although the occurrence of axons in the heart nerve was variable. When these axons were present, the heart excitatory action of these neurones was always resistant to perfusion of the ganglia with 3Mg, Ca-free solution. This result suggests that the action of these cerebral neurones is not dependent on other neurones. Thus, at least in some preparations, d-RCDN and d-LCDN may also act as heart excitors like PON, TAN, TAN-2 and TAN-3. In the present preparations, these two cerebral cells did not necessarily show coordinated activity. However, since common excitatory and inhibitory inputs were found, these cells may be, in some cases, driven to fire at the same time in intact animals.

There is no obvious reason for the variable occurrence of the d-RCDN and d-LCDN axon in the heart nerve. There may be a seasonal variation because most of the experiments about cerebral cells were carried out during winter to early spring (1985-1986). In its natural habitat (Okinawa, Japan), this snail hibernates during these periods. The hibernation would produce a decrease of body fluid and this

may induce the sprouting or retraction of axons as indicated in *Helisoma* neurones (Bulloch, 1984; Maetzold & Bulloch, 1986). Further investigations are needed to clarify this problem.

The four heart excitatory neurones reported for *Helix pomatia* (S.-Rôzsa, 1981) are not considered to be homologous to any heart excitors described in this chapter. The heart inhibitory motoneurones, V12 and V13, the heart regulatory neurone, V21, and RPa1 of *Helix pomatia* (S.-Rôzsa & Salánki, 1973; S.-Rôzsa, 1979a) have been considered to have homologues in *Achatina* (S.-Rôzsa, 1979b). In the present experiments, homologous neurones to V12 and V13 were not found.

The newly identified VG1 may be homologous to V21 of *Helix pomatia* which also has an axon in the intestinal nerve and whose tonic activity arrested the heart beat (S.-Rôzsa, 1979a; S.-Rôzsa, 1979b), although VG1 usually increased beat amplitude at low firing frequency (Fig. 4.12). Since the actions of VG1 were variable, this neurone is not considered to be a motoneurone. Again, the contrast between the present observation and the earlier one may reflect the difference of experimental conditions. In the earlier experiments, heart activity was monitored by a photo-optic method without dissecting the pericardium; in this condition, the tension imposed on the heart is considered to be lower than that in the present experimental condition. In my experience, it seems that the more the tension of the heart was increased, the less explicit the heart inhibitory action of VG1 became.

As described in chapter II , the *Achatina* heart receives the dual innervations (see Fig. 2.4). Spontaneous depression

of the heart activity was frequently observed in the present preparation, which was not usually seen in the isolated heart preparation. Moreover, the high firing frequency of VG1 induced depression of the heart activity in most cases. However, direct inhibitory neurones were not identified in the present experiments. The heart inhibitory motoneurones may be small and missed in the present examinations.

CHAPTER V

INTERCONNECTIONS AMONG THE HEART REGULATORY NEURONES

In chapter IV , the features of identified heart regulatory neurones of *Achatina* were discussed. In this chapter, the features of the heart regulatory network of *Achatina* will be described based on the synaptic interactions between those neurones.

Summary of the heart regulatory network

Synaptic connections between the heart regulatory neurones were examined by recording from two or three cells together in various combinations. The heart regulatory network is complex and its features are briefly described here before the presentation of evidence (see also Fig. 5.12).

Four heart excitatory motoneurones (PON, TAN, TAN-2 and TAN-3) and a 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. 5.1A shows an example in the case of TAN-3. In Fig. 5.1B, 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 analyze the synaptic responses as the amplitude of an individual excitatory post-synaptic potential (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 3Ca, 3Mg 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 owing to 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 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 *et al.*, 1978; Colmers *et al.*, 1982) and to raise the membrane input resistance, thus producing an increase in the amplitude of post-synaptic potential. Fig. 5.1C 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

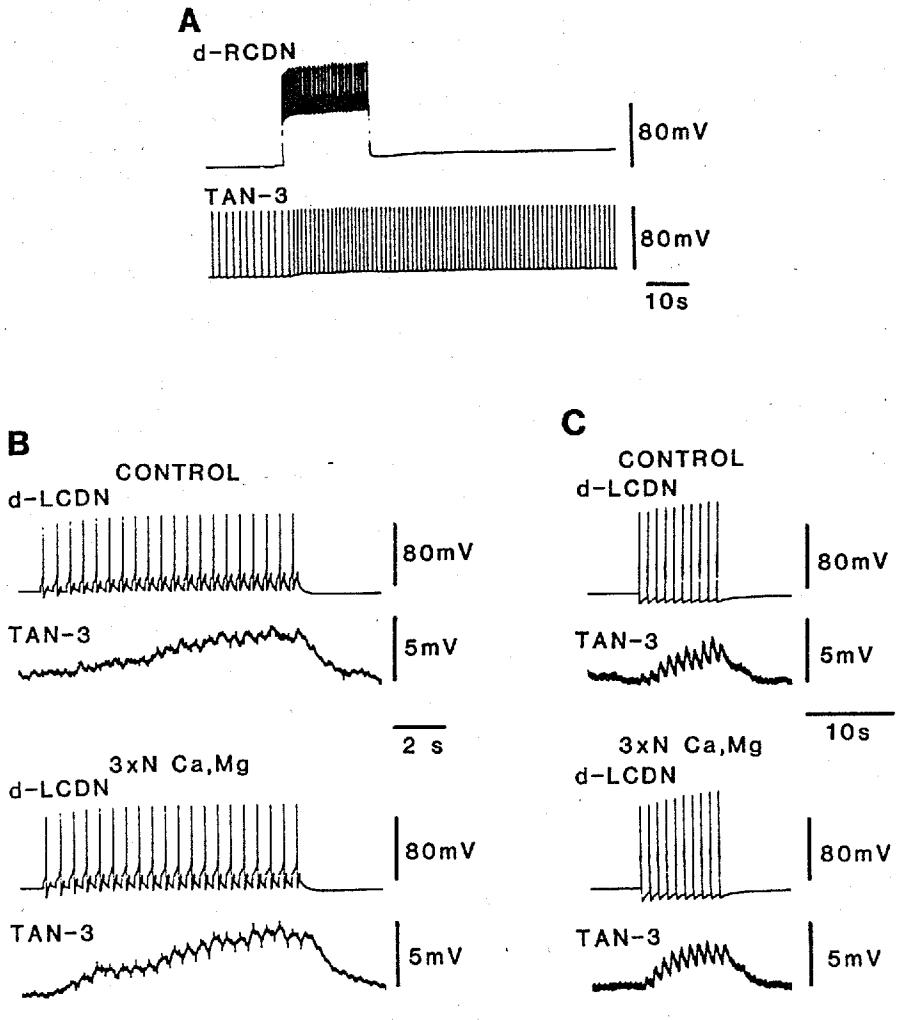

Fig. 5.1. A:Effect of a burst of spikes in d-RCDN on the spontaneous activity of TAN-3. d-RCDN was driven to fire by current injection (20 s, 16 nA). B:EPSPs of TAN-3 produced by the evoked spikes in d-LCDN and the effect of 3Ca, 3Mg solution (3xN Ca, Mg) on these EPSPs. d-LCDN was driven to fire by 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 KCH_3COO. C:EPSPs of Cs^+-loaded TAN-3 produced by evoked spikes in d-LCDN and the effect of 3Ca, 3Mg solution (3xN Ca, Mg) 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.

they were not blocked by perfusion of the ganglia with 3Ca, 3Mg solution. The latency between 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. 5.2A). A longer duration burst in d-LCDN drove PON into activity and this was followed by delayed inhibitory synaptic inputs (Fig. 5.2B). These inhibitory inputs to PON were presumably due to activation of interneurone(s) driven by the burst of spikes in d-LCDN. Fig. 5.2C 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 of d-RCDN produced a slow depolarization in PON which was not blocked by perfusion of the ganglia with 3Ca, 3Mg solution, but increased in amplitude.

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

VIN is the intraganglionic neurone, i.e. this cell has no output axons to the periphery (Fig. 5.3) as reported previously (Goto *et al.*, 1986). The primary axon of this cell bifurcates and each axon goes to the pedal ganglia through the neuropile of left and right sides in the suboesophageal ganglia. VIN is a periodically firing neurone in the isolated ganglia preparation (Ku & Takeuchi,

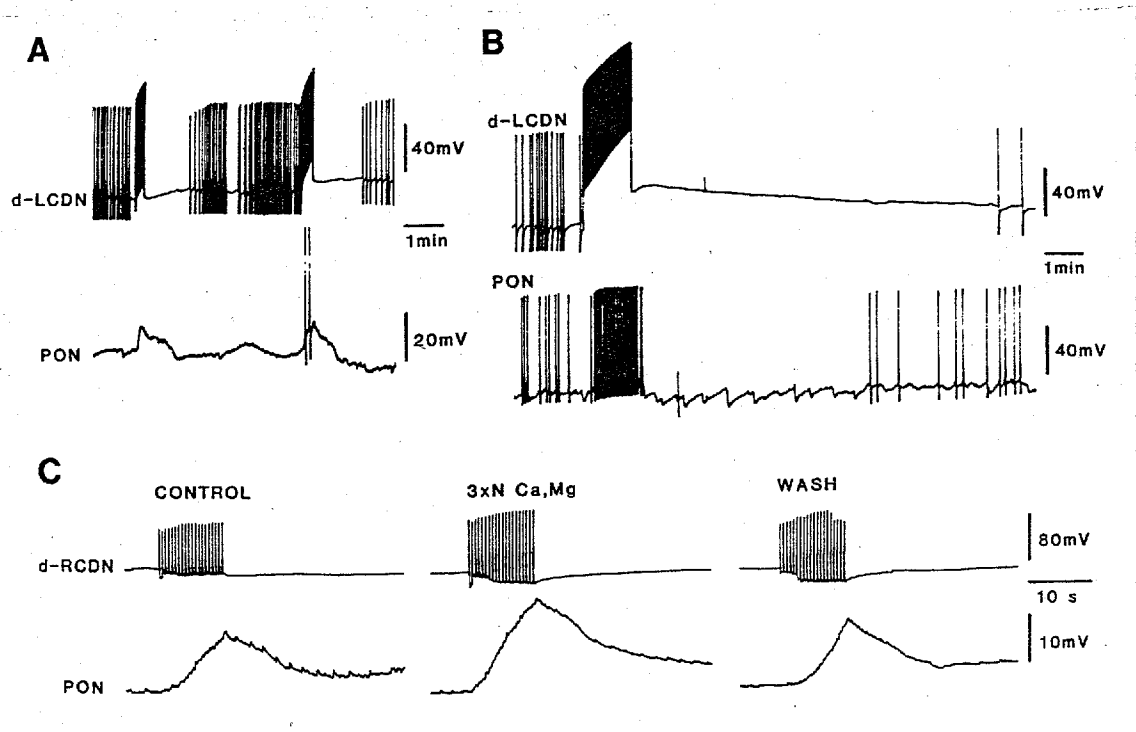

Fig. 5.2. The excitatory action of d-RCDN and d-LCDN on PON. A:Slow depolarizing response in PON induced by a burst of spikes in d-LCDN. B:Effects of longer duration burst in d-LCDN on PON. C:Effect of 3Ca, 3Mg solution (3xN Ca, Mg) on the slow depolarizing response in PON induced by d-RCDN. d-RCDN was driven to fire at 2Hz. The membrane potential of PON was set at -100 mV. The recording electrode for PON was filled with CsCl.

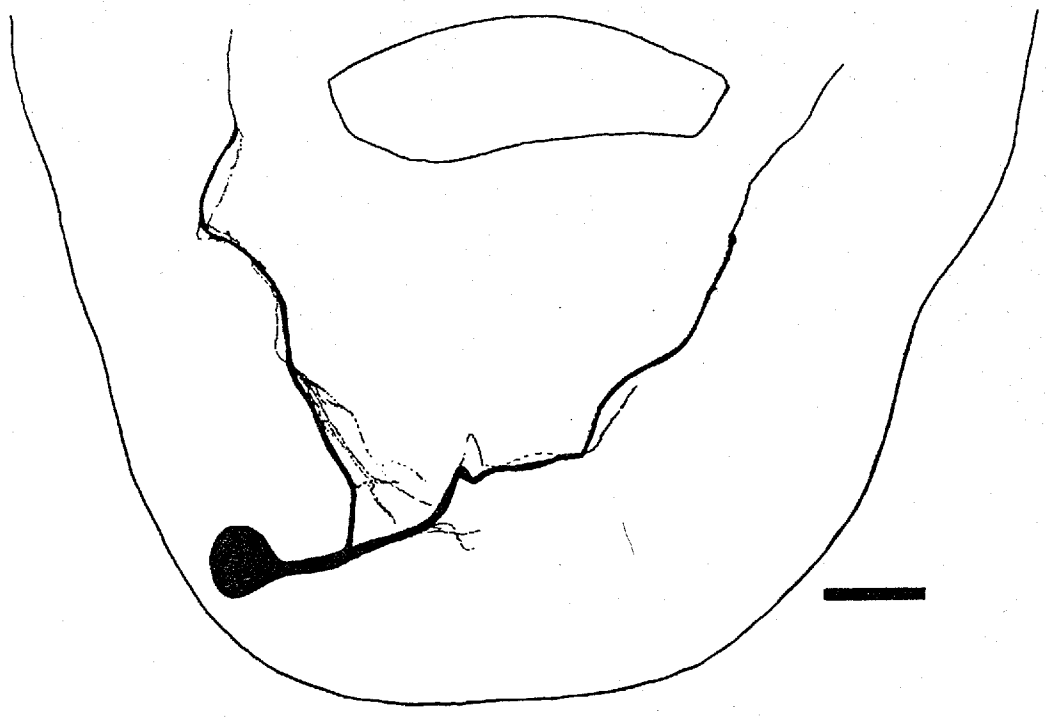

Fig. 5.3. Morphology of VIN stained by the injection of Lucifer Yellow. Scale: 200 μm.

1983). However, in the present preparation that included the heart, the spontaneous activity of VIN was greatly reduced like that of PON. When VIN and PON were recorded simultaneously, many common inhibitory inputs were seen and the active phase of both neurones was correlated (Fig. 5.4A). These two neurones were weakly electrically coupled (Fig. 5.4B). 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. 5.4C. In this experiment, VIN was penetrated by a CsCl-filled electrode and the membrane potential of VIN was set at -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 3Ca, 3Mg solution (Fig. 5.4C).

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. 5.5). In the experiment shown in Fig. 5.5B, the ganglia were perfused with 3Ca, 3Mg 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 chapter Ⅳ .

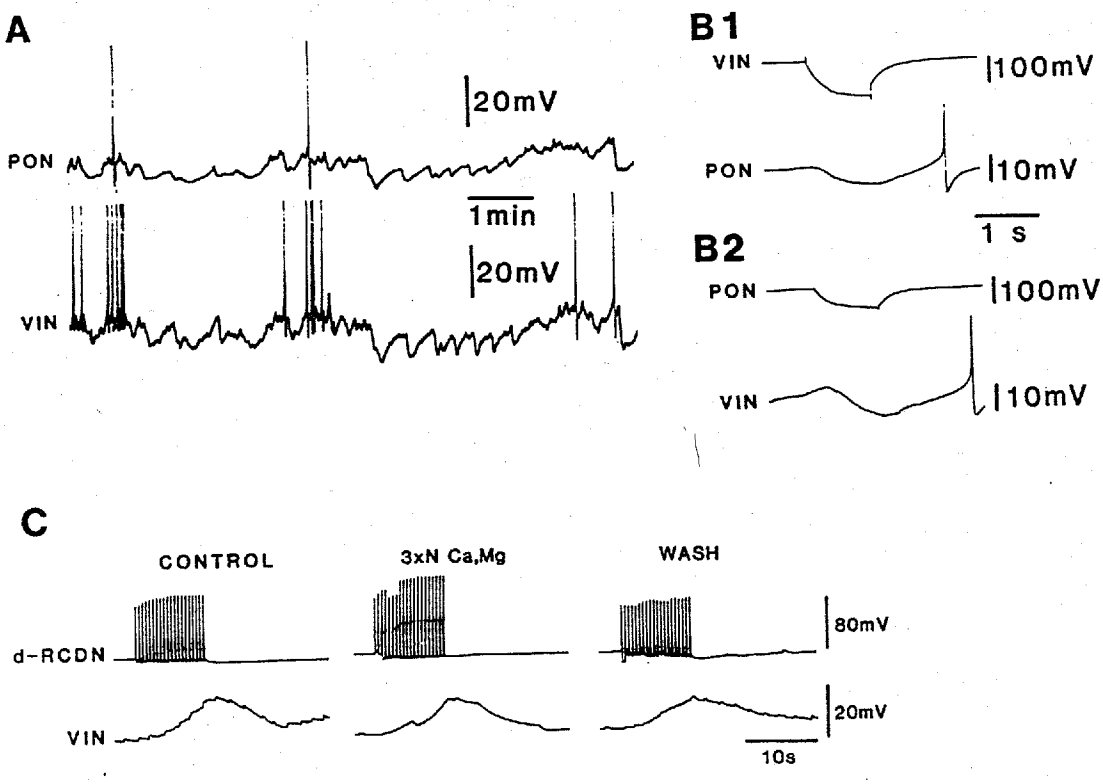

Fig. 5.4. A:Simultaneous recording from PON and VIN. Note the numerous common inhibitory inputs. B:Weak electrical coupling between PON and VIN. Hyperpolarizing current was injected into VIN (B1) or PON (B2). C:Slow depolarizing response in VIN induced by d-RCDN and the effect of 3Ca, 3Mg solution (3xN 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.

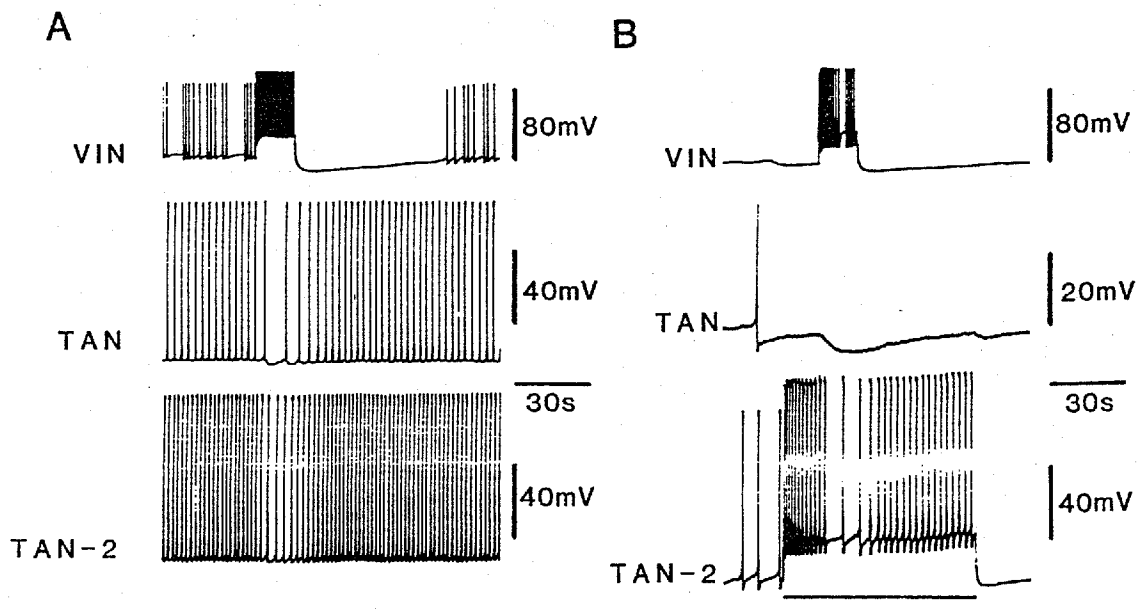

Fig. 5.5. Inhibitory action of VIN on TAN and TAN-2. A: Inhibition of the activity in TAN and TAN-2 produced by the burst of spikes in VIN. B: Effect of 3Ca, 3Mg solution on the inhibitory response. TAN-2 was depolarized by current injection during the period indicated by the bar. VIN was driven to fire by current injection (15 s, 6 nA).

