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THE EFFECTS OF HYPERCAPNIA ON FORCE AND RATE OF CONTRACTION AND INTRACELLULAR pH OF PERFUSED VENTRICLES FROM THE LAND SNAIL *HELIX LUCORUM* (L.)

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Summary

The effects of hypercapnia, together with low and high levels of extracellular Ca^{2+}, on heart activity and intracellular pH were examined in isolated perfused hearts from the land snail *Helix lucorum*. In addition, the intracellular level of Ca^{2+} was determined in slices of ventricles superfused with both normal and hypercapnic salines, containing low and high concentrations of Ca^{2+}, to investigate whether low extracellular pH affects the entry of Ca^{2+} into the heart cells. We also examined the effect of a saline that simulated the composition of the haemolymph of snails after estivating for 3 months on the heart activity and intracellular pH. The results showed that hypercapnia causes decreases in the rate and force of heart contraction, and these are more pronounced in the presence of low levels of extracellular Ca^{2+}. Moreover, the present results indicate that Ca^{2+} maintains the contractility of the heart muscle under acidic conditions and seems to act by competing with protons for the Ca^{2+} binding sites on sarcolemma. The negative effect of hypercapnia on heart activity appears to be due to a reduction in extracellular pH rather than to changes in intracellular pH.

Key words: land snail, *Helix lucorum*, heart perfusion, hypercapnia, force and rate of contraction, intracellular pH.

Introduction

The effect of hypercapnic acidosis on heart performance has been studied extensively in vertebrates, and it is known that an increase in the extracellular partial pressure of carbon dioxide (P_{CO_2}) reduces the force and rate of heart contraction (Orchard and Kentish, 1990; Driedzic and Gesser, 1994). An increase in P_{CO_2} in extracellular fluids causes a decrease in the intracellular pH (pH_i) of myocytes, which is thought to reduce contractile force through H^+ competing for intracellular Ca^{2+} binding sites (Williamson et al., 1976). On the other hand, it has been shown by Langer (1985) and Langer et al. (1989) that low extracellular pH (pH_e) reduces the rate of Ca^{2+} entry into heart cells. The mechanisms by which H^+ might reduce Ca^{2+} entry into heart cells are not well known. It is believed that Ca^{2+} and H^+ act in a competitive manner for the same binding sites on sarcolemma and indeed, several studies have shown that, when increased extracellularly, Ca^{2+} improves cardiac performance during hypercapnic acidosis (Gesser and Poupa, 1979; Lagerstrand and Poupa, 1980; Williamson et al., 1976). However, some data indicate that low extracellular pH causes conformational changes in Ca^{2+} channels or transporters, resulting in decreased Ca^{2+} entry into myocytes (Iijima and Hagiwara, 1986a); Krafte and Kass, 1988; Klockner and Isenberg, 1994).

Although hypercapnia is a common response of many molluscs to various environmental conditions, little is known regarding the effects of hypercapnic acidosis on the pH_i of molluscan hearts and on cardiac activity. Hypercapnia and respiratory acidosis are greatly developed in land pulmonate snails during periods of estivation (Barnhart, 1986; Barnhart and McMahon, 1987; Rees and Hand, 1990) and the elevation of P_{CO_2} in the haemolymph of estivating snails results in a decrease in pH_e and pH_i (Barnhart and McMahon, 1988; Rees et al., 1991). On the other hand, mobilization of $CaCO_3$ stores caused by hypercapnia causes an increase in Ca^{2+} levels in the haemolymph of estivating snails (Burton, 1976; Barnhart, 1986). Increases in Ca^{2+} levels in the haemolymph of estivating land snails play an important role in the acid–base balance (Burton, 1976); however, the exact role of Ca^{2+} ions on heart activity in snails during estivation remains unknown.

In the present work, we studied the effect of hypercapnic salines, containing different concentrations of Ca^{2+}, on the force and rate of contraction of isolated ventricles from the land snail *Helix lucorum*. This was done in order to elucidate how heart activity is influenced by hypercapnia in land pulmonates during estivation. In addition, the pH_i of the perfused ventricles was determined in order to examine

whether it plays any key role in the regulation of heart activity. Moreover, we examined whether low extracellular pH affects entry of Ca^{2+} into the heart cells. To examine this, using fura-2 we determined the intracellular concentration of Ca^{2+} in slices of heart muscle superfused under normal and hypercapnic conditions.

It is known that, as well as Ca^{2+}, the levels of some other solutes (e.g. Mg^{2+}, K^+) increase in the haemolymph of estivating snails (Barnhart, 1986). We measured the concentrations of Na^+, Mg^{2+}, K^+ and HCO_3^- in the haemolymph of snails estivating for 3 months and then we created a saline simulating the composition of the corresponding haemolymph. Afterwards hearts were perfused with this saline in order to obtain some data concerning the effect of hypercapnia in relation to the changes of solutes in haemolymph on the heart activity.

Materials and methods

Adult specimens of *Helix lucorum* (L.) were collected in the vicinity of Edessa, in northern Greece. The snails were kept in an active state at a temperature of 25±0.5 °C and subjected to a 10.00 h:14.00 h L:D photoperiod in large glass boxes, with a daily supply of lettuce leaves and water. High humidity (85±1 %) was maintained by sprinkling the interior of the boxes with water every day. To induce estivation, active snails were removed and transferred to glass boxes without food and water, but with ample aeration. The snails were kept for 3 months in a dormant state at the same conditions of temperature and photoperiod as described above.

