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Effect of acute temperature change on lung respiration of the mollusc *Lymnaea stagnalis*

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Abstract

1. Temperature-dependent effects on respiratory behaviour as well as the corresponding temperature-dependent activities of identified neurons within the respiratory network of the pulmonate snail *Lymnaea stagnalis* were investigated.
2. *Lymnaea* lung ventilation terminated at low temperatures (under 10 °C) while temperature elevation increased ventilation rates. The respiratory central pattern generator (CPG) functioning was relatively quiescent at temperatures under 12.5 ± 0.44 °C.
3. Identified CPG neurons (RPeD1, VD4, VD1/RPaD2) and the respiratory network motor neurons (Vi- and RPa-cells) were found to exhibit varied temperature-dependent electrophysiological parameters (action potential frequency and amplitude, resting potential value) between cell types.
4. The observed alterations in the electrical activity of the *Lymnaea* respiratory network neurons underlie the marked changes of respiratory behaviour observed in the intact animal during temperature changes.

Keywords: Identified neurons; Behaviour; Central pattern generator; Gastropoda

1. Introduction

The study of temperature effects on the nervous system was historically focused on nerve conductivity (Hodgkin and Katz, 1949), synaptic processes (Katz and Miledi, 1965) and neuron functioning (Marmor, 1971; Carpenter, 1981). Changes of various types of animal behaviour following temperature changes were also frequently reported (see observations in Prosser, 1973). The various species of molluscs (*Aplysia*, *Helix*, *Lymnaea*, *Limax* and others) are widely used for neurophysiological investigations as relatively simple model nervous systems (Kandel, 1976). *Lymnaea* has proved to be a useful model for gaining insights into the neuronal basis of different types of behaviour. One of the well-investigated parts of the CNS in fresh-water pond snail *Lymnaea stagnalis* is the respiratory network underlying the processes of pulmonary respiration. Its main components have been identified during the past two decades (Syed and Winlow, 1991; Moroz, 1991). Thus, the question of how the activation of *Lymnaea* respiratory network neurons is accomplished is still uncertain (Moroz, 1991; Inoue et al., 2001). In the literature there is a nearly complete absence of studies investigating the influence of temperature on the functioning of neuronal circuits as well as on the various types of *Lymnaea* behaviour (some data have been

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reported previously (Janse, 1981, 1982; Sidorov and Kazakevich, 2001; Sidorov, 2003)), although it is well known that molluscs live in nature deal with a wide variation of temperature over both daily and seasonal time courses (Vlastov and Matekin, 1988), which is unstable during the day and/or seasons in the natural habitation of *Lymnaea stagnalis*. Temperature can vary widely from 0 °C (in winter some molluscs may even survive in ice) to 35 °C (on hot summer days). Such large variations may contribute to the changes in activity of the respiratory CPG and subsequently may determine the respiratory behaviours this network serves.

The *Lymnaea* respiratory act (lung ventilation) consists of a series of stereotyped complex reactions. It begins with the mollusc moving to the water surface. Weak tactile stimulation of the tentacles and the pneumostome with the water surface induce respiratory movements. When the edges of the lips touch the surface film, anticlockwise shell movements begin. This is often accompanied by inhibition of locomotion. Parallel to the shell movements, pneumostome erection occurs (Moroz, 1991; Vlastov and Matekin, 1988; Syed et al., 1991) followed by pneumostome opening and a small air bubble is subsequently expelled that breaks the surface film. This stereotypical behavioural sequence marks the start of respiration. The pneumostome will remain open for at least 45–60 s occasionally reaching durations of over 2 min. After pneumostone closure, the animal, as a general rule, leaves the water surface.

Fig. 1A shows the location of neurons used in the study. The rhythmic activity of *Lymnaea* respiratory network is a close representation of the "half-centre" model central pattern generator described by Euler von (1985) and Syed et al. (1990). In this case, neurons VD4 and IP3I are the half-centres (Fig. 1B). Electrical stimulation of VD4 results in occurrence of excitatory post-synaptic potentials (EPSPs) in the pneumostome closer motor neurons and simultaneous inhibitory post-synaptic potentials in the pneumostome opener motor neurons. Electrical stimulation of IP3I results in opposite effects. Thus, these two neurons have reciprocally inhibitory connections. The CPG structure, besides two working beginnings (half-centres), includes a component responsible for all neuronal network activation. Two candidates for this role are now offered: neuron RPeD1 and a pair of electrically coupled neurons VD1/RPaD2. The increase in RPeD1 electrical activity initiates IP3I activation due to a conjoint inhibitory-excitatory synapse from RPeD1 to IP3I. Once activated by RPeD1, IP3I excites RPeD1 while inhibiting VD4. VD4 and RPeD1 have reciprocal inhibitory connections. Electrical stimulation of RPeD1 causes EPSP in pneumostome closer motor neurons. For the duration of IP3I activation RPeD1 fails to induce its effects on follower cells. It is hypothesized that IP3I decouples RPeD1 from its follower cells by presynaptic