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. 5.6A1 and 5.6B1). However, if the heart was dissected away by cutting the intestinal nerve, this inhibition was not seen (Fig. 5.6A2 and 5.6B2). 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 peripheral systems. 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. 5.7). In a few preparations, only one action potential of VG1 was enough to produce the hyperpolarizing responses in PON and VIN (Fig. 5.7A1 and 5.7C). In most cases, a burst of spikes was needed to inhibit these postsynaptic cells (Fig. 5.7B1 and 5.7D). In either case, PON and VIN appeared to receive common inhibitory input (Fig. 5.7C and 5.7D). This inhibitory action of VG1 was also lost if the heart was dissected away (Fig. 5.7B2), 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 inhibition of PON and VIN by the stimuli of the intestinal nerve and the tactile stimulation of the pericardium

The inhibitory actions of TANs/VG1 on PON/VIN are complex as described above, indicating some intervening

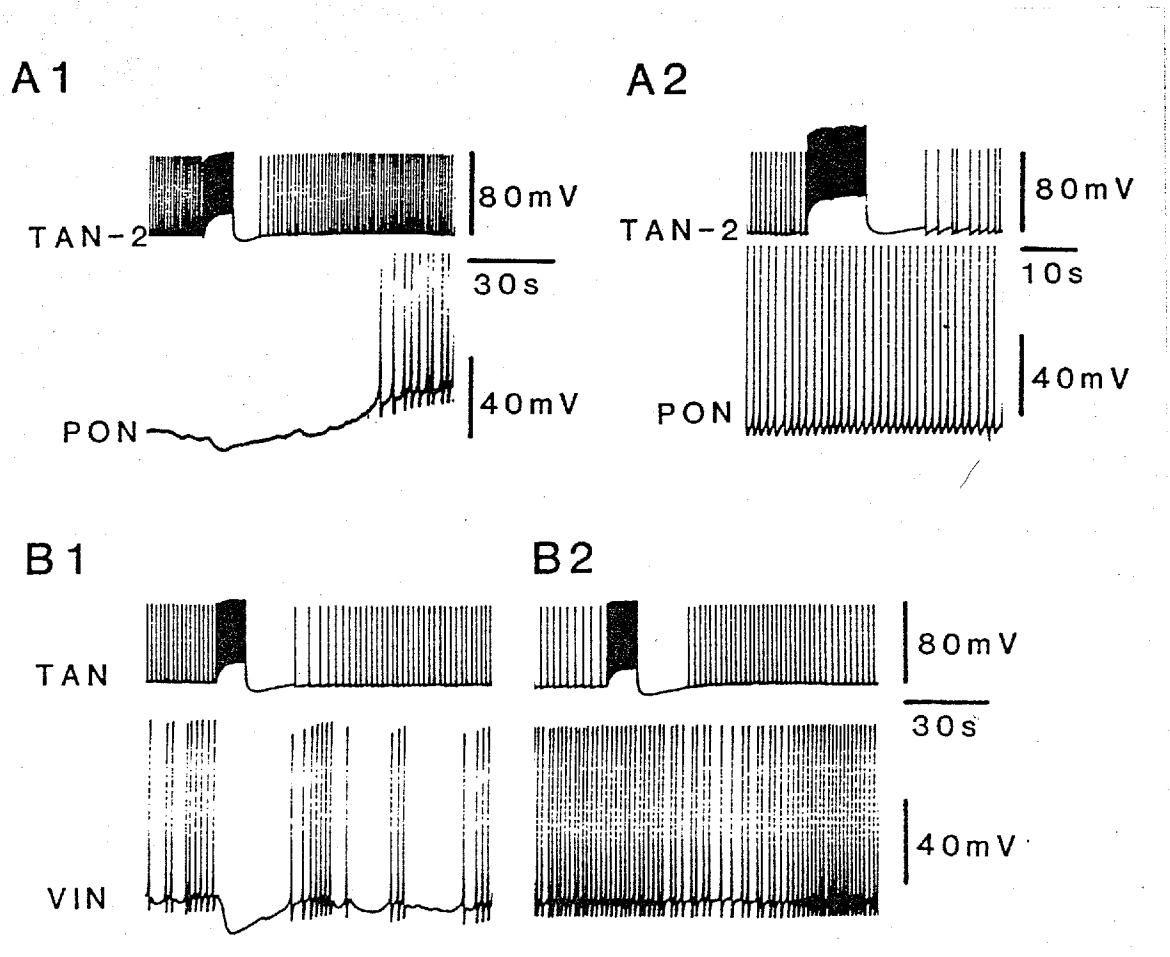

Fig. 5.6. A: Inhibitory action of TAN-2 on PON (A1) and the effect of cutting the intestinal nerve on this response (A2). The firing frequency of TAN-2 was increased by current injection (10 s, 6 nA). B: Inhibitory action of TAN on VIN (B1) and the effect of cutting the intestinal nerve on this response (B2). The firing frequency of TAN was increased by current injection (10 s, 6 nA).

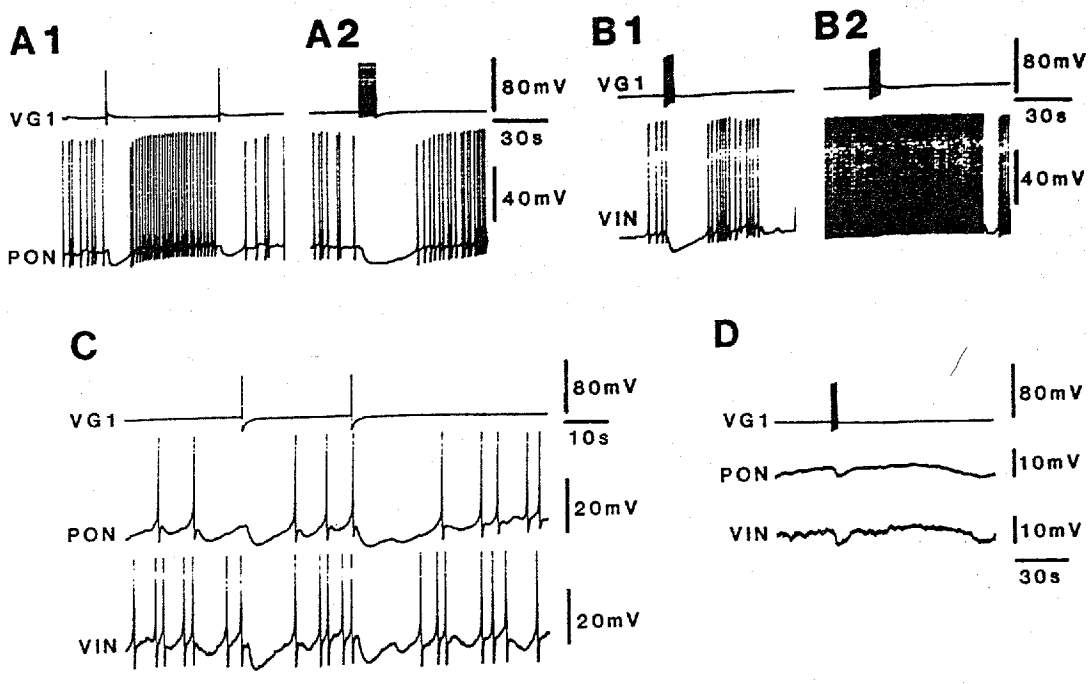

Fig. 5.7. Inhibitory action of VG1 on PON and VIN. A: Inhibitory action of VG1 on PON. VG1 was driven to fire by current injection. A1 shows the effect of single action potential, and A2 shows that of repetitive spikes (1 Hz, 10 spikes) on PON. Both records are taken from the same preparation. B: Inhibitory action of VG1 on VIN (B1) and the effect of cutting the intestinal nerve on this response (B2). 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).

neurones which are not in the ganglia. Thus, the effects of stimulation of the intestinal nerve on PON and VIN were examined. A single stimulus of the branch of the intestinal nerve going to the heart produced the inhibition of long-duration (ILD) in both cells. Fig. 5.8 shows the ILD of PON. The constant hyperpolarizing pulses which can be seen as the small vertical deflexions in this figure were applied to monitor the change of membrane conductance. The early part of ILD was clearly accompanied by the conductance increase and was reversed by the hyperpolarization of postsynaptic cells (Fig. 5.8A). However, the later part did not appear to accompany the change of conductance, and was depressed but not reversed by the hyperpolarization. The ILD was resistant to the perfusion of the ganglia with 3Ca, 3Mg solution, suggesting the monosynaptic nature (Fig. 5.8B).

Stronger ILD was observed if the proximal portion of the intestinal nerve (which contained the axons of other neurones as well as those of heart regulatory neurones; see Fig. 2.3) was stimulated (Fig. 5.8C). The feature of this stronger ILD was similar to that described above except one point, i.e. this ILD was reduced by the perfusion of the ganglia with 3Ca, 3Mg solution. Thus, it was suggested that a part of this ILD was produced by the activation of polysynaptic pathways.

A probable origin of above mentioned ILD may be some sensory output from the periphery. However, detailed examinations of the sensory elements in the heart regulatory network of *Achatina* were not carried out at present since the presumed sensory stimulations (e.g. the change of intra-heart pressure) are difficult to control. Here, some

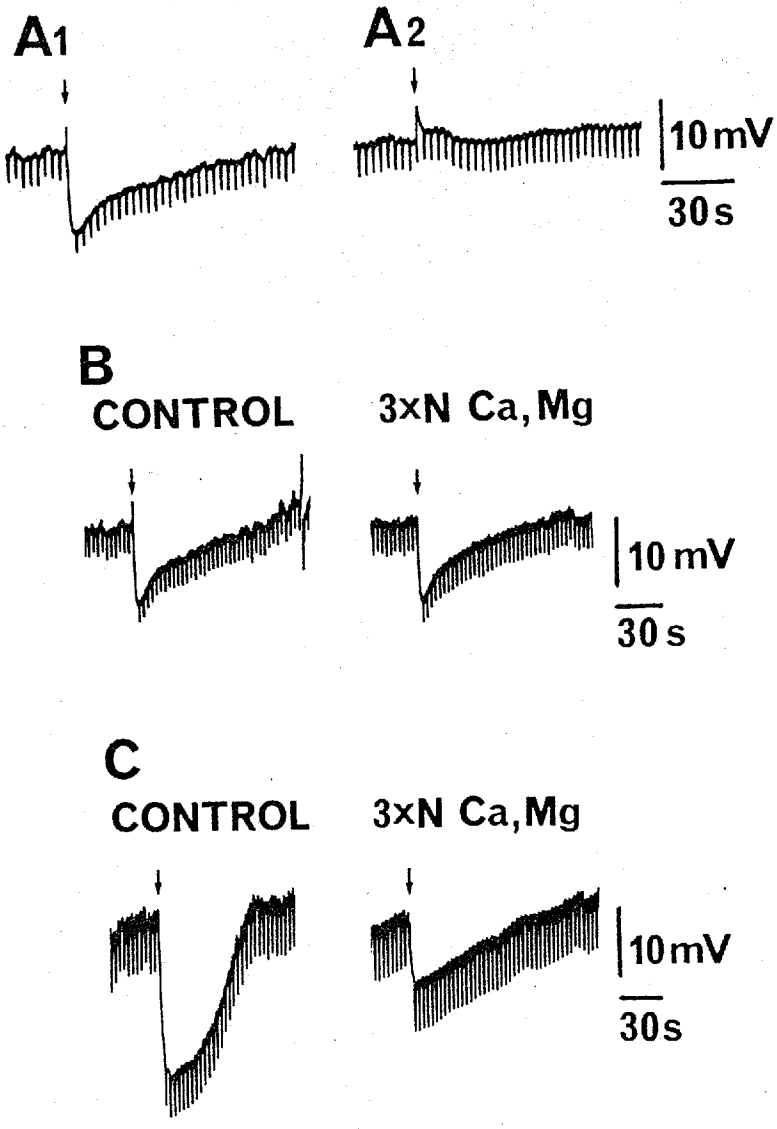

Fig. 5.8. The inhibition of long-duration (ILD) of PON. Hyperpolarizing current pulses were applied every 3 s to monitor the change of membrane conductance in all experiments. All ILD were produced by a single stimulation of the intestinal nerve (indicated by an arrow). A:ILD of PON at -60 mV (A1) and at -100 mV (A2). Stimulus point and factor; a branch in the pericardium, 1 ms, 11 V. B:Effect of 3Ca, 3Mg solution (3xN Ca, Mg) on ILD of PON. Membrane potential was -60 mV. Stimulus point and factor; a branch in the pericardium, 1 ms, 11 V. C:Effect of 3Ca, 3Mg solution on ILD of PON. Membrane potential was -60 mV. Stimulus point and factor; proximal region before the first bifurcation, 1 ms, 14 V.

results about the effect of mechanical stimulation of the pericardium are presented.

Single light rubbing of the pericardium by the hand-held glass rod (about 500 μm in tip diameter) produced the common hyperpolarization in PON and VIN. This hyperpolarization was accompanied by the conductance increase (Fig. 5.9A). Fig. 5.9B1 shows the simultaneous recording of PON and VIN. The tactile stimulus to the vein produced similar but much smaller response. This result indicates the inputs to both cells are common. When VIN was hyperpolarized by current injection, the early part of the response in VIN reversed without affecting the response in PON, and the late part of the response in VIN became to null but did not reverse (Fig. 5.9B2). A similar result was obtained when PON was hyperpolarized. These results suggest the chemical nature of these synaptic responses.

Because the stimulus method was rather rough in spite of its effectiveness, the applied stimulation did not always produce reproducible responses in PON and VIN even in the same preparation. Thus, it was difficult to test the nature of synaptic connections by comparing two responses obtained before and after perfusing the ganglia with 3Ca, 3Mg solution. However, the hyperpolarization of PON and VIN induced by the tactile stimulation was never blocked by the perfusion of the ganglia with such solution (Fig. 5.9C). These results suggest that the hyperpolarization is at least in part due to the activation of monosynaptic inhibitory pathways.

The hyperpolarization produced by the tactile-stimulation seems to be similar to that induced by the stimulation of the intestinal nerve. It may be suggested

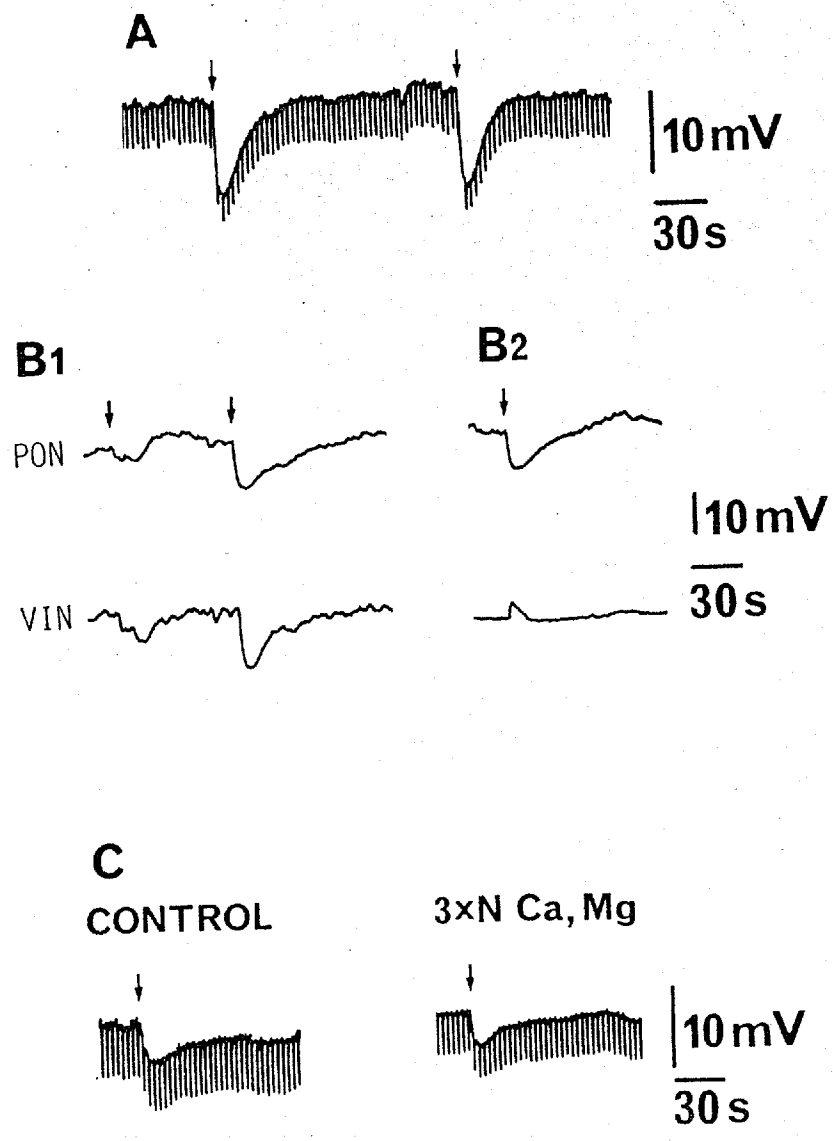

Fig. 5.9. The hyperpolarization of PON and VIN induced by the tactile stimulation of the pericardium or vein. The tactile stimulation (a light rubbing) was applied by the hand-held glass plobe. Hyperpolarizing current pulses were applied every 3 s to monitor the change of membrane conductance in the experiments of A and C. A:The hyperpolarization of PON induced by the tactile stimulation of the pericardium (arrow). Membrane potential (MP); -52 mV. B1:Simultaneous hyperpolarization of PON and VIN induced by the tactile stimulation of the vein (first arrow) and the pericardium (second arrow). MP: PON, -70 mV; VIN, -64 mV. B2:Reversal of the early hyperpolarization of VIN induced by the tactile stimulation of the pericardium. MP: PON, -76mV; VIN, -100 mV. C:Effect of 3Ca, 3Mg solution (3xN Ca, Mg) on the hyperpolarization of PON induced by the tactile stimulation of the pericardium. MP; -52 mV.

that the activity of TANs/VG1 can activate this inhibitory pathway which originates in the mechano-afferents in the pericardium and/or the vein.