Collection of haemolymph and determination of cations, pH and P_{CO_2}

Haemolymph samples from active snails and those estivating for 1, 2 and 3 months were collected as described by Pedler et al. (1996). The concentrations of monovalent (Na^+, K^+) and divalent (Ca^{2+}, Mg^{2+}) cations in the haemolymph of active and estivating snails were measured by atomic absorption spectrophotometry as described by Wieser (1981).

Haemolymph pH (pH_e) was measured with a capillary pH electrode (G299A, BMS Mk2, Radiometer, Copenhagen) calibrated at 25 °C with Radiometer precision buffers. P_{CO_2} was extrapolated after determination of total CO_2 (C_{CO_2}) according to the method of Cameron (1971) using a Radiometer P_{CO_2} electrode (E5037) and calibrated with known concentrations of $NaHCO_3$. Haemolymph P_{CO_2} was calculated by the modified equation of Henderson–Hasselbach:

$$P_{CO_2} = C_{CO_2} / [10^{pH-pK} \times \alpha CO_2 + \alpha CO_2], \quad (1)$$

using a pK value of 6.189 and solubility of CO_2 (αCO_2) of 0.044 mmol l^{-1} $mmHg^{-1}$ at 25 °C (Barnhart, 1986). The concentration of bicarbonate ($[HCO_3^-]_e$) in haemolymph samples was calculated according to the following equation:

$$[HCO_3^-]_e = C_{CO_2} - (\alpha CO_2 \times P_{CO_2}). \quad (2)$$

Perfusion of the ventricles of Helix lucorum

Perfusion of isolated ventricles was performed as described by Wernham and Lukowiak (1983). After removing the shell, the heart was dissected out and a cannula was placed in the ventricle through the auricle. The auricle–ventricle junction was ligated so that the auricle attached to the ventricle was not filled with the perfusion buffer. A small hook, connected by a thread to a force-displacement transducer, was placed on the tip of the ventricle. The perfusion saline was delivered into the ventricle through a three-way valve at a pressure head of 8 cm H_2O. The normal saline used was composed according to measured concentrations of monovalent and divalent cations

Table 1. *Haemolymph solute concentrations, extracellular pH and* P_{CO_2} *in haemolymph and* pH_i *of ventricles in active and estivating* Helix lucorum

		Estivation time		
	Active	1 month	2 months	3 months
Solute				
$[Na^+]$	67.17±2.02[a]	75.00±5.00[b]	89.00±1.00[c]	69.39±2.01[d]
$[HCO_3^-]_e$	20.84±0.73[a]	21.58±1.48[b]	31.48±0.00[c]	19.72±0.04[b]
$[K^+]$	3.22±0.11[a]	6.10±1.00[b]	5.20±0.40[c]	4.77±0.43[c]
$[Mg^{2+}]$	1.23±0.01[a]	2.43±0.14[b]	3.46±0.01[b]	12.63±1.43[b]
$[Ca^{2+}]_e$	6.85±0.28[a]	8.97±0.19[a]	9.52±0.65[a]	27.25±1.89[b]
pH_e	7.75±0.04[a]	7.48±0.04[b]	7.56±0.02[b]	7.44±0.03[b]
P_{CO_2}	13.06±0.41[a]	26.73±2.45[b]	29.08±0.41[b]	25.66±1.52[b]
pH_i	7.052±0.017[a]	7.057±0.017[a]	7.088±0.018[a]	7.033±0.016[a]

Haemolymph solutes are expressed in mmol l^{-1}, P_{CO_2} in mmHg.

pH_e, extracellular pH; pH_i, intracellular pH.

Values are means ± S.E.M., N=10 determinations from separate preparations of animals.

Different subscript letters indicate significant differences within the same row as determined by applying Bonferroni t-test: for $[Na^+]$: [a–b] NS; [a–c] $P<0.001$; [a–d] NS; [b–c] $P<0.05$; [b–d] NS; [c–d] $P<0.001$; for $[HCO_3^-]_e$: [a–b] NS; [a–c] $P<0.001$; [b–c] $P=0.001$; for $[K^+]$: [a–b] $P<0.01$; [a–c] NS; [b–c] NS; for $[Mg^{2+}]$: [a–b] $P<0.001$; for $[Ca^{2+}]_e$: [a–b] $P<0.001$; for pH_e: [a–b] $P<0.001$; for P_{CO_2}: [a–b] $P<0.001$.

NS, not significant.

and the $[HCO_3^-]_e$ calculated in the haemolymph of normal snails (Table 1). The composition of the normal saline (saline A) was: 46 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 1.25 mmol l^{-1} $mgCl_2$, 6.85 mmol l^{-1} $CaCl_2$ and 21 mmol l^{-1} $NaHCO_3$ (see also Table 2). Saline A was equilibrated with air and its pH was adjusted to pH 7.75 prior to the experiments.

Experimental protocols

Four experimental protocols (all performed at 25 °C) were applied to the isolated perfused ventricles. The purpose of the first experiment was to examine the effect of hypercapnic salines, in the presence of normal levels (6.85 mmol l^{-1}) of extracellular Ca^{2+}, on the force and rate of heart contraction as well as on pH_i. The first hypercapnic saline had the same composition as saline A except that it was equilibrated with 10 % CO_2 in air, pH 7.2 (saline B) (Table 2). Two other hypercapnic salines used had the same composition as saline A except that they were equilibrated with 10 % of CO_2 in air and contained either 15 mmol l^{-1} $NaHCO_3$ (pH 7.0) (saline C) or 5 mmol l^{-1} $NaHCO_3$ (pH 6.6) (saline D; Table 2). Gas mixtures were obtained using a Woesthoff (Bochum, Germany) gas-mixing pump. Before perfusion of ventricles with any of the above hypercapnic salines, ventricles were preincubated with saline A until a stable heart frequency was obtained. Ventricles were perfused with the above hypercapnic salines for 1 h and recordings of ventricle beats were monitored continuously on a chart recorder. To determine the pH_i of ventricles perfused under the conditions described above, we repeated the experiment and ventricles were removed from the perfusion system at intervals of 10, 30 and 60 min after perfusion with all of the above hypercapnic salines. Hearts were frozen in liquid nitrogen and held thus until pH_i was determined. Ventricles perfused with the saline A were used as controls (0 min).

