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Studies of Intracellular pH Regulation in Cardiac Myocytes From the Marine Bivalve Mollusk, *Mercenaria campechiensis*

W. ROSS ELLINGTON

Department of Biological Science, B-157, Florida State University, Tallahassee, Florida 32306

Abstract. Myocytes were isolated from the ventricle of the marine clam *Mercenaria campechiensis* by enzymatic dispersion procedures. Intracellular pH (pH_i) was measured via fluorescence imaging techniques using an inverted microscope interfaced with a high sensitivity television camera. Myocyte pH_i was similar to values observed in other molluscan muscles measured by weak acid distribution and nuclear magnetic resonance (NMR) techniques. Myocytes displayed a good capacity for defending pH_i against changes in extracellular pH (pH_e) as the pH_i remained unchanged in the pH_e range of 7.1 to 8.0, but gradually declined at lower pH_e values. Myocytes had a relatively high non-bicarbonate intracellular buffering capacity. Further, these cells showed recovery from imposed acid loads. This recovery was accelerated by increasing HCO_3^- concentrations, was not dependent on external Na^+ and was blocked by a stilbene transport inhibitor, suggesting that a HCO_3^-:Cl^- transporter plays a central role in regulation of pH_i. Collectively, these data show that ventricular myocytes of *M. campechiensis* have a relatively high capacity for dealing with potential metabolic proton loads associated with environmental anaerobiosis.

Introduction

Environmental hypoxia or anoxia imposes important energetic and acid/base stresses on marine invertebrates. When anaerobic energy yielding processes prevail, there appears to be an uncoupling of proton production and consumption. The extent of excess production of protons is dependent on the specific pathways operating (Pörtner *et al.*, 1984a; Pörtner, 1987a, 1989). The major evolutionary trajectory in highly anoxia-tolerant marine invertebrates (bivalve/gastropod mollusks and certain worm groups) is the development and use of anaerobic metabolic pathways with a lower H^+/ATP ratio, the ratio of proton release to ATP produced (Gnaiger, 1980).

Because proton production will continue throughout anoxia, it is readily apparent that specific mechanisms are present in these organisms to minimize reductions in intracellular pH (pH_i). Rates of intracellular acidification during anoxia (or air exposure) are generally quite low in the muscles of bivalves (Barrow *et al.*, 1980; Ellington, 1983a; Walsh *et al.*, 1984) and gastropods (Ellington, 1983b; Graham and Ellington, 1985). This is also true of the sipunculid *Sipunculus nudus* (Pörtner *et al.*, 1984b; Pörtner, 1987b). Muscles of many of these species have moderately high non-bicarbonate intracellular buffering capacities (β_{NB}) (Eberlee and Storey, 1984; Morris and Baldwin, 1984; Pörtner *et al.*, 1984a; Wiseman and Ellington, 1989). Furthermore, there is good evidence for ion exchange of acid/base equivalents between the intra- and extracellular compartments. For instance, in *S. nudus* Pörtner and coworkers (Pörtner *et al.*, 1984b; Pörtner, 1987b) have shown that the extracellular compartment serves as sink during anoxia for metabolically produced protons. This is also true of bivalves where the calcareous shell serves as an external buffering agent (Crenshaw and Neff, 1969; Booth *et al.*, 1984). In terms of ion exchange processes in marine invertebrates, a sodium-dependent Cl^--HCO_3^- exchanger appears to be the predominant effector of regulation of pH_i in two well-studied systems—squid giant axon (Boron and Russell, 1983) and the giant muscle fibers of barnacles (Boron *et al.*, 1979). These exchangers are blocked by stilbene derivatives and have low Kms for HCO_3^- (around 2–3 m*M*). Recently, it has been shown that bivalve anterior byssus retractor muscle (ABRM) has a stilbene-sensitive anion exchanger (Zange *et al.*, 1990).

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In the present study, regulation of pH_i has been investigated in myocytes isolated from the ventricle of the marine bivalve *Mercenaria campechiensis*. This study uses fluorescent ratio imaging technology, which permits the observation of the dynamics of change in pH_i in individual myocytes. Experiments focus on the measurement of β_{NB} and observation of defense of pH_i after exposure of cells to acid/base stress.