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.

d-LPeLN is situated in the left pedal ganglion (Fig. 5.10), 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. 5.11, the isolated ganglia preparation was used so that PON and VIN were showing periodical bursting activity. A burst of spikes in d-LPeLN induced by current injection clearly increased PON (Fig. 5.11A1) and TAN (Fig. 5.11A2) activity. The effect on VIN was not so striking because of its strong spontaneous activity (Fig. 5.11A3). In Fig. 5.11B, 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 3Ca, 3Mg solution (Fig. 5.11B), suggesting that this connection is

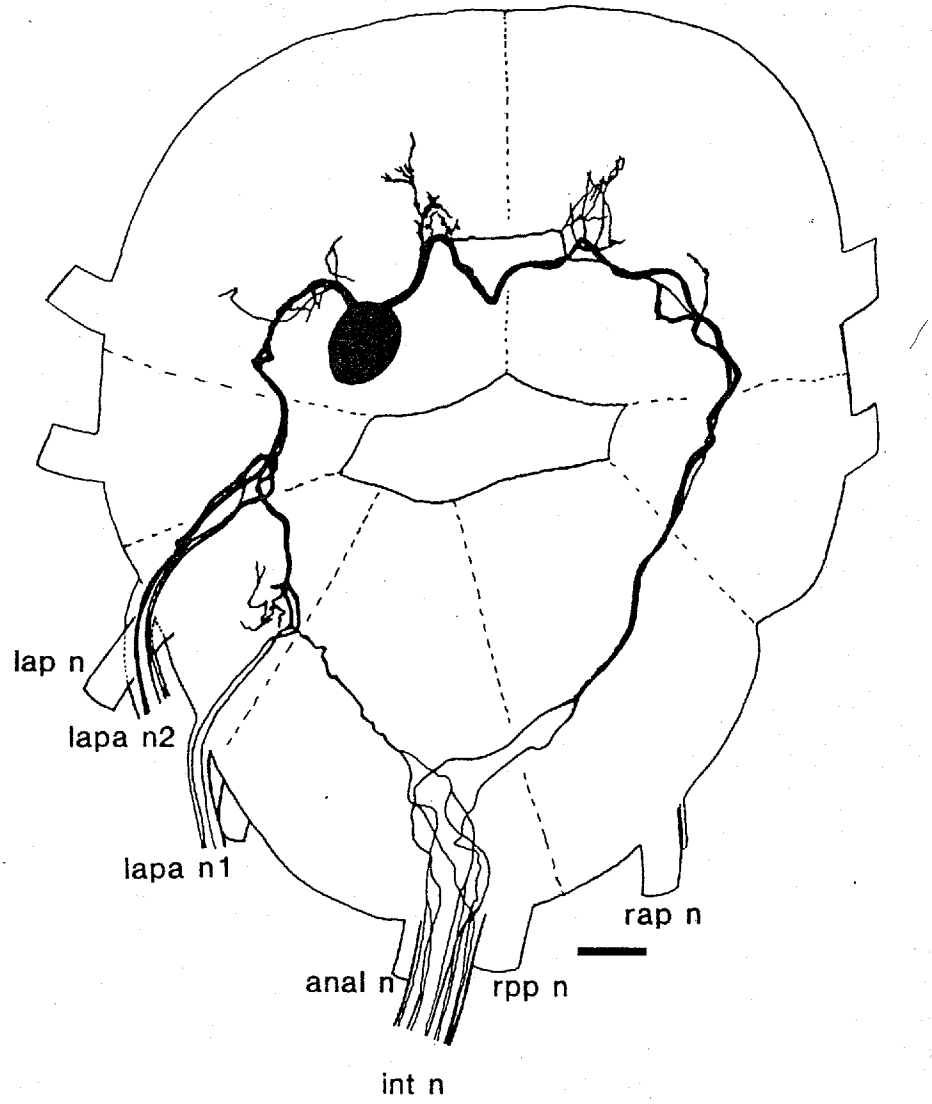

Fig. 5.10. Morphology of d-LPeLN stained by the injection of Lucifer Yellow. lap n (left anterior pallial nerve), lapa nl (left anterior pallial accessory nerve 1), lapa n2 (left anterior pallial accessory nerve 2), anal n (anal nerve), int n (intestinal n), rpp n (right posterior pallial nerve), rap n (right anterior pallial nerve). Scale: 200 μ m

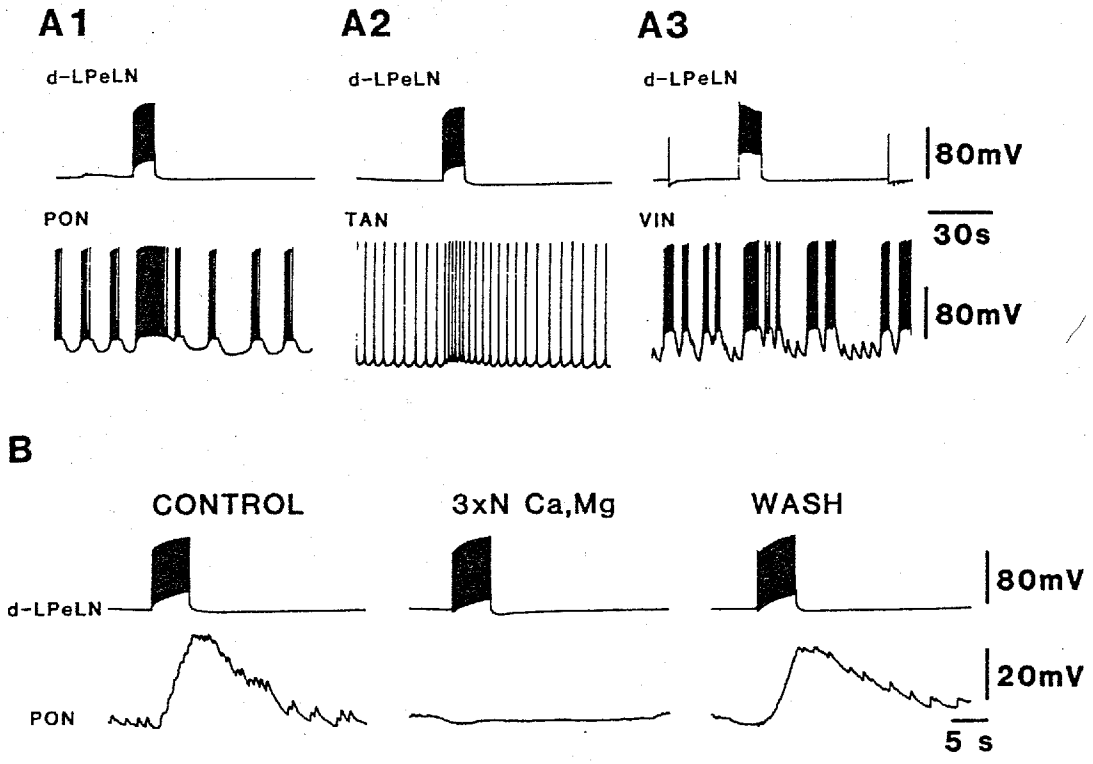

Fig. 5.11. Excitatory action of d-LPeLN on PON, TAN and VIN. A: Effects of a burst of spikes in d-LPeLN on the spontaneous activity of PON (A1), TAN (A2) and VIN (A3). d-LPeLN was driven to fire by current injection (10 s, 10 nA). B:Slow depolarizing response in PON induced by d-LPeLN and the effect of 3Ca, 3Mg solution (3xN 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).

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.

Discussion

In this chapter, synaptic connections between the heart regulatory neurones of *Achatina* were described, as summarized in Fig. 5.12. As there was 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 chapter Ⅳ . 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. Both types of response were not blocked when the ganglia were perfused with 3Ca, 3Mg 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 burst of spikes in these cells produced

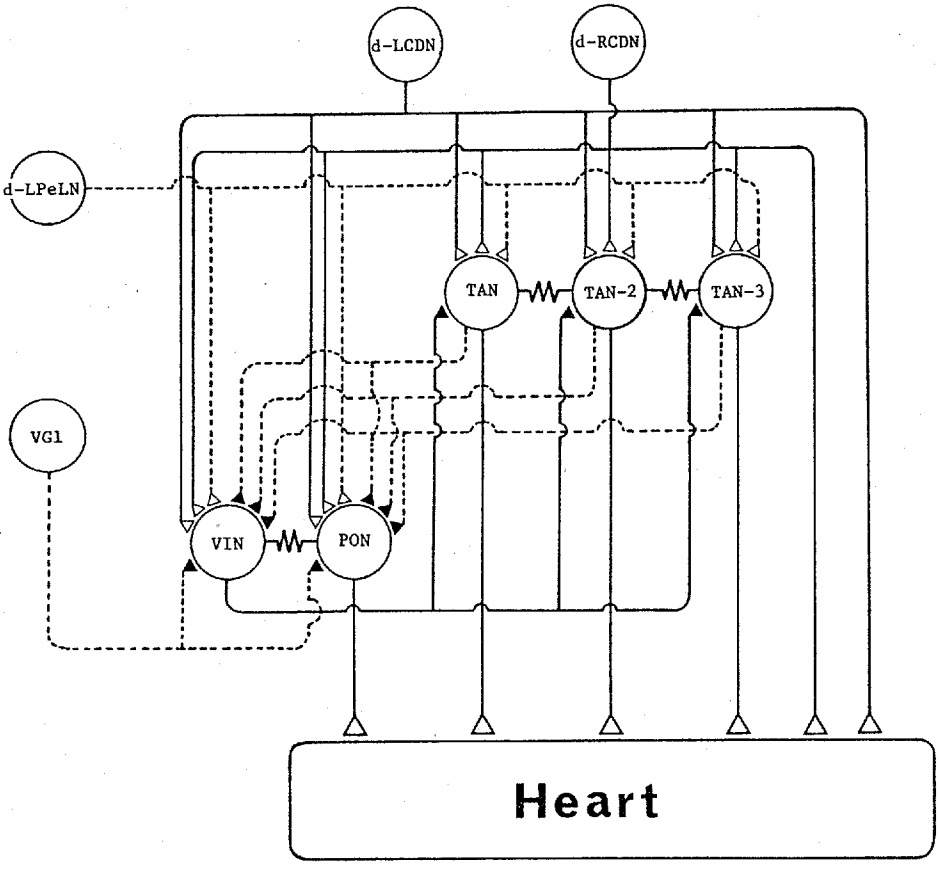

Fig. 5.12. Schematic diagram showing synaptic connections between the heart regulatory neurones. Open and closed triangles indicate excitatory and inhibitory synapses, respectively. Zigzag lines indicate weak electrical couplings. Broken lines indicate the involvement of the other neurones in the pathways.

delayed inhibition of PON (see Fig. 5.2B). 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 in other gastropods, the function of these neurones 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 *et al.*, 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. Each branch goes to the left and right pedal ganglion, respectively. 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, the 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. The connection from VIN to TAN is considered to be monosynaptic as the response was not blocked by perfusion of the ganglia with 3Ca, 3Mg 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.

VG1 is the only identified neurone whose activity produces heart inhibition and this inhibitory action is

probably mediated by other neurones. 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.

The tactile stimulus applied to the pericardium produced the hyperpolarization in PON and VIN, which resembled the ILD induced by a single electrical stimulus of the branch of the intestinal nerve entering the pericardium. Both the ILD and the hyperpolarization produced by the tactile stimulation were resistant to the perfusion of the ganglia with 3Ca, 3Mg solution, suggesting the monosynaptic nature of the responses. These results indicate the existence of the inhibitory pathways from the pericardium to the central heart regulatory neurones. When the proximal portion of the intestinal nerve was stimulated, the larger ILD was observed and this ILD was not blocked but depressed by the perfusion of the ganglia with 3Ca, 3Mg solution. These results suggest the existence of polysynaptic inhibitory pathways in addition to the monosynaptic ones. The activity of TAN or VG1 may inhibit PON and VIN through the activation of these inhibitory pathways. The identified inhibitory pathways to PON and VIN and the presumed connections between these pathways and TAN and VG1 are summarized in Fig. 5.13. The monosynaptic inhibitory pathways are considered to be the mechano-afferents

themselves in this figure, although there may be "peripheral" interneurones. It is hypothesized that TAN and VG1 inhibit PON and VIN through the activation of the mechano-afferents. In this diagram, the delayed inhibition of PON produced by the strong burst of the cerebral neurones is also explained by the activation of the polysynaptic inhibitory pathways. As described previously, PON and VIN receive many common inhibitory inputs, if the heart (including the pericardium) is left intact. Some of them may originate from the mechano-afferents in the pericardium.

The pericardium is not merely the protective sack of the heart, but is known to possess some crucial functions in the circulation (see review, Jones, 1983). The pericardial pressure is consistently less than the pressure in the heart at all stages of the beat cycle in any molluscan hearts in which the intrapericardial pressure was measured simultaneously with the intraheart pressure. This property has been considered to be essential for the cardiac refilling of molluscs. Thus, the mechano-afferents in the pericardium may function as the sensor of intrapericardial pressure and play an important role in the hemodynamics.

In *Helix pomatia*, several neurones respond to the tactile stimulus applied to the cardio-renal system (S.-Rôzsa, 1979a; S.-Rôzsa, 1981). Similar results have also been reported in *Achatina* (S.-Rôzsa, 1979b) and in *Aplysia* (S.-Rôzsa *et al.*, 1980). Although the results obtained in different gastropods as well as the present results indicate the involvement of the mechano-afferents in the heart regulatory network of gastropods, the sensory modalities for such mechano-afferents are still not clear. Further

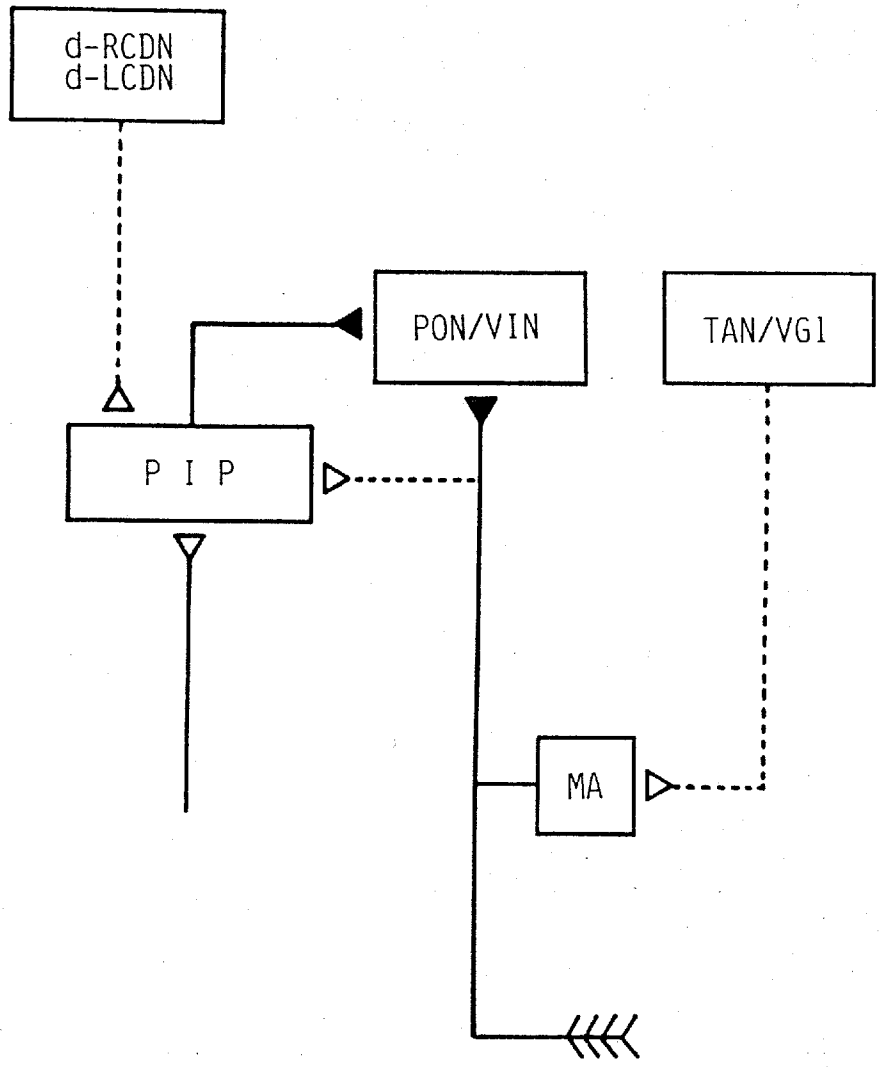

Fig. 5.13. Schematic diagram showing the inhibitory pathways from the pericardium and other periphery to PON/VIN. Presumed connections are indicated by the broken lines. Open and closed triangles indicate excitatory and inhibitory synapses, respectively. MA;mechano-afferents. PIP;polysynaptic inhibitory pathways.

experiments, especially the development of the experimental methods to apply the *quantitative physiological stimulus*, will be needed to elucidate the sensory elements in the heart regulation of gastropods.

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 3Ca, 3Mg 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.-Rôzsa, 1981).

In *Aplysia* (Mayeri *et al.*, 1974; Koester *et al.*, 1974) and *Helix* (S.-Rôzsa, 1979a), 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 heart regulatory motoneurones in this snail are connected monosynaptically or polysynaptically. 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. 5.12).

CHAPTER VI

MODULATION OF IONIC CURRENTS BY SYNAPTIC ACTION AND 5—HT IN THE IDENTIFIED HEART EXCITOR

Introduction

In the previous chapter, the two cerebral cells (d-RCDN and d-LCDN) were shown to be the presynaptic excitatory neurones for several heart excitatory neurones in the heart regulatory network of *Achatina*. Considering such synaptic connections and their own heart-excitatory role, the two cerebral neurones may be not only members of the heart regulatory network but also the component of "arousal" system of this snail. Thus, these synaptic pathways are interesting and seem to be important for heart regulation of *Achatina*.

In this chapter, the ionic mechanisms underlying a slow depolarization of PON (the most effective heart excitor; see Chap. IV) induced by the activity of the cerebral cells were examined under the voltage-clamped condition. A similar slow depolarization was induced by application of 5-HT and the actions of 5-HT upon PON were compared with those of the cerebral cells.

