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Giant multimodal heart motoneurons of *Achatina fulica*: a new cardioregulatory input in pulmonates

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Abstract

The regulation of the heartbeat by the two largest neurons, d-VLN and d-RPLN, on the dorsal surface of visceral and right parietal ganglia of Giant African snail, *Achatina fulica,* was examined. Using the new method of animal preparation, for the first time, discrete biphasic inhibitory–excitatory junction potentials (I-EJPs) in the heart and several muscles of the visceral sac were recorded. The duration of hyperpolarizing phase (H-phase) of biphasic I-EJPs was 269 ± 5.6 ms ($n = 5$), which is 2–3 times less than that of the cholinergic inhibitory JPs (682 ± 68.5 ms, $n = 5$). The H-phase of I-EJPs was not altered by the application of atropine, picrotoxine, succinylcholinchloride, D-tubocurarine and tetraethylammonium or substitution of Cl^- ions. Even the low-frequency neuronal discharges (1–2 imp/s) evoked significant facilitation and potentiation of the H-phase. Between the multimodal neurons d-VLN/d-RPLN and mantle or visceral organs there is evidence of direct synaptic connections. These neurons were found to have no axonal branches in the intestinal nerve as once suspected but reach the heart through several other nerves. New giant heart motoneurons do not interact with previously identified cardioregulatory neurons.

Keywords: Cardioregulatory control; Multimodal neurons; Snail; Molluscan heart; Biphasic junction potentials; Potentiation; Facilitation; *Achatina*

1. Introduction

Cardioregulatory neuronal networks have been well characterized in several gastropods. For example, in *Aplysia* at least six different functional groups of the heart's motoneurons are known (Skelton et al., 1992). However, in land pulmonates only four groups of cardiostimulating nervous cells have been identified (S.-Rózsa, 1987; Furukawa and Kobayashi, 1987a,b; Zhuravlev et al., 1997). The population of cardiostimulating motoneurons includes both serotonergic (Goldstein et al., 1984) and various peptidergic neurons (Schot and Boer, 1982; Santama et al., 1995). The inhibition of heartbeat in gastropods is carried

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out by cholinergic motoneurons. Moreover, in the ganglia of *Aplysia*, *Helix* and *Achatina* only two inhibitory motoneurons of heart are known.

All of these cells have been identified and investigated using traditional semi-intact preparations. For this study, we used the modified method of preparation of Giant African snail, *Achatina fulica*. In this preparation the connection between some of the previously identified neurons and the heart have been tested. It was found that the two largest neurons on the dorsal surface of both visceral and right parietal ganglia have direct outputs in the heart. Previously, these neurons were characterized in both an isolated ganglia preparation and in a semi-intact one. Munoz et al. (1983) denoted these neurons as VG1 and RPr1, and Goto et al. (1986) as d-VLN and d-RPLN, respectively. However, the connection between these neurons and heart was not investigated.

In modified preparation all nerves remain intact. Apparently, under these conditions both neurons evoke discrete biphasic inhibitory–excitatory junction potentials (I-EJPs) in the myocardium. These junction potentials differ from the well-known inhibitory cholinergic JPs in gastropod mollusks (Kuwasawa and Hill, 1972; Kuwasawa, 1979; Kuwasawa and Yazawa, 1981; Bychkov et al., 1997; Zhuravlev et al., 1997).

The aim of this study was to characterize the new cardioregulatory input and I-EJPs in the heart and visceral muscles of *Achatina fulica*. In addition, the effect of several pharmacological tools on the mechanism involved in the generation of hyperpolarizing phase (H-phase) of I-EJPs was investigated.

2. Materials and methods

Experiments were carried out on adult Giant African snails, *Achatina fulica* (Gastropoda, Pulmonata, Stylommatophora). Animals were kept at 25–27°C and fed a vegetable diet.

2.1. Animal preparation

All experiments were performed at room temperature (22–26°C). Snails (30–50 g) were anaesthetized by injection of 0.3–0.4 ml succinylcholinchloride (SucCh; 1%) into the visceral sinus through a body stem (Croll and Baker, 1990). Experimental procedures were performed as described previously (Zhuravlev et al., 1999) with the following exceptions: the shell was removed and the snail was fixed to the experimental chamber. The preparation procedure was carried out in physiological solution modified for *Achatina* (see Section 2.5). During the preparation only one incision on the mediodorsal surface of a body was made. The edges of the incision were stretched to the side and pinned on silicon. This type of preparation we will denote as 'whole-mount preparation', which is similar to an intact animal, as almost all of neuronal connections to the heart and visceral organs remain unaffected.

In order to avoid excess manipulation of the organism we recorded the intracellular activity of neurons in animals without removing their shell. In this case, the wound at the end of the experiment was stitched and the animals were transferred to a tank lined with wet cotton paper (Croll and Baker, 1990). Recovery was seen in 2–4 days as the subjects began to eat and after 2 months the initial distonia had almost completely disappeared.

2.2. Electrograms of muscles

The cardiac monophasic electrograms were recorded using flexible suction electrodes filled with 0.15 M KCl (Zhuravlev et al., 1991a; Li et al., 1997). The perfusing pressure of the heart was approximately 2–3 cm H_2O. The extracellular electrical activity from mantle muscles was recorded by means of bevelled glass micropipettes (tip diameter 100–200 μm) and a flexible light connective tube both filled with *Achatina*'s solution. The electrode was manually impaled into the mantle muscles. The mechanical stimulation of somatic and visceral afferents was made by a thin brush.

2.3. Stimulation of nerves

The local electrical stimulation of nervous trunks was performed by a coaxial electrode with an external diameter of 0.5 mm and an internal 0.05-mm silver wire. For the stimulation of extended areas of tissues (*field stimulation*) linear electrodes sized 0.5–1 × 3–7 mm were applied.

2.4. Electrical activity of neurons

The electrical activities of two giant neurons, d-VLN and d-RPLN, were recorded simultaneously using unbevelled (10–15 MΩ) or a bevelled (1–5 MΩ) microelectrodes. In order to impale the neuron through ganglionic sheets our patented electromechanical device was used (Zhuravlev et al., 1991b).