The purpose of second experiment was to examine whether changes in extracellular level of Ca^{2+} affect the heart activity as well as pH_i under hypercapnic acidosis. We therefore perfused ventricles in the presence of extracellular concentrations of Ca^{2+} lower or higher than 6.85 mmol l^{-1}. Specifically, ventricles were perfused initially with saline A containing 6.85 mmol l^{-1} Ca^{2+} and then with the saline B containing one of the following concentrations of Ca^{2+}: 3 mmol l^{-1}, 11 mmol l^{-1}, 15 mmol l^{-1} or 30 mmol l^{-1}. The pH_i of ventricles was determined after perfusing them with the above hypercapnic salines for 1 h.

Table 2. *Composition and pH of normal saline A and hypercapnic salines B, C and D*

	Constituents (mmol l^{-1})					
Saline	NaCl	KCl	$MgCl_2$	$CaCl_2$	$NaHCO_3$	pH
A	46	3.2	1.25	6.85	21	7.75
B	46	3.2	1.25	6.85	21	7.20*
C	52	3.2	1.25	6.85	15	7.00*
D	62	3.2	1.25	6.85	5	6.60*

*The salines B, C and D were equilibrated with 10 % CO_2 in air before use.

The importance of extracellular Ca^{2+} on pH_i and heart activity under hypercapnic acidosis was examined further in the third experiment. Specifically, we examined the effect of an organic and inorganic blockers of Ca^{2+} entry into cells on the force and rate of ventricle contraction and on the pH_i of ventricles perfused under hypercapnic conditions. Ventricles were perfused initially with saline A containing 6.85 mmol l^{-1} Ca^{2+} and then the perfusate was changed to saline B containing the same concentration of Ca^{2+} as saline A plus 10^{-4} mol l^{-1} verapamil or 10^{-4} mol l^{-1} Co^{2+}, both of which are known to affect Ca^{2+} entry into molluscan heart cells (Devlin, 1993a,b). Ventricles were perfused under the above conditions for 1 h and recordings were taken as described in the first experiment. To determine the pH_i of ventricles perfused under the above conditions, we repeated the experiment and ventricles were removed from the perfusion system at intervals of 10, 30 and 60 min and kept frozen in liquid nitrogen until pH_i was determined. Ventricles perfused with saline A were used as controls (0 min).

In the fourth experiment, we examined the effect of a saline that simulated the composition of haemolymph of snails after estivating for 3 months on the force and rate of ventricle contraction and on pH_i. This saline consisted of measured concentrations of divalent and monovalent cations and the $[HCO_3^-]_e$ calculated in the haemolymph of estivating snails (Table 1): 49 mmol l^{-1} NaCl, 4.8 mmol l^{-1} KCl, 12.6 mmol l^{-1} $mgCl_2$, 27 mmol l^{-1} $CaCl_2$ and 20 mmol l^{-1} $NaHCO_3$. The pH of this saline was adjusted to a value of 7.4. P_{CO2} of the saline was determined using a P_{CO2} electrode after pH adjustment and was found to be about 27 mmHg. Before applying the above saline, ventricles were perfused initially with saline A in the presence of normal levels of Ca^{2+} (6.85 mmol l^{-1}). Ventricles were perfused with this saline for 1 h and recordings of ventricle beats were taken as described previously. To determine the pH_i of ventricles perfused under the above conditions we repeated the experiment and the ventricles were removed from the perfusion system at intervals of 10, 30 and 60 min. They were frozen in liquid nitrogen and thus stored until pH_i was determined. Ventricles perfused with saline A were used as controls.

Determination of pH_i

The pH_i was determined by the homogenate method developed by Pörtner et al. (1990). In brief, ventricles were ground under liquid nitrogen and then 100 mg of tissue powder were put into an Eppendorf vial (600 µl) containing 200 µl ice-cold medium (160 mmol l^{-1} KF, 1 mmol l^{-1} nitrilotriacetic acid, pH 7.4). After completely filling the vial with the medium, the mixture was first stirred with a needle in order to release air bubbles, mixed in a Vortex mixer, and then centrifuged for 30 s. Within 3 min after thawing of the tissue powder in the medium, the pH of the supernatant was measured at 25 °C using a capillary pH electrode G299A, as described previously for the determination of pH_e. In addition to determining pH_i in

the perfused ventricles, we also determined pH_i of hearts from active snails and those estivating for 1, 2 or 3 months.