Cerebral neurones or 5-HT induce the slow depolarization in PON

The activity of two cerebral neurones (d-RCDN & d-LCDN) produced a slow depolarizing response in PON (Chap. V). This slow depolarization was found to be depressed reversibly by the 5-HT antagonist, methysergide (Fig. 6.1A),

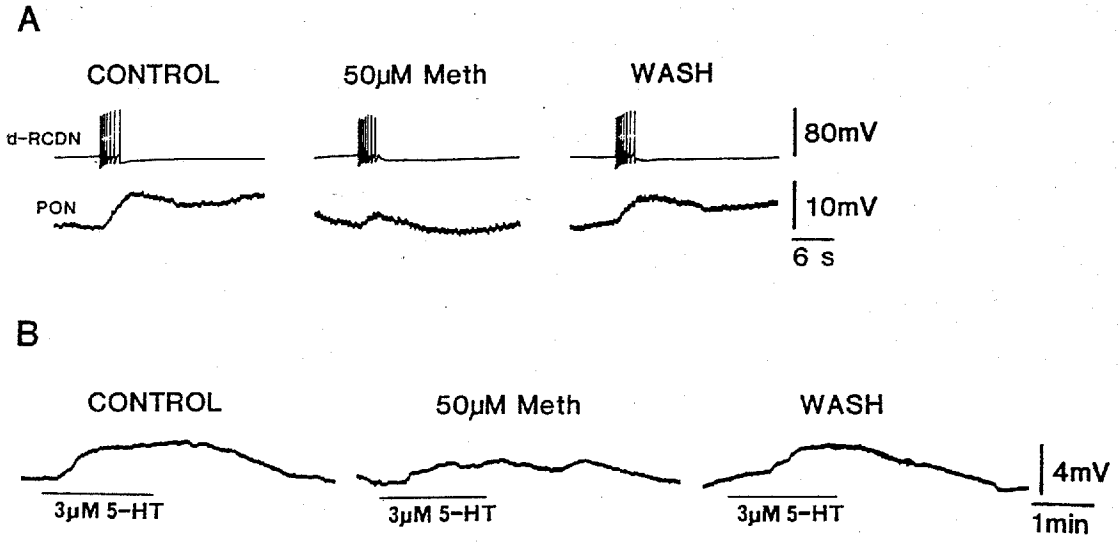

Fig. 6.1. A:Blocking action of methysergide (Meth) on the slow depolarization of PON induced by activity of d-RCDN. PON was hyperpolarized to -80 mV. d-RCDN was driven to fire by a depolarizing current injection. Spike number was 8 in CONTROL, and 9 in 50 μM Meth and WASH. B:Blocking action of methysergide on the slow depolarization of axotomized PON induced by 5-HT. Membrane potential of PON was -40 mV.

in six preparations. The cerebral neurones were driven to burst by a depolarizing current pulse, and the number of spikes in the burst was not strictly controlled in this and the following experiments. However, a given duration of depolarizing pulse evoked a fairly constant number of spikes and produced reproducible synaptic responses in PON. 5-HT produced a similar slow depolarization in axotomized PON, which was also depressed by methysergide (Fig. 6.1B) in four preparations. 5-HT appeared to be acting directly upon PON since the neurone was isolated by axotomy, and the soma of molluscan neurones is known to lack synaptic contacts. Higher concentrations of methysergide produced a complete block which was not reversible over the experimental time-course (less than two hours). These results suggest that 5-HT is the neurotransmitter of the two cerebral neurones. To examine this hypothesis, the ionic mechanism of the synaptic action was compared with that of the 5-HT action under voltage-clamp.

Ionic mechanisms of the slow depolarization of PON by cerebral neurones

When the membrane potential of PON was held at -50 mV, activity in the cerebral neurone induced an inward shift in the holding current accompanied by a decrease of conductance (Fig. 6.2A1). At a more negative holding potential, less current shift was produced (Fig. 6.2A2). After the burst in the cerebral neurone, there was a slow recovery of the current level. Similar results were obtained in ten preparations out of twelve. In the other two preparations a change of conductance was not observed. These results may be explained if this response is produced by a decrease in

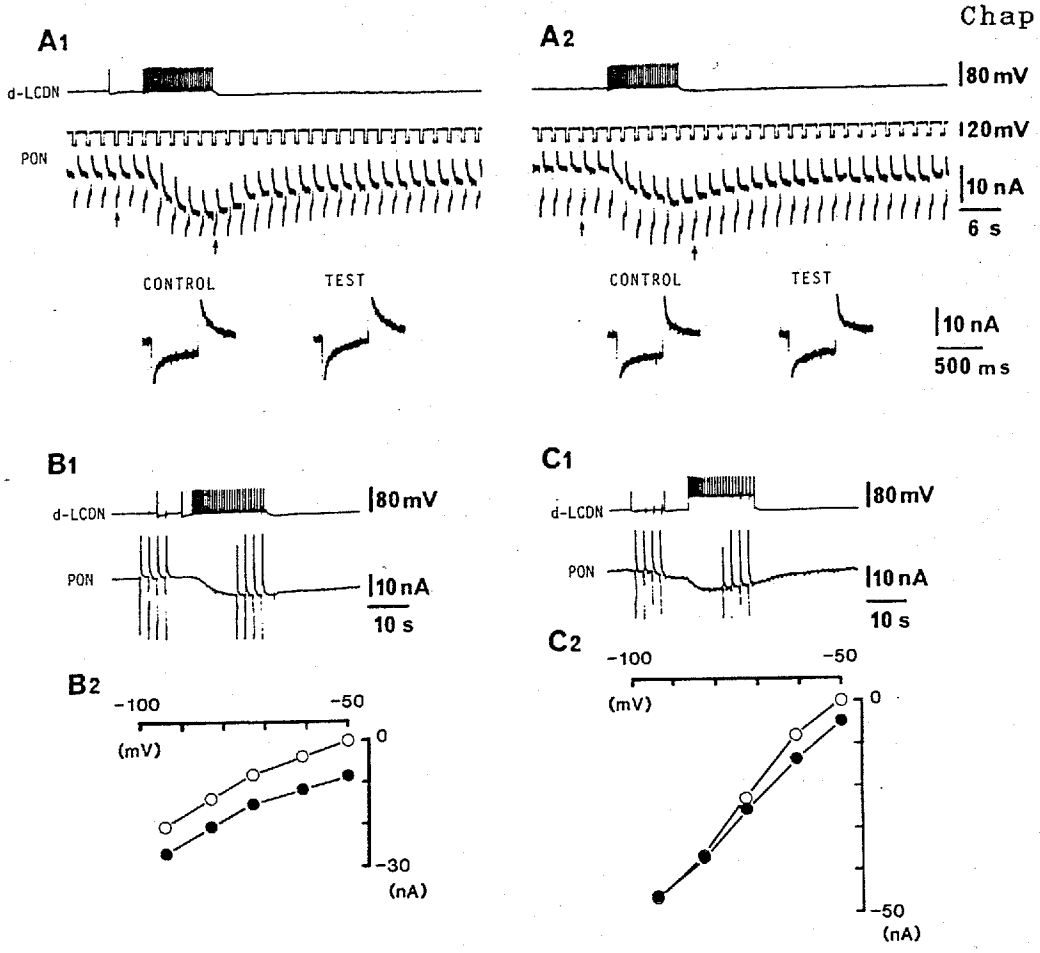

Fig. 6.2. A: Inward shift in holding current of PON induced by activity of d-LCDN. Holding potential of PON was -50 mV in A1 and -70 mV in A2. Hyperpolarizing command pulses (20 mV, 500 ms duration) were applied at 0.5 Hz to monitor the change of conductance. Arrows indicate selected currents which are displayed in lower insets at expanded time scale. Note the decrease of conductance in PON produced by activity of d-LCDN. Spike number was 42 in A1 and 39 in A2. A1 and A2 were obtained from the same preparation. B: I-V relationships of PON before and during a burst of d-LCDN in the normal physiological solution. C: I-V relationships of PON before and during a burst of d-LCDN in 3 K solution. Holding potential was -50 mV in both B and C. I-V relationships were measured by applying command pulses (200 ms duration) before and during a burst of d-LCDN which are seen as vertical deflections in B1 and C1. Amplitude of current at the end of the pulse is plotted against the command voltage in B2 and C2. The holding current before the burst of d-LCDN is denoted to 0 nA. Open circle; before the burst. Closed circle; during the burst. d-LCDN was driven to fire by a depolarizing current injection. Spike number was 35 in B and 31 in C. B and C were from the same preparation.

K^+ conductance. Thus, the synaptic response in the normal physiological solution was compared with that in 3K solution (Table 3.1). The inward shift in holding current produced by the d-LCDN burst was larger in the normal physiological solution (Fig. 6.2B1) than in 3K solution (Fig. 6.2C1). The current-voltage (IV) relationships in normal solution, measured using 200 ms hyperpolarizing pulses, showed that the extra membrane current induced by the activity of d-LCDN (difference between open and closed circles in this figure) was reduced by hyperpolarization but not reversed (Fig. 6.2B2). In 3K solution, the effect of d-LCDN activity upon I-V curve was much smaller and showed a null effect at -90 mV (Fig. 6.2C2). Similar results were obtained in all tested preparations (n=5). These results suggest that a decrease of K^+ conductance is involved in the slow depolarization induced by the cerebral neurones.

In some preparations (n=3), the activity in the cerebral cells produced a transient increase of conductance before the decrease in conductance (Fig. 6.3A1), which can clearly be seen when conductance is plotted as a proportion of the value before the d-LCDN burst (Fig. 6.3A2). Thus activity of the cerebral cells induces a further change in PON, besides a decrease in K^+ conductance. To further investigate whether the inward shift in holding current induced by cerebral cell activity was dependent on K^+ conductance, Cs^+ was injected into PON since this ion blocks K channels (Akaike *et al.*, 1978; Colmers *et al.*, 1982). Before Cs^+-injection, PON showed a steady inward shift in holding current during the burst of d-LCDN and the response was recovered quite slowly after the burst (Fig. 6.3B1). After Cs^+-injection, the synaptic response began to decay

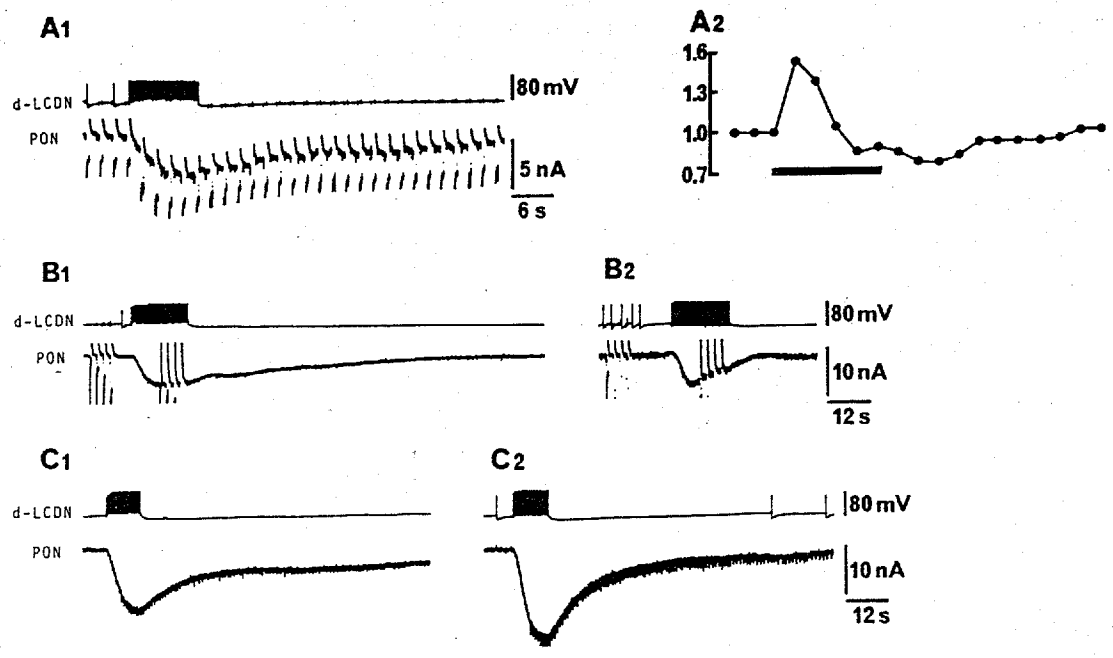

Fig. 6.3. A1: Inward shift in holding current of PON induced by activity of d-LCDN, preceded by a transient increase in conductance. Holding potential was -60 mV, and hyperpolarizing command pulses (20 mV, 500 ms duration) were applied at 0.5 Hz. d-LCDN was driven to fire by depolarizing current injection. Spike number was 50. A2: Change in conductance of PON produced by a burst of d-LCDN, obtained from the same record as displayed in A1. Vertical scale indicates the conductance and control conductance is denoted as 1.0. Horizontal interval between points is 2 s. Bar indicates the duration of a burst of d-LCDN. B: Effect of Cs^+-injection into PON on the inward shift in holding current induced by activity of d-LCDN. Holding potential was -60 mV. B1; Control. B2; After Cs^+-injection. d-LCDN was driven to fire by depolarizing current injection. Spike number was 61 in B1 and 68 in B2. Vertical deflections in the current traces are currents in response to the command pulses. C: Effect of EGTA-injection into PON on the inward shift in holding current induced by activity of d-LCDN. Holding potential was -50 mV. C1; Control. C2; After EGTA-injection. d-LCDN was driven to fire by a depolarizing current injection. Spike number was 37 in C1 and 40 in C2.

during the d-LCDN burst, and there was rapid recovery (Fig. 6.3B2). Although the result is consistent with the notion that the slow depolarization induced by the cerebral cells is due to a decrease in K^+ conductance, the peak level of the synaptic response did not change (Fig. 6.3B2). This result, together with the transient increase in conductance (Fig. 6.3A), suggests that the slow depolarization of PON induced by the cerebral cells is due to two different conductance mechanisms: a decrease of K^+ conductance which persists after activity in the cerebral cells, and a transient increase in conductance of an unknown ion. As Mg^{2+} can be neglected, possible ions are Na^+, Ca^{2+} and Cl^-. Na^+ can be excluded as there was no effect on this response when half of Na^+ was replaced by $Tris^+$. Cl^- cannot be involved because Cl^--injection into PON had no effect. Thus, the only plausible mechanism appears to be an increase of Ca^{2+} conductance.

The possible involvement of Ca^{2+} could not be investigated by ionic substitution since this would modify the transmitter release. Instead, the effect of EGTA-injection into PON upon the synaptic response was investigated. Because the Ca channel is known to be inactivated by an increase in $[Ca^{2+}]_{in}$ and that kind of inactivation can be depressed by the injection of EGTA (Plant *et al.*, 1983), the peak amplitude of the synaptic response in PON may be increased by EGTA-injection if the increase of Ca^{2+} conductance is involved. As expected, the peak amplitude of the response was clearly increased by EGTA-injection into PON (Fig. 6.3C). This result is consistent with the notion that an increase of Ca current, in addition to a decrease of K current, is concerned in the

slow depolarization of PON induced by the cerebral cell activity.

Ionic mechanisms of the slow depolarization of PON by 5-HT

With the membrane potential of PON clamped at -50 mV, application of 5-HT, at concentrations above about 1 μM, produced an inward shift in holding current with a decrease in conductance. Preliminary experiments showed that the response was unaffected by a decrease in Na concentration, but was reduced by an increase in K concentration.

The effect of K concentration upon the 5-HT response was investigated in the absence of Na^+ and Ca^{2+}, and in the presence of Co^{2+} (the Ca channel blocker). The solutions were made by mixing the Na-, K-, Ca-free solution and the Na-, Ca-free, 10K solution (see Table 3.1). Quasi-steady-state I-V relationships were measured using 300 ms command pulses. Sodium-free conditions were employed because it was easier to measure the 5-HT-sensitive current in the absence of the Na current. The measurements were made in the absence of Ca^{2+}, and the presence of Co^{2+}, because 5-HT was found to increase the voltage-dependent Ca current (as shown later).

5-HT produced an inward shift in holding current of PON (see upper inset of Fig. 6.4A1) and decreased outward current at every tested voltage at a potassium concentration of 3.3 mM (Fig. 6.4A1). This 5-HT-sensitive current (i.e. difference current) showed little time-dependency (see lower inset of Fig. 6.4A1). The 5-HT-sensitive current became smaller with hyperpolarization but did not reverse (Fig. 6.4A2; Fig. 6.5, circles). At a potassium concentration of 33 mM, 5-HT produced an outward shift of

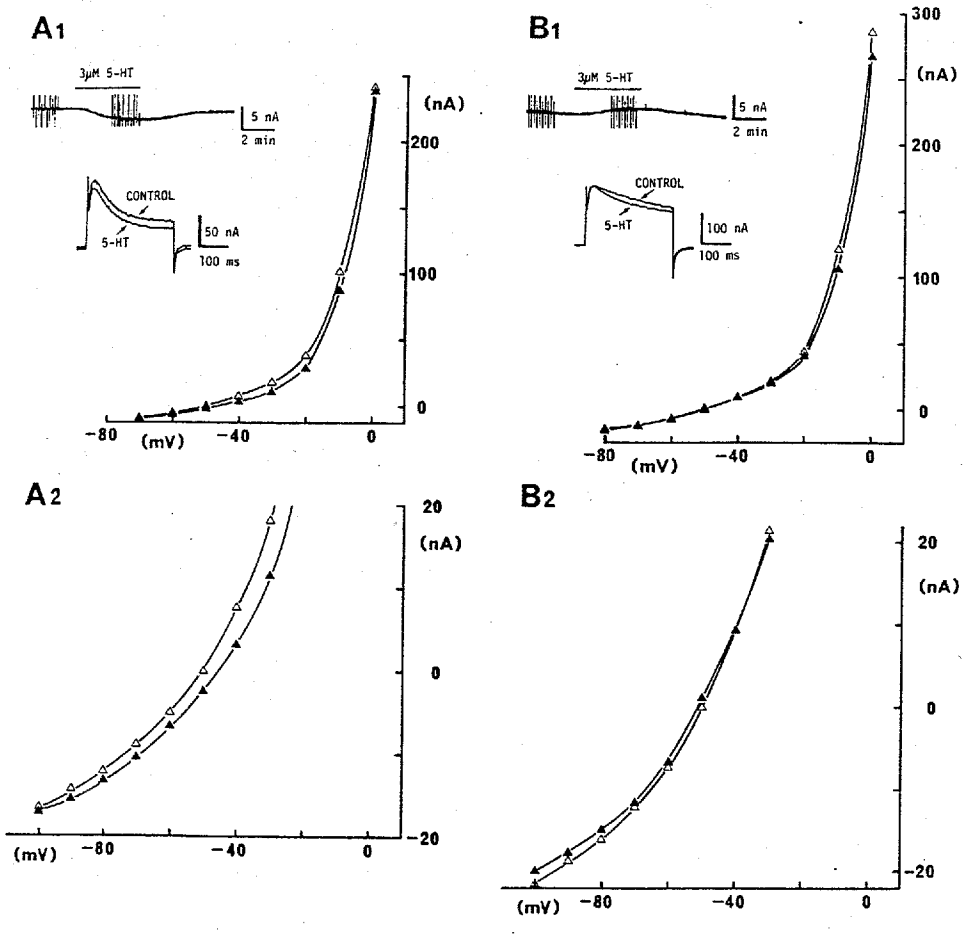

Fig. 6.4. Effects of 5-HT on the quasi-steady membrane current of PON. Amplitude of current at the end of the command pulse (300 ms duration) was plotted against the command voltage. Holding potential was -50 mV. Open-triangle; I-V relationship before application of 3 μM 5-HT. Closed-triangle; I-V relationship during application of 3 μM 5-HT. The holding current before application of 5-HT is denoted to 0 nA. Vertical deflections in upper insets of A1 and B1 are current signals in response to command pulses at 0.1 Hz. A and B are from the same preparation. All records were made in Na-, Ca-free solution as described in the text. A1: I-V relationships in 3.3 mM K^+ solution. Upper inset shows the effect of 5-HT on the holding current. Lower inset shows currents in response to the command pulse to -20 mV with and without 5-HT. A2: I-V relationships at enlarged vertical scale. B1: I-V relationships in 33 mM K^+ solution. Upper inset shows the effect of 5-HT on the holding current. Lower inset shows currents in response to the command pulse to -10 mV with and without 5-HT. B2: I-V relationships at enlarged vertical scale.