2.5. Solutions and chemicals

Most of experiments were performed in a modified physiological solution for *Achatina* containing (in mmol/l): 61 NaCl, 3.3 KCl, 10 $CaCl_2$, 9 $MgSO_4 \times 7H_2O$ and 10 HEPES. The pH was adjusted to 7.6 with NaOH. For the Cl^--free solution, the equivalent concentration of the gluconate salts of sodium, potassium and calcium were used. The heart was perfused with solution containing either 4 mM $MnCl_2$ or 2 mM $CdCl_2$ in order to block chemical synapses. In these solutions, the concentration of Ca^{2+} was reduced to 1 mM. The effect of the following agents was tested on the H-phase of biphasic I-EJPs: D-tubocurarine (D-TC; Neopharm, Finland), tetraethylammonium (TEA; BDH Chemicals Ltd, England), atropine, picrotoxine and succinylcholinchloride (SucCh). All other chemicals were purchased from Sigma (USA).

2.6. Comparison of facilitation and potentiation of JPs in muscles

The coefficients of facilitation and potentiation of the H-phase of biphasic I-EJPs in both the heart and mantle muscles were determined according to Magleby (1973a,b). The magnitude of facilitation (F_t) was obtained using the following equation:

$$F_t = A_n / A_0 \quad (1)$$

where A_n is the amplitude of a current JP and A_0 is that of the first JP (the 'zero JP'). A standard neuronal burst (AP_0, AP_1 … AP_n) consisted of five action potentials (AP). The polarizing current was adjusted so that the burst duration was close to 4 s. All measurements were made with respect to this average, though the momentary interspike interval in burst could vary from 0.8 up to 1.2 s (Fig. 1c). After a standard series of neuronal APs, i.e. five APs with an interspike interval average of 1 s, one test AP (AP_{test}) was evoked. The amplitude of a test JP (A_{test}) for the calculation of potentiation (P_t) was used. The change in the potentiation was estimated using the equation:

$$P_t = A_{test} / A_0 \quad (2)$$

The testing interval (t_{test}) varied from 1 s to 4 min. Following measurements of a standard series of APs and one AP_{test} was performed, when the potentiation was fully eliminated, i.e. after approximately 5–6 min. During this interval, only one test AP was evoked. Both facilitation and potentiation of JPs were measured only in the resting state. Spontaneous heartbeats were suppressed by increasing the perfusing pressure up to 10–12 cm H_2O.

The coefficients of afferental asymmetry (K) were obtained using the equation:

$$K = A_{\text{d-VLN}} / A_{\text{d-RPLN}}, \quad (3)$$

where $A_{\text{d-VLN}}$ and $A_{\text{d-RPLN}}$ are the maximal amplitudes of EJPs in giant neurons d-VLN and d-RPLN, respectively. These EJPs were evoked by mechanical stimulation of different areas of skin or viscerae.

Origin 4.0 (Microcal Software, MA, USA) and CorelDraw 9.0 software were used for statistical analyses and drawing of figures. The regression for potentiation was estimated using standard methods. Data are presented as means ± S.E.M. Student's paired t-test was used for the examination of the differences between data groups. A P value < 0.05 was considered to be statistically significant.

3. Results

3.1. The biphasic I-EJPs in the heart during either d-VLN or d-RPLN activation

A typical experimental set-up is shown in Fig. 1a. Simultaneous intracellular recordings from two neurons and an extracellular recording from the heart (mantle or visceral muscle) were performed. Fig. 1b represents the location of the identified heart motoneurons on the dorsal surface of subesophageal ganglia in *Achatina*. The