Materials and Methods

Animals and materials

Specimens of *M. campechiensis* were collected via a dredge by a commercial fisherman from St. Joseph's Bay, Gulf County, Florida, and were transported to the Florida State University Marine Laboratory within a few hours after collection. Animals were maintained in raw (unfiltered, unsettled), continuously flowing seawater. Prior to experiments, animals were transported to the main university campus and maintained in recirculating aquaria 20 ± 1.5°C under a 12:12 (L:D) photoperiod.

Dispersion enzymes and buffers were purchased from Sigma Chemical Co. (St. Louis, Missouri). Nigericin (free acid), 2′,7′-bi-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein-acetoxymethyl ester (BCECF/AM) and BCECF (free acid) were purchased from Molecular Probes (Eugene, Oregon). The anion transport inhibitor, 4-acetamido-4′-isothiocyanatostilbene-2, 2′-disulfonic acid (SITS), was obtained from Sigma Chemical Co. All other chemicals were of reagent grade quality.

Isolation of myocytes

Procedures for myocyte dispersion were adapted from suggestions made by C. Bruce (Department of Pharmacology, University of British Columbia, Vancouver, BC). Ventricles were dissected from 3 to 5 specimens of *M. campechiensis*. After removal of the intestine, tissue was cut into very small pieces (1 mm^3), suspended in 45 ml myocyte artificial seawater (MASW, 440 m*M* NaCl, 10 m*M* KCl, 7.5 m*M* $CaCl_2$, 23 m*M* $MgCl_2$, 25 m*M* $MgSO_4$ and 10 m*M* HEPES adjusted to pH 7.75 with NaOH), and gently washed in a rotary shaker for 1 h. The suspension was placed in a 50 ml conical centrifuge tube and the tissue pieces were allowed to settle by gravity for a few minutes. After aspirating off the MASW, tissue was resuspended in 20 ml 0.1% protease VIII (Sigma) in MASW and incubated with gentle agitation for 30 min. The tissue was again placed in a 50 ml centrifuge tube followed by 30 ml MASW. After settling of the tissue, the MASW was aspirated off and 50 ml MASW added. After settling and aspiration, the tissue pieces were resuspended in 20 ml 0.1% collagenase (type 2, Sigma) and incubated with agitation for 90 min. Periodically during incubation, tissue was gently sucked in and out of a flame-polished Pasteur pipette. After incubation, the cell suspension was centrifuged for 45 s at low speed (400 rpm) in a clinical centrifuge. The supernatant was carefully decanted without disturbing the loose pellet and centrifuged for 4 min as above. The supernatant was discarded and the pellet resuspended in MASW and centrifuged for 4 min. The final pellets were resuspended in a small volume of MASW. Cells were seeded on circular coverslips (Nicholson Precision Instruments, Gaithersburg, Maryland) which were immersed in 15 ml MASW in 10 cm plastic culture dishes. Coverslips had been previously washed in acid, rinsed exhaustively and then polished with ethanol using lens paper. Dishes were placed in a humidified culture chamber. Cells were always prepared during the afternoon and then used for imaging the following morning. All isolation and incubation procedures were conducted at 18–21°C.

Fluorescent ratio imaging

The overall rationale and approach for BCECF imaging has been previously described (Rink *et al.*, 1982; Bright *et al.*, 1987). Cells were loaded with 5 μM BCECF/AM for 45 min. After washing, the coverslip was mounted in a Dorvak-Stottler chamber (Nicholson Precision Instruments) which was attached to a Peltier device (Physitemp, Clifton, New Jersey) mounted on the stage of a Zeiss IM-35 inverted microscope. Cells were superfused (0.1 ml/min) by gravity flow from a manifold device consisting of a Hamilton (Reno, Nevada) eight-way valve, microbore tubing and eight reservoir chambers. Temperature was controlled at 20°C.

A xenon lamp (Optiquip, Highland Mills, New York) provided illumination. Fluorescence excitation was controlled via a dual filter wheel/shutter assembly (Ludl, Hawthorne, New York). One wheel contained excitation filters (490, 450 nm) while the other had a range of neutral density filters. A dichroic (530 nm) was positioned on the fluorescence emission side. All filters were from Omega Optical (Brattleboro, Vermont). Phase and fluorescence images were obtained using an Achrostigmat LD 32X/.4 PH1 objective with light passing onto an iCCD camera (QUANTEX, Sunnyvale, California). Video signals were digitized and processed by IMAGE 1/FL software from Universal Imaging Corp. (Westchester, Pennsylvania) using a 486-based computer with output on a color monitor (Trinitron, SONY). Software controlled the operation of the filterwheel/shutter system.