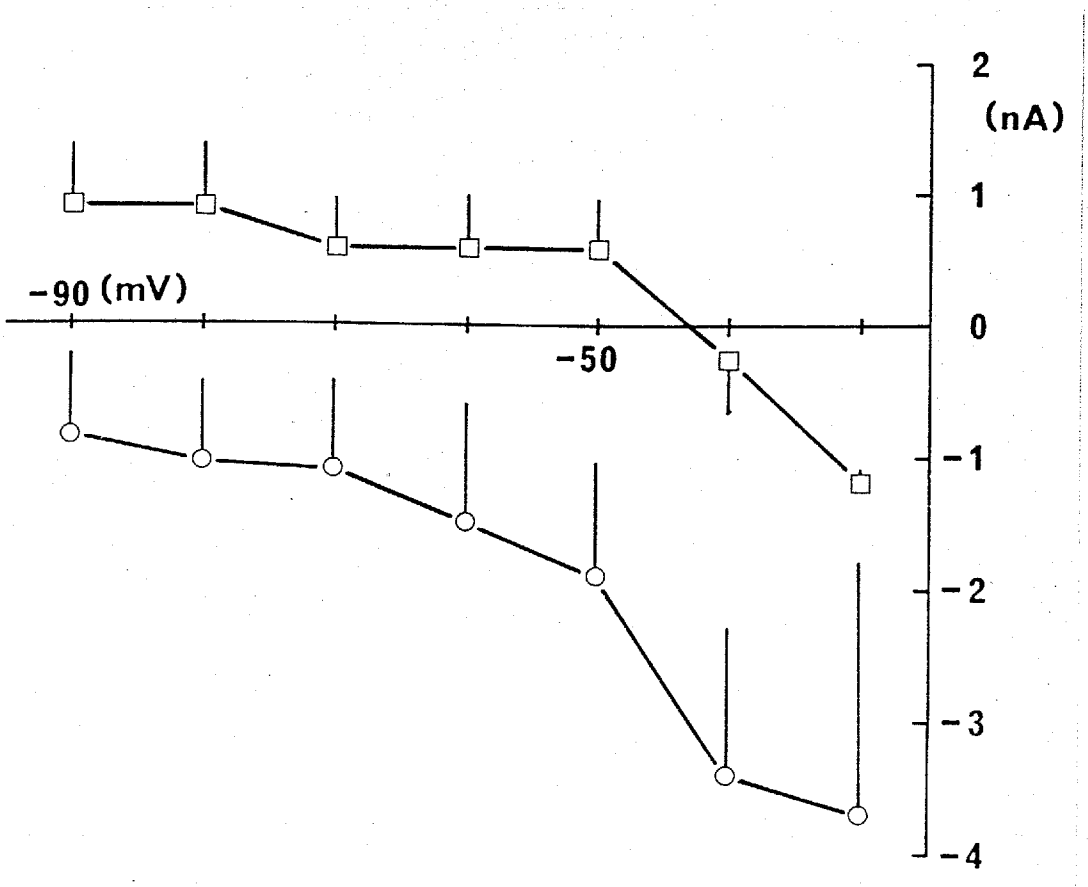

Fig. 6.5. 5-HT-sensitive current in 3.3 mM K^+ solution (circle) and 33 mM K^+ solution (square). 5-HT-sensitive current was obtained as the difference in currents obtained before and during the 5-HT application in an experiment like that in Fig. 6.4. Amplitude of the mean 5-HT-sensitive currents is plotted against the command voltage; each vertical bar is the S.D. of the mean. The number of preparations was 9 in the case of 3.3 mM K^+ and 3 in the case of 33 mM K^+.

holding current (see upper inset of Fig. 6.4B1). The 5-HT-sensitive current reversed at about -40 mV (Fig. 6.4B2; Fig. 6.5, squares). The results also indicate that the ion channel carrying the 5-HT-sensitive current can function over a wide range of voltages, around the resting level (-50 mV). A plot of reversal potential as a function of the log of K concentration, using extrapolation to obtain a value for 3.3 mM, is in good agreement with the Nernst equation (Fig. 6.6) and suggests that the current is a K current. The current was also found to be blocked by injection of Cs^+ into PON (data not shown), producing further evidence that the current is a K current. These results indicate that the slow depolarization by 5-HT is mainly due to a decrease in K^+ conductance.

Two 5-HT-sensitive K channels identified by the patch-clamp experiments

By the whole-cell voltage clamp, 5-HT-sensitive K current could be identified as described above. However, it is not clear whether 5-HT-sensitive K current represents the activity of a single species of K channel, because the whole-cell current reflects the sum of the activities of many ion channels. Thus, the single channel recording by the patch clamp technique was carried out in PON.

Single channel recording was made by the cell-attached configuration of the improved patch clamp technique (see Chap. Ⅲ ; see also Hamill *et al.*, 1981). One difficulty of the cell-attached configuration in PON was its unstable membrane potential as PON is the bursting neurone. Since the membrane potential of the cell-attached patch (patch potential) is the resting potential of a cell minus the

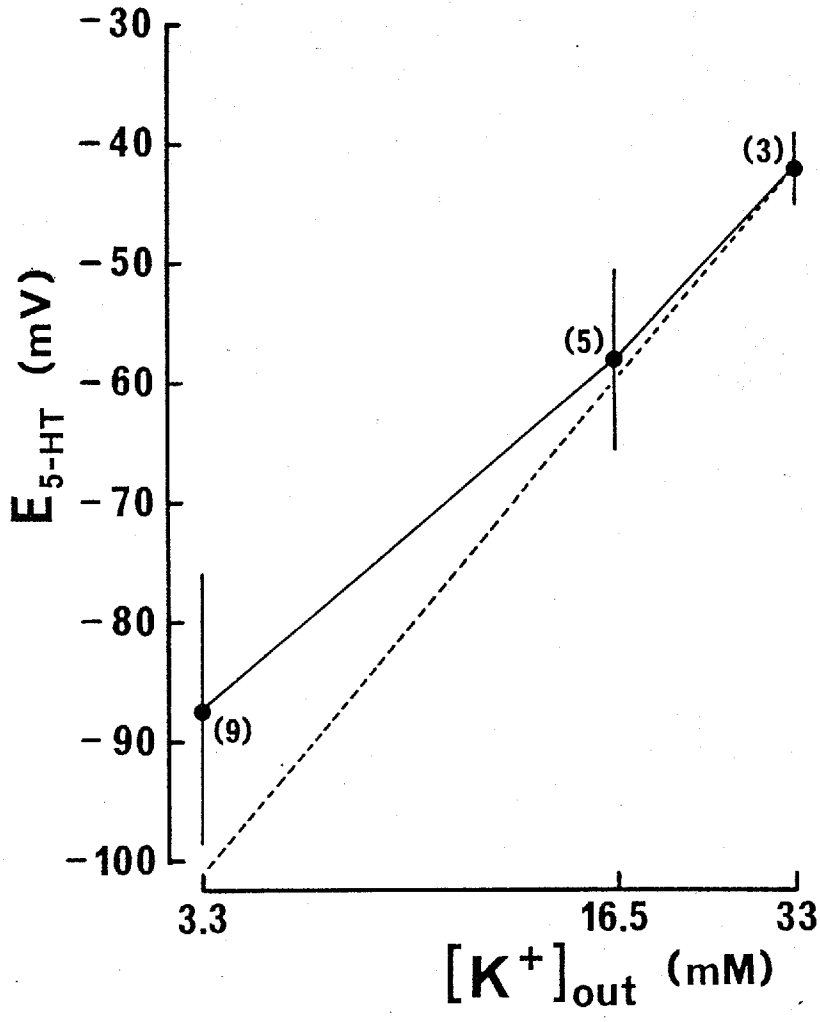

Fig. 6.6. Effect of $[K^+]_{out}$ upon reversal potential of 5-HT-sensitive current in PON. Closed circles are mean values of 3 to 9 preparations and bars are S.D. of the mean. The value in 3.3 mM K^+ was obtained by the extrapolation. The number in the parentheses indicates the number of preparations. The dotted line represents the change of E_K anticipated by the Nernst equation. It was drawn through the mean value at 33 mM $[K^+]_{out}$.

pipette potential, the patch potential cannot be clamped if the resting potential of a cell is not stable. To overcome this problem, the bursting activity of PON was stopped by perfusing the ganglia with Tris solution (see Table 3.1). The concentration of K^+ in this solution is the same as that of the normal physiological solution (3.3 mM). In Tris solution, the resting potential of PON was stable, and the averaged value was -63.3 ± 6.7 mV (mean $\pm$ S.D., n=20). The patch pipette was usually filled with Tris or 85 mM K solution. In some experiments, the pipette filled with the normal solution was used to ascertain whether $Tris^+$ has any action on the activities of the channels. The results were not different from those obtained by the pipette filled with Tris solution. The single channel recording was made under the steady state, since the 5-HT-sensitive K current was seen in the steady state (see Fig. 6.4). In such condition, other voltage-dependent ion channels which have voltage- and time-dependent inactivations would not be recorded.

Fig. 6.7 shows single channel activities of the two distinct 5-HT-sensitive channels which have different single channel conductances and gating kinetics (see also Fig. 6.8). The channel shown in Fig. 6.7A, having larger unitary current, is tentatively called SL-channel (serotonin-sensitive large channel), and the channel in Fig. 6.7B is called SS-channel (serotonin-sensitive small channel) in this work, because a single 5-HT-sensitive K channel has been identified in *Aplysia* and named S-channel (Siegelbaum *et al.*, 1982). Both SL- and SS-channels were functioning in the steady depolarized state and showed the flickering kinetics; i. e. the fast transition between the open state and the closed one. When 5-HT was applied by

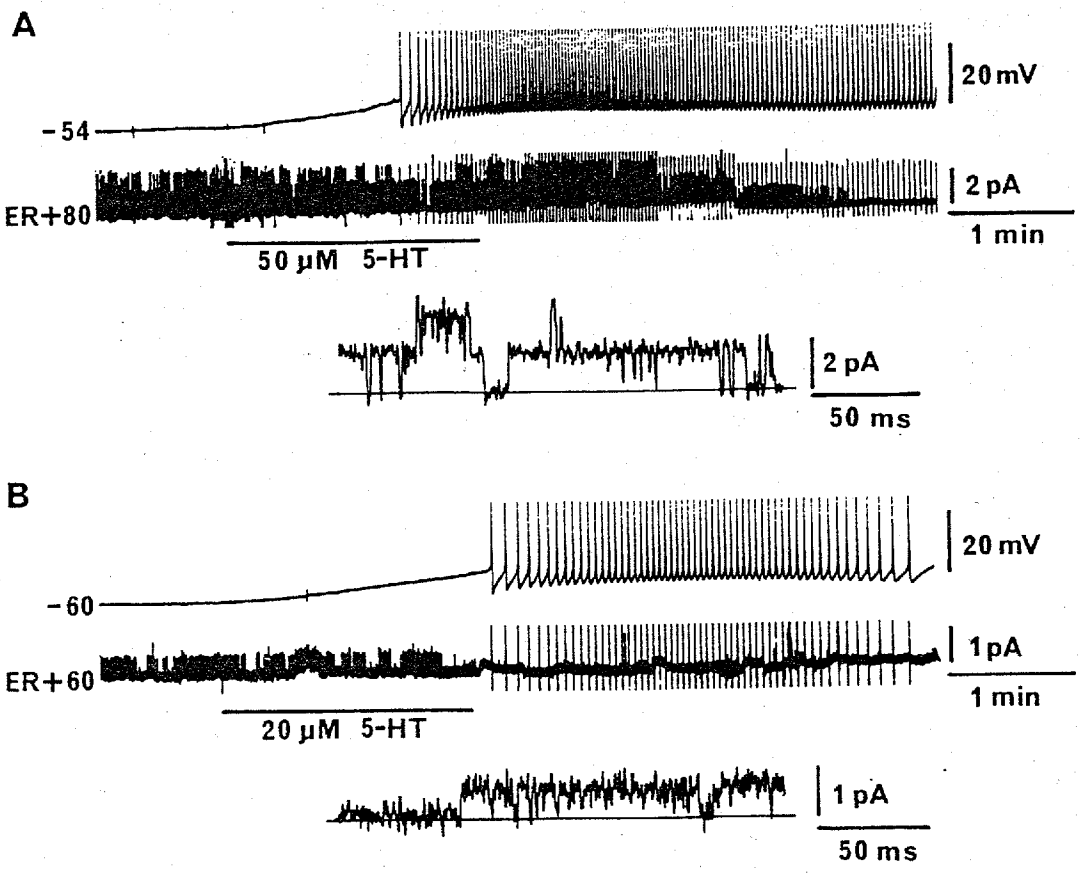

Fig. 6.7. Two 5-HT-sensitive channels recorded by the patch clamp technique. A:SL-channel. B:SS-channel. In both A and B, upper trace is the membrane potential of PON, middle trace is activity of the ion channel in the cell-attached patch, and lower trace is unitary currents before application of 5-HT at the expanded time scale. The pipette was filled with Tris solution in both experiments. Left-side figure in each upper trace indicates the resting potential of PON before the application of 5-HT. ER; the resting potential. The patch potential was 80 mV more positive than the resting potential in A and 60 mV more positive in B. Straight line in each lower trace shows the closed level of the channel. 5-HT was applied by bath perfusion.

bath perfusion, the activities of both channels disappeared with some delay. Similar results were obtained in three other preparations for SL-channel and in seven for SS-channel. The recovery of channel activity was not usually seen during the recording period; the recovery was seen in one patch for SL-channel and two patches for SS-channel. The irreversibility of 5-HT action is probably because of the high concentration of 5-HT being used. Because the limit of stable single channel recording was 10-20 min in most experiments, rather high concentration of 5-HT was used to ensure its effect. The result of the cell-attached patch clamp experiments also suggests the mode of action of 5-HT. When a giga-seal is established, the distance between the glass-rim and the membrane is in the order of Å (Hamill *et al.*, 1981). Thus, the bath-applied 5-HT cannot reach the channels in the cell-attached patch. Accordingly, the 5-HT action should be mediated by the second messenger system(s). Since the activities of both channels remained unchanged in the inside-out patch where the intracellular surface was perfused with high-K, Ca-free solution containing 5 mM EGTA (Table 3.1), these channels are not Ca^{2+}-dependent.

I-V relationships of these two channels are illustrated in Fig. 6.8. I-V relationship of SL-channel was non-linear and showed clear outward-rectification when a pipette was containing 3.3 mM K^+; i.e. Tris or the normal solution (Fig. 6.8A2, circle). The data could be fitted with the Goldman-Hodgkin-Katz equation (GHK-equation) for K^+ (Hodgkin & Katz, 1949).

That is:

$$I_K = P_K E \cdot \frac{F^2}{RT} \cdot \frac{[K^+]_{out} - [K^+]_{in} e^{FE/RT}}{1 - e^{FE/RT}}$$

where I_K is the single channel current amplitude, E is the patch potential, and P_K is the permeability of K^+. F, R, and T have their usual thermodynamic means. In the experiment of Fig. 6.8A1, P_K and $[K^+]_{in}$ were estimated to be 9.2×10^{-14} cm^3/s and 147 mM by the least square method. Thus, E_K was calculated to be -97.3 mV. The mean$\pm$ S.D. of P_K and E_K were $9.4 \pm 0.1 \times 10^{-14}$ cm^3/s and -96.6 ± 0.75 mV (n=4). The slope conductance at 0 mV was 23.2$\pm$ 5.2 pS (n=4). In two patches, the reversal of the SL-channel current could be obtained when the pipette was filled with 85 mM K solution (Fig. 6.8A2). The reversal potential of them was -7.9 mV and -4.2 mV, and the slope conductance at 0 mV was 34.0 pS and 27.3 pS. I-V relationship in such condition was almost linear (Fig. 6.8A2, triangle) and could be fitted with the GHK-equation using the values of P_K and $[K^+]_{in}$ obtained by the fitting of the data in 3.3 mM K^+. These results indicate that SL-channel is K channel and its outward-rectifying property is due to the constant-field rectification. Although detailed kinetic analyses are not yet done, the inspection of single channel data revealed that SL-channel was almost voltage-independent. The activity of the channel was not markedly different in the wide range of voltage and the single channel current could be recorded at the hyperpolarized potential as well as the depolarized potential if the driving force for K^+ was

