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THE REGULATION OF INTRACELLULAR pH ESTIMATED BY ^{31}P-NMR SPECTROSCOPY IN THE ANTERIOR BYSSUS RETRACTOR MUSCLE OF *MYTILUS EDULIS* L.

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Summary

The regulation of the intracellular pH (pHi) in the anterior byssus retractor muscle (ABRM) of *Mytilus edulis* L. during an acidic and alkaline load was studied by ^{31}P-NMR spectroscopy. Under aerobic conditions, a total intracellular buffer capacity of $26.5\pm0.7\,mmol\,l^{-1}\,pH\,unit^{-1}$ ($N=3$) was estimated. In the absence of serotonin, active acid extrusion was by a Na^+-independent, primary active transport mechanism coupled to anion transport. Base equivalents appear to be extruded by a Cl^-/HCO_3^- exchanger. Serotonin ($10^{-5}\,mol\,l^{-1}$) induced an increase in pHi through additional activation of a Na^+/H^+ exchanger.

Introduction

The anterior byssus retractor muscle of *Mytilus edulis* belongs to a special group of molluscan smooth muscles called catch muscles. After an active contraction elicited by acetylcholine, the ABRM is able to maintain muscle tension and shortening by a passive mechanism called catch (for a review, see Rüegg, 1971). The stretch resistance of the ABRM during catch depends on changes in the intracellular pH within a range of 0.2 pH units. During catch, acidification of the intracellular milieu decreases the velocity by which an isotonically contracted ABRM is stretched, whereas alkalinization increases it (Zange *et al.* 1988; Zange *et al.* 1990). At concentrations above $10^{-6}\,mol\,l^{-1}$, serotonin elicits a rapid relaxation of catch and induces a permanent increase in pHi (Twarog, 1954).

Changes in pHi can also be elicited in all tissues by stress factors originating from the mussel's habitat, the intertidal zone of northern seashores. During low tide, when mussels are exposed to air, they close their shells tightly to avoid desiccation. During this period the oxygen stored within the shell is used within minutes and CO_2 cannot be released. Therefore, the mussels survive low tide in an anaerobic state (Brinkhoff *et al.* 1983). The resulting hypercapnic acidosis is

Key words: ABRM, intracellular pH, serotonin, sodium/proton exchange, chloride/bicarbonate exchange.

increased by a metabolic acidosis resulting from anaerobic metabolism (Pörtner, 1987). During 8 h of exposure to air the pH of the haemolymph decreases from 7.65 to 7.24 and the intracellular pH of the posterior adductor muscle decreases from 7.38 to 7.15 (Booth *et al.* 1984; Walsh *et al.* 1984). At the onset of high tide, rapid release of CO_2 could cause an intracellular alkalinization. Thus, since maintenance of catch depends at least partially on the pHi (Zange *et al.* 1990), the ABRM must have effective mechanisms of pH regulation to avoid pronounced changes of pHi.

This study was designed to investigate the mode of pHi regulation in the ABRM. We concentrated on the intracellular buffering capacity and the activity of ion-exchange mechanisms in the absence and presence of serotonin.

Materials and methods

Animals

Mussels (*Mytilus edulis* L.) were obtained from the Nederlands Instituut voor Onderzoek der Zee, Den Helder, The Netherlands. The animals were kept in recirculating, filtered artificial sea water (32 ‰) for 3–12 weeks at 15±1 °C until used in the experiments.

Preparation and incubation

The shell was opened and the byssus, which is held within the shell by two anterior and two posterior byssus retractor muscles, was isolated from the surrounding tissue. Silk threads were tied to the byssal ends of the two ABRMs and to those ends where the muscles were attached to the shell. Finally, the ABRMs were separated from byssus and shell.

For a single experiment four or five ABRMs with a total wet mass of 100–150 mg were used. The muscles were tied at their shell ends to a muscle holder which was placed at the bottom of a 10 mm NMR tube. Using the threads tied to the byssal ends of the muscles, the ABRMs were arranged vertically within the NMR tube. The tube was filled with 4 ml of air-equilibrated standard medium at 15±1 °C and a pH of 7.65±0.02. A gas-inlet capillary was placed 0.5 cm above the sensitive range of the receiver coil. The medium was mixed by gassing until a homogenous distribution of oxygen was achieved.

Medium exchange

To determine the dependence of pHi on extracellular pH (pHe) the incubation medium was changed outside the spectrometer. pHe was measured before and after a period of data acquisition using a normal laboratory pH meter.

For CO_2- and NH_4Cl-pulse experiments, the medium was changed in the spectrometer using an additional glass capillary placed on the muscle holder. The medium was removed quantitatively by a vacuum pump and replaced by new medium pre-equilibrated either with air or with an air/10 % CO_2 mixture.

Media

The standard medium had the following composition (in mmol l^{-1}): NaCl, 438; KCl, 10; $MgCl_2$, 24; $MgSO_4$, 28; $CaCl_2$, 10 and HEPES/NaOH, 30. HCO_3^- was formed during the equilibration with air (P_{CO_2}=0.31 kPa) or 10% CO_2 (P_{CO_2}=10.1 kPa) depending on the prevailing pHe.