Isolated myocytes did not display any auto-fluorescence. Preliminary experiments showed that BCECF-loaded myocytes went into contracture when illuminated with intense monochromatic light (490 or 450 nm). Unloaded cells did not respond to light in this way. Furthermore, the ability to regulate pH_i was impaired in

loaded cells at high light intensities. Thus, during all experiments we used high range neutral density filters to reduce light intensities compensating for the reduced fluorescence by employing high camera gain and intensity settings. Furthermore, the period of irradiation of cells with monochromatic light was reduced to a minimum consistent with camera lag. Images were acquired using shade (shade "mask" obtained using 25 μl of 25 μM BCECF sandwiched between coverslip and slide) and background correction capabilities of the IMAGE 1/FL software. Image pairs were acquired at specific time intervals (usually every 20 s). Individual cells were selected and fluorescence ratios (I_{490}/I_{450}) for each cell *versus* time were stored in a spread-sheet data base. Numerical data were transferred as ASCII files to a Macintosh IIci and processed and analyzed using Sigmaplot (Jandel, San Rafael, California).

In vivo *calibration of ratios*

Fluorescent ratios with respect to pH were calibrated by the nigericin pH clamp approach of Thomas *et al.* (1979). The calibration solution was identical to MASW except that it contained 290 mM NaCl, 160 mM KCl and nigericin (5 μg/ml). The concentration of KCl chosen brackets values for intracellular K^+ as determined in the muscles of marine mollusks (Potts, 1958; Robertson, 1965; Burton, 1983). Cells were allowed to equilibrate with each solution until ratios stabilized (generally <5 min). In routine experiments, pH_i was estimated for individual myocytes. Mean values for acid-base measurements in each physiological treatment represent data from individual cells from up to two independent cell dispersions.

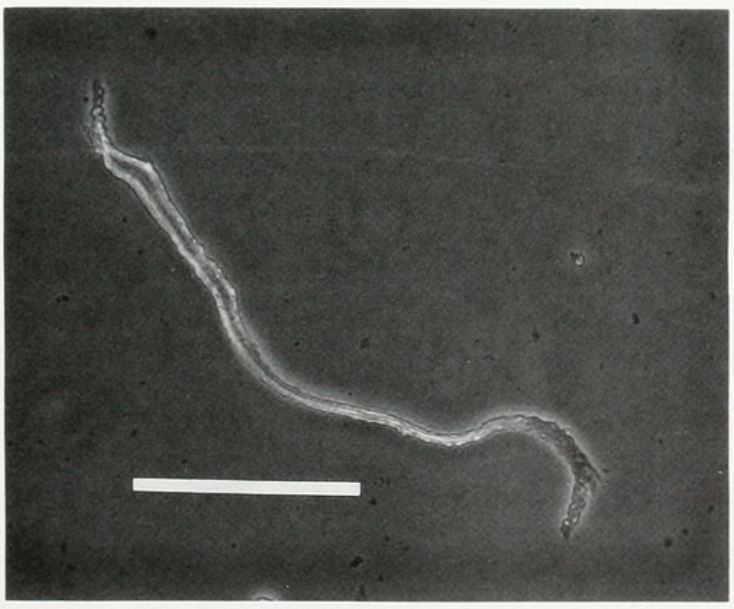

Figure 1. Cardiac myocyte from the ventricle of the clam *Mercenaria campechiensis*. Photograph was taken with Kodak TMAX 100 film. Bar corresponds to 100 μm.

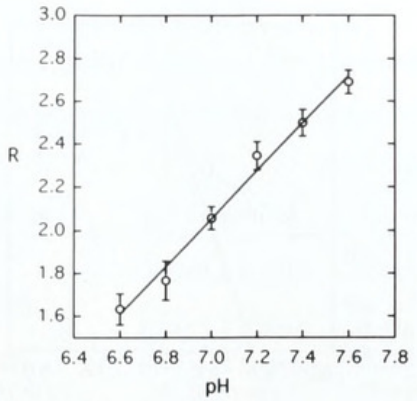

Figure 2. Relationship between the fluorescence intensity ratio (R = I_{490}/I_{450}) and pH obtained using the nigericin approach with cardiac myocytes from *Mercenaria campechiensis*. Each value corresponds to a mean ± 1 SD (n ranges from 8 to 12).