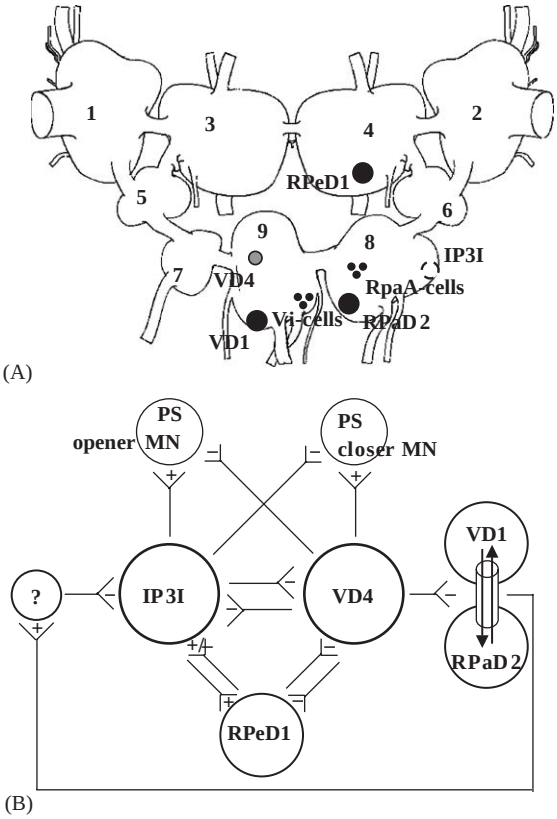

Fig. 1. (A) Diagrammatic representation of the nervous system of *Lymnaea* and the location of the neurons used in the present study. The figure shows the dorsal view of the nervous system. Buccal ganglia have been omitted and the cerebral commisure has been sectioned. 1,2—left and right cerebral; 3,4—left and right pedal; 5,6—left and right pleural; 7,8—left and right parietal ganglia; 9—visceral ganglion. (B) The respiratory network of *Lymnaea* (modified from Syed et al., 1990; Moroz, 1991). Excitatory chemical connections marked by a plus, inhibitory—by minus; electrotonic coupling between VD1/RPaD2 by the double arrow. The ? symbol represents hypothetical neural elements. MN—pneumostome (PS) opener and closer motor neurons.

inhibition (Syed and Winlow, 1991). The pair of electrically coupled neurons VD1 and RPaD2 reacts to decreases of pO_2 by strong hyperpolarization (Janse et al., 1985). It is supposed (Moroz, 1991) that powerful (about 10 mV) hyperpolarization of these neurons leads to inhibition of the neuron(s) normally inhibiting IP3I activity. Such "double inhibition" results in IP3I disinhibition and, as a consequence, in the activation of the whole respiratory program. Meanwhile VD4 forms inhibitory connections with VD1/RPaD2 neurons (Janse et al., 1985) that can stimulate IP3I activity after VD4 burst generation.

Previously, we have described the changes in synaptic transmission within *Lymnaea* respiratory CPG neurons (Sidorov, 2002) at different temperatures, but the reactions of identified respiratory network neurons were unexplored. Here, we suggest that these cells also exhibit an unequal temperature dependence of their electrical activity and this phenomenon underlies the change in *Lymnaea* pulmonary respiration during acute temperature changes.

2. Materials and methods

Animals. Specimens of *Lymnaea stagnalis* (L.) were collected locally during spring–autumn period, kept for up to 4 weeks in tap water at 16–18 °C and fed lettuce. The shortest period the animals were used in experiments after collection was at least 2 weeks. All experiments were done on snails weighing 2–4 g.

Behavioural experiments. The respiratory behaviour was observed in different temperature ranges: 4–6, 16–18, 24–26, 34–36 °C. The water was equilibrated with pO_2 at all temperatures used. The molluscs were placed in 51 vessels (five in each) filled with settled tap water. The number of respiratory cycles (pneumostome opening–closing movements) during 1 h of observation was recorded. The pneumostome open time and the total time of lung ventilation per hour were measured as well. Tests were begun 10 min after animals had been placed in new temperature conditions. The number of respiratory cycles and pneumostome open time made by each snail were recorded.