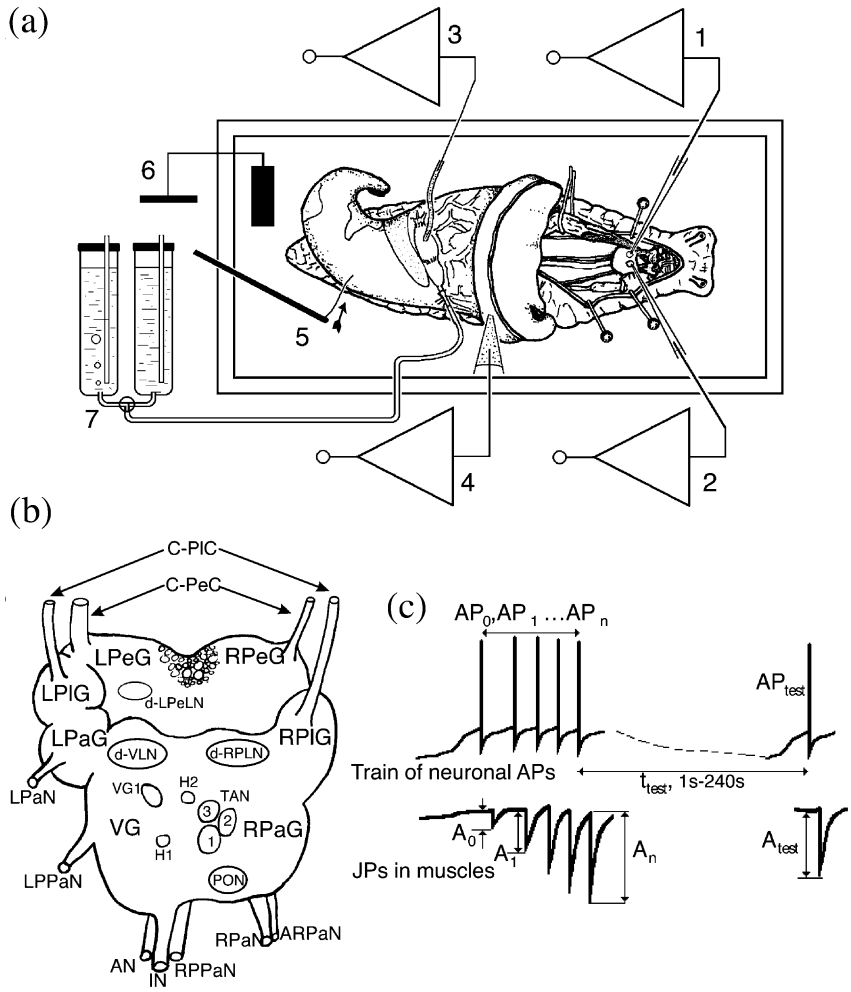

Fig. 1. (a) Experimental set-up: 1 and 2, intracellular microelectrodes and preamplifiers; 3, suction electrode; 4, extracellular glass microelectrode; 5, hair for mechanical stimulation; 6, reference electrode; 7, perfusion system. (b) The position of cardioregulating neurons in the dorsal surface of suboesophageal ganglia of *Achatina fulica* (Furukawa and Kobayashi, 1987a,b; Zhuravlev et al., 1997). Ganglia: LPeG and RPeG, left and right pedal ganglia, respectively; LPlG and RPlG, pleural ganglia; LPaG and RPaG, parietal ganglia; VG, visceral ganglion. Nerves: C-PlC, cerebro-pleural connectives; C-PeC, cerebro-pedal connectives; LPaN, left pallial nerve; LPPaN and RPPaN, left and right posterior pallial nerves; AN, anal nerve; IN, intestinal nerve; RPaN, right anterior pallial nerve; ARPaN, right anterior pallial accessory nerve. Neurons: d-LCDN and d-RCDN, dorsal left and right cerebral distinct neurons; d-LPeLN, dorsal left pedal large neuron; d-VLN, dorsal visceral large neuron; d-RPLN, dorsal right parietal large neuron; VG1, visceral ganglion neuron; HI1 and HI2, heart inhibitory neurons; TAN1, TAN2 and TAN3, tonically autoactive neurons; PON, periodically oscillating neuron. (c) Analysis of trains of neuronal APs and I-EJPs (description in Section 2).

names of nerves and identified neurons are given according to Croll (1988) and Takeuchi et al. (1996).

A series of evoked APs either in d-VLN or d-RPLN generate discrete junction potentials in the heart (Fig. 2a). These JPs exhibited distinct facilitations. If there was an overlap of activity in both neurons, as shown in Fig. 2a, the heart reacted with an additional JP (indicated by an arrow). This particular JP and those that appeared before and after the APs in neuron d-RPLN were generated by d-VLN and have no effect on the amplitude of JPs evoked by another neuron. Moreover, the facilitation of JPs developed independently according to the increasing number of APs in each neuron. We theorized that these junction potentials in the heart could be inhibitory JPs. However, further experiments revealed that they are biphasic inhibitory–excitatory junction potentials (I-EJPs) consisting of a

short, high-amplitude hyperpolarizing phase (H-phase) followed by a long, small amplitude depolarizing phase (D-phase). The D-phase was often difficult to distinguish on oscillograms because of small shifts in membrane potential and/or spontaneous APs in the heart. In the absence of these APs, as well as after rhythmic discharges of giant neurons, the D-phase was readily distinguished (see, for example, Fig. 2a,d and Fig. 6a). For this paper we have studied and analyzed only the characteristics of H-phase of biphasic I-EJPs.

The duration of the H-phase of JPs (Fig. 2b) was shorter (269 ± 5.6 ms, $n = 5$) than the duration of well-known inhibitory JPs (682 ± 68.5 ms, $n = 5$) mediated by cholinergic inhibitory neurons HI1 and HI2 (Bychkov et al., 1997). Because of the rather short duration, the JPs summation

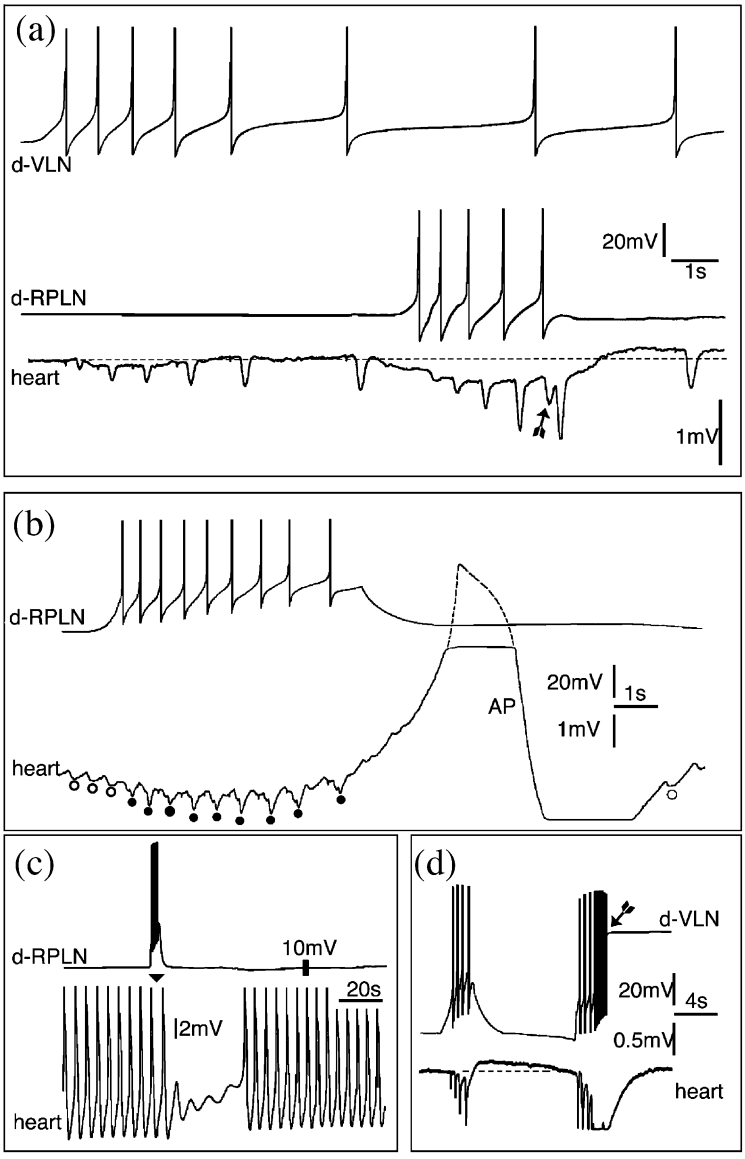

Fig. 2. The activity of giant neurons and junction potentials in the heart. (a) Simultaneous recordings from neurons and heart. (b) The background cholinergic and I-EJPs during burst of APs in d-RPLN. Open and close circles show the peak of IJPs and H-phase of I-EJPs, respectively. The myocardial AP was restricted by a pen recorder (indicated by dashed lines). (c) The heart's monophasic APs (lower trace) before and after high-frequency discharges in the giant neuron (upper trace). Triangle shows the increased intersystolic interval during neuronal discharges ($\sim 20\%$). (d) The summation of the H-phases of I-EJPs evoked by d-VLN. The arrow indicates the moment of electrode withdrawal from the neuron. Dashed line indicates the baseline in order to better present the D-phase of I-EJP.