In Na^+-free media, Na^+ was replaced either by choline chloride and KOH or by *N*-methyl-D-glucamine. DIDS (4,4′-diisothiocyanostilbene-2,2′-disulphonic acid) was dissolved in standard medium to make a saturated solution (between 0.5 and 1 mmol l^{-1}). In Cl^--free medium, Cl^- was replaced by glucuronate. The ABRMs were pre-equilibrated with these media for 30 min before an experiment was started.

Standard conditions

A muscle was considered to be kept under 'standard conditions' when it was incubated in aerated standard medium at pH 7.65.

Determination of the intracellular pH by ^{31}P-NMR spectroscopy

Recordings of ^{31}P-NMR spectra and the determination of pHi from the chemical shift of the intracellular inorganic phosphate were performed as described by Zange *et al.* (1990). The titration curve (Fig. 1) was used to measure the relationship between the chemical shift of inorganic phosphate and pHi.

Statistical analysis of the results

The significance of a difference between two values of pHi (means±S.D.) was tested using Student's *t*-test (P<0.05). A rate of recovery was calculated from the time course of pHi change within an appropriate interval using linear regression analysis. The rate of recovery was calculated as the regression coefficient (±S.D.)

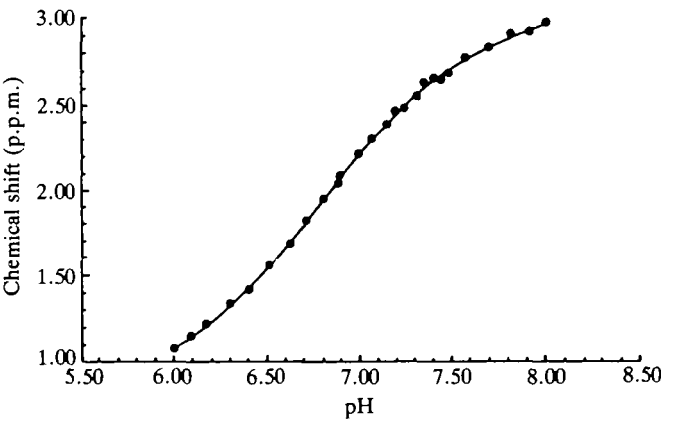

Fig. 1. Titration curve showing the dependence of the chemical shift on the pH of a muscle extract with Ca^{2+} complexed by EGTA.

when $r \geq 0.90$. Deviation from a rate of zero and the difference between two rates were again tested for significance using the appropriate t-tests.

Results

The steady-state pHi under standard conditions

A typical ^{31}P-NMR spectrum is shown in Fig. 2. The pHi of the ABRMs incubated in air-equilibrated standard medium at pH 7.65 was found to be 7.45 ± 0.02 ($N=7$) in the absence of serotonin.

The dependence of the steady-state pHi on extracellular pH

The dependence of pHi on the extracellular pH (pHe) was studied within the pH range 6.00–8.30. In standard medium pHi was regulated within a range from 7.11 ± 0.03 to 7.49 ± 0.04 ($N=4$). At pHe values more alkaline than 7.65, pHi did not deviate as much from its standard value as it did within the more acidic range of pHe. The steady-state values of pHi over the whole range are shown in Fig. 3A.

A Na^+-free medium was used to determine whether a Na^+-dependent,

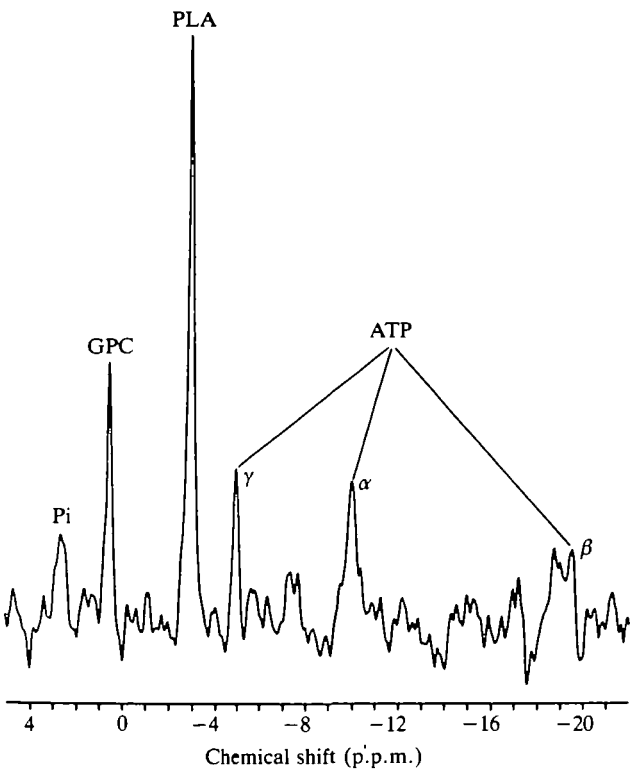

Fig. 2. A typical ^{31}P-NMR spectrum (161.5 MHz) obtained from four ABRMs within 2.9 min (512 scans). Signals were observed from ATP (α, β and γ phosphate), phospho-L-arginine (PLA), glycerophosphorylcholine (GPC) and inorganic phosphate (Pi).