Electrophysiological experiments. Animals were anaesthetized in 0.2 M $MgCl_2$. Isolated brains were bathed in normal physiological saline (at 20 °C), which consisted of (mM): NaCl 44.0, KCl 1.7, $CaCl_2$ 4.0, $MgCl_2$ 1.5, HEPES 10.0. The pH was adjusted to 7.5 with 0.1 M NaOH. Protease E-type XIV (Sigma) was used to soften the perineurium (1.5 mg/ml for 10 min at 20 °C). The preparation was perfuse (perfusion rate—0.1 ml/min) by normal physiological saline. Individual neurons were impaled with glass microelectrodes filled with 2.5 M KCl (electrode resistance 20–50 MΩ). Central neurons were identified according to their location, size, colour and electrophysiological characteristics (see the maps in Syed and Winlow, 1991; Moroz, 1991; Benjamin and Winlow, 1981; Janse et al., 1985). Electrophysiological signals were amplified, displayed on an oscilloscope and recorded by a pen chart recorder. The temperature was maintained and changed using a laboratory-made thermostat based on a Peltier assembly.

Statistics. Data were processed by standard methods of variational statistics. The significance was evaluated with Students *t*-test or χ^2-test.

3. Results

Respiratory behaviour. We observed that the temperature has the following influence on respiratory behaviour of *Lymnaea* (Figs. 2 and 3). First the water temperature was lowered from 14–16 °C to 4–6 °C over a period of minutes. Decreasing the water temperature by 10 °C from 14–16 °C caused respiration to stop. Only 3 of 31 molluscs demonstrated active (lung) respiration: two cycles with a duration about 15 and 30 s, respectively (this phenomenon was measured in the first 15 min of observation). Increasing the water temperature by 10 °C (from initial 14–16 °C level) led to an increased oxygen consumption. This resulted in an increased number of respiratory cycles as well as an increase in the total respiratory time. In contrast, pneumostome-open time was reduced at 24–26 °C. As a rule, *Lymnaea* prefer not to visit the bottom of the experimental vessels, remaining active near the water surface. Further water temperature elevation (34–36 °C) led to a number of

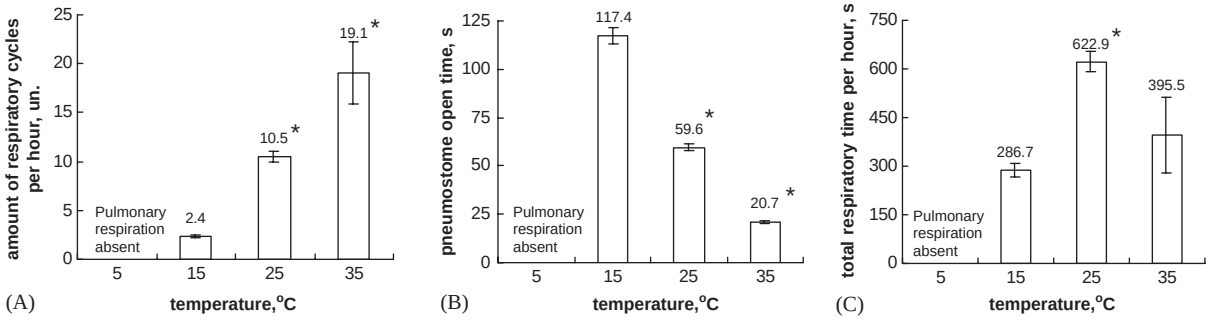

Fig. 2. Effect of temperature on (A) the number of respiratory cycles per hour, (B) pneumostome open time and (C) total respiratory time per hour in intact freely moving *Lymnaea*. Data present mean value (number above the column) ± SEM. *Significant relative to the value at 15 °C ($P < 0.05$). Number of animals (trials) studied: 5 °C $n = 31$ molluscs, 15 °C $n = 20$ molluscs (288 observations), 25 °C $n = 100$ molluscs (1096 observations), 35 °C $n = 15$ molluscs (292 observations).

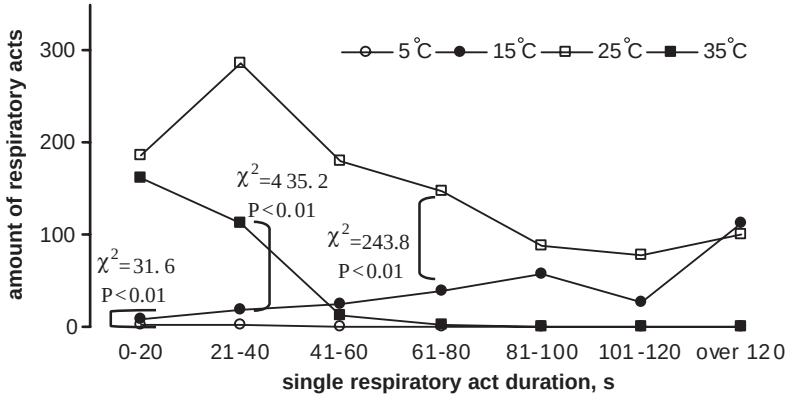

Fig. 3. Effect of temperature on distribution pattern of pneumostome open time in intact freely moving *Lymnaea*. Data present χ^2 meaning and significance in comparison with control at 15 °C. Number of animals (trials) studied: 5 °C $n = 31$ molluscs, 15 °C $n = 20$ molluscs (288 observations), 25 °C $n = 100$ molluscs (1096 observations), 35 °C $n = 15$ molluscs (292 observations).