Measurement of intracellular Ca^{2+}

Measurement of intracellular Ca^{2+} was performed in superfused slices of ventricles in a manner similar to that described by Bickler (1992). Before superfusing the ventricle slices, they were incubated at 30 °C for 2 h in 10 ml of normal saline containing 10 μmol ml^{-1} fura-2 acetoxymethylester (fura-2/AM) (Sigma Chemical Co, USA). The slices were then transferred to Petri dishes filled with normal saline and washed for 5 min. Afterwards they were mounted on a mesh baffle and fitted into a fluorometer cuvette. A cap, fitted with stainless steel inlet and outlet tubing, was then used to seal the cuvette. The cuvette was then placed in the cuvette holder of a Shimadzu fluorometer and positioned so that the excitation light fell within the confines of the slices. The inlet tubing was connected to chambers containing the perfusates. Ventricle slices were superfused continuously with saline B, containing either a low (3 mmol l^{-1}) or high concentration (27 mmol l^{-1}) of Ca^{2+}. Before superfusing slices with saline B in the presence of 3 mmol l^{-1} or 27 mmol l^{-1} of Ca^{2+}, ventricles were perfused with saline A, containing, respectively, either 3 mmol l^{-1} or 27 mmol l^{-1} of Ca^{2+}. The cuvette holder and perfusing solution were both temperature-controlled at 25 °C. Slices were alternately excited at 340 and 380 nm wavelengths and fluorescence intensity at 510 nm was recorded every 0.5 s using the fluorometer. Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$; nmol l^{-1}) were calculated from fluorescent ratios (340/380) using the equation

$$[Ca^{2+}]_i = K_d\,[(R - R_{min}) / (R_{max} - R)]\,(F_0/F_1)\,, \qquad (3)$$

where K_d is the dissociation constant (224 nmol l^{-1}) and R is the ratio of fluorescence intensity excited by 340/380 nm. F_0/F_1 is the ratio of the 380 nm excitation intensity at zero and saturating $[Ca^{2+}]_i$ levels. The fluorescence at 340 and 380 nm for Ca^{2+}-saturated (R_{max}) and free dye (R_{min}) was determined by applying digitonin to a final concentration of 50 μmol l^{-1} to the cuvette, followed by 10 mmol l^{-1} Tris-HCl (pH 7.4) or 20 mmol l^{-1} EGTA, respectively.

Statistical analysis

The results are presented as means ± S.E.M. Significance of differences was tested using Bonferonni's test, which permits multiple comparisons to be taken into consideration. The limit of significance was set at various levels, as indicated in the corresponding Tables and Figures.

Results

The effect of estivation on acid–base variables and the concentrations of divalent and monovalent cations are given in Table 1, which also shows the pH of haemolymph (pH_e) and the intracellular pH (pH_i) of hearts from both active and estivating snails for 1, 2 and 3 months. pH_e, although variable, declined progressively during estivation and was determined to be 7.44±0.03 after 3 months. P_{CO_2} increased significantly

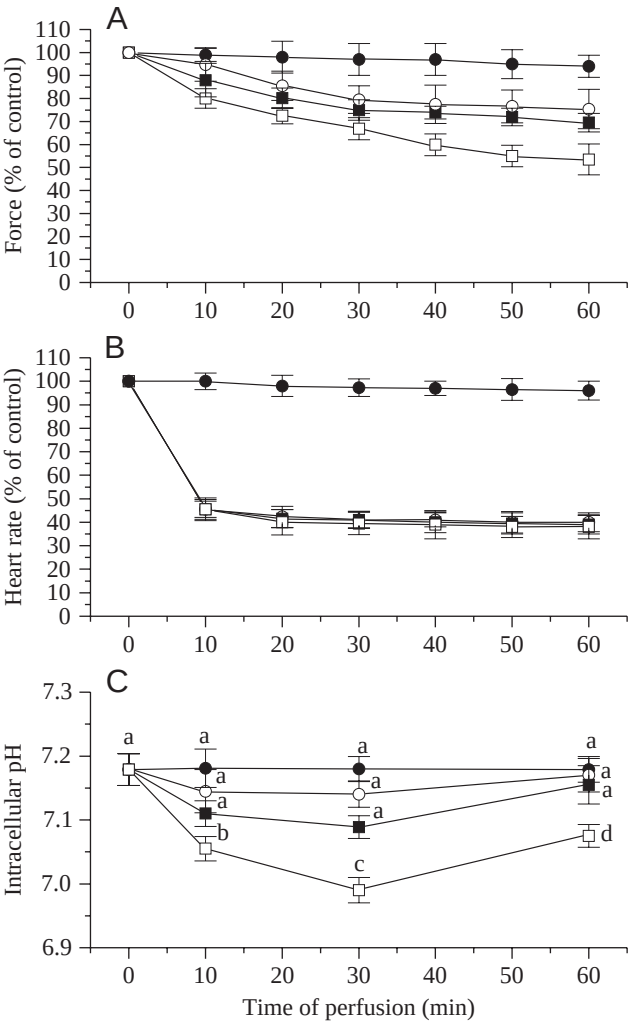

Fig. 1. Effect of hypercapnic salines in the presence of 6.85 mmol l^{-1} Ca^{2+} on (A) the force, (B) the rate of contraction and (C) the intracellular pH of isolated perfused ventricles. (●) saline A, pH 7.75; (○) saline B, pH 7.2; (■) saline C, pH 7.0; (□) saline D, pH 6.6. Values are means ± S.E.M., N=10 determinations from separate preparations of animals. In C, different subscript letters indicate significant differences within the same plot; for □, pH 6.6: $^{a–b}$ P<0.001; $^{a–c}$ P<0.001; $^{a–d}$ P<0.05; $^{b–c}$ NS; $^{b–d}$ NS; $^{c–d}$ P<0.05. NS, not significant.

within the first 2 months and afterwards it decreased slightly. Similarly, the concentration of $[HCO_3^-]_e$ increased within the first 2 months, but afterwards it decreased to control levels. From the determined divalent and monovalent cations, Ca^{2+} and Mg^{2+} showed the most pronounced changes in the haemolymph of estivating snails. Both Ca^{2+} and Mg^{2+} increased during estivation up to about 27.25±1.89 mmol l^{-1} and 12.63±1.43 mmol l^{-1} after 3 months, respectively. In contrast, the pH_i of hearts remained at control levels during estivation.