occurred only during high-frequency discharges in giant neurons d-VLN/d-RPLN (Fig. 2a,c,d). Such trains of APs could be obtained exclusively after a strong depolarization of neuron by depolarizing current application (10–12 nA). The summation of H-phases led to the short-term time failure of heart frequency (see triangle in Fig. 2c). Furthermore, the distinct summation of H-phase during extremely high-frequency neuronal discharges was observed (Fig. 2d). These discharges in d-VLN were elicited by slow withdrawal of electrode from the neuron (shown by an arrow). However, during a normal synaptic activation of both d-VLN and d-RPLN these long and high-frequency trains did not take place.

Thus, during the burst of spikes in neurons d-VLN/d-RPLN, no long-lasting inhibitory effects on heartbeat were observed. However, after these discharges the inhibition of heart activity with a latency of 2–5 s occurred (Fig. 2c). This inhibition was related to IJPs from neurons HI1 and HI2. It is known that the giant neurons d-VLN/d-RPLN do not interact with HI1 and HI2 in isolated ganglia (Bychkov et al., 1997). The inhibition of heartbeat after a series of APs in giant neurons was possibly evoked according to mechanisms similar to those of viscerocardial reflexes (see Section 4).

We suggested that the axons of all previously identified cardioregulating neurons in land pulmonates reach the heart as a component of intestinal nerve. Therefore, for the initial examination of the nervous control of heartbeat in *Achatina fulica*, a complex of three nerves was saved. This complex included the intestinal, anal and right posterior pallial nerves (Fig. 1b), which have a main pathway to the viscerae. The remaining nerves were frequently cut. By mean of the electrical stimulation and sequential cutting, the basic pathway between neurons d-VLN/d-RPLN and the heart was found. Thus, the left posterior pallial nerve (LPPaN) as the main pathway was identified. Fig. 3 represents the biphasic I-EJPs in the heart and their modulation by neuron d-VLN and nerve LPPaN. In preparations where LPPaN remained intact, a series of APs in neuron d-VLN led to the generation of JPs in the heart with an increasing amplitude (Fig. 3a). Stimulation of intact left pallial nerve caused the activation of different elements of the cardioregulating network, which subsequently evoked the burst of JPs both in myocardium and giant neuron. The duration and the amplitude of each these JPs were different (Fig. 3b). Two JPs in the ventricle before and after AP in giant neurone followed the first stimulus of LPPaN. As the nerve was cut, the stimulation of the distal part of the LPPaN evoked a series of JPs (Fig. 3c). The later stimulation had no effect on neuron d-VLN. The minor parts of neuronal endings possibly reach the heart also through the other nerves. After cutting of LPPaN the activation of d-VLN evoked I-EJPs in the heart, but their amplitude was essentially decreased (Fig. 3d). The amplitude of JPs in the heart after a stimulation of distal ends of other pallial nerves varied from preparation-to-preparation, which reflects the variability in distribution of nervous endings. During a rhythmic stimulation the desynchronization of JPs was observed. This could be explained by taking into account that the heart is innervated by two giant neurons, i.e. d-VLN and d-RPLN, sending their axons to the heart through many nerves. Axons of giant neurons in the anal, left and right anterior pallial, left anterior pallial accessory and left posterior pallial nerves were found by both Munoz et al. (1983) and Goto et al. (1986). However, axons of these neurons were not present in intestinal nerve.

By means of both a sequential cutting of nerves and stimulation of various zones close to the heart it was found that the majority of axons of giant neurons enter the heart through the aortic end of the ventricle. We could find neither a certain nerve nor a compact path via which the axons of giant neurons enter the heart. The local stimulation of various zones close to the aortic part of ventricle evokes only small JPs, i.e. the

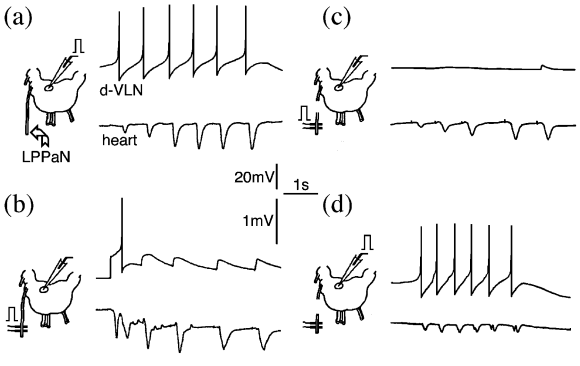

Fig. 3. Junction potentials generated by cardioregulatory neurons in the heart. JPs were recorded during the stimulation of either neuron d-VLN or nerve LPPaN before (a, b) and after the nerve cutting (c, d).

amplitude of junction potentials evoked by this procedure was less than the ones evoked during an intracellular stimulation of giant neuron. Only a field stimulation of lung cavity floor close to the aortic part of ventricle evokes nearly normal JPs in the heart. Linear field electrodes were more effective when they were situated perpendicularly to the axis of the heart. Thus, using a different method of stimulation, it was found that axons of giant neurons arrive diffusely (from various directions) from the aortic part of the ventricle through both the floor and wall of the lung cavity.

3.2. Pharmacological profile of I-EJPs

The polarity of quasi-intracellular transepicardially recorded junction potentials did not differ from those recorded intracellularly. The latter recordings from the myocardium were performed using floating glass electrodes. The suction electrode adequately mimics both the polarity and time course of junction potential compared to those recorded intracellulary. The amplitudes of JPs recorded intracellularly were 2–3-fold greater than those recorded using suction electrodes (data not shown).