deviations from the standard respiratory behaviour model. Nine of the 24 animals shared no respiratory behaviour. This group was characterized by the complete absence of pulmonary respiration. During the first 30 min of observation, these animals were active both at the surface and in the bottom areas. Additionally, their locomotory activity was terminated, and these animals were relatively insensitive to tactile stimulation of the skin and tentacles. About 15 of the 24 animals demonstrated normal pulmonary respiration activity characterized by a further increase in the number of respiratory cycles with the notable difference that the pneumostome-open time and total respiratory time were decreased.

On returning to the normal temperature range (14–16 °C) stereotypical respiratory behaviour recovered to previous values.

Temperature caused the following changes in electrical activity of the respiratory network neurons in *Lymnaea*.

*Neuron RPeD*1 ($n = 18$) (Fig. 4).

At 14–16 °C RPeD1 was spontaneously active. A temperature decrease to as low as 6.2 ± 0.44 °C resulted in significant hyperpolarization and reduction of action potential (AP) frequency. At temperatures below 6.2 ± 0.44 °C, RPeD1 tonic activity disappeared (only irregular, single spikes were observed). A temperature increase of 10 °C (24–26 °C range) depolarized RPeD1 and increased AP frequency. Further temperature elevations resulted in a sharp suppression of RPeD1 spontaneous activity and greater depolarization. The dependence of the (AP) frequency on temperature was non-linear with a maximum at 24–26 °C. Changes in resting potential (RP) and AP amplitude with temperature alterations were characterized by a closely linear dependence with a negative coefficient of correlation (-0.99 ± 0.01 and -0.97 ± 0.02, respectively) with increasing temperature.

*Neuron VD*4 ($n = 5$) (Fig. 4B).

In all experiments, we failed to observe a single AP from VD4. Thus, we analysed the changes of RP value only. Both increases and decreases in temperature by 10 °C resulted in cell depolarization. A change in RP of VD4 was non-linear with a maximum at 14–16 °C.

Electrically coupled neurons *VD*1/*RPaD*2 ($n = 15$) (Fig. 4).

At 14–16 °C, these cells were spontaneously active. A temperature decrease caused sharp fall of AP frequency. Both VD1 and RPaD2 were depolarized with decreasing temperature. The lowest temperature that permitted spontaneous spike activity for these neurons was 8.6 ± 0.42 °C. A temperature increase by 10 °C above 15 °C resulted in an insignificant increases in the AP frequency of VD1/RPaD2. Both cells were depolarized with elevated temperatures and the amount of depolarization was comparable with those observed during cooling. Further temperature increases resulted in AP frequency reduction comparable with those observed at 24–26 °C. RP was also decreased. The dependence of AP frequency on a temperature change was non-linear with a maximum at 24–26 °C. The dependence of the rest potential value and AP amplitude on a temperature followed this same relation.

Neuron IP3I (Input 3) ($n = 10$).

Simultaneous recording of IP3I activity with the activity of other respiratory network neurons had the inherent difficulty of simultaneous access with multiple electrodes given that the soma of this cell is situated on the ventral surface of the right parietal ganglion, the surface opposite to that of other respiration neurons (Syed et al., 1990). Nevertheless, indirect evidence of IP3I activity could be obtained by recording from known follower cells Vi, j, k-cells of the visceral ganglion, VD4, RPeD1 and VD1/RPaD2. The following effects on IP3I activity were observed with changes in