Under normal conditions of perfusion, ventricles beat at a stable rate (29±2 beats min^{-1}, N=10) and only a slight reduction (6 %) in force of contraction was observed after 1 h of perfusion

(Fig. 1A). However, there was a gradual reduction of force of contraction as a function of pH_e (Fig. 1A), while rate of contraction (beats min^{-1}) declined between pH 7.75 and 7.2, without further changes at lower pH values (Fig. 1B). Specifically, the contractile force of ventricles decreased by about 25 %, 30 % and 47 % after 60 min of perfusion with salines B, C and D, respectively, in the presence of 6.85 mmol l^{-1} Ca^{2+}. The pH_i (7.179±0.025) remained stable in the ventricles perfused for 60 min with saline A (Fig. 1C). Also, perfusion of ventricles with the hypercapnic salines B and C did not cause any significant reduction in pH_i. Perfusion of ventricles with saline D, however, caused a significant reduction in pH_i compared to control values (P<0.001) within the first 30 min of perfusion. After 30 min of perfusion, however, pH_i was recovering slowly towards the control level (Fig. 1C).

Perfusion of ventricles with the hypercapnic saline B in the presence of 3 mmol l^{-1} Ca^{2+} caused greater decreases in the contractile force (66 %) compared to ventricles perfused with the corresponding hypercapnic saline, but containing 6.85 mmol l^{-1} Ca^{2+} (25 %) (Fig. 2). Increases in the concentration of Ca^{2+} in the hypercapnic saline B to >6.85 mmol l^{-1} did not further improve the contractility and rate of contraction of ventricles and had nearly the same effects as perfusion in the presence of 6.85 mmol l^{-1} Ca^{2+}. pHi was determined to be 7.174±0.03, 7.21±0.035, 7.20±0.025 and 7.185±0.036 after 60 min of perfusion with saline B containing 3 mmol l^{-1}, 11 mmol l^{-1}, 15 mmol l^{-1} or 30 mmol l^{-1} of Ca^{2+}, respectively.

The effects of the combination of hypercapnia and veparamil or hypercapnia and Co^{2+} on the contractile force, rate of contraction and pH_i of ventricles are shown in Fig. 3. Perfusion of ventricles with the saline B in the presence of verapamil (10^{-4} mol l^{-1}) caused a marked reduction in the force and rate of ventricle contraction (Fig. 3A). Similar results were obtained in the presence of Co^{2+} (10^{-4} mol l^{-1}) (Fig. 3B). In both cases, however, the pH_i of ventricles, after an initial increase, remained stable during perfusion (Fig. 3C).

The effects of the saline simulating the composition of haemolymph of snails estivating for 3 months on the perfused ventricles are shown in Fig. 4. The contractile force of ventricles was reduced by about 18 % (Fig. 4A) and the rate of ventricle beating by about 60 % (Fig. 4B) after perfusion with the above saline. The pH_i remained stable in the ventricles perfused under the same conditions (Fig. 4C).

To examine whether low extracellular pH affects the influx of Ca^{2+} into the heart cells, we determined the intracellular concentration of Ca^{2+} in ventricles perfused with salines A and B, containing either 3 mmol l^{-1} Ca^{2+} or 27 mmol l^{-1} Ca^{2+} (Fig. 5). There was no effect on the level of intracellular Ca^{2+} in ventricles perfused with the hypercapnic saline B containing 27 mmol l^{-1} Ca^{2+}, while perfusion of ventricles in the presence of 3 mmol l^{-1} Ca^{2+} caused a significant decrease in the level of intracellular Ca^{2+}.