The activation of neuron d-VLN under control conditions consistently led to the generation of JPs in the myocardium (Fig. 4a). The perfusion of heart with the solution containing 2 mM Cd^{2+} significantly decreased these JPs (Fig. 4b). The blocking effects of this agent were manifested by a failure of JP amplitude, as well as a decrease of facilitation of synaptic transmission. The significant effect was observed after 5–10 min. In order to better present the constant latency between neuronal AP and the initiation of I-EJP in the heart, both APs ($n = 10$) and corresponding I-EJPs were superimposed in an expanded time and amplitude scales (Fig. 4ai, bi).

Evidently, neurons d-VLN and d-RPLN are not cholinergic because the H-phase of biphasic JPs and cholinergic IJP has clear distinctions. The duration of the H-phase of I-EJP was significantly less than the duration of previously investigated cholinergic inhibitory JP (IJP). To appreciate the nature of this difference the time constant of myocardial syncytium was measured. The rectangular current pulse was passed through a suction electrode using a bridge-circuit. It was found that the time constant of ventricular syncytium (279 ± 4.6 ms, $n = 5$) is very close to those of the H-phase of I-EJP (269 ± 5.6 ms, $n = 5$) but not to the cholinergic IJP (682 ± 68.5 ms, $n = 5$; Fig. 5a). The much longer duration of the rising and falling phases of cholinergic IJP compared to the time constant of myocardial syncytium suggests that the deletion of acetylcholine from the intercellular space is less effective than those in the classical neuromuscular synapse, for example, in frog.

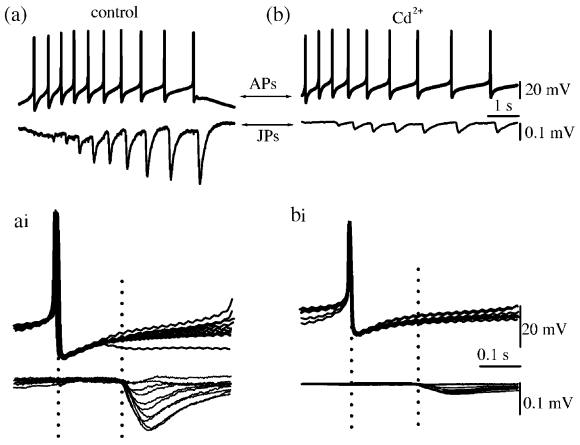

Fig. 4. (a) Simultaneous recordings of APs and I-EJPs in the neuron d-VLN and ventricle, respectively. I-EJPs were recorded extracellularly using suction electrode. (b) Effects of Cd^{2+} on JPs evoked by d-VLN stimulation. Perfusion of the heart with a solution containing 2 mM Cd^{2+} significantly decreased the amplitude of JPs. The resting membrane potential of neuron d-VLN was −43 mV. ai and bi are the superimposed APs ($n = 10$) and corresponding JPs shown in a and b.

Furthermore, the effect of some known ganglioblockers on I-EJPs was tested. Neither the amplitude nor the duration of the H-phase of I-EJPs was affected by 10^{-3} M SucCh, 2×10^{-5} M d-TC and 5×10^{-5} M TEA (Fig. 5b). Also, both atropine (up to 10^{-5} M) and 10^{-4} M picrotoxine have no effect on these JPs (data not shown). On the contrary, after a long perfusion (10–20 min) of heart with either SucCh or d-TC the suppression of cholinergic IJPs was observed. The cholinergic IJPs in the heart of gastropods are Cl^--dependent (Kuwasawa and Yazawa, 1981; Kuwasawa et al., 1987), i.e. they are inverted in Cl^--free solution. In the 'whole-mount' preparation the perfusion of heart with Cl^--free solution inverted the polarity of cholinergic IJPs (Fig. 5c, open circles). This effect was reversible with washout. However, the polarity of the H-phase of biphasic JPs remained unaffected (Fig. 5c, filled circles).

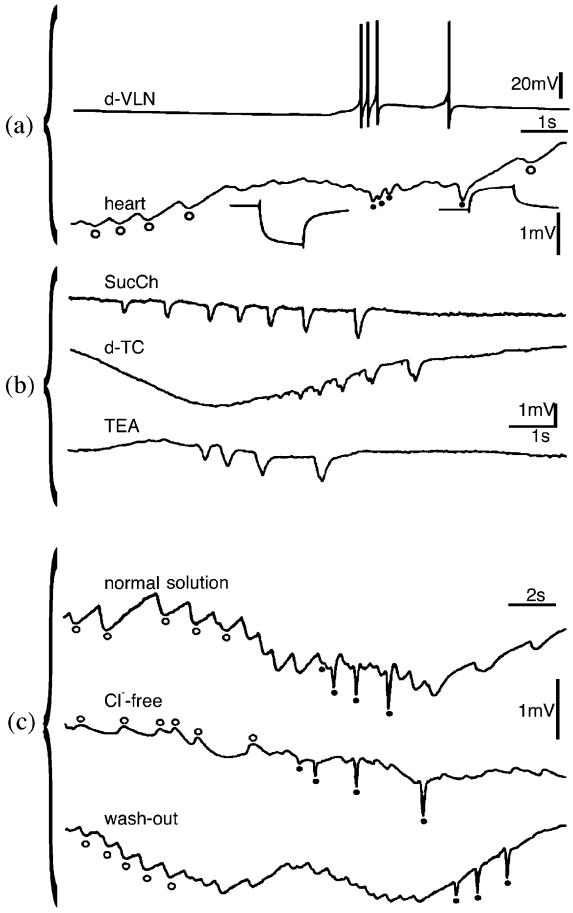

Fig. 5. (a) The comparison of duration of cholinergic IJPs (open circles) and H-phase of I-EJPs (filled circles). The response of myocardium to the rectangular hyperpolarizing current shown in inset as inverted in order to best compare with the H-phase of I-EJPs (the last filled circle); (b) the effects of 10^{-3} M SucCh, 2×10^{-5} M d-TC and 5×10^{-5} M TEA on JPs; (c) junction potentials in normal and Cl^--free solution. The peaks of cholinergic IJPs and the H-phases of I-EJPs are marked with open and filled circles, respectively.