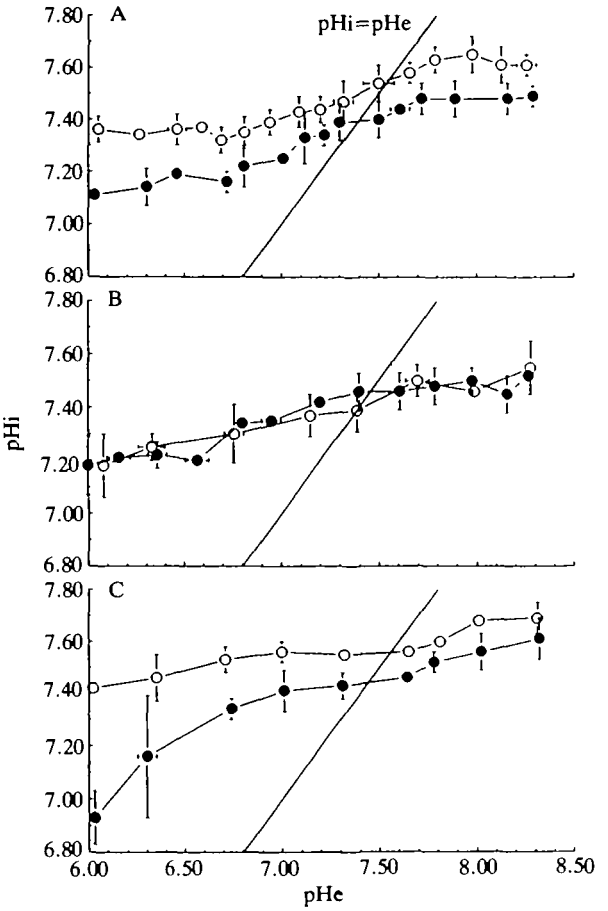

Fig. 3. The dependence of the steady-state pHi on the extracellular pH in the absence (●) and in the presence (○) of serotonin. (A) Aerated standard medium (N=4). (B) Na^+-free medium with Na^+ being replaced by choline and *N*-methyl-D-glucamine (N=3). (C) Standard medium containing DIDS at saturating concentrations (0.5–1.0 mmol l^{-1}; N=3).

secondary active transport mechanism was present in ABRMs (Fig. 3B). Na^+ was replaced by choline and *N*-methyl-D-glucamine. In Na^+-free media, in the absence of serotonin, pHi was regulated between 7.18±0.11 and 7.49±0.07 (N=3). Consequently, the regulation of pHi was not inhibited by the removal of Na^+ and, therefore, did not depend on a secondary active mechanism.

DIDS, which was used as a probe for an anion exchanger, inhibited the regulation of pHi in the absence of serotonin (Fig. 3C). The resulting pHi values were significantly different from those measured in the absence of DIDS at pHe 6.03±0.03 (pHi 6.93±0.10, N=5 *versus* 7.11±0.03, N=4) and pHe 8.32±0.01 (pHi 7.61±0.08 *versus* 7.49±0.04).

In the presence of 10^{-5} mol l^{-1} serotonin (Fig. 3A), ABRM preparations,

incubated in standard medium at pHe 6.0–8.3, regulated their pHi at significantly higher levels than in the absence of serotonin. pHi was regulated between 7.36±0.05 and 7.61±0.04 (N=3).

In Na^+-free medium (Fig. 3B) the serotonin-induced alkalinization was completely inhibited. The levels of pHi were not significantly different from those measured in the absence of serotonin in Na^+-free medium. DIDS, however, did not inhibit the alkalinization induced by serotonin (Fig. 3C).

Effect of CO_2 on pHi

A group of five ABRMs was subjected to a medium equilibrated with a mixture of 95 % air and 5 % CO_2. Under these conditions changes of pHi in the ABRMs could not be detected because the regulation of pHi was too fast. Therefore, we chose an equilibration with 10 % CO_2 to change pHi. pHe in aerated medium was titrated to 7.65. After equilibration with 10 % CO_2, pHe decreased to 6.55±0.05 (N=13).

pHi changes in the absence of serotonin following the addition and removal of 10 % CO_2 are presented in Fig. 4. The pHi of the ABRMs in aerated standard medium was found to be 7.45±0.02 (N=4). When they were exposed to 10 % CO_2, pHi fell to a minimum of 6.85±0.02 (N=4; ΔpHi=−0.60±0.03). pHi recovered slowly at 0.006±0.001 units min^{-1} (Fig. 4A).

Since the rate of pHi recovery was low during a CO_2 pulse under standard conditions, the intrinsic buffering capacity (β_i), defined as $\Delta[HCO_3^-]_i/\Delta$pHi, could be calculated from ΔpHi at the minimum pHi, assuming that CO_2 is equilibrated between the extra- and intracellular spaces in the ABRM (Heisler, 1986). The value of β_i under standard conditions was 26.8±1.6 mmol l^{-1} pH $unit^{-1}$.