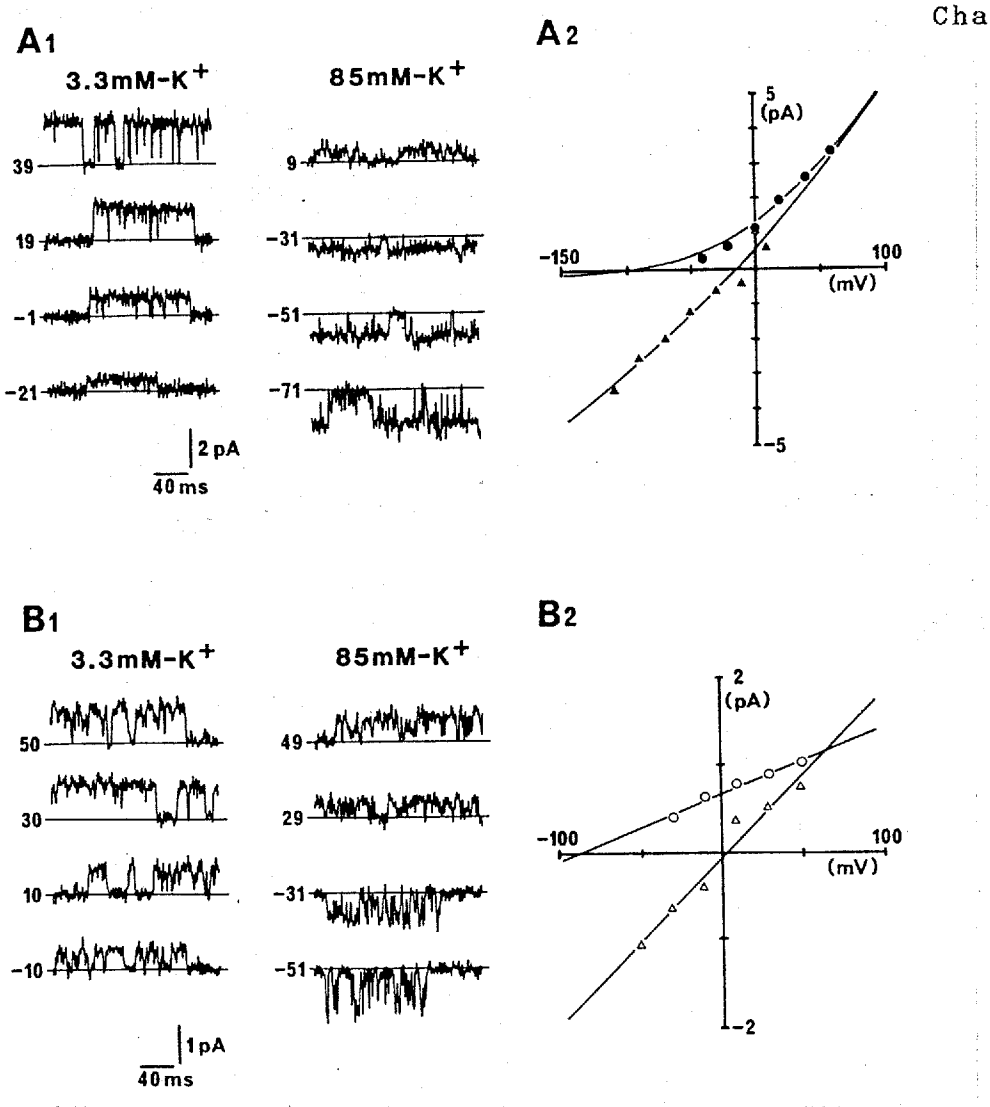

Fig. 6.8. A1:Unitary currents of SL-channel with the pipette containing 3.3 mM K^+ or 85 mM K^+. Left-side figure in each trace indicates the patch potential. A2:I-V relationships of SL-channel. The same data as shown in A1. Circle; 3.3 mM K^+. Triangle; 85 mM K^+. The smooth line for the data in 3.3 mM K^+ was the regression line of the GHK-equation by the least square method. P_K and $[K^+]_{in}$, were estimated to 9.2×10^{-14} cm^3/s and was 147 mM. The smooth line for the data in 85 mM K^+ was drawn by the GHK-equation using the same P_K and $[K^+]_{in}$. B1:Unitary currents of SS-channel with the pipette containing 3.3 mM K^+ or 85 mM K^+. Left-side figure in each trace indicates the patch potential. B2:I-V relationships of SS-channel. The same data as shown in B1. Circle; 3.3 mM K^+. Triangle; 85 mM K^+. A straight line was drawn by the least square method. Single channel conductance; 7.5 pS in 3.3 mM K^+ and 18.9 pS in 85 mM K^+.

appropriate (see the data in 85 mM K^+ in Fig. 6.8).

I-V relationship of SS-channel was linear (Fig. 6.8B2). When the pipette solution was containing 3.3 mM K^+, the extrapolated reversal potential was -86.4± 12.3 mV and the single channel conductance was 8.5± 1.7 pS (n=13). When the pipette was filled with 85 mM K solution, the SS-channel current reversed at about 0 mV (0.0± 3.4 mV, n=4). The single channel conductance was increased to 17.1± 2.4 pS (n=4) in 85 mM K^+. These results indicate that SS-channel is also K channel. In contrast to SL-channel, SS-channel was voltage-dependent. Its activity was not seen until the patch potential was depolarized from the resting potential even though the pipette was filled with 85 mM K solution and the opening probability was clearly increased by the depolarization.

5-HT modulates the active currents in PON

The effects of 5-HT on the active currents were examined in the axotomized PON. This preparation is more suitable than the intact neurone for this purpose because it provides much better conditions for space-clamp.

The membrane currents of axotomized PON, measured using depolarizing command pulses, were characterized by a transient inward current and slowly developing outward current (Fig. 6.9A). Holding potential was set at -40 mV to inactivate A-current (Thompson, 1977). Peak inward current and delayed outward current were raised by 5-HT (Fig. 6.9) and the transient outward current (A-current) which was seen after turning off the hyperpolarizing command pulse also showed a slight increase (Fig. 6.9B). Similar results were obtained in all tested cells (n=11) and threshold

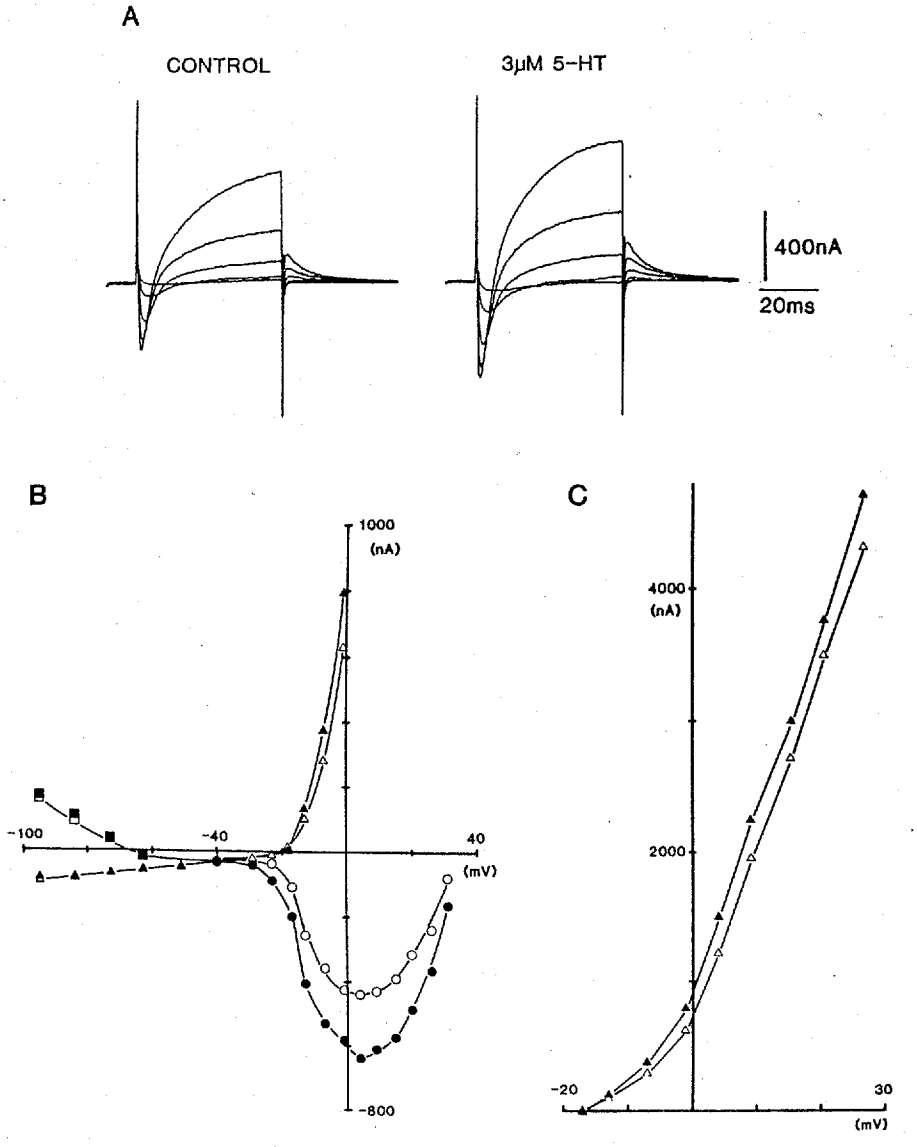

Fig. 6.9. Effects of 5-HT on the active currents of PON in the normal physiological solution. A:Membrane currents with and without 3 μM 5-HT. Holding potential was -40 mV. The command pulses were 50 ms in duration and stepped to -23, -17, -13, -7 and -1 mV. B:I-V relationships with (closed symbols) and without (open symbols) 3 μM 5-HT. Circle; peak inward current. Triangle; current at the end of the pulse. Square; peak transient outward current activated after the hyperpolarizing pulse. C:I-V relationships of outward currents measured at the end of the pulse with (closed triangle) and without (open triangle) 3 μM 5-HT. A part of them (below 0 mV) is plotted in B at enlarged vertical scale.

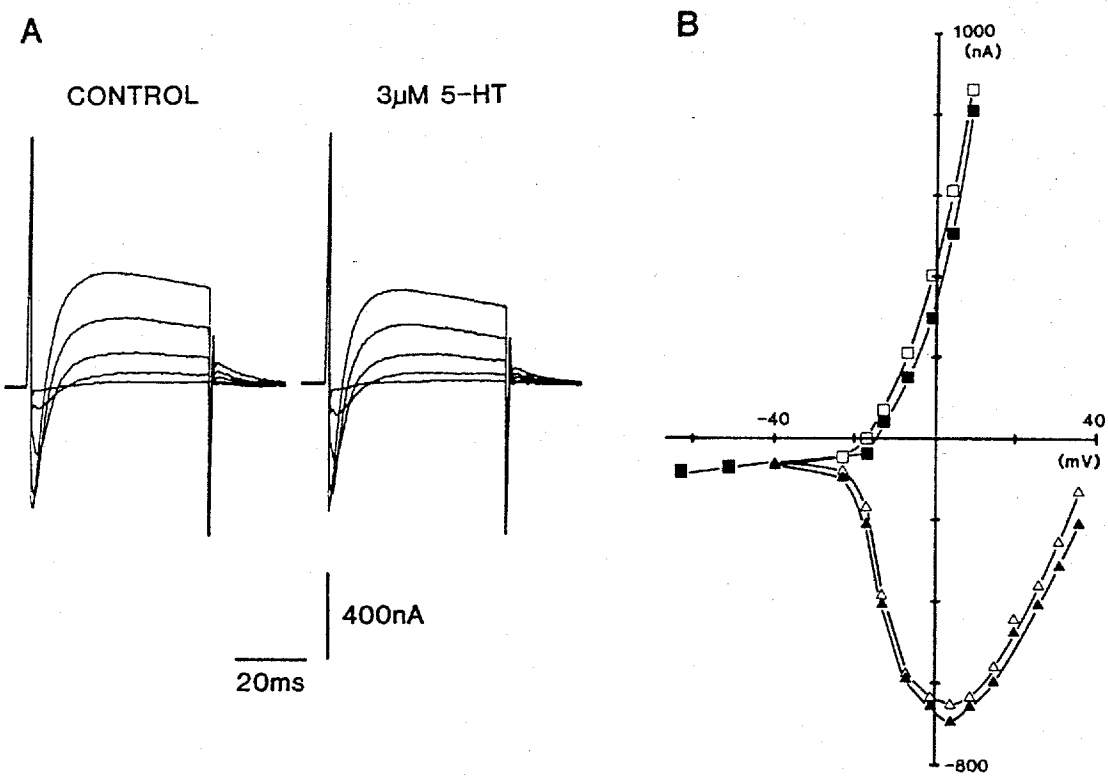

Fig. 6.10. Effects of 5-HT on the active currents of PON in Ca-free solution. A:Membrane currents with and without 3 μM 5-HT. Holding potential was -40 mV. The command pulses were 50 ms in duration and stepped to -23, -17, -13, -7 and -1 mV. B:I-V relationships with (closed symbols) and without (open symbols) 3 μM 5-HT. Triangle; peak inward current. Square; current at the end of the pulse.

concentration of 5-HT was about 1 μM.

In the normal physiological solution, outward current increased during command pulse (see Fig. 6.9A). In Ca-free solution, however, outward current came to a peak followed by a slight decay during the command pulse, and total outward current was greatly reduced (Fig. 6.10A). These results suggest that the delayed outward current of PON in the normal solution includes a substantial Ca^{2+}-dependent K current (Meech, 1978). 5-HT reduced the outward current and slightly raised the peak inward current in Ca-free solution (Fig. 6.10), indicating that 5-HT lowered the K^+ conductance. Similar results were obtained in three other preparations. It is therefore proposed that 5-HT increases Ca current, and that the increased Ca^{2+}-influx produces an increase of Ca^{2+}-dependent K current secondarily, although the possibility that 5-HT directly increases the Ca^{2+}-dependent K current remains to be tested.

5-HT increases the activity of the K channel having a large conductance

By the path clamp technique, single Ca^{2+}-dependent K channel currents have been recorded in several tissues (see review, Petersen & Maruyama, 1984). The single channel currents show flickering (Pallotta *et al.*, 1981; Maruyama *et al.*, 1983) and the conductance of such channels is usually very large, i.e. 100 to 250 pS in symmetrical high-K^+ solution (Petersen & Maruyama, 1984). In PON, a single K channel current which showed similar features with the Ca^{2+}-dependent K channels was recorded. In this work, this channel is called LK-channel (large K channel) as the Ca^{2+}-sensitivity of the channel is not yet tested. Fig. 6.11

shows I-V relationships of LK-channel recorded by the pipette filled with Tris solution (3.3 mM K^+) or 85 mM K solution. In 3.3 mM K^+, the activity of LK-channel was not seen until the patch potential was depolarized to more than 0 mV. The activity was increased with further depolarization. These results are consistent with the notion that LK-channel is Ca^{2+}-dependent. Ca^{2+}-influx through the patch membrane by a large depolarization is considered to be required for the activation of Ca^{2+}-dependent channels. I-V relationship of LK-channel seemed to be linear in the recorded voltage range and the single channel conductance was 64 pS. LK-channel current showed a marked flickering between the open and the closed states, especially at the highly depolarized potential. Even if the pipette was filled with 85 mM K solution, the LK-channel activity was not seen at the resting potential. However, the long-lasting depolarization (e. g. for one minute) of the patch to +40 mV or more induced the channel activity and the activity continued for several minutes even after the repolarization. The data in 85 mM K^+ in Fig. 6.11 was obtained after such depolarization. I-V relationship in 85 mM K^+ was nearly linear and the conductance was 83 pS (Fig. 6.11B). The reversal potential was -9.3 mV, which is well within the expected E_K range when the pipette was filled with 85 mM K solution. This result suggests that LK-channel is K channel. However, the reversal potential at 3.3 mM K^+ estimated by the linear extrapolation in Fig. 6.11B (dotted line) was far less than the expected E_K. This result may suggest the non-linearity of the LK-channel current in the physiological K^+-gradient, although it is also possible that LK-channel is not completely selective to

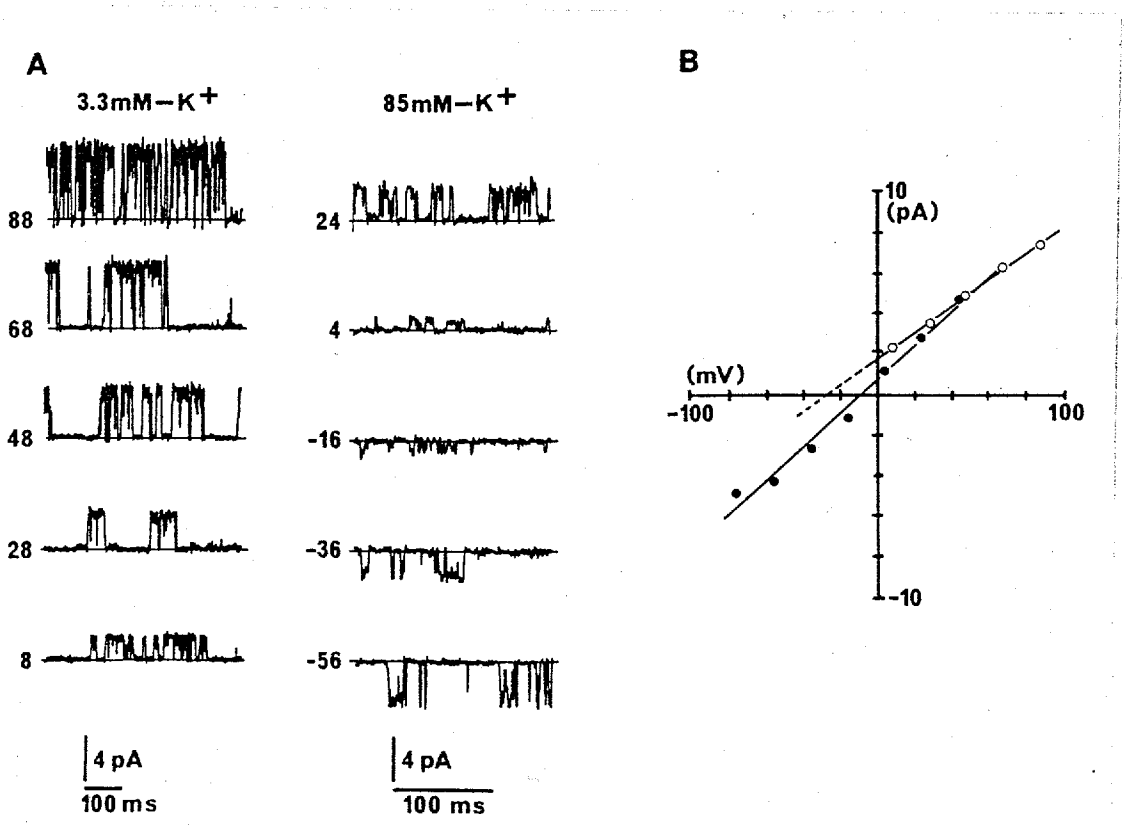

Fig. 6.11 A: Unitary currents of LK-channel with the pipette containing 3.3 mM K^+ or 85 mM K^+. Left-side figure in each trace shows the patch potential. B: I-V relationships of LK-channel. Open circle; 3.3 mM K^+. Closed circle; 85 mM K^+. A straight line and a dotted line were drawn by the least square method.