3.3. The functional characteristics of giant heart motoneurons

The cells d-VLN and d-RPLN innervate visceral organs, muscles of body wall and mantle in *Achatina*. A series of discharges in these cells evokes clear contractions of many visceral and somatic muscles. Even the short burst of spikes in d-VLN and d-RPLN increases the basic tone of muscles in the 'whole-mount' preparation. Using intra- and extracellular glass microelectrodes, the neuronal spikes and JPs in different muscles were simultaneously recorded. A train of APs in giant neurons evoke biphasic JPs in heart and mantle muscle (Fig. 6a). The amplitude of JPs in mantle was larger than those in heart but both demonstrated facilitation. Similar junction potentials were recorded in a body wall, mantle edge, ventricle, and lung vessels. The cardiac components (APs) in electromyograms obtained from the ventricle and large lung vessels were always present (Fig. 6b). Neurons d-VLN and d-RPLN either in semi-intact or 'whole-mount' preparations were silent. The burst of spikes in the neuron as a consequence of its depolarization evoked the tonic contractions of various muscles of the visceral sac, as well as the body wall and foot muscles. We also observed a contraction of the mantle edge in a 1:1 manner to each AP in any of the neurons.

The coefficient of facilitation was large; therefore, the first JP was sometimes difficult to distinguish. This might be the reason for the lack of noticeable response of muscles to single stimulus (Munoz et al., 1983). At a frequency average of 1 AP/s, the amplitude of the fifth JP was approximately 5–7-fold larger than the first one. The quantitative analysis revealed similar coefficients of facilitation for JPs in both heart and mantle (Fig. 7a). The coefficient of facilitation of JPs generated by neuron d-VLN in both heart (7 ± 1.4) and mantle muscles (6.5 ± 0.7) were significantly ($P < 0.01$) larger than those generated by d-RPLN (4.9 ± 0.5 and 4.7 ± 0.4 in heart and mantle, respectively). However, the values of facilitation in different muscles were close in respect to both neurons. The above results confirm the presence of direct connection between neurons and peripheral muscles.

It has been shown that the facilitation causes long-term potentiation (Magleby, 1973a,b). The distribution of amplitudes of testing JPs was well approximated by exponential curves (Fig. 7b). Both single and averaged values of regressions were close. The time constants for elimination of potentiation for JPs generated by neurons d-VLN/d-RPLN in both mantle and heart muscles were similar, i.e. 172.4, 166.7, 178.6 and 175.4 s (Fig. 7b). These values were determined after a series of five spikes in giant neurons. Therefore, during the recording of 'zero-JP', it was necessary to install an interval value either two or threefold more than the duration of the potentioning effect. Thus, the potentiation of previous APs was eliminated.

Giant neurones d-VLN and d-RPLN are symmetrical cells, although they are situated in non-

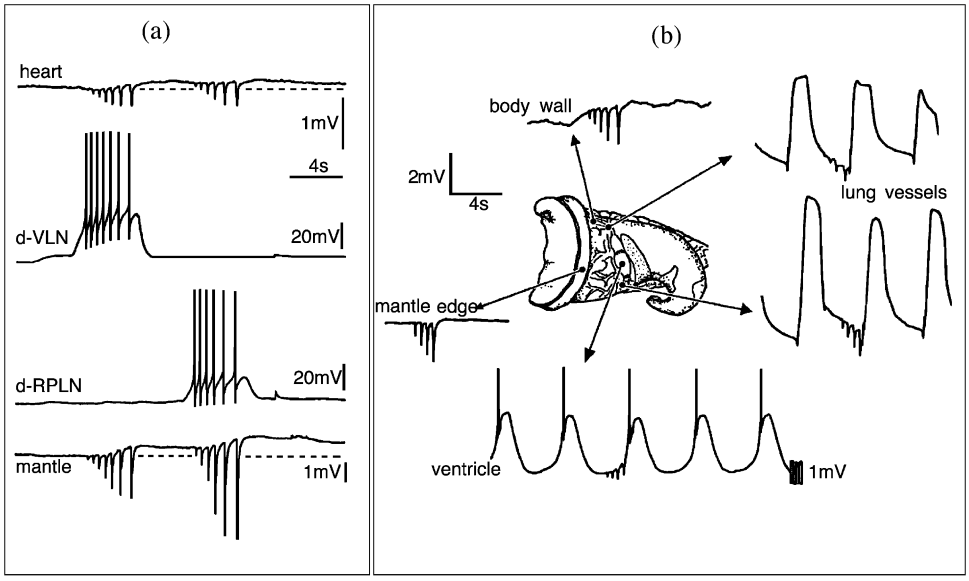

Fig. 6. (a) Simultaneous recordings of APs in a pair of giant neuron d-VLN/d-RPLN and I-EJPs in the heart and mantle muscle. Dashed lines are baselines that also indicate the magnitude of amplitudes of D-phase (of I-EJPs); (b) extracellularly recorded JPs in the heart and different points of viscerae in 'whole-mount' preparation. These JPs mimic the spike activity in giant neurons.

symmetrical ganglia (Munoz et al., 1983). The mirror symmetry of these neurons suggests the presence of both symmetric afferental inputs and efferental outputs. These cells have extensively overlapped receptive fields and react to the mechanical stimulations of body wall and viscera. The afferent stimulation similarly evoked either single or repetitive postsynaptic potentials (PSPs) in both neurons. However, the amplitude of PSPs generated by the stimulation of afferents in the left and right sides of the body is slightly different. We have compared amplitudes of PSPs in two neurons after mechanical stimulation of several symmetric points in both left and right sides (Fig. 8). Evidently, the stimulation of points from the left side mostly evokes PSP with higher amplitude in the left neuron (d-VLN), and vice versa (Table 1).