Results

Myocytes

Dispersion procedures produced a very high yield of myocytes. Cells were generally long and spindly (50–400 $\mu \times 10\ \mu$) (Fig. 1). Immediately after dispersion, a large fraction of cells showed spontaneous contractile activity but were generally quiescent after 12 h. Dispersed cells excluded trypan blue and were responsive to addition of 10^{-5} M 5-hydroxytryptamine (5-HT). In fact, many cells remained viable and responded to 5-HT up to 7 days after isolation. The addition of antibiotics (penicillin G, bacitricin) and 5 mM D-glucose did not enhance survival in the short term nor influence results of pH_i determinations. Thus, these components were not added to MASW. Ventricles of the congeneric clam *Mercenaria mercenaria* have extremely high glycogen levels, on the order of 240 μmoles/g wet wgt (Ellington, 1985). Thus, it is clear that there is a sufficient endogenous reserve of metabolic fuels in these myocytes for the period over which they were used (15–18 h).

Nigericin pH clamp

Myocytes were subjected to a nigericin pH clamp protocol encompassing the range of pH from 6.6 to 7.6 in 0.2-unit increments. Typically, most cells responded to the clamping medium by contracting to approximately 60% of their initial length and remained so throughout the protocol. Fluorescence ratios varied linearly with pH (Fig. 2). A regression equation was calculated (RATIO = 1.111 pH −5.724, R = 0.994) and used to transform all ratios from the ratio *versus* time spreadsheets to pH_i. The compressed ratio range was due to differential

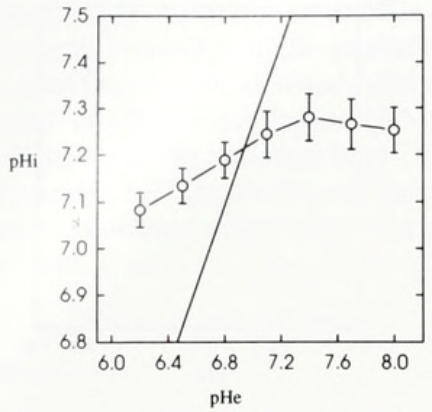

Figure 3. The relationship between pH_i and pH_e in cardiac myocytes from *Mercenaria campechiensis*. The continuous diagonal line corresponds to the iso-pH line. Each value corresponds to a mean ± 1 SD (n = 11).

gain/level adjustments of the analog-digital converter at the two excitation wavelengths as well as the fact that the neutral density filter at 490 nm was higher than the one used at 450 nm. Under routine superfusion conditions, pH_i of myocytes was observed to be 7.22 ± 0.08 (mean ± 1 SD, n = 20).

Relationship between intracellular and extracellular pH

Cells were superfused with MASW (containing 20 m*M* HEPES) adjusted to various pH values (pH_e). Media were equilibrated with air. Ratios were observed for 15–20 min at each pH. pH_i was essentially constant in the pH_e range from 7.1 to 8.0 (Fig. 3). At lower pH_e values, the pH_i declined linearly but still was considerably above the iso-pH line indicating good capacity for defense of pH_i against pH_e (Fig. 3).

Non-bicarbonate buffering capacity (β_{NB})

Non-bicarbonate buffering capacity was estimated by the NH_4Cl prepulse method of Boron (1977). Myocytes were superfused with MASW and then subjected to a pulse of 15 m*M* NH_4Cl-MASW (pH 7.75). After peak alkalinization, myocytes were superfused with MASW resulting in a pronounced acidification. β_{NB} values were calculated as described by Boron (1977). Buffering capacity is expressed as Slykes (dH^+/dpH). Since the MASW was equilibrated in air, $[HCO_3^-]$ was low (around 0.7 m*M*) so the contribution of this species to total buffering capacity is negligible. Figure 4 is an example of a typical pre-pulse experiment. Following alkalinization, there was a gradual decline in pH_i. After NH_4Cl wash-out, there was a characteristic alkalinization back towards the initial condition (Fig. 4). β_{NB} values for individual myocytes were somewhat variable ranging from 22 to 65 Slykes with a mean of 39.98 ± 8.86 (±1 SD, n = 31). The inherent limitation of the NH_4Cl prepulse approach is that some recovery of pH_i could occur during the early phase of the wash-out therebye producing an overestimate of β_{NB} (Boron, 1977). In the present study, wash-out was extremely