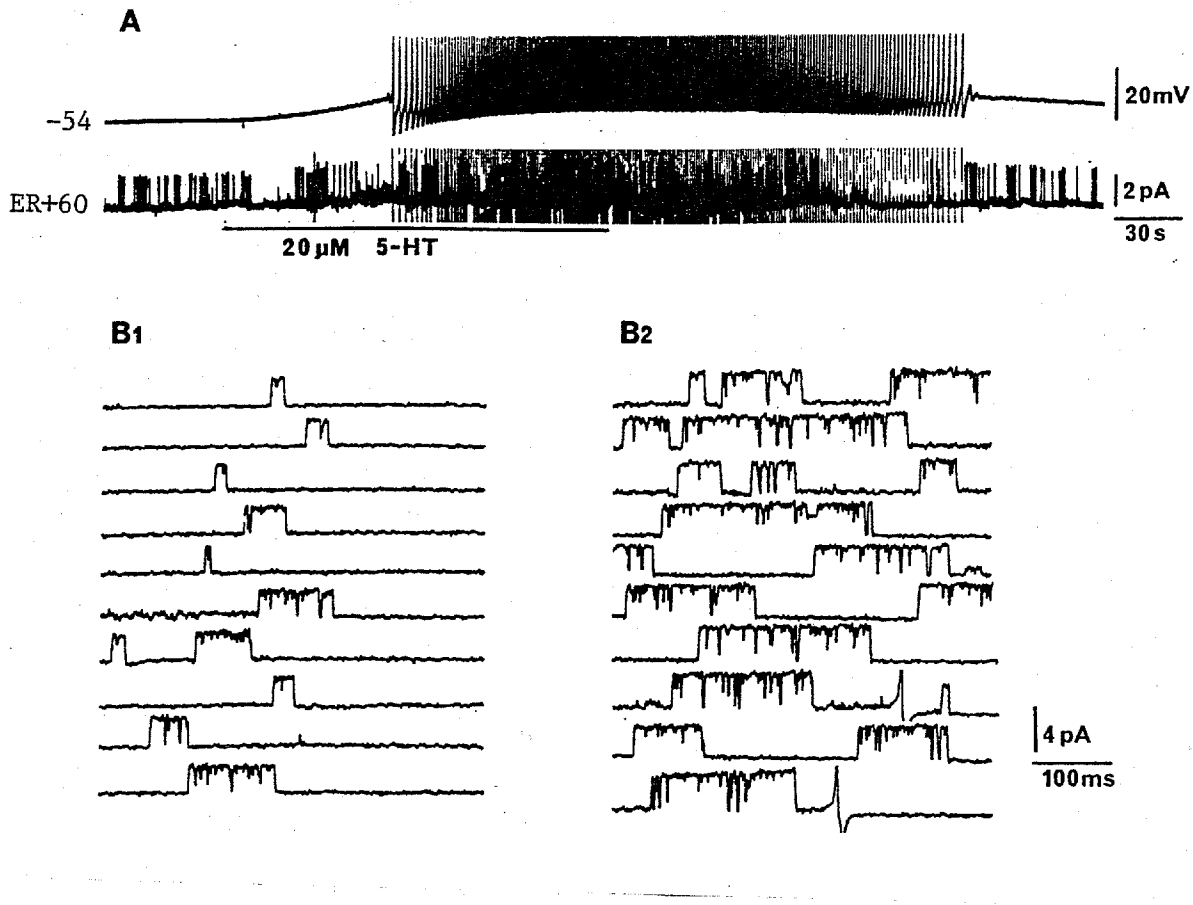

Fig. 6.12 The increased activity of LK-channel by 5-HT. The pipette was filled with Tris solution. A: Simultaneous recording of the membrane potential of PON and the activity of LK-channel. ER; the resting potential. The patch potential was 60 mV more positive than the resting potential. B1; Unitary currents of LK-channel before application of 5-HT. B2; Unitary currents of LK-channel during application of 5-HT. Slight increase of a single channel current in B2 was due to the depolarization of PON.

potassium.

The activity of LK-channel was increased by application of 5-HT (Fig. 6.12). Both the probability of opening and the burst time appeared to be increased by 5-HT (Fig. 6.12B). This result was not simply due to the change of the patch potential by the depolarization of PON, as the gating of LK-channel was not markedly changed in the voltage range encountered in the experiment of Fig. 6.12 (about 14-24 mV). At present, it is not clear whether the increased activity of LK-channel was due to the modulation of the channel by 5-HT or due to the influx of Ca^{2+} during a burst of impulses of PON produced by 5-HT. However, as seen in Fig. 6.12, the change of the LK-channel activity was correlated to a burst of impulses of PON and the activity of the channel was recovered rather quickly in contrast to the results in SL- and SS-channel. This result seems to favor the hypothesis that the increased activity of LK-channel by 5-HT is due to the Ca^{2+}-influx during a burst of spikes.

5-HT increases Ca current in PON

To investigate further whether 5-HT raised Ca current, the effect of 5-HT was examined in TEA, Ba solution (see Table 3.1), in which all Na^{+} was replaced by TEA^{+} to block Na and K currents. Ba^{2+} does not activate Ca^{2+}-dependent K current and a larger current can be recorded since Ba^{2+} is more permeable than Ca^{2+} in the Ca channel (Hagiwara & Ohmori, 1982). Ba^{2+} is also known to block some K channels (Hille, 1984). In the experiment shown in Fig. 6.13, 5 mM 4-AP was also added to block A-current (Thompson, 1977). In response to the depolarizing steps, slowly activating Ba currents were recorded and these currents showed little

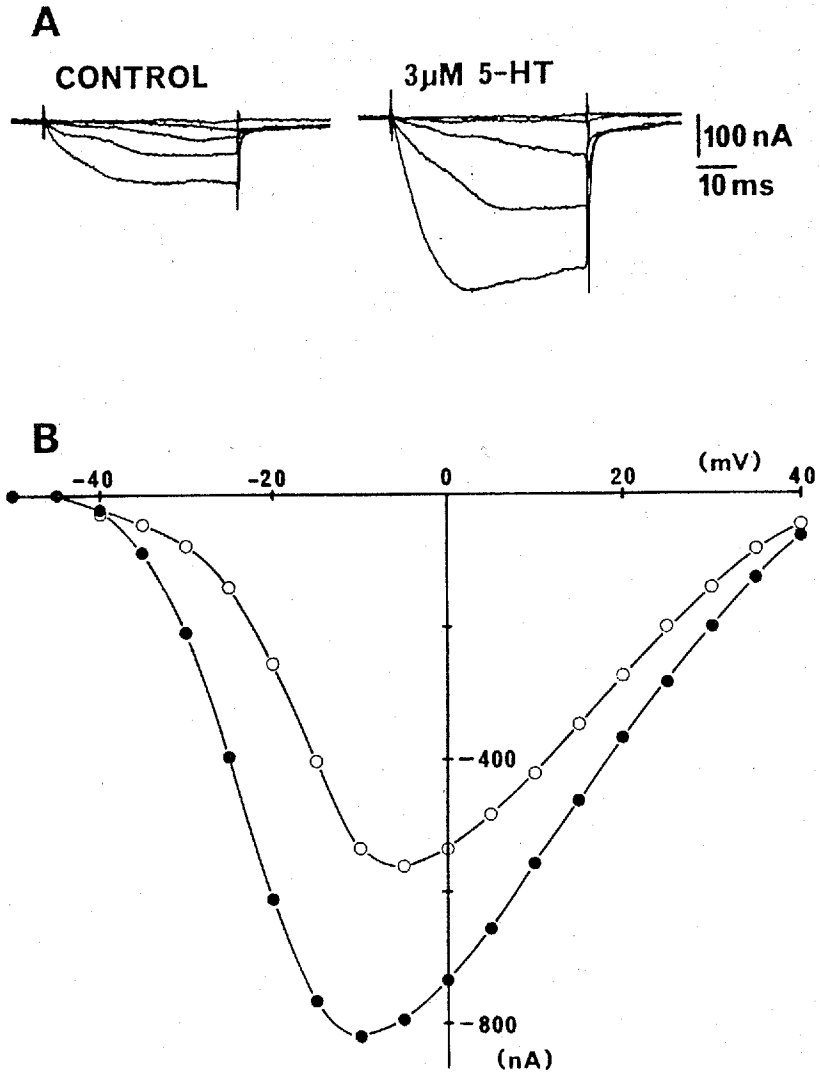

Fig. 6.13. Effect of 5-HT on Ba current of PON in the TEA, Ba solution with 5 mM 4-AP. A:Ba currents with and without 3 μM 5-HT. Holding potential was -50 mV. The command pulses were 50 ms in duration and stepped to -45, -40, -35, -30 and -25 mV. Capacitive and linear leak currents were subtracted. B:I-V relationships of the peak Ba current with (closed circle) and without (open circle) 3 μM 5-HT. The slight shift of the I-V relationship along the voltage axis in 5-HT-containing solution is not typical.

inactivation during the command pulse (Fig. 6.13). This Ba current was depressed by the addition of 2 mM Co^{2+} (not shown), which suggests that this current is carried through the Ca channel. The Ba current was greatly increased by 5-HT (Fig. 6.13) and this was confirmed in six other cells. These results indicate that 5-HT increases the voltage-dependent Ca current of PON.

Single Ca channel current was recorded by the pipette filled with 70 mM Ba solution (see Table 3.1), because single Ca channel current is too small in the physiological condition. Ca channel current could be seen by the steady depolarization or the step depolarization. The change of the patch potential was limited to the small depolarization from the holding potential, since the large depolarization induced the simultaneous openings of several Ca channels and single channel currents could not be recognized in such condition. Fig. 6.14 shows a single Ca channel current in PON and its modulation by 5-HT. In the control, the depolarizing command pulse of 30 mV induced rare opening of Ca channel. The amplitude of unitary currents was about 1 pA. By the application of 5-HT, the opening probability of the channel was markedly increased. The opening probability of Ca channel was increased by a larger depolarizing step, but it was further increased by 5-HT (data not shown). These results obtained by the whole-cell clamp and the patch clamp indicate that 5-HT increases the voltage-dependent Ca^{2+} current in PON through the modulation of the gating of Ca^{2+} channel.

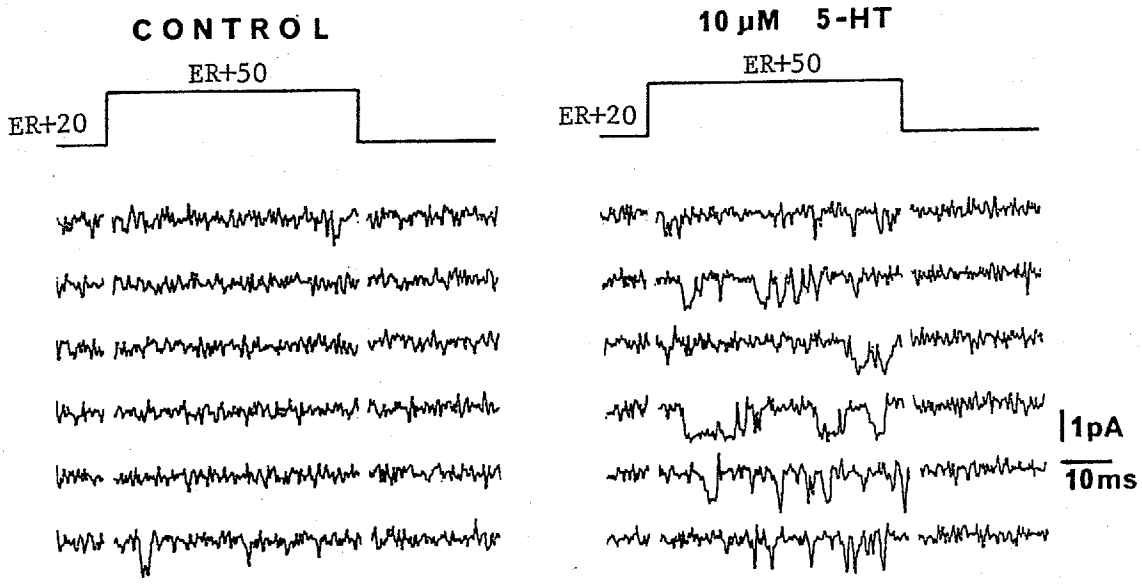

Fig. 6.14 Unitary Ca channel currents produced by the step depolarization of 30 mV. The duration of command pulses was 50 ms. ER indicates the resting potential of PON. The resting potential recorded by a microelectrode before the experiment was -64 mV. The pipette was filled with 70 mM Ba solution.

5-HT increases the inward rectifying K current

In the axotomized PON, 5-HT was also found to increase another conductance. In the normal physiological solution, 5-HT produced an inward shift in holding current, and the membrane current in response to a hyperpolarizing step was decreased by 5-HT (Fig. 6.15A1). However, in 3K solution, the membrane current in response to the same hyperpolarizing step was increased by 5-HT (Fig. 6.15A2). I-V curve showed an inward rectification in 3K solution and this inward rectifying current was increased by 5-HT (Fig. 6.15B). The inward rectifying current in 3K solution was depressed by the addition of 1 mM Ba^{2+} (not shown). These features suggest that this current is an inward rectifying K current (Hagiwara, 1983). This current was not studied further as it was too small in the normal physiological solution.

Spike broadenings of PON by synaptic action and 5-HT application

A burst of impulses in the cerebral neurones, or application of 5-HT, produced spike broadening in PON (Fig. 6.16). PON was slightly hyperpolarized to depress spontaneous activity and was driven to fire by injection of depolarizing current at 0.5 Hz. The intensity of the current was chosen so that each pulse produce a single spike (multiple spikes in this cell easily induced spike broadening). A burst of impulses in d-LCDN produced a slight depolarization and spike broadening in PON (Fig. 6.16A). Spike broadening could also result from the depolarization produced by a constant current injection (Fig. 6.16B). Thus, it was not clear to what extent the synaptically induced conductance changes were concerned with

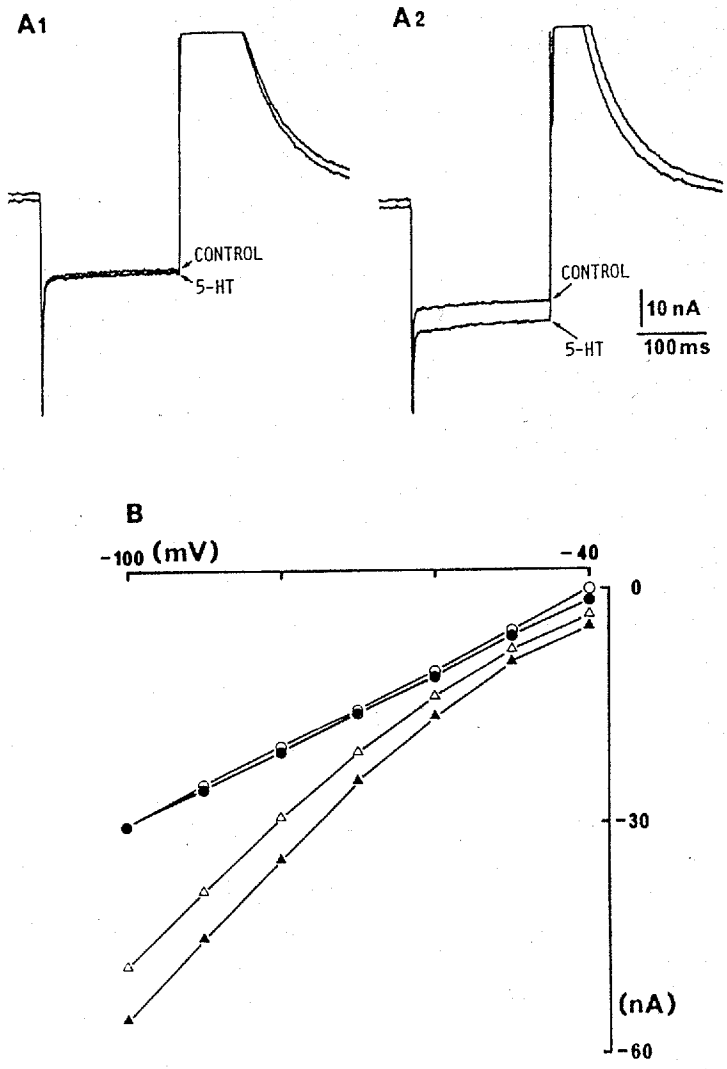

Fig. 6.15. Effect of 5-HT on the inward rectifying K current of PON. Holding potential was -40 mV and the command pulses were 200 ms in duration. A1:Membrane currents in the normal physiological solution, with and without 4 μM 5-HT. A2:Membrane currents in 3K solution, with and without 4 μM 5-HT. The command pulse was stepped to -80 mV in A1 and A2. The large A-current after the command pulse is clipped in both A1 and A2. B:I-V relationships at the end of the pulse, with (closed symbol) and without (open symbol) 4 μM 5-HT. Circle; currents in the normal physiological solution. Triangle; currents in 3K solution.

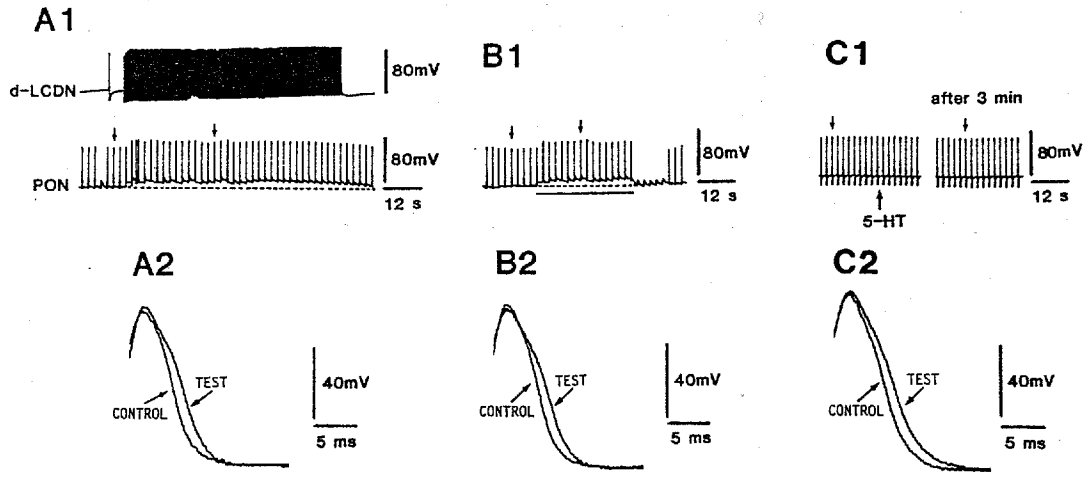

Fig. 6.16. Spike broadening of PON produced by a burst of impulses in d-LCDN (A), the steady depolarization by a constant current injection (B), or application of 5-HT at 5 μM (C). PON was hyperpolarized to -50 mV and driven to fire by a depolarizing current injection at 0.5 Hz. Dotted lines in A1 and B1 indicate -50 mV level and the bar in B1 indicates the duration of constant depolarizing current injection. Arrows in A1, B1, and C1 indicate selected spikes which are displayed at expanded time scale in A2, B2, and C2.

the spike broadening recorded in the soma. However, synaptic action had another effect, i.e. the excitability of PON was greatly increased by the burst of the cerebral cells. In this experiment, the intensity of injected depolarizing current to PON had to be reduced during the burst of d-LCDN to prohibit the generation of multiple spikes by the injected current. Fig. 6.16C illustrates the spike broadening of axotomized PON by 5-HT. 5-HT did not depolarize PON at this potential (-50 mV), because the 5-HT-sensitive K^+ conductance was small in the axotomized preparation and became prominent at a more depolarized potential.