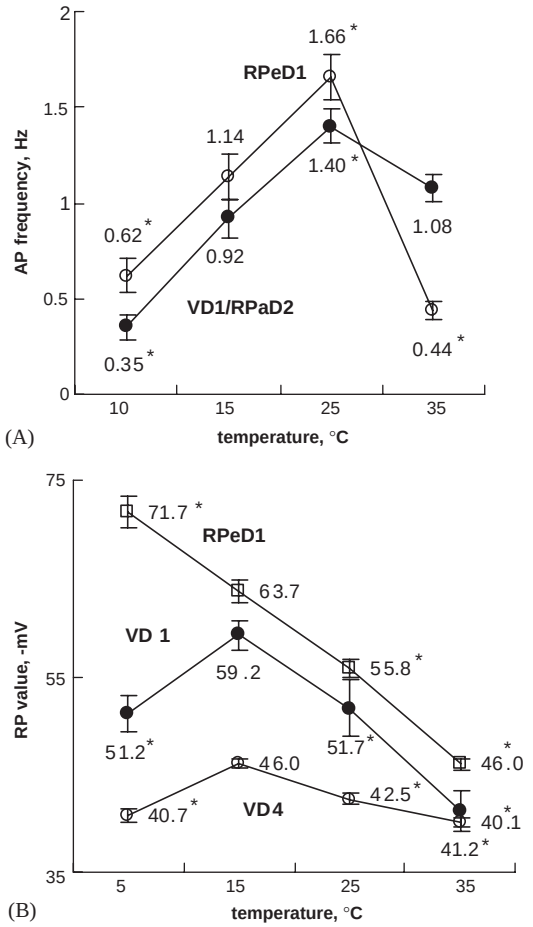

Fig. 4. Effect of temperature changes on AP generation frequency (A) and the RP value (B) of respiratory CPG interneurons in *Lymnaea*. (A) Open cycles—RPeD1, close cycles—VD1/RPaD2, (B) Open cycles—VD4, close cycles—VD1, open squares—RPeD1. Number of neurons studied: RPeD1: $n = 18$, VD1/RPaD2: $n = 15$, VD4: $n = 5$. Data present mean values (number near the point) ± SEM. *Significant ($P < 0.05$) relative to the value at 15 °C ($P < 0.05$).

temperature (Fig. 5). At 16–18 °C, IP3I burst frequency was 0.8 ± 0.08 burst/min. At temperatures below 12.5 ± 0.44 °C, the effects of IP3I activity on its followers were absent. Returning to room temperature restores IP3I follower activity at 14.0 ± 0.11 °C. A temperature increase of 10 °C increased the IP3I burst frequency to 1.4 ± 0.15 burst/min at 24–26 °C. At temperatures above 25.4 ± 0.18 °C, the regular inhibitory component of respiratory CPG work (i.e. VD4 activity) that divides IP3I bursts, was absent. This rhythmic activity was restored after the temperature was returned to 23.1 ± 0.50 °C. In nervous system preparations with spontaneous CPG neuronal activity, maintained at the above-mentioned temperature paradigms (25 °C and above), marked (20–30 mV, 5 s), hyperpolarization of VD1/RpaD2 was observed (Fig. 6).

Vi-cluster cells ($n = 7$) (Fig. 7).

At 14–16 °C, these neurons were spontaneously active. A temperature decrease by 10 °C led to a sharp reduction of AP frequency. In contrast to the above neurons, the RP did not significantly change with cooling. Vi-cells retained the ability to generate AP at temperatures as low as 2 °C. A temperature increase of 10 °C from 15 °C did not result in significant changes of AP frequency although these neurons were depolarized. Greater temperature increases (34–36 °C) led to a sharp rise in AP frequency and depolarization of the cells. The dependence of AP frequency on temperature changes was characterized by a sigmoid curve with a positive coefficient of correlation (0.98 ± 0.02) with increasing temperature. The dependence of RP and AP amplitude on temperature changes was characterized by a linear curve and had a negative coefficient of correlation (−0.99 ± 0.02 and −0.98 ± 0.02, respectively) with increasing temperature.

RPaA-cluster cells ($n = 6$) (Fig. 7).

At 14–16 °C these neurons were spontaneously active. Temperature decreases resulted in a sharp reduction of AP frequency and strong hyperpolarization. RPaA-cells retained the ability to generate AP at temperatures as low as 2 °C. Although elevated temperatures resulted in increased AP frequencies, the RP did not change. Further temperature increases (34–36 °C) resulted in arhythmic spontaneous spike activity and changes in burst pattern compared with those initially observed at room temperature. The dependence of spike activity on temperature was non-linear with a maximum at 24–26 °C. RP and AP amplitude had the negative coefficient of correlation (–0.96 ± 0.05 and –0.94 ± 0.06, respectively) with temperature.

Returning the preparation to room temperature led to a complete restoration of the initial parameters of spontaneous electrical activity of the respiratory network neurons.

4. Discussion

The periodic activity of VD4 is due to the inhibitory influence of IP3I (Syed and Winlow, 1991; Syed et al., 1990). The IP3I firing frequency during temperature changes (particularly during its increase from 15 to 25 °C) correlating ($r = 0.98 \pm 0.02$) with respiratory rate increases in the same temperature range. At temperatures of 24–26 °C and higher, IP3I activity is no longer interrupted by inhibitory input from the VD4 neuron. In this case, we hypothesize that IP3I bursts follow practically one after another, and that combined with the low RP value in VD4, interferes with the occurrence