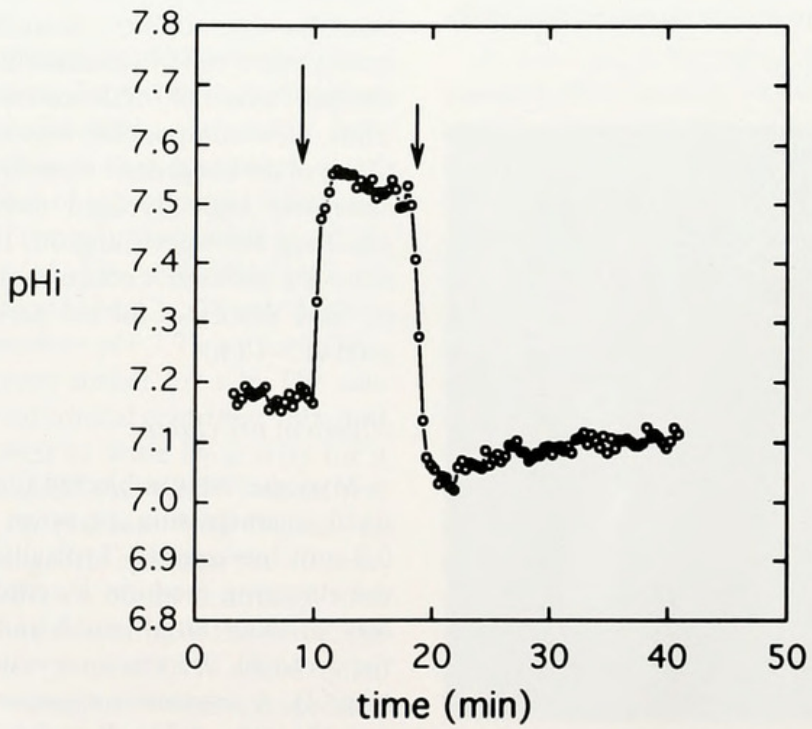

Figure 4. Time course of changes in pH_i in a single *Mercenaria campechiensis* cardiac myocyte during a typical NH_4Cl pre-pulse experiment. The first arrow indicates the onset of superfusion with 15 m*M* NH_4Cl-MASW. The second arrow indicates the onset of washing with MASW.

rapid as the peak acidosis occurred within 3 min (Fig. 4). Thus, it is likely that pH_i recovery processes would potentially produce only minor errors in β_{NB} determination in this system.

Recovery from acid loading

Isolated myocytes regulate pH_i after acid-loading as evidenced by the slow alkalinization following NH_4Cl wash-out using normal MASW ($[HCO_3^-]$ approximately 0.7 m*M*) (Fig. 5A; Table 1). However, the rate of alkalinization was greatly accelerated during wash-out using 0.3% CO_2/4 m*M* HCO_3^- MASW (Fig. 5B; Table I). Recovery from acid loading did not appear to be dependent on external Na^+, as the recovery rate was essentially the same for MASW and Na^+-free MASW (Table I). SITS completely blocked recovery. In fact, there was a gradual reduction in pH_i once the plateau acidification after wash-out had been attained (Table I). Collectively, these results show that it is likely that a SITS-sensitive HCO_3^-:Cl^- exchanger plays a major role in recovery from acid loading in *M. campechiensis* myocytes.