Multiple axons of PON and the effect of spike broadening on the conduction in those axons

PON has multiple axons only in the intestinal nerve which goes to the heart (Chap. IV ; see also Goto *et al.*, 1986). However, it is not known whether all axons go to the heart. In the present study, the existence of multiple axons was tested electrophysiologically. When the soma of PON is hyperpolarized beyond a certain point, an antidromic action potential elicited by stimulation of a branch of the intestinal nerve going to the heart (heart nerve) can be recorded as depolarizing wave (Fig. 4.5A). If only one axon of PON is going to the heart, the amplitude of the depolarizing wave recorded at the soma should be constant when the stimulus intensity is increased. If several axons of PON go to the heart, graded depolarizing waves should be recorded by increasing the stimulus intensity. In the experiment shown in Fig. 6.17A, at least four different depolarizing waves were discriminated by increasing the

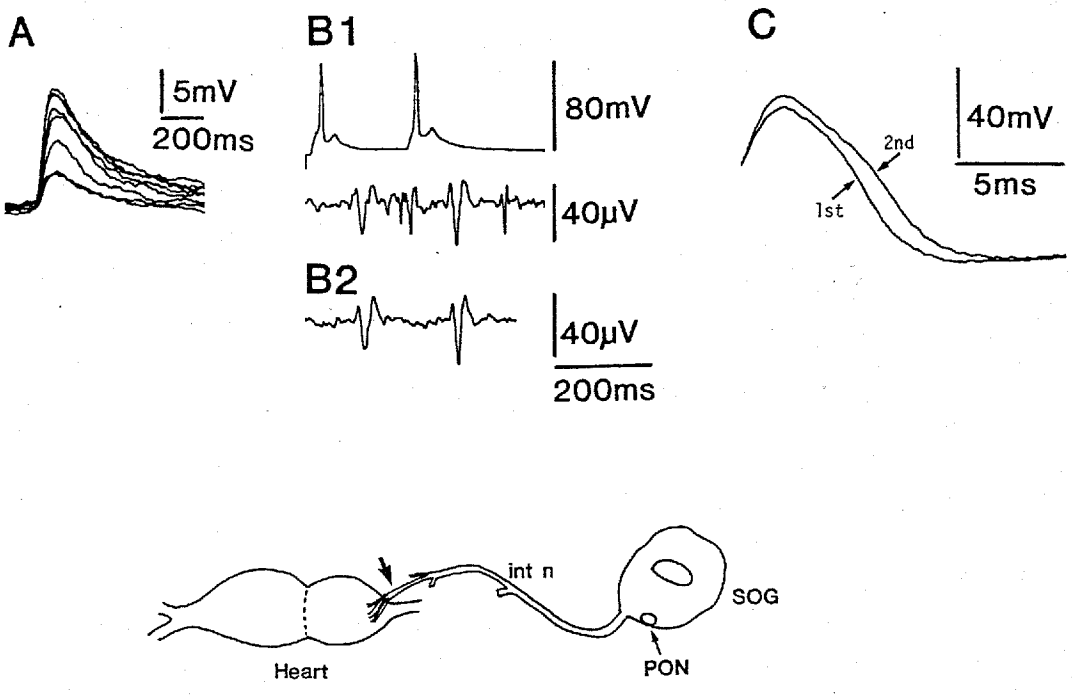

Fig. 6.17. A:Superimposed graded depolarizing waves of PON produced by stimulation of the heart nerve. PON was hyperpolarized to prevent generation of a regenerative spike. The duration of stimuli was 0.5 ms and the stimulus intensity was increased from 3 V to 7.5 V by 0.5 V steps. B:Simultaneous recording from PON and the heart nerve. PON was driven to fire twice every 3 s by two successive current injections and the interval of two succeeding spikes was 200 ms. One example of such spikes is shown in B1. B2:Extracellularly recorded spikes of PON averaged from 10 trials as those shown in B1. C:Superimposed soma spikes of PON shown in B1. Schematic drawing in this figure shows the position of the soma of PON and the stimulating or recording point (arrow) in the heart nerve. int n; intestinal nerve. SOG; suboesophageal ganglia.

stimulus intensity, indicating the existence of multiple axons of PON in the heart nerve.

Fig. 6.17B illustrates simultaneous recordings of the membrane potential of PON and extracellular activity of the heart nerve. PON was driven to fire at 3 s intervals with a pair of spikes separated by 200 ms. This separation was determined to produce spike broadening at the second spike (see Fig. 6.17C). In this condition, the amplitude of extracellulary recorded spikes of PON in the heart nerve was not the same, second spike being larger than the first (Fig. 6.17B1). This was also seen in the averaged signal (Fig. 6.17B2). These results suggest that there may be conduction block in some axons of PON, and these failures may be overcome by spike broadening.

Discussion

In addition to the classical "fast" synaptic transmission leading to the opening of previously closed ion channels, "slow" synaptic transmission which often includes the modulation of the already functioning ion channels has been found in the vertebrate and invertebrate tissues (see reviews, Kehoe & Marty, 1980; Hartzell, 1981; Siegelbaum & Tsien, 1983). The synaptic response of PON induced by the cerebral cells was found to be this "slow" type.

Activity of the cerebral cells produced an inward shift in the current required to hold the membrane potential of PON at the resting level, together with a decrease in K^+ conductance. Thus, the K current which was decreased by the burst of the cerebral cells contributes to the resting potential of PON, i.e. this K current is the background K current. A similar synaptically mediated decrease of the

background K^+ conductance has been reported in sensory neurones of *Aplysia* (Klein & Kandel, 1980), in which the application of 5-HT also produces a similar response and a single 5-HT-sensitive K^+ channel (S-channel) has been first identified (Siegelbaum *et al.*, 1982).

In some preparations, activity of the cerebral cells produced an increase of conductance in PON, preceding the decrease of K^+ conductance. This increased conductance is considered to be a Ca^+ conductance for the following reasons. (1) The slow depolarization of PON was not depressed by replacing Na^+ with impermeant $Tris^+$. (2) Injection of Cl^- into PON had no effect on the slow depolarization. (3) Injection of EGTA into PON increased the peak amplitude of the apparent inward current. Although these results are not straight-forward, an increase of Ca^{2+} conductance seems a likely explanation.

The slow depolarization induced by 5-HT was also mainly due to a decrease of K^+ conductance which was also chiefly responsible for the background K current. Thus the current which is sensitive to 5-HT is quite similar to the K current which is decreased by activity of the cerebral cells. The 5-HT-sensitive K current is not Ca^{2+}-dependent as it could be recorded in Ca^{2+}-free solution containing Co^{2+}. Because the 5-HT-sensitive K current of PON was seen over a wide voltage range around the resting level and showed little time-dependency, it is not either the delayed rectifying K current or A-current. Similar 5-HT-sensitive K currents have been reported in some molluscan neurones (Klein & Kandel, 1980; Klein *et al.*, 1982; Paupardin-Tritsch *et al.*, 1981; Pollock *et al.*, 1985; Jacklet & Acosta-Urquidi, 1985). Modulations of K channels seem to be a rather general

mechanism for the action of neurotransmitter (or neuromodulator). Actually, there are increasing evidences indicating such modulations in vertebrate and invertebrate neurones (Abrams *et al.*,1984; Belardetti *et al.*, 1986; Paupardin-Tritsch *et al.*, 1985; Benson & Levitan, 1983; Cottrell *et al.*, 1984; Brown & Adams, 1980; Akasu *et al.*, 1983).

Two distinct 5-HT-sensitive K channels (SL-channel & SS-channel) could be identified in PON by the patch-clamp experiments. Single channel current of SL-channel showed clear outward rectification in the normal K^+-gradient and could be fitted by the GHK-equation. Thus, the outward rectification of the SL-channel current can be explained by the constant-field rectification; i.e. measurable inward current cannot flow in the normal K^+-gradient. SL-channel seems to be the homologous channel to S-channel of *Aplysia* (Siegelbaum *et al.*, 1982; Pollock & Camardo, 1987; Brezina *et al.*, 1987). Both SL-channel of PON and S-channel of *Aplysia* are functioning in a wide range of voltage near the resting potential and do not inactivate with prolonged depolarization. S-channel also has the outward rectifying property and the I-V relationship can be fitted by the GHK-equation. Although the slope conductance at 0 mV of SL-channel (about 23 pS) is less than that of S-channel (about 50 pS), P_K of both channels is quite similar. The published values for P_K of S-channel are 8.7×10^{-14} cm^3/s (Siegelbaum *et al.*, 1982), 1.66×10^{-13} cm^3/s (Pollock & Camardo, 1987) and 1.31×10^{-13} cm^3/s (Brezina *et al.*, 1987). These values are quite similar to the value obtained for SL-channel of PON (9.3×10^{-14} cm^3/s). The difference of the slope conductance at 0 mV may result from the difference of $[K^+]_{in}$

and $[K^+]_{out}$ in both species, because the conductance of several K channels is dependent on the concentration of K^+ (Pallotta *et al.*, 1981; Ohmori *et al.*, 1981; Kakei & Noma, 1984; Sakmann & Trube, 1984; Standen *et al.*, 1985).

SS-channel was the voltage-dependent K channel and its single channel conductance was about 8.5 pS in the normal K^+-gradient. This value and the flickering kinetics of this channel are comparable to those of the delayed rectifier K channel identified in the giant axon of squid (Conti & Neher, 1980) and the frog skeletal muscle (Standen *et al.*, 1985), although detailed kinetic analyses of SS-channel should be done.

The action of 5-HT on SL- and SS-channel is considered to be mediated by the second messenger system(s) since 5-HT applied by bath perfusion cannot reach the ion channels in the cell attached patch (Hamill *et al.*, 1981). In *Aplysia*, several evidences suggest that the modulation of S-channel by 5-HT is mediated by cyclic AMP-dependent protein kinase (Siegelbaum *et al.*, 1986).

5-HT-sensitive K current of PON in the whole-cell clamped condition did not reverse in the solution containing 3.3 mM K^+ (standard K concentration of the normal physiological solution for *Achatina*). Indeed, the reversal of this current was not seen until $[K^+]_{out}$ was raised more than 5-fold. These results can be explained by the constant-field rectification of the SL-channel current and the voltage dependency of SS-channel.

5-HT also increased the voltage-dependent Ca current in the axotomized PON and the modulation of gating of a single Ca channel by 5-HT was demonstrated by the patch clamp experiments. According to the same reasoning for the cases

of SL- and SS-channels, this modulation is also considered to be mediated by the second messenger(s). It is possible that this modulation of the Ca channel by 5-HT is related to the presumed increase of Ca^{2+} conductance of PON by the burst of cerebral cells. Similar 5-HT-induced increase of Ca current has been reported in *Helix* and *Hermissenda* neurones (Paupardin-Tritsch *et al.*, 1986a; Jacklet & Acosta-Urquidi, 1985) and may be exist in the vertebrate neurone (Hounsgaard & Kiehn, 1985). 5-HT-induced increase of Ca current of *Helix* neurone is probably mediated by cyclic GMP-dependent protein kinase (Paupardin-Tritsch *et al.*, 1986b). Modulations of Ca channel by other neurotransmitters have been described in some invertebrate and vertebrate preparations. The well known example is Ca channel of vertebrate heart muscle, where β-adrenergic agents increase Ca current and this is considered to be mediated by cyclic AMP-dependent protein kinase (Osterrieder *et al.*, 1982; Cachelin *et al.*, 1983). In some *Helix* neurones, Ca current is decreased by the neuropeptide, FMRFamide (Colombaioni *et al.*, 1985). In chick dorsal root ganglion cells, γ-aminobutyric acid, dopamine and noradrenaline decrease Ca current (Deisz & Lux, 1985; Marchetti *et al.*, 1986). The modulation of Ca current in neuronal membrane is important; i.e. it is considered to underlie the several plasticities of synapses (Klein *et al.*, 1980).

LK-channel was a K channel having the largest conductance in PON and its activity was increased by 5-HT. The properties of this channel were consistent with those of the Ca^{2+}-dependent (or activated) K channels identified in several tissues (see review, Petersen & Maruyama, 1984), although Ca^{2+}-sensitivity of LK-channel should be tested in

the cell-free patch. It seems likely that the Ca^{2+}-dependent K current in PON which is increased by 5-HT results from the activity of LK-channel.

In 3K solution, substantial inward rectifying K current was revealed in PON, and this K current was also increased by 5-HT. The enhancement of the inward rectifying K current by 5-HT has been reported in *Aplysia* R15 (Benson & Levitan, 1983) which is considered to be a homologous neurone to PON (Chap. IV).

The slow depolarization of PON induced by activity of the cerebral cells was depressed by methysergide. Methysergide is known to be a rather specific 5-HT antagonist in the molluscan central nervous system (Leake & Walker, 1980). 5-HT induced a similar slow depolarization in the axotomized PON which was also depressed by methysergide. Furthermore, the ionic mechanisms underlying both types of depolarization were found to be similar. These results suggest that the neurotransmitter of the two cerebral neurones is 5-HT.

Depression of the background K^+ conductance of PON led to an increase in excitability. As described in chapter IV , PON is the most effective heart excitor in *Achatina*, but its activity is usually reduced by many inhibitory synaptic inputs. Thus, PON is considered to be in the inactive mode in usual. The increased excitability of this cell by the modulation of K channel may overcome such inhibition and PON may go into the active mode, which would significantly increase the heart activity of this snail.

Activity of the cerebral cells or application of 5-HT produced spike broadening in PON as to be expected from the results of the voltage-clamp experiments. This spike

broadening may also have a role in heart regulation. The physiological significance of spike broadening has been thoroughly studied in *Aplysia* sensory neurones (Klein & Kandel, 1978; Hochner, Klein, Schacher & Kandel, 1986). In *Aplysia*, spike broadening of sensory neurones produced by connective stimuli or by application of 5-HT results in an increase of EPSPs in its follower cell, the gill-motoneurone, probably through increased Ca^{2+} influx at the terminal during the broadened spike. Such modulation of synaptic efficacy may also occur at the terminals of PON, although the long distance from the ganglia to the heart (usually more than 3 cm) makes a deduction from the phenomena recorded in the soma difficult.

It was also demonstrated in this chapter that at least some of the multiple axons of PON reach the heart, and the spike broadening in the soma of PON appeared to correlate with an increased amplitude of PON spikes in the heart nerve, recorded extracellularly. This may be because the multiple axons have different thresholds, so that the spike broadening recruits more axons. If this hypothesis is correct, the number of terminals activated by a single somatic action potential would be increased by spike broadening, which would augment the heart excitatory action of PON. To test this hypothesis, a quantitative investigation of the relationship between the width of spike of PON and its heart excitatory action would be necessary.

CHAPTER Ⅶ

SUMMARY AND CONCLUSION

The purpose of this work was to understand the neural control of the heart activity in the African giant snail, *Achatina fulica* Fèrussac. Although the neural network for the control is fairly complex and needs further studies to clarify the whole aspects, the present work revealed several important features of the neural control mechanisms of the heart in this snail.

Heart regulatory network of Achatina

Seven heart regulatory neurones (PON, TAN, TAN-2, TAN-3, d-RCDN, d-LCDN and VG1) were identified in the central nervous system of *Achatina*. Among these neurones, PON was the most effective heart excitor and it produced heart excitation at rather low firing frequencies. TAN, TAN-2 and TAN-3 were tonically firing neurones and their spontaneous activity was found to produce tonic heart excitation which supplemented the myogenic heart activity. There were some evidences that two cerebral ganglion cells (d-RCDN and d-LCDN) were also likely to be heart excitors although the direct connection to the heart was a little doubtful in some specimens. No direct inhibitory neurone was found, but the firing of VG1 at a high frequency usually produced heart inhibition.

Two cerebral ganglion cells, d-RCDN and d-LCDN, were found to have monosynaptic excitatory connections with several neurones in the suboesophageal ganglia (PON, TAN, TAN-2, TAN-3 and VIN). 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 VG1 inhibited PON and VIN although the connections seemed to be polysynaptic. Another neurone in the pedal ganglia, d-LPeLN, was found to excite PON, VIN, TAN, TAN-2 and TAN-3. These connections were not monosynaptic.

There were the inhibitory pathways originating from the periphery in the heart regulatory network and many inhibitory inputs which depressed the activity of PON and VIN were considered to arise from these pathways. The mechano-afferents in the pericardium were found to be the components in these inhibitory pathways. Such mechano-afferents may be important as the pericardium is known to be essential for the hemodynamics.

The heart regulatory network of *Achatina* was somewhat different from that of the previously investigated gastropods (*Aplysia* and *Helix*) in that the heart regulatory motoneurones are connected mono- and poly-synaptically. Thus, in *Achatina*, the higher order neurones can not activate a single motoneurone without affecting other motoneurones.

Synaptic mechanisms between PON and the two cerebral neurones (d-RCDN and d-LCDN)

Under voltage-clamp, activity of the cerebral neurones usually produced an inward shift in the holding current of PON with a decrease of conductance. Ionic substitution experiments and injection of Cs^+ into PON showed that the response was mainly due to a decrease in K^+ conductance. In some cases, this inward shift showed two components: an

early component with increased conductance and a late one with decreased conductance. The early component was not decreased by Cs^+-injection but was augmented by EGTA-injection into PON, suggesting the involvement of a Ca^{2+} conductance in this synaptic response.

Application of 5-HT produced a similar inward shift in holding current, which was also mainly the result of a decrease in the background K current. Two different 5-HT-sensitive K channels (SL-channel & SS-channel) were identified by the patch-clamp experiments, and the behavior of 5-HT-sensitive K current in the whole-cell clamp could be explained by the activity of these two channels at least qualitatively. 5-HT was also found to increase the voltage-dependent Ca current by the whole-cell clamp and the single channel recording. The Ca^{2+}-dependent K current and the inward rectifying K current were also increased by application of 5-HT. A presumed Ca^{2+}-dependent K channel current was recorded by the patch clamp.

The slow depolarization of PON induced by the activity of the cerebral neurones was blocked by the 5-HT antagonist, methysergide. Bath application of 5-HT to the axotomized PON produced a similar slow depolarization and it was also blocked by methysergide. These results as well as the similarity of the ionic mechanisms of both responses suggest that the neurotransmitter of d-RCDN and d-LCDN is 5-HT.

These synaptic and 5-HT actions increased the excitability of PON and produced the spike broadening. As PON is the strongest heart excitatory neurone and its activity may be usually depressed by the inhibitory inputs from the periphery, the modulation of the activity of this cell would have significant effect on the heart regulation.

Conclusion

In this work, nine neurones have been identified to be involved in heart regulation of *Achatina*. They are giant neurones of more than 100 μm in diameter and can be easily identified. These situations are contrast to those in previously studied two other snails (*Aplysia* and *Helix*), in which most identifiable heart regulatory neurones are not giant except for some interneurones. Thus, the nervous system of *Achatina* is a promising system for future analysis of the neural control of heart regulation and the relationships between heart regulation and other behaviors, and also for the detailed biophysical and biochemical analyses of the neuronal membrane.

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