When the medium was changed to CO_2-free, aerated medium, the release of CO_2 from the intracellular spaces was followed by a rapid increase of pHi, reaching a maximum of 7.67±0.09 (N=4), 0.22±0.11 units above the initial level. Thereafter, pHi significantly recovered at −0.034±0.001 units min^{-1}, reaching its initial value after 8.8 min.

In Na^+-free medium, the effects of CO_2 on pHi in the absence of serotonin were not significantly different from those on muscles in standard medium (Fig. 4B). In the presence of CO_2, pHi decreased significantly from 7.37±0.05 to 6.80±0.02 (N=3, ΔpHi=−0.57±0.06; β_i=26.4±2.2 mmol l^{-1} pH $unit^{-1}$). The rate of re-alkalinization was again +0.006±0.001 units min^{-1}. When CO_2 had been removed from the muscles, pHi significantly increased to 7.54±0.04 (ΔpHi=+0.17±0.05) followed by a re-acidification of −0.032±0.009 units min^{-1}.

In the presence of DIDS and in the absence of serotonin the fast decrease of pHi during the initial phase of CO_2 application was not followed by a period of recovery (Fig. 4C). pHi continued to decrease slowly during the whole period of CO_2 application. When CO_2 was removed, pHi rose to a significantly higher value than in the absence of DIDS (maximum pHi=7.80±0.05, ΔpHi=+0.39±0.07;

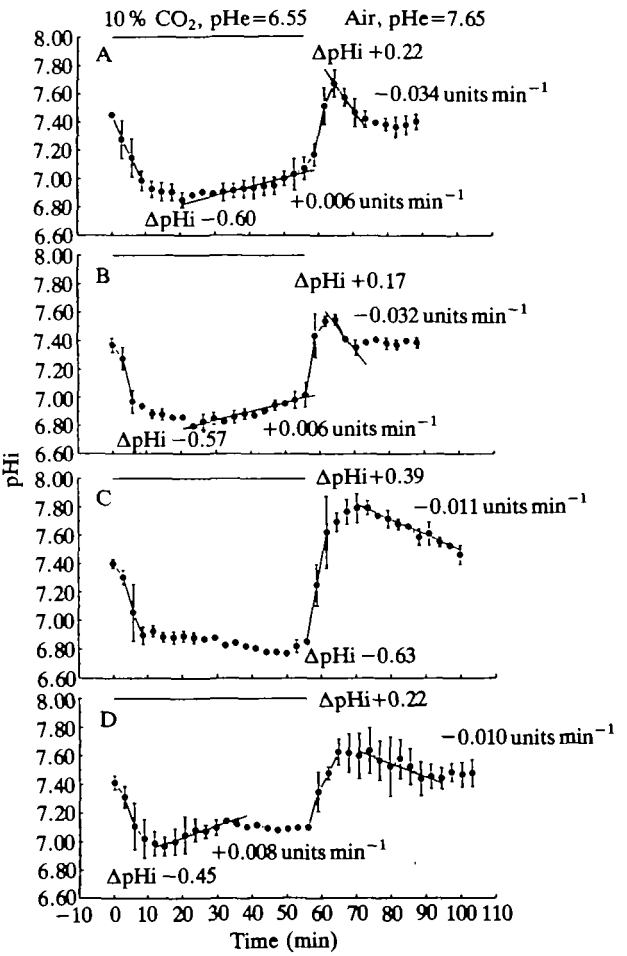

Fig. 4. Time course of changes in pHi in ABRMs during and after exposure to a mixture of air and 10% CO_2 in the absence of serotonin. ΔpHi values are the difference between the minimum or maximum pHi, respectively, and the steady-state pHi of controls. Recovery rates have been calculated by linear regression. (A) Standard medium (N=4); (B) Na^+-free medium (N=3; Na^+ replaced by N-methyl-D-glucamine); (C) standard medium containing saturating concentrations of DIDS (0.5–1.0 mmol l^{-1}; N=3); (D) Cl^--free medium (N=3; Cl^- replaced by D-glucuronate).

N=3). The rate of recovery was significantly reduced to −0.011±0.001 units min^{-1} (34% of the rate in the absence of DIDS).