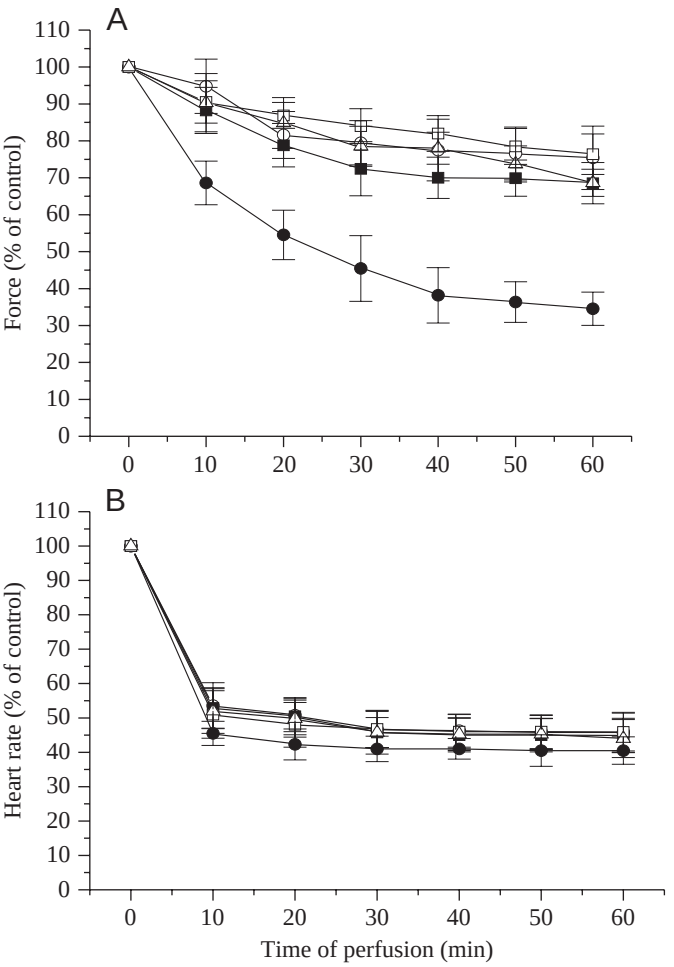

Fig. 2. Effect of hypercapnic saline B (pH 7.2) in the presence of low and high concentrations of Ca^{2+} on (A) the force and (B) the rate of contraction of isolated perfused ventricles. (●) 3 mmol l^{-1} Ca^{2+}; (○) 6.85 mmol l^{-1} Ca^{2+}; (■)11 mmol l^{-1} Ca^{2+}; (□) 15 mmol l^{-1} Ca^{2+}; (△) 30 mmol l^{-1} Ca^{2+}. Values are means ± S.E.M., N=10 determinations from separate preparations of animals.

Discussion

Similar to other land snails (Barnhart, 1986; Rees and Hand, 1990), *Helix lucorum* experiences hypercapnia and respiratory acidosis during estivation (Table 1). The results from in vitro experiments on isolated perfused ventricles indicate that hypercapnia may negatively affect the heart activity in estivating snails. Specifically, it seems that hypercapnia causes a marked decrease in the rate and, to a lesser extent, in the ability of heart muscle to generate force. However, these changes in heart activity seem to be correlated with a reduction in extracellular pH rather than with that of intracellular pH. As shown in Fig. 1, a reduction of pH in the perfusates, by changing the concentration of bicarbonates, resulted in a significant reduction in the contractility (Fig. 1A) and rate of ventricle beating (Fig. 1B). However, there does not seem to be any correlation between heart activity and changes in pH of intracellular fluids, since perfusion of ventricles with hypercapnic salines does not appear to cause a significant reduction of pH_i (Fig. 1C). Only when ventricles were perfused with hypercapnic saline containing 5 mmol l^{-1}

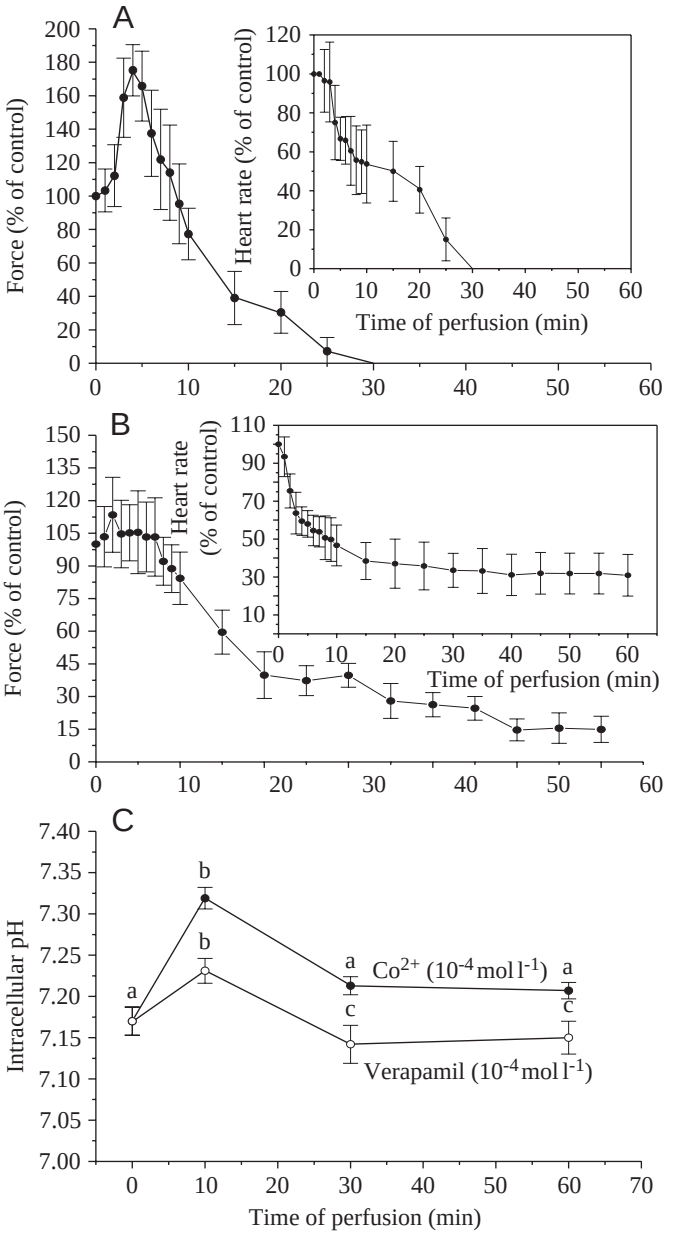

Fig. 3. Effect of hypercapnic saline B (pH 7.2) in the presence of 10^{-4} mol l^{-1} verapamil (A) and 10^{-4} mol l^{-1} Co^{2+} (B) on the force, rate of contraction and intracellular pH of isolated perfused ventricles. Values are means ± S.E.M., *N*=10 determinations from separate preparations of animals. In C, different subscript letters indicate significant differences in the same plot: (●) Co^{2+}: $^{a–b}$ $P<0.001$; (○) verapamil: $^{a–b}$ NS; $^{a–c}$ NS; $^{b–c}$ $P<0.05$. NS, not significant.