Discussion

Molluscan myocytes have been used on a number of occasions as experimental systems for investigating physiological phenomena ranging from ion channels (Brezden *et al.*, 1986) to changes in intracellular Ca^{2+} concentrations during contraction (Ishii *et al.*, 1989). In this regard, cardiac myocytes from *M. campechiensis* appear to be an ideal model system for studies of regulation of pH_i in that these cells are easily isolated, retain viability for extended time periods and, of course, can maintain acid-base balance in spite of extracellular and intracellular pH disturbances. The average pH_i of 7.22 in these cells as determined by BCECF-imaging is comparable to values observed in various marine gastropod muscles (Ellington, 1983b; Graham and Ellington, 1985; Wiseman and Ellington, 1989) and is slightly lower (0.1–0.2 units) than what has been observed for squid giant axon (Boron and Russell, 1983) and various tissues of the mussel *Mytilus edulis* (Walsh *et al.*, 1984; Zange *et al.*, 1990). The above data on other species were obtained by a variety of techniques including NMR, weak acid distribution and microelectrode methods.

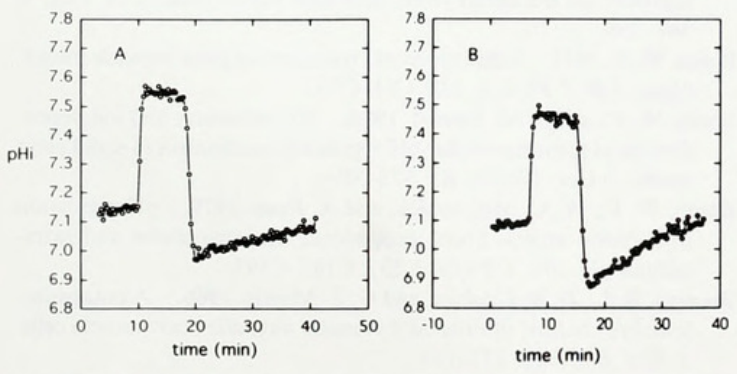

Figure 5. Typical records of change of pH_i in single *Mercenaria campechiensis* myocytes during NH_4Cl pre-pulse experiments when MASW (A) or 0.3% CO_2:4 m*M* HCO_3^--MASW (B) were used in washing.

Table I

Recovery from acid loading in myocytes from Mercenaria campechiensis

Washing medium	dpH/dt (pH units/min)	Acid/base transport (μmoles/min)	n
MASW	0.0040 ± 0.0018	0.157 ± 0.055	10
MASW-0.3% CO_2: 4 m*M* HCO_3^-	0.0152 ± 0.0012	0.576 ± 0.115	9
Na^+-free MASW	0.0054 ± 0.0030	0.236 ± 0.159	9
MASW-0.5 m*M* SITS	No Recovery (−0.0073 ± 0.0056)	Not estimated	10

Myocytes were superfused with MASW followed by 15 m*M* NH_4Cl-MASW. After the plateau alkalinization was acheived, myocytes were washed with various media. The rate of recovery (dpH/dt) was calculated using a regression (Sigma Plot) of the initial, linear portion of the recovery curve after NH_4Cl wash-out. Buffering capacity values (dH^+/dpH) were calculated according to Boron (1977). Rates of acid/base equivalent transport (μmoles/min) were calculated for each myocyte by multiplying the measured individual β_{NB} value times the corresponding dpH/dt value (μmoles/min = β_{NB} · dpH/min). Each value represents a mean ± 1 SD. Sample size (n) is indicated.

MASW-0.3% CO_2: 4 m*M* HCO_3^- was prepared by gassing MASW with 0.3% CO_2 (balance air), addition of solid $NaHCO_3$ followed by adjustment of pH. The reservoir was continuously gassed with hydrated 0.3% CO_2 in air and the superfusion line was contained within a gas jacket. Concentrations were calculated using appropriate apparent dissociation (Mehrbach *et al.*, 1973) and solubility (Riley and Skirrow, 1975) constants. Na^+-free MASW was prepared by replacing NaCl with three times crystallized choline chloride (Sigma Chemical Co.). SITS solutions were shielded from light to prevent photodecomposition.

Changes in pH_e have minimal effect on the pH_i of *M. campechiensis* myocytes over what can be viewed as a physiologically realistic range of pH_es (7.1–8.0). A similar high capacity for defending pH_i against changes in pH_e has been observed in *M. edulis* ABRM preparations (Zange *et al.*, 1990) as well as in hemocytes from the squid *Sepiateuthis lessoniana* (Hemming *et al.*, 1990). In the present study, we have further seen that clam myocytes display recovery from experimentally imposed acid-base disturbances. Walsh and Milligan (1989) have pointed out that there are three potential avenues of regulation of pH_i available to cells—(a) intracellular physico-chemical buffering, (b) ion exchange of acids/bases between intra- and extracellular compartments, and (c) metabolic production or consumption of acids and bases. The present results with *M. campechiensis* myocytes provide strong

evidence for operation of the first two of these mechanisms.