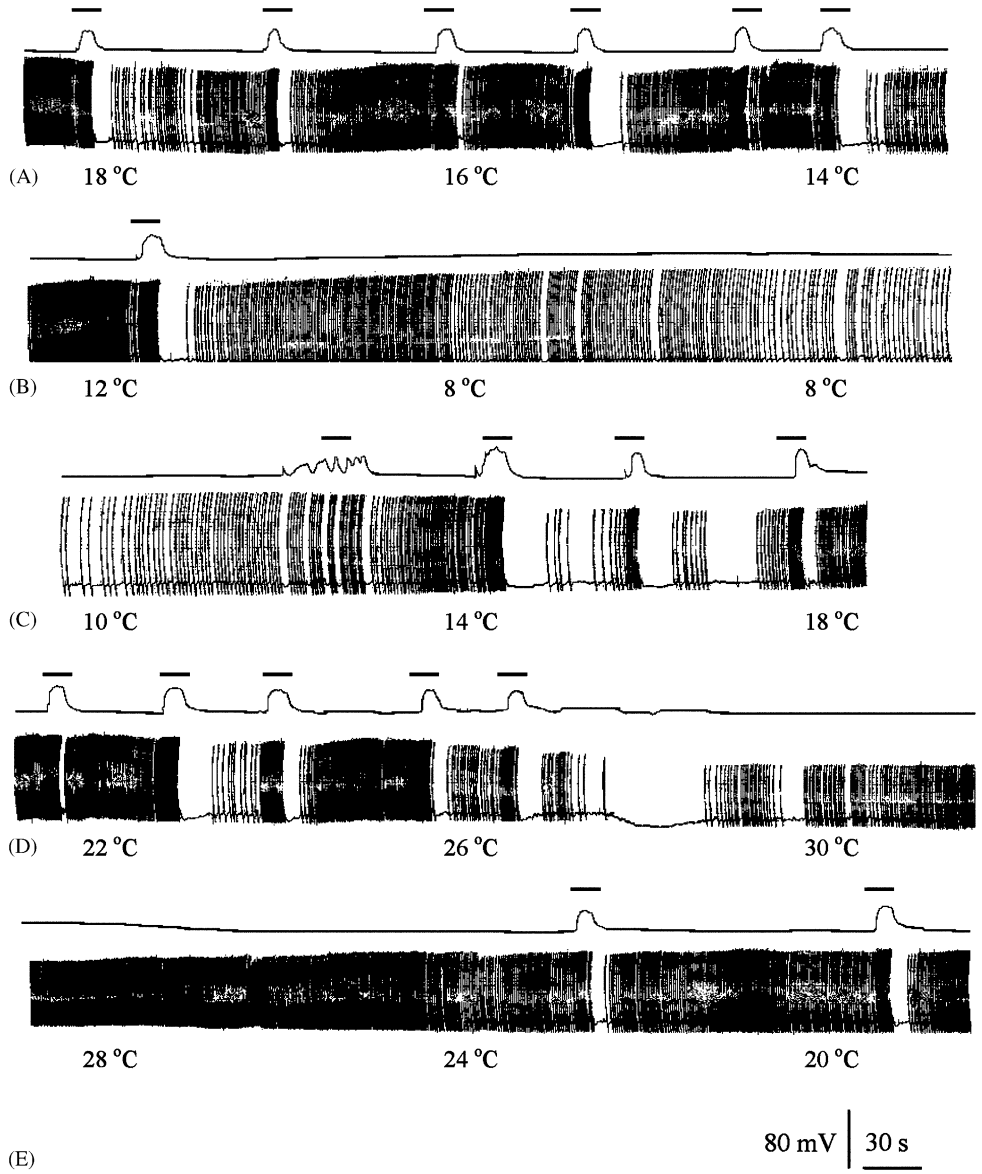

Fig. 5. Effect of temperature on spontaneous electrical activity of respiratory CPG interneurons in *Lymnaea*: Continuous traces of electrical activity (A–E) are given (simultaneous recording). Membrane potential in VD4—top trace, in RPeD1—bottom trace. The output of VD4 from the inhibitory connection of IP3I is marked by the bar.

of an inhibitory component of the respiratory rhythm. Neuron VD4 is a unique, quickly adapting cell with a relatively low RP value that opposes AP initiation. Both decreases and increases of temperature depolarize VD4 resulting in incompatible VD4 activation and its subsequent lack of effect on the appropriate pneumostome motor neurons. As a consequence, pneumostome remains in an open condition for a long period of time, as is observed at 24–26 °C.

Decrease in water temperature (from 15 to 5 °C) resulted in total reduction of pulmonary respiration. The energy needs, which have sharply decreased at these temperatures, are supplied by the simple oxygen dissolved in water (the amount of dissolved O_2 is higher as the temperature decreases) across the animal's skin (Vlastov and Matekin, 1988). The effects of IP3I activity, as observed on its follower cells, ceases at temperature below 12.5 ± 0.44 °C. This does not suffocate the animal taking into consideration the absence of lung ventilation via the decreased oxygen requirements at lower water temperatures. The similar blockade of spontaneous neuronal activity during temperature

