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REGULATION OF INTRACELLULAR pH BY TOADFISH (*OPSANUS BETA*) HEPATOCYTES

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Summary

The BCECF [2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein] method for measurement of intracellular pH (pHi) was successfully applied to toadfish (*Opsanus beta* Goode and Bean) hepatocytes and used for investigating the pHi regulatory properties of the hepatocytes. As in previous studies of fish hepatocytes, toadfish hepatocyte pHi showed a marked dependence on extracellular pH (pHe) with characteristic values of about 0.15–0.2 units below pHe. Measurement of membrane potential (-33.76 ± 3.07 mV, $N=3$) in toadfish hepatocytes by the $S^{14}CN^-$ distribution method allowed calculation of equilibrium pHi values and, despite the close tracking of pHi and pHe, proton equilibration across the membrane was never observed. Furthermore, when toadfish hepatocytes were acidified by the NH_4Cl prepulse method, recovery to normal pHi values could be inhibited by addition of 0.5 mmol l^{-1} amiloride or replacement of extracellular Na^+, indicating that these cells possessed a Na^+/H^+ exchanger. These experiments led to the conclusion that pHi is regulated in toadfish hepatocytes. I next exposed toadfish hepatocytes to a physiological range of several extracellular variables that change during exercise (pHe, P_{CO_2}, [lactate], [catecholamine], [glucose]), in the presence and absence of amiloride, in an attempt to determine the importance of Na^+/H^+ exchange to hepatocyte pHi regulation in a physiological context. The exchanger plays a significant role in post-exercise recovery from acidosis induced by changes in P_{CO_2} and pHe. Further experiments employing inhibitors of lactate metabolism (3-mercaptopicolinic acid and amino-oxyacetic acid) and amiloride established that the increased lactate metabolism associated with 'post-exercise' recovery creates an intracellular acid load, and that Na^+/H^+ exchange protects the cell from changes in pHi caused by lactate metabolism.

Introduction

Various physiological states and environmental conditions produce extracellular and tissue acid–base disturbances in fish (Heisler, 1986). For example, following strenuous exercise in fish, blood pH can decrease by up to 0.5 units (Wood and Perry, 1985), and certain tissues (such as active muscles) can also incur substantial

Key words: *Opsanus beta*, intracellular pH, hepatocytes, exercise.

acid loads (Milligan and Wood, 1986, 1987). Several *in vivo* studies of acid–base status in fish during extracellular acidosis have shown that intracellular pH (pHi) of liver tissue is often regulated within relatively narrow limits and in some cases pHi may actually become more alkaline (Walsh and Moon, 1982; Milligan and Wood, 1986, 1987; Perry *et al.* 1988). The mechanisms of these responses *in vivo* are not well understood. As in other animal cells (Roos and Boron, 1981; Walsh and Milligan, 1989), fish hepatocytes have specific plasma membrane transporters that exchange acid–base equivalents with the extracellular space (i.e. Na^+/H^+ exchange, Cl^-/HCO_3^- exchange; Walsh, 1986; Hugentobler *et al.* 1987) and whose rates of function are sensitive to changes in intracellular acid–base state (Walsh, 1986). However, these *in vitro* studies of intracellular pH regulation in fish hepatocytes have focused primarily on descriptions of mechanisms, employing largely non-physiological perturbations. In general, studies of pHi regulation have placed little emphasis on the physiological context in which these acid–base transporters operate or on other potential means of pHi regulation (i.e. buffering capacity and metabolism: Roos and Boron, 1981; Walsh and Milligan, 1989).

The purpose of the present study, therefore, was first to characterize the acid–base transporter(s) involved in pHi regulation in hepatocytes from a marine teleost, the gulf toadfish (*Ospanus beta*). I then attempted to simulate the *in vivo* acid–base perturbations associated with a specific physiological state (exercise) in suspensions of hepatocytes *in vitro* in the presence and absence of inhibitors of pHi regulation. By this approach I hoped to gain insight into the relative importance and *in vivo* physiological role of acid–base transporters in pHi regulation in fish liver tissue.