Cl^--free medium was used to demonstrate the presence of a Cl^-/HCO_3^- exchanger (Fig. 4D). In Cl^--free medium, pHi decreased significantly from 7.41±0.05 to 6.97±0.07 (N=3) during the initial period of CO_2 exposure in the absence of serotonin. As pHi only decreased by −0.45±0.14 units, a buffering capacity of 52±21 mmol l^{-1} pH $unit^{-1}$ was calculated, a significant increase above

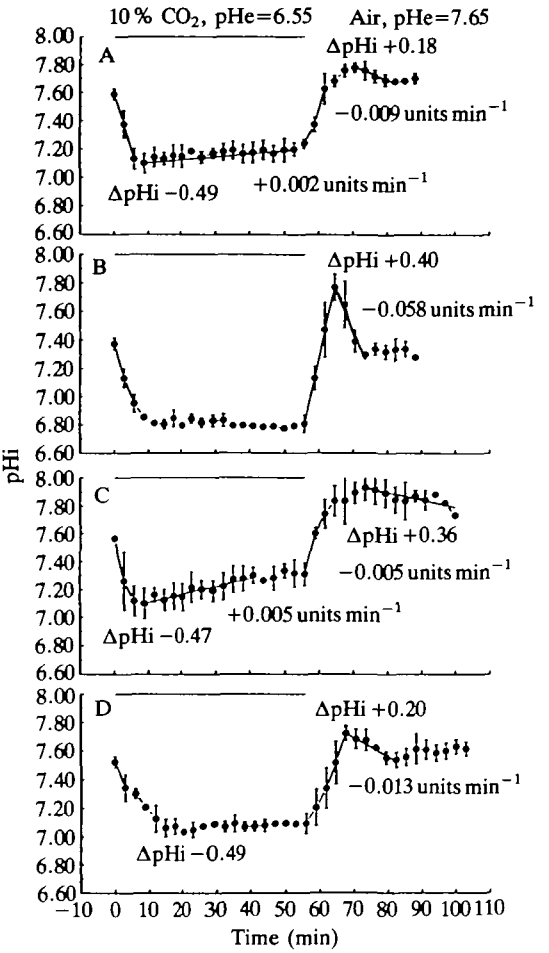

Fig. 5. Time course of changes in pHi of ABRMs during and after exposure to media equilibrated with a mixture of air and 10% CO_2 in the presence of serotonin. ΔpHi values are the difference between the minimum or the maximum pHi and the control steady-state pHi. Recovery rates have been calculated by linear regression. Media are standard medium (A, N=4), Na^+-free medium (B, N=3, Na^+ replaced by N-methyl-D-glucamine), standard medium saturated with DIDS (C, N=3, 0.5–1.0 mmol l^{-1}) and Cl^--free medium (D, N=3, Cl^- replaced by D-glucuronate).

β_i estimated under standard conditions. When pHi reached its minimum, a transient but significant re-alkalinization of +0.008±0.001 units min^{-1} was observed until pHi reached a value of 7.10±0.05. It remained at this value for the remainder of the period of CO_2 application. When CO_2 was removed from the ABRMs, pHi rose to 7.64±0.16 (ΔpHi=+0.22±0.16). The rate of recovery was only −0.010±0.002 units min^{-1} (31% of the rate in standard medium).

The changes in pHi in the presence of serotonin (10^{-5} mol l^{-1}) in response to addition or removal of 10% CO_2 are presented in Fig. 5. When ABRMs were

exposed to CO_2 in standard medium containing serotonin, pHi decreased from 7.59±0.04 to 7.10±0.07 (N=3; Fig. 5A). The ΔpHi of −0.49±0.06 was correlated with a buffer capacity of 63.0±14.1 mmol l^{-1} pH $unit^{-1}$, which was significantly increased above β_i calculated for standard conditions (2.5 times). When pHi had reached its minimum, a slow re-alkalinization of 0.002±0.001 units min^{-1} was observed. pHi did not recover as long as the ABRMs were subjected to CO_2. When CO_2 was removed, pHi increased to 7.78±0.03, followed by a slow re-acidification of −0.009±0.001 units min^{-1} (28% of the rate in absence of serotonin).

In Na^+-free medium (Fig. 5B) pHi dropped by 0.56±0.02 units from 7.37±0.04 to 6.81±0.03 (N=3). The calculated buffering capacity of 26.4±2.2 mmol l^{-1} pH $unit^{-1}$ was equal to β_i calculated for standard conditions. After the initial acidification, pHi remained at constant levels for the whole period of CO_2 exposure. Removal of CO_2 was followed by a rapid alkalinization (pHi 7.77±0.09) and a subsequent rapid re-acidification of −0.058±0.007 units min^{-1}.

DIDS and Cl^--free medium (Fig. 5C,D) did not inhibit the effect of serotonin on the regulation of pHi during exposure to CO_2. When CO_2 was removed in the presence of DIDS and serotonin, pHi rose to significantly higher levels (7.93±0.09) than those reached in the absence of DIDS. This was followed by a slow re-acidification of −0.005±0.013 units min^{-1} (56% of the uninhibited rate in the presence of serotonin). In Cl^--free medium containing serotonin, pHi increased to 7.72±0.05 when CO_2 was removed. This alkalinization was followed by a re-acidification of −0.013±0.001 units min^{-1}.