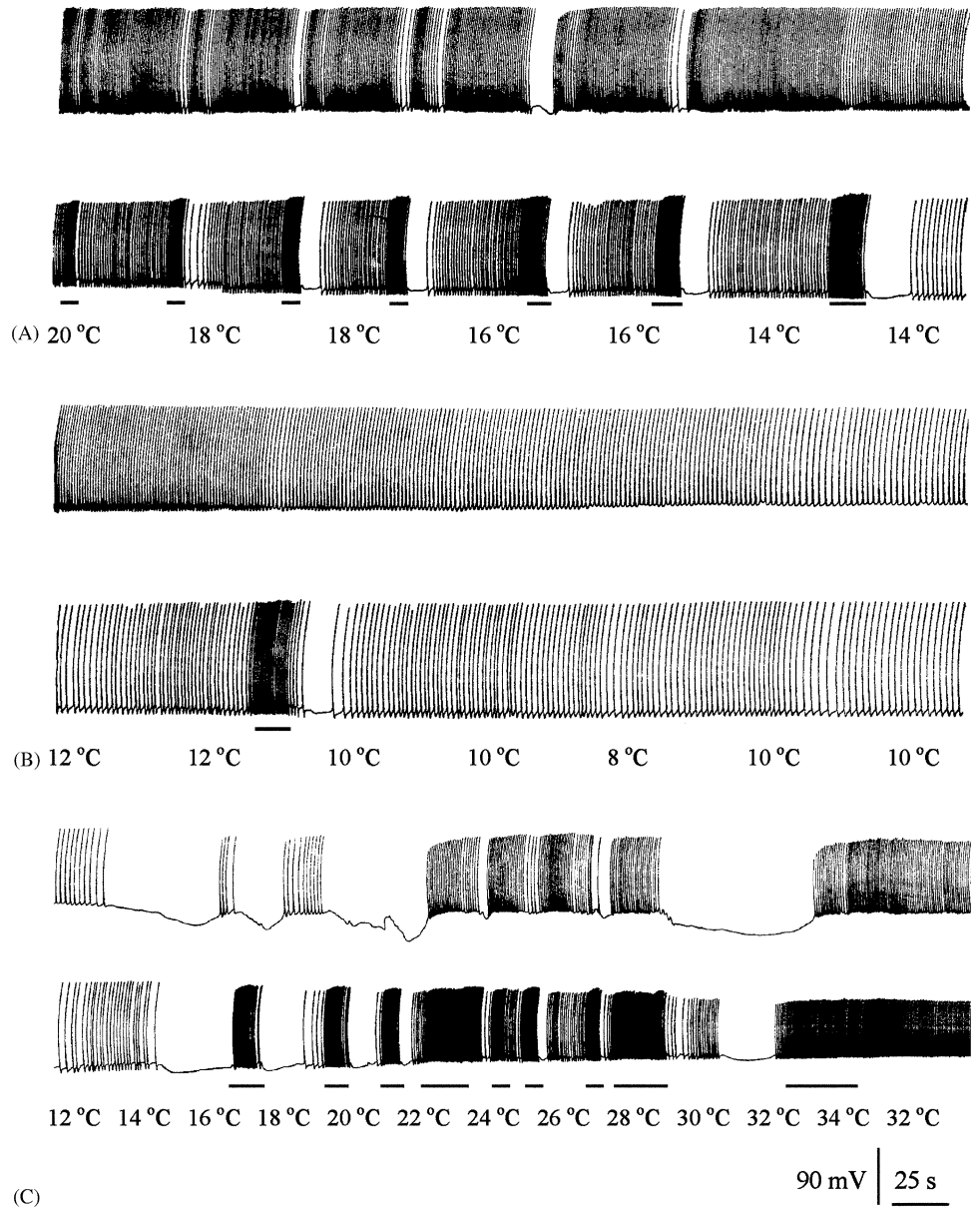

Fig. 6. Effect of temperature on spontaneous electrical activity of RPeD1 and RPaD2. Continuous traces of electrical activity (A–C) are given (simultaneous recording). Membrane potential in RPaD2—top trace, in RPeD1—bottom trace. Indirect observation of IP3I activity is delineated by the bar.

decreases has been reported in various invertebrates including *Limax* feeding neurons (Prior and Grega, 1982) and lobster neurons (Konishi and Kravitz, 1978).

The hyperpolarization observed in RPeD1 during temperature decreases suppresses IP3I activation due to an elevation of the AP generation threshold. At 30 °C and above, RPeD1 spontaneous electrical activity sharply falls, and VD1/RPaD2 RP is at its lowest value. Strong VD1/RPaD2 depolarization leads to IP3I inhibition. The factor that normally stimulates respiration in molluscs is decreased hemolymph pO_2 level. It is thought that decreased pO_2 causes the increase in RPeD1 firing rate that results in respiratory CPG activation (Syed and Winlow, 1991). At the same time, in the presence of pure nitrogen, a slow hyperpolarization of RPeD1 and decreased firing rate are observed. The recovery of normal atmosphere O_2 leads to the restoration of RPeD1 firing patterns (Janse et al., 1985).