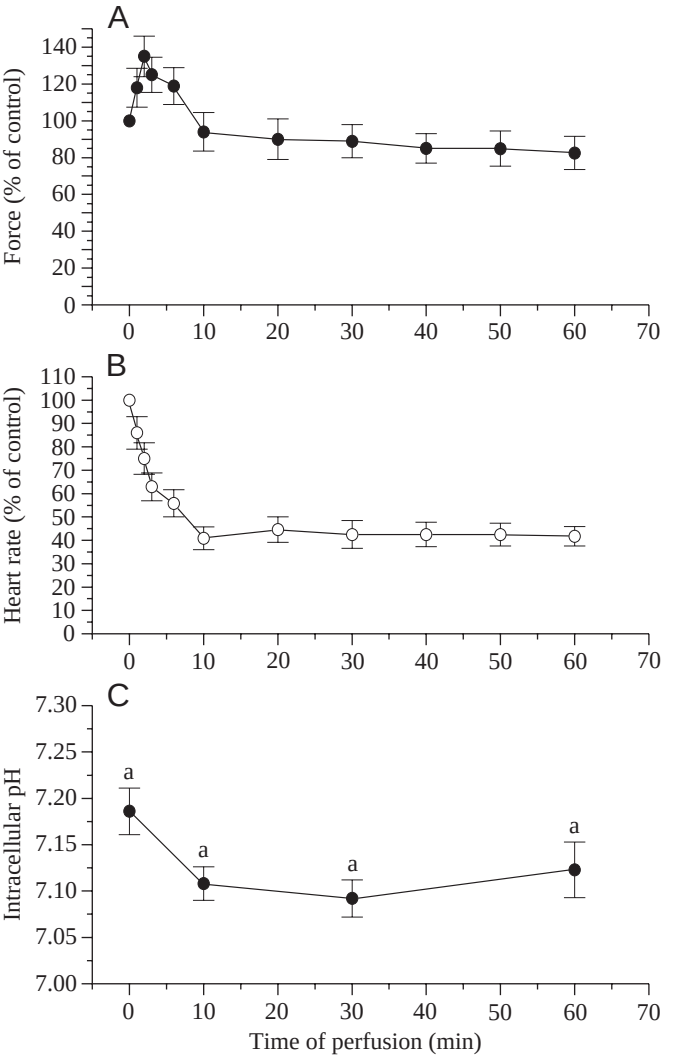

Fig. 4. Effect of a saline simulating the composition of hymolymph of snails estivated for 3 months, on (a) the force, (b) rate of contraction and (c) intracellular pH of isolated perfused ventricles. Values are means ± S.E.M., *N*=10 determinations from separate preparations of animals. [a] Differences within the plot in C are not significant.

$NaHCO_3$, did pH_i fall significantly within the first 30 min; thereafter it recovered at a slow rate (Fig. 1C).

The reduction in force was more significant when ventricles were treated with hypercapnic salines containing 3 mmol l^{-1} Ca^{2+} (Fig. 2). Nevertheless, even in this case, the changes in ventricle activity did not appear to correlate with changes in the pH_i, since pH_i did not change after 60 min of perfusion in the presence of low levels of Ca^{2+}. The above results indicate that high extracellular concentrations of Ca^{2+} may counteract the acidic effect of hypercapnia on heart contractility in land snails during estivation. On the contrary, increased extracellular levels of Ca^{2+} do not seem to restore the rate of heart contraction during hypercapnia. However, concentrations of Ca^{2+} higher than 6.85 mmol l^{-1} did not completely restore the contractility of the heart during perfusion with hypercapnic salines (Fig. 2). This response of ventricles' contractility to increasing extracellular Ca^{2+} under hypercapnic acidosis seems to be similar to that observed under normal conditions of perfusion. As reported, the contractile tension of ventricles rises to a plateau level with increasing concentrations of Ca^{2+} (Burton and Mackay, 1970).

The data obtained using verapamil and Co^{2+} (Fig. 3) show a high dependence of heart activity on the rate of Ca^{2+} entry

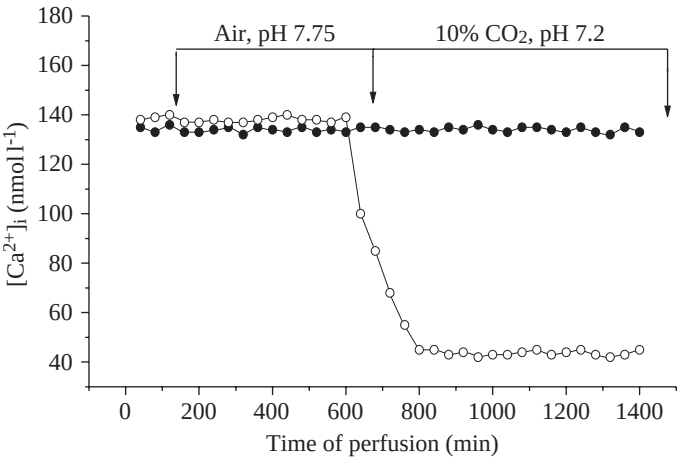

Fig. 5. Typical recordings of the effect of hypercapnic saline B equilbrated in air (pH 7.75) and in 10% CO_2 (pH 7.2) in the presence of low (3 mmol l^{-1}) (○) and high (27 mmol l^{-1}) (●) concentrations of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ in isolated superfused slices of ventricles.