On the basis of the current findings, the previous view of the cardioregulatory neuronal network of *Achatina* have been modified. The newly discovered connections between giant neurons and heart are summarized in Fig. 9. Thus, the present study using the 'whole-mount' preparation had supplemented the scheme of cardioregulatory network of the Giant African snail by two more cells, d-VLN and d-RPLN (Fig. 9). These neurons and heart are connected separately and their interaction with other heart motoneurons was not observed. Now five types of nervous cells in subesophageal ganglia innervating the heart of *Achatina* are described. Beside the giant neurons d-VLN/d-RPLN, the cardioregulatory network includes the group of heterogeneous excitatory cells and two identical cholinergic heart inhibitory motoneurons, HI1 and HI2 (Bychkov et al., 1997). The tonically autoactive neurons TAN1, TAN2 and TAN3, the periodically oscillating neuron PON and neuron VG1 belong to cardioexcitatory cells (Furukawa and Kobayashi, 1987a; Zhuravlev et al., 1997). There are neuronal connections between dorsal left pedal large neuron (d-LPeLN), PON and TANs (Furukawa and Kobayashi, 1987b).

4. Discussion

Using 'whole-mount' preparation a new function of some previously identified neurons has been described. Neurons d-VLN and d-RPLN appear multimodal, i.e. participating simultaneously in the regulation of several physiological processes. Multimodality of neurons is a recurrent theme in the nervous system of molluscs. Usually, only visceral organs and somatic muscles

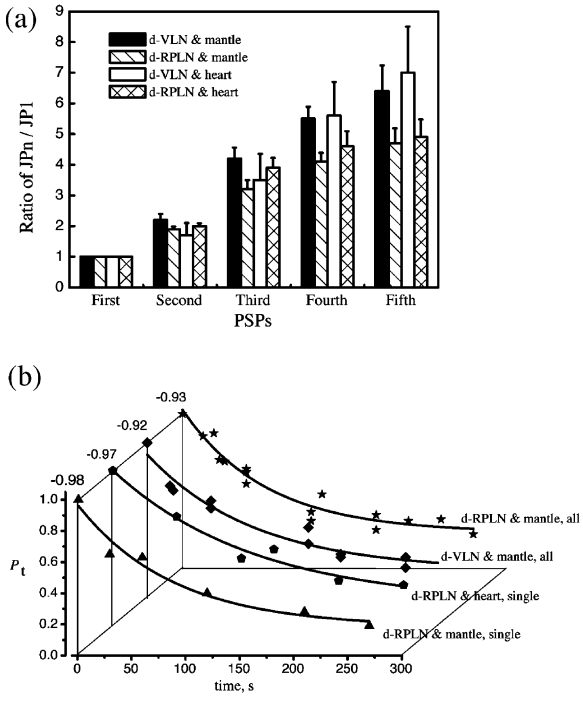

Fig. 7. (a) The facilitation of H-phases of I-EJPs during standard bursts in giant neurons. JPs in mantle muscle and heart were recorded during stimulation of d-VLN and d-RPLN. (b) Regression curves were calculated for data obtained from both a single (d-RPLN and mantle/heart, single) and all of experiments (d-RPLN/d-VLN and mantle, all; $n = 7$). Values of regression were similar in all data groups.

limit the activity of previously identified multimodal neurons (Alevizos et al., 1989). In this case, neurons modulate the functions of both systems.

Because of the high-amplitude H-phase of I-EJPs, evoked by d-VLN and d-RPLN in the heart, these cells were first denoted as new non-cholinergic heart inhibitory motoneurons. The hyperpolarizing phase of I-EJPs is Cl^--independent. Probably, the hyperpolarization is governed by potassium ions that could be determined by measuring the reversal potential of JPs. Although, by mean of extracellular recordings it is difficult to determine the changes in reversal potential, at least accurately.

However, the summation of H-phase of I-EJPs in contrast to cholinergic IJPs occurred only at maximal frequency of spikes in giant neurons. Cells d-VLN and d-RPLN are silent in isolated ganglia, semi-intact and 'whole-mount' preparations. Therefore, these cells can hardly inhibit the heart in situ as a result of direct hyperpolarization of myocardium. Nevertheless, the heartbeat was obviously abolished for longer periods after a short discharge in giant neurons (Fig. 2c). The specific experiments (Zhuravlev et al., 1999) revealed that (1) train of APs in giant cells d-VLN/d-RPLN leads to the contraction of different visceral muscles; (2) subsequently, the contraction of muscles or shift of viscerae can irritate the mechanosensitive endings of cholinergic heart inhibitory motoneurons HI1/HI2; and (3) the afferent train in these cells returned to the heart and evoked a train of cholinergic inhibitory JPs in the myocardium. Thus, the inhibition of heartbeat after train of APs in giant neurons is the viscerocardial reflex, which is organized by sensorymotor cholinergic heart inhibitory neurons HI1 and HI2 (Zhuravlev et al., 1993). The cutting of the intestinal nerve abolishes this reflex, but does not affect the JPs in the heart evoked by giant neurons.

Activation of giant neurons after cutting of the intestinal nerve slightly increased ($< 10\%$) the heartbeat. The heart rate remained unchanged (Zhuravlev et al., 1999). This is consistent with the observation of Ripplinger (1957) who has previously described a small increase in the tonus and amplitude of heartbeats under the stimulation of the left pallial nerve in *Helix*.

Based on studies of Munoz et al. (1983) it was suggested that neurons d-VLN/d-RPLN in

Table 1
The coefficients of afferental asymmetry during mechanical stimulation of different points in the whole-mount preparation (see Fig. 8).

Point no.	1	2	3	4	5	Pericard
Left side		1.07 ± 0.16, $n = 7$	0.86 ± 0.15, $n = 6$	1.32 ± 0.1, $n = 7$		
Centre	0.86 ± 0.15, $n = 6$			1.34 ± 0.23, $n = 6$	1, $n = 2$	1.77 ± 0.49, $n = 4$
Right side		0.85 ± 0.13, $n = 5$	0.63 ± 0.29, $n = 3$	0.89 ± 0.1, $n = 9$		

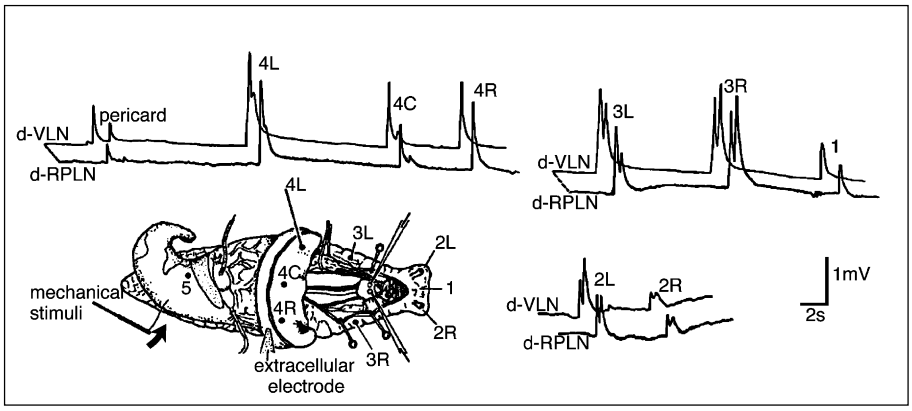

Fig. 8. Synaptic potentials in giant neurons evoked by mechanical stimulation of different points of body wall and viscerae (see also Table 1).