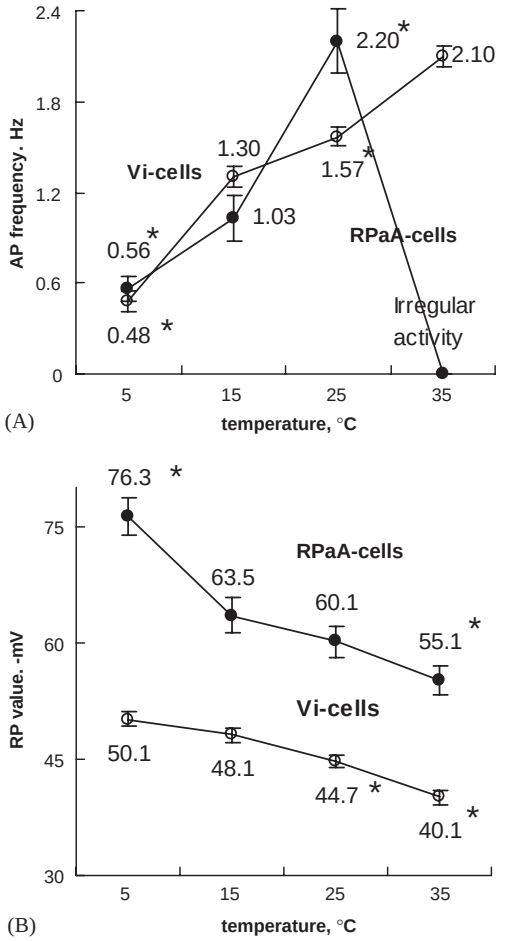

Fig. 7. Effect of temperature changes on AP generation frequency (A) and the RP value (B) of the respiratory motor neurons in *Lymnaea*. Open cycles—Vi-cells, close cycles—RPaA cells. Number of neurons studied: Vi-cells: $n = 7$, RPaA-cells: $n = 6$. Data present mean value (number near the point) $\pm$ SEM *Significant relative to the value at 15 °C ($P < 0.05$).

Vice versa, VD1/RPaD2 hyperpolarized during pO_2 decrease. Thus, VD1/RPaD2 is thought to be the element for activation of the respiratory network of *Lymnaea*. Sharp decreases of spontaneous activity in the Vi-cells (pneumostome opening motor neurons (Syed et al., 1991)) during temperature decreases is most likely caused by inactivation of IP3I, which shares monosynaptic connections with these neurons. At temperatures of, and exceeding, 24–26 °C, these neurons sustain a relatively high frequency of AP activity due to increases in burst activity of IP3I. The RPeD1 inhibitory outputs, practically, are completely suppressed due to a sharp decrease of their own firing rate and presynaptic inhibition from IP3I (see Fig. 1B). RPaA-cells (mantle cavity motor neurons) directly participate in the lung ventilation process (Syed et al., 1991). A sharp decrease of their spontaneous activity is observed at low (4–6 °C) temperatures when the need for lung ventilation is absent. Practically, complete suppression of the activity of RPaA-cells at 34–36 °C and higher can be one of the causes of death at high temperatures. The increase in oxygen consumption by the animal's tissues at these elevated temperatures could not be sustained by lung respiration due to the inactivation of RPaA-cells (and subsequently the respiratory CPG) by elevated heat. Thus, the lethal outcome can be a consequence of oxygen deficiency and subsequent ishemic collapse of *Lymnaea* nervous system.

It is likely that within the *Lymnaea* CNS, there are specialized neuronal elements reacting to temperature changes, particularly to its increase. They can appropriately modulate the activity of the neurons supervising various types of behaviour. Indirect confirmation of this statement is suggested by the VD1/RPaD2 pattern at temperatures 25 °C and above. Simultaneously with VD1/RPaD2 hyperpolarization, the inhibitory part of respiratory program was absent. Previously, it has been shown that dopamine causes VD1/RPaD2 hyperpolarization (Wildering, 1992). Yet, no synaptic connections were revealed between dopaminergic neuron RPeD1 and VD1/RPaD2 (Benjamin and Winlow, 1981). These findings prompted the author to suggest the existence of an unknown, dopaminergic (?) neuron activating the *Lymnaea* respiratory CPG neurons due to its inhibitory influence on VD1/RPaD2. This hypothetical neuron had to be also sensitive both to temperature and pO_2 changes.

5. Summary

The respiratory network neurons in *Lymnaea* are characterized by unequal temperature dependence of their electrophysiological parameters. Temperature is the powerful factor supervising pulmonary respiration of the mollusc *Lymnaea stagnalis*. The reactions of appropriate neurons within the *Lymnaea's* CNS underlie the temperature effects on mollusc behaviour.

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