The total intracellular buffering capacity

In three series of experiments ABRMs were subjected to 5, 10, 15 or 20 mmol l^{-1} NH_4Cl dissolved in standard medium titrated to pH 7.65. Muscles were exposed to each concentration of NH_4Cl for 29.4 min followed by a period of recovery in standard medium. At the beginning of exposure to NH_4Cl, pHi increased until it reached a maximum value within 6–12 min. During the remaining period of NH_4Cl exposure pHi decreased. When NH_4Cl was removed from the ABRMs, pHi rapidly recovered to its initial levels. Even when the muscles had been subjected to 15 or 20 mmol l^{-1} NH_4Cl, no transient decrease below the initial levels occurred. Fig. 6 gives an example of such an experiment.

In each experiment we calculated the total intracellular buffering capacity, defined as $\Delta[NH_4^+]_i/\Delta$pHi, from the increase of pHi at its maximum (Roos and Boron, 1981). A pK_{NH_4} of 9.6 was taken from Boutilier *et al.* (1984). The rate of transport of base equivalents (mmol l^{-1} min^{-1}) was calculated from the product of buffering capacity (mmol l^{-1} pH $unit^{-1}$) and recovery rate (ΔpH min^{-1}). The results are summarized in Table 1.

Discussion

In the haemolymph of mussels (*Mytilus edulis*) living in aerated sea water Booth

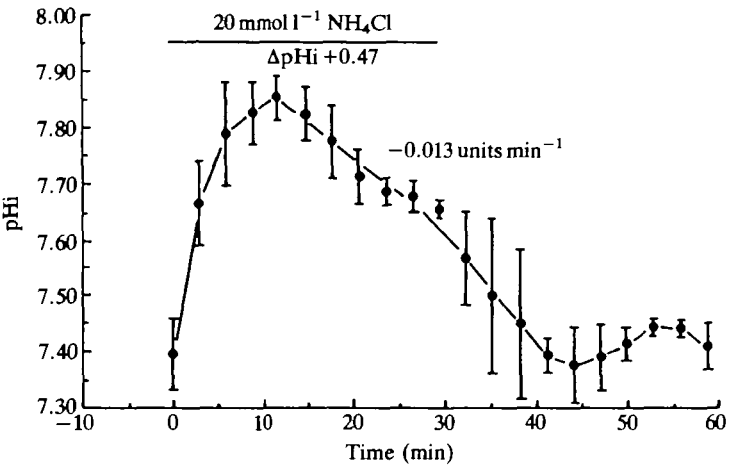

Fig. 6. Time course of changes in pHi in ABRMs during and after exposure to 20 mmol l^{-1} NH_4Cl in standard medium (pHe=7.65). Values are mean±S.D. (N=3).

Table 1. *The intracellular buffer capacity (β_i) of the ABRM calculated by the alkaline load during exposure to 5, 10, 15 or 20 mmol l^{-1} NH_4Cl and the rate of recovery of pHi*

NH_4Cl (mmol l^{-1})	Maximum pHi±S.D.	pHi±S.D.	β_i±S.D. (mmol l^{-1} pH $unit^{-1}$)	Rate of recovery (ΔpH min^{-1})	Transport of base equivalent (mmol l^{-1} min^{-1})
20	7.85±0.04	0.47±0.04	27.1±4.0	0.013	0.35
15	7.81±0.01	0.39±0.03	26.6±2.8	0.009	0.24
10	7.71±0.05	0.32±0.02	26.8±3.7	0.007	0.19
5	7.63±0.04	0.20±0.01	25.6±3.7	0.008	0.20
Mean±S.D.			26.5±0.7		

Aerated medium, 15°C, pHe=7.65, N=3, pK_{NH_4}=9.6 (Boutilier *et al.* 1984).

et al. (1984) measured a pH of 7.65 and a P_{CO_2} slightly above the P_{CO_2} in the sea water. The standard conditions, defined in our study ($P_{CO_2}\approx 0.031$ kPa; pH=7.65; $[HCO_3^-]\approx 0.4$ mmol l^{-1}) are close to physiological conditions in the haemolymph of aerobic mussels. Under these conditions the pHi of the ABRMs was found to be 7.45±0.02 (N=7).

The ABRM contains a uniform type of muscle cell and a very small number of nerve endings (Twarog *et al.* 1973). Therefore, an overall estimation of pHi within the whole muscle by ^{31}P-NMR spectroscopy reflects mainly the mean pHi within muscle cells.

In agreement with the results in our study, Walsh *et al.* (1984) estimated a pHi of 7.38 in the posterior adductor muscle and a pHi of 7.42 in the foot muscle of *Mytilus edulis* using [^{14}C]DMO. Using ^{31}P-NMR spectroscopy, Ellington (1983),

however, estimated a pHi of 6.96 in the posterior adductor of *Mytilus edulis* and Chih and Ellington (1985) measured a pHi of 7.06 in the phasic adductor of the clam *Agropecten irradians concentricus*. These distinctly more acid values of pHi can be explained by the calibration curves which, in both cases, had been derived using muscle extracts obtained without a Ca^{2+} complexing agent. Apart from H^+, the cations which affect the chemical shift of inorganic phosphate (Pi) are mainly divalent (Mg^{2+} and Ca^{2+}; Hamm and Yue, 1987). During the extraction of muscle tissue, Ca^{2+} is released from Ca^{2+}-storing vesicles (Atsumi and Sugi, 1976). Therefore, we homogenized the ABRMs using EGTA to complex Ca^{2+}, but leaving the concentration ratio of Mg^{2+} to Pi unchanged.