into the cells. These results are similar to those obtained from other land snails and they show that extracellular Ca^{2+} plays an important role in the snail heart, both in excitation–contraction and in the generation of the action potentials (Elekes et al., 1973; Kiss and S.-Roza, 1973; Paul, 1961). Our results indicate that H^+ ions might negatively influence Ca^{2+} entry into the heart cells of *Helix lucorum* and that increases in extracellular Ca^{2+} might counteract this negative effect (Fig. 5). The intracellular concentration of Ca^{2+} was about 135±16 nmol l^{-1} in the ventricles of normal snails, a value which is similar to that reported for muscles from other invertebrates (Ishii et al., 1989), vertebrates (Batle et al., 1993) and also for the brain of the turtle *Trachemys scripta* (Bickler, 1992). Superfusion of ventricle slices under hypercapnia in the presence of 27 mmol l^{-1} Ca^{2+} did not cause any change in the intracellular concentration of Ca^{2+}. In contrast, the latter decreased significantly when slices of ventricles were superfused under the same hypercapnic conditions but in the presence of 3 mmol l^{-1} Ca^{2+} (Fig. 5). The above results are similar to those reported for vertebrates, where hypercapnic acidosis depresses cardiac activity. The mechanisms by which low extracellular pH modulates heart activity in vertebrates are not well understood. It has been reported that low pH reduces contractile force through competition by H^+ for Ca^{2+}-binding sites intracellularly and, possibly, extracellularly (Williamson et al., 1976). Competition of H^+ for the Ca^{2+} binding sites is supported by studies demonstrating a reversal of acidosis depression by increasing extracellular Ca^{2+} (Williamson et al., 1976; Yee and Jackson, 1984; Lagerstrand and Poupa, 1980; Gesser and Poupa, 1979). On the other hand, it has been shown that the strength of cardiac muscle contraction is determined by the magnitude of Ca^{2+} bound to sarcolemmal surface receptors (Langer, 1985; Philipson et al., 1980; Bers et al., 1981) which, in turn, corresponds to the concentration of extracellular Ca^{2+} (Philipson et al., 1980). Moreover, it has been reported that conformational changes in Ca^{2+} channels or transporters and changes in the voltage dependence of channel gating caused by low pH might decrease Ca^{2+} entry into heart cells (Iijima and Hagiwara, 1986; Ohmori and Yoshii, 1977). Besides, H^+ can block the channel, possibly in the channel pore (Krafte and Kass, 1988; Klockner and Isenberg, 1994).

Although it becomes obvious from the results presented that low extracellular pH has a negative effect on heart activity, it is unclear what is the exact role of extracellular pH on the modulation of heart activity in *Helix lucorum* during estivation. This difficulty is due to the fact that the levels of acid–base parameters vary in the haemolymph of *Helix lucorum* during estivation (Table 1) and, as has been reported previously, these changes may reflect periodic bursts of ventilation in land snails (Barnhart and McMahon, 1987; Rees and Hand, 1990). Moreover, the levels of several solutes change in the haemolymph of estivating snails and they may also be involved in the modulation of heart activity. Specifically, the levels of of Ca^{2+} and Mg^{2+}, which have opposite effects on heart activity (Burton and Mackay, 1970; Burton and Loudon, 1972) increase significantly in the haemolymph of estivating snails compared to controls (Table 1). Perfusion of ventricles with the saline simulating the haemolymph of estivating snails caused a reduction in contractile force of about 18 % (Fig. 4A) and a reduction in the rate of contraction of about 59 % (Fig. 4B). However, the above conditions of perfusion did not cause any change in pH_i of ventricles (Fig. 4C). Taking into consideration all the above data, it could be concluded that it is not only the extracellular pH but the combination of the acid–base status and the various solutes which may affect heart activity in land snails during estivation. Moreover, recent data indicate that the biogenic amines serotonin and dopamine are involved in the modulation of heart activity in estivating snails (Rofalikou et al., 1999).

According to the results presented, the heart of *Helix lucorum* seems to defend itself against a drop in extracellular pH and maintains a stable pH_i during estivation (Table 1). The maintenance of pH_i at stable levels seems to be in conflict with the effect of artificial hypercapnia on the pH_i of whole body of other land snail species. Determination of pH_i of the whole body either by DMO (Barnhart and McMahon, 1988) or by NMR (Rees et al., 1991) has shown that artificial hypercapnia causes decreases in the pH_i. The acid–base variables in the haemolymph of *Helix lucorum* (Table 1) are in accordance with those reported for the haemolymph from other land snails (Barnhart, 1986; Rees and Hand, 1991) and the pH_i in the heart of *Helix lucorum* is similar to that reported for the hearts of other molluscs (Ellington, 1993; Kinsey and Ellington, 1995). Perhaps pH_i fluctuations in tissues as small as the heart cannot be recorded when pH_i is determined by DMO and NMR in the whole body. The recovery of pH_i in the perfused ventricles of *Helix lucorum* under hypercapnia indicates that mechanisms of ion exchange of acid–base equivalents between intracellular and extracellular compartments may exist in cardiac muscles. This suggestion is in accordance with data which indicate that regulation of pH_i in the nervous and muscular system of land

snails may involve tightly linked Cl^-–HCO_3^- and Na^+–H^+ exchange (Thomas, 1977; Ellington, 1993; Zange et al., 1990). The physiological importance of pH_i maintainance at stable levels in the heart of land snails during estivation is not clear. However, recent data indicate that the circulatory system of snails is involved in gas exchange during estivation (Rofalikou et al., 1999). Consequently, maintainance of heart pH_i at stable levels may be of great physiological importance since it might preserve heart activity.

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