The presence of non-bicarbonate intracellular buffers constitutes the first line of defense against acid/base stress in cells (see reviews by Burton, 1978, and Roos and Boron, 1981). In vertebrate-muscles, there appears to be a general correlation between the magnitude of the β_{NB} and the potential for anaerobic function (Castellini and Somero, 1981). This general correlation has been suggested for molluscan muscles (Eberlee and Storey, 1984; Morris and Baldwin, 1984), however, the validity of these conclusions is somewhat in doubt due to the artifacts imposed by homogenate titration methods used for β_{NB} determinations (Wiseman and Ellington, 1989; Pörtner, 1990). In the present study we used the NH_4Cl-prepulse approach and obtained a value of 40 Slykes, which is in the range of values determined by NMR-prepulse for whelk radula muscle (33 Slykes; Wiseman and Ellington, 1989) and mussel ABRM (26.5 Slykes; Zange *et al.*, 1990), both of which have impressive capacities for anaerobic metabolism. In contrast to these observations, the average β_{NB} for squid giant axons was 11.2 (Boron and Russell, 1983). Thus, it is clear that *M. campechiensis* myocytes have a relatively high β_{NB}, which is consistent with the natural history of this species where exposure to hypoxic stress may be a regular phenomenon.

The pH_e in bivalves is alkaline relative to pH_i under normal conditions (Booth *et al.*, 1984). Given a slightly alkaline pH_e, the pH_i of 7.22, and the undoubtedly negative sign of the membrane potential in *M. campechiensis* myocytes, it is clear that protons are not at equilibrium. These cells must continuously export protons or bring in base equivalents to maintain pH_i. This problem becomes greatly exacerbated when the pH_e is reduced (therebye decreasing or even reversing the transmembrane proton gradient) or when acid or base loads are imposed on the cells. The relative constancy of pH_i with pH_e and recovery from experimentally imposed acidosis in clam myocytes clearly show that such ion exchange processes are operating in these cells.

Our results show that *M. campechiensis* myocytes appear to regulate pH_i via a SITS-sensitive ion exchanger which does not have a requirement for external Na^+. Most likely, this transporter is a HCO_3^-:Cl^- exchanger as has been seen in *M. edulis* ABRM (Zange *et al.*, 1990). Under the routine, normocapnic conditions in the present study the concentration of HCO_3^- was estimated to be around 0.7 m*M*, which appears to be sufficient to promote recovery of pH_i after acidosis. However, recovery was greatly accelerated when HCO_3^- concentration was increased to around 4 m*M*. Booth *et al.* (1984) estimated that [HCO_3^-] in *M. edulis* hemolymph was 1.8 m*M* in normoxia and rose to nearly 3 m*M* during hypoxic stress. It is likely that physiological [HCO_3^-] in *M. campechiensis* spans a higher range that 0.7 m*M*, implying greater overall transport rates *in vivo*. The above results are in contrast to the work of Boron and Russell (1983) who found that there was an absolute Na^+ requirement (Km = 77 m*M*) for HCO_3^-:Cl^- exchange in squid axons. However, Hemming *et al.* (1990) found that acid recovery in squid hemocytes was Na^+-independent. Zange *et al.* (1990) found that addition of 5-hydroxytryptamine (5-HT) elicited activation of a Na^+:H^+ exchanger in *M. edulis* ABRM. It was not possible to investigate this possibility in *M. campechiensis* myocytes, as addition of 5-HT caused rather violent contractions of myocytes, which interfered with imaging experiments.

The present results show that myocytes from the clam *M. campechiensis* have a good capacity for regulation of pH_i. This capacity is based a relatively high β_{NB} and the presence of a SITS-sensitive anion exchanger. Clam myocytes also appear to be excellent candidates for long-term primary culture. Thus, future studies will focus on potential phenotypic plasticity of β_{NB} and ion exchange capacity in cells cultured under conditions which might induce such changes (altered pH_e, hyper- or hypo-capnia or hypoxia).

Acknowledgments

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