An important parameter affecting pHi is the intracellular buffering capacity, which consists of two components, the bicarbonate and the non-bicarbonate or intrinsic buffering system. The latter component consists mainly of charged groups of proteins and other organic molecules. In previous studies Walsh *et al.* (1984) estimated an intrinsic buffering capacity of $23.9\pm2.6\,mmol\,l^{-1}\,pH\,unit^{-1}$ by titrating tissue homogenates of the posterior adductor muscle of *Mytilus edulis*. Monitoring pHi by ^{31}P-NMR in intact ABRMs treated with NH_4Cl pulses, we measured a total intracellular buffering capacity of $26.5\pm0.7\,mmol\,l^{-1}\,pH\,unit^{-1}$ ($N=3$). Under normocapnic conditions ($P_{CO_2}=0.031\,kPa$) the bicarbonate buffering capacity ($0.4\,mmol\,l^{-1}\,pH\,unit^{-1}$), which was calculated according to Heisler (1986), could be neglected. Consequently, the total buffering capacity was found to be nearly equal to the intrinsic buffering capacity ($26.8\pm1.6\,mmol\,l^{-1}\,pH\,unit^{-1}$) estimated in intact ABRMs by CO_2 pulse experiments. The buffering capacity in ABRMs becomes important when, for example during the working phase of an isotonic contraction, the transphosphorylation of phospho-L-arginine restricts the alkalinization to a change by about 0.1 pH units.

The buffering capacity in the ABRM is similar to buffer values in muscle tissues from other marine invertebrates, which have been estimated with pH-sensitive microelectrodes (for a review, see Roos and Boron, 1981). In a recent ^{31}P-NMR study Wiseman and Ellington (1989) estimated a total intracellular buffering capacity of about $30\,mmol\,l^{-1}\,pH\,unit^{-1}$ in the radular protractor muscle of the whelk *Busycon canaliculatum*. The study of Wiseman and Ellington (1989) and this study demonstrate the validity of ^{31}P-NMR spectroscopy for estimating intracellular buffering capacities in isolated tissues.

Generally, the pHi of a living cell is more alkaline than it should be if distribution of H^+ was passive and followed the Nernst equation (Thomas, 1984). Since the membrane potential of the ABRM was found to be $-65\,mV$ (Twarog, 1967; Hidaka and Goto, 1973), the equilibrium pHi of the ABRM at a pHe of 7.65 should be 6.52. Therefore, cells must continuously extrude acid equivalents or take up base equivalents by primary active H^+ transporters (H^+-ATPases) or secondary active transporters coupled with the Na^+ gradient being produced by a Na^+/K^+-ATPase.

Our results show that, both in the presence and in the absence of serotonin, ABRMs of *Mytilus edulis* are able to regulate their steady-state pHi within a

narrow range when subjected to changes in pHe above and below 7.65. It remains to be shown by what mechanism this muscle regulates its pHi.

When pHe was decreased to 6.03±0.02 in the absence of serotonin, pHi was maintained at a significantly more acidic pHi (7.11±0.03, N=4) than it was under standard conditions. This decrease of pHi was enhanced by the addition of DIDS (pHi=6.93±0.10), indicating involvement of an anion transport mechanism in regulation of pHi (Cabantchik *et al.* 1978). To keep the steady-state pHi constantly above the equilibrium value, H^+ must be extruded or HCO_3^- must be taken up against a pH and HCO_3^- gradient. The mechanisms of pHi regulation in the absence of serotonin have been shown to be independent of the presence of extracellular Na^+. Therefore, secondary active mechanisms, such as a Na^+/H^+ exchanger, a Na^+/HCO_3^- cotransporter or a $(Na^+ + HCO_3^-)/(H^+ + Cl^-)$ exchanger, should not be involved in the regulation of pHi. Amiloride (Benos *et al.* 1976) could not be used as a probe for Na^+-dependent exchangers because it was almost insoluble (<0.05 mmol l^{-1}) at 15 °C and in the presence of 438 mmol l^{-1} Na^+.

Further information was obtained from CO_2-pulse experiments. During exposure to 10% CO_2 (pHe=6.55) in the absence of serotonin, pHi decreased rapidly to 6.85±0.02. After the minimum pHi had been reached, a slow re-alkalinization of +0.006±0.001 units min^{-1} was observed, from which an acid extrusion rate of 0.38±0.02 mmol l^{-1} min^{-1} was calculated. This acid extrusion, which was again performed against a pH gradient, was completely inhibited by DIDS, but it was not inhibited in Na^+-free medium. Consequently, we have to assume the presence of an, as yet unidentified, active but Na^+-independent transport mechanism coupled to an anion transporter. H^+-ATPases are not directly inhibited by DIDS (Pedersen and Carafoli, 1987), but an electrogenic H^+-ATPase coupled to Cl^- transport could be indirectly blocked. An electroneutral K^+/H^+-ATPase, however, would not be affected by DIDS.