Achatina and the pneumostoma command neurons LPa3 and RPa3 in *Helix* (Maximova and Balaban, 1983) are homologous. The command neurons of pneumostoma in *Helix* also have extensive zones of afferental and efferental innervations in the body wall. These neurons activate many muscles including the heart (van Wilgenburg and Milligan, 1976; Iniushin et al., 1987). The dendritic trees of d-VLN and d-RPLN are similar to those of command neurons in *Helix* (Babmindra et al., 1979; Goto et al., 1986; Takeuchi et al., 1996). In the pedal, pallial and visceral nerves the axons of giant neurons are included. The axonal connections of giant neuron pairs in both species have mirror symmetry. Each neuron dominates on its own side for afferental inputs and efferental outputs. The branches of these neurons are absent in the intestinal nerve. Apparently, the giant symmetrical cells are involved in the mantle–heart interactions in both *Helix* (Ripplinger, 1957; van Wilgenburg and Milligan, 1976) and *Achatina*.

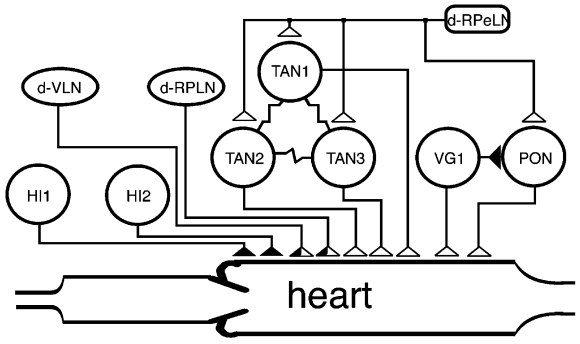

Fig. 9. Schematic representation of cardioregulatory neuronal network in *Achatina fulica*. Closed triangles indicate the inhibitory connections, open triangles are the excitatory connections, and mixed triangles show biphasic JPs. The heart inhibitory motoneurons (HI1 and HI2) are cholinergic, cells d-RPeLN, TAN1, TAN2 and TAN3 are serotonergic, and periodically oscillating neuron PON is peptidergic. Neurons VG1, d-VLN and d-RPLN are probably also peptidergic.

Neurons LPa3 and RPa3 in *Helix* show a positive immunoreactivity to FMRFamide-antibodies (Elekes and Nassel, 1990). In contrast, cells d-VLN and d-RPLN do not show immunoreactivity to FMRF-amide, or to the cardioexcitatory peptide of *Achatina* ACEP1 (Fujiwara-Sakata and Kobayashi, 1994; Satake et al., 1999). They are also negatively immunoreactive to a family of α-peptides of neurons VD1 and RPD2 in pond snail *Lymnaea stagnalis* (Kerkhoven et al., 1993). Moreover, these cells do not contain biogenic monoamines (Croll, 1988).

Possibly, the neurotransmitter of the neurons d-VLN and d-RPLN can directly activate any of regulatory mechanisms in cardiomyocytes, visceral and somatic muscles. It is known that many peptidergic neurons (including FMRFamidergic neurons) induce only small JPs in muscles, modulating the contractility of muscles through the second messenger pathways (Benjamin et al., 1988; Buckett et al., 1990a; Brezden et al., 1991).

There are also the cardioregulatory neurons in *Achatina* that have distinct homologues in other species. The PON is a homologue of the well-known bursting neuron RPa1 (F1) in *Helix pomatia* and *Helix aspersa*, neurons VD1/RPD2 in *Lymnaea* (Kerkhoven et al., 1991) and neuron R15 in *Aplysia*. The number of serotonergic cardioregulating cells differs in *Aplysia* (only one

neuron) and land pulmonates (three cells). In *Achatina* the stimulation of serotonergic cells causes only slow summated depolarization of the myocardium (Zhuravlev et al., 1997). However, in *Aplysia* discrete serotonergic EJPs in the heart were observed (Mayeri et al., 1974). A two-phasic EJPs and simple small EJPs in the heart of *Achatina* correspond to spikes in PON and VG1, respectively (Zhuravlev et al., 1997).

The cholinergic cells represent the networks of heart inhibitory motoneurons in all investigated gastropod mollusks. In ganglia of *Aplysia*, *Helix*, *Achatina* and *Lymnaea* only two cholinergic cardioinhibitory motoneurons are known (Buckett et al., 1990b; Skelton et al., 1992; Zhuravlev et al., 1991a,b; Bychkov et al., 1997). These cholinergic cells in land pulmonates are multifunctional, i.e. they are sensory-motor neurons (Zhuravlev et al., 1993).

Many of multimodal cardioregulating cells participate in the creation of common humoral background, which plays a role in oscillatory activity and other parameters of myocardium (Smith, 1987; Nassel, 1996). The neuronal networks that are involved in the viscerocardial reflexes are organized around these both multimodal and multifunctional cells playing an integrative role. Elements of the networks do not only limit to the fixed paths; the dynamic networks are based on combinations of included elements activated by the various inputs. The same element can play a various role in the dynamically organized networks, which increases the functional plasticity of nervous system (S.-Rózsa, 1981, 1987). Thus, the giant multimodal cells d-VLN/d-RPLN and cardiorespiratory system in *Achatina* is a unique model for the study of both the myocardial noncholinergic I-EJPs and the viscerocardial reflexes.

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