Na^+-independent, primary active acid extrusion has not been reported from invertebrates. Muscle fibres of the barnacle *Balanus nubils*, axons from the squid *Loligo pealeii* and neurones of the terrestrial snail *Helix aspera* extrude acid equivalents by a Na^+-dependent Cl^-/HCO_3^- exchanger. In neurones of the crayfish *Procambarus clarkii* additional activity of a Na^+/H^+ exchanger was observed (for a review, see Thomas, 1984). In glial cell from the leech *Hirudo medicinalis* Deitmer and Schlue (1989) found both a Na^+/H^+ exchanger and an electrogenic $Na^+ + HCO_3^-$ cotransporter.

At the onset of exposure to 10% CO_2 in Cl^--free medium in the absence of serotonin, the pHi of the ABRM decreased significantly less than under standard conditions. The buffering capacity calculated by this lower ΔpHi was significantly greater than β_i estimated under standard conditions. The apparent increase of β_i in Cl^--free medium could be explained by fast regulation processes. The initial rate of re-alkalinization following the minimum pHi was higher than under standard conditions, but re-alkalinization stopped suddenly and pHi remained constant thereafter. Possibly, in Cl^--free medium, intracellular Cl^- might be trapped

within the cells. During exposure to CO_2, the activity of the Cl^-/HCO_3^- exchanger increased and the large gradient between intracellular Cl^- and the Cl^--free medium allowed a fast, but transient, uptake of HCO_3^- as long as the intracellular stores of Cl^- were depleted.

The regulation of pHi against an alkaline load in the absence of serotonin was more efficient than the regulation against an acid load. When pHe was increased to 8.30 steady-state pHi (7.49±0.04, N=4) was not significantly increased. The rapid increase of pHi elicited by the removal of CO_2 after a 10% CO_2 pulse was followed by a re-acidification of −0.034±0.001 units min^{-1} until pHi had regained its initial value.

The following lines of evidences indicate that the recovery of pHi after an alkaline load was due to Cl^-/HCO_3^- exchange. At a pHe of 8.30 the steady-state pHi (7.61±0.08, N=3) was significantly increased when ABRMs were incubated in the presence of DIDS and in the absence of serotonin. The activity of the Cl^-/HCO_3^- exchanger, after an alkaline load elicited by the removal of CO_2, was inhibited by DIDS and depended on extracellular Cl^-. The Cl^-/HCO_3^- exchanger used the gradient between the high (about 15 mmol l^{-1}) intracellular and the low (about 0.2 mmol l^{-1}) extracellular concentrations of bicarbonate. When pHi was increased by NH_4Cl under normocapnic conditions, the intracellular concentration of HCO_3^- did not increase beyond 0.5 mmol l^{-1}. Consequently, the extrusion of HCO_3^- was slow compared to that in CO_2-pulse conditions.

When serotonin was applied to the ABRM, pHi was significantly increased. This increase in pHi was independent of pHe within a range from 6.0 to 8.3 (pHi 7.36±0.05 to 7.61±0.08 *versus* 7.11±0.03 to 7.49±0.04 in the absence of serotonin). As the pHi in the absence of serotonin was actively regulated above the equilibrium pHi, serotonin must turn on another active transport mechanism eliciting a further increase of pHi. In the initial phase of an acid load elicited by exposure to 10% CO_2, rapid, active H^+ extrusion was indicated by the high apparent buffering capacity (2.5 times β_i in the absence of serotonin) calculated by the ΔpHi of −0.49±0.06, N=3). During CO_2 exposure, pHi did not reach its initial levels because pHe was 6.55.

All effects induced by serotonin were completely inhibited in Na^+-free medium. Therefore, it can be concluded that serotonin activates a Na^+-dependent, secondary active transport mechanism. DIDS and Cl^--free medium were used to indicate and distinguish between $(Na^+ + HCO_3^-)/(H^+ + Cl^-)$ exchanger and $Na^+ + HCO_3^-$ cotransport. As the effects of serotonin on the regulation of pHi were inhibited neither by DIDS nor by Cl^--free medium, it can be concluded that serotonin induces the additional activation of a Na^+/H^+ exchanger.

Within the physiological range of pHe and CO_2 concentrations, the ABRM is well equipped to regulate its pHi under normoxic conditions. It is particularly important for the ABRM to control its pHi because small changes in the activity of protons affect the stretch resistance of the muscle during catch (Rüegg, 1964; Zange *et al.* 1988; Zange *et al.* 1990). In the absence of serotonin, acid extrusion is performed by an active, but Na^+-independent, mechanism which is coupled to

anion transport. We suggest that this mechanism might be an electrogenic H^+-ATPase coupled to a Cl^- transporting system. Of course, further evidence for this assumption must be provided. The extrusion of base equivalents, however, is performed by a Cl^-/HCO_3^- exchanger. Serotonin (10^{-5} mmol l^{-1}) elicits an increase in pHi by the additional activation of a Na^+/H^+-exchanger.

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