Materials and methods

Experimental animals and hepatocyte isolation

Specimens of mature gulf toadfish, *Opsanus beta* (body mass 40–490 g), were collected by roller trawl in Biscayne Bay, Florida, by a local fisherman during August to December 1988. Upon return to the laboratory, toadfish were held in running saltwater aquaria (salinity 33‰, temperature 23–28°C) and fed a diet of chopped squid twice a week. Fish were fasted for 3 days before use. Hepatocytes were isolated by collagenase perfusion of the liver, as previously described (Walsh, 1987). Media were identical to those in Walsh (1987), except that bovine serum albumin (BSA) was omitted (see below); [lactate]=0.4 mmol l^{-1} and [glucose]= 1.3 mmol l^{-1}, unless noted.

Measurement of pHe, C_{CO_2}, pHi and buffering capacity

Extracellular pH (pHe) was measured in suspension media with a Radiometer PHM 84 pH meter and capillary glass pH electrode. Total CO_2 (C_{CO_2}) content was measured with a Corning 965 total CO_2 analyser. Intracellular pH was measured by an adaptation of the BCECF-fluorescence method for measuring cytoplasmic

pHi (Rink *et al.* 1982). Hepatocytes were incubated for 1 h with 10 μmol l^{-1} BCECF-AM [2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl-ester; 1 μl of a stock solution of 10 mmol l^{-1} in dimethylsulphoxide (DMSO) was added per ml of cell suspension] (Calbiochem, La Jolla, CA), a non-fluorescent, permeant form of the dye (converted to the fluorescent impermeant BCECF intracellularly by endogenous hepatic esterases). Insufficient dye accumulated in cells during shorter periods, whereas longer periods resulted in no further significant accumulation. Cells were washed (centrifuged at 200 *g* for 3 min and resuspended in fresh suspension medium) several times to remove any extracellular BCECF-AM or BCECF, and then approximately 15–30 mg of cells was suspended in 3.5 ml of suspension medium with a small magnetic flea in a cuvette mounted on a small magnetic stirrer (Hellma, Cuv-O-Stir, model 333, Jamaica, NY) in a Perkin–Elmer LS3B fluorescence spectrophotometer. Fluorescence was continuously measured at 518 nm and cells were excited primarily at 490 nm (a pH-sensitive wavelength) with periodic switching to excitation at 440 nm (a pH-insensitive wavelength). After correction for autofluorescence by the cells (measured prior to BCECF loading, see Results) and leaked dye (by monitoring the medium after cells had been centrifuged at 13 000 *g* for 1 min), the ratio of fluorescence when excited at 490 nm to that when excited at 440 nm was used to calculate pHi. pHi is calculated by comparing ratios obtained for the experimental cells with ratios obtained for cells exposed to high-potassium medium (as for normal toadfish saline, Walsh, 1987, except NaCl and KCl concentrations are 5 mmol l^{-1} and 130 mmol l^{-1}, respectively) in the presence of 10 μg ml^{-1} nigericin, a K^+/H^+ ionophore (1 μl of a stock solution of 10 mg ml^{-1} 75 % dimethyl formamide/25 % ethanol was added per ml of cell suspension). This treatment equilibrates pHe and pHi, and allows placement of the cell ratio values on a standard curve *vs* pHe. These curves were also compared with those obtained for pure BCECF (Calbiochem) in K^+-rich suspension medium. BSA was omitted from all media since it binds nigericin and renders it ineffective in the calibration protocol.

In selected experiments, pHi was measured by the dimethyloxazolidine (DMO) method with [^{14}C]DMO (Amersham, Arlington Hts, IL), as previously applied to fish hepatocytes (Walsh, 1986). Non-bicarbonate buffering capacity (β_a) was measured by a modification of the methods of Boron (1977) using the difference between pHi at the end of a 30 min NH_4Cl prepulse and immediately after NH_4Cl washout with normal medium plus 0.5 mmol l^{-1} amiloride. The addition of amiloride ensured that Na^+/H^+ exchange did not raise pHi and lead to overestimates of β_a.

When kinetic experiments were performed on the effects of various agents on pHi, cells were exposed either by adding small volumes of concentrated stock solutions to the cuvette, or by low-speed centrifugation followed by rapid resuspension in the new medium and transfer to the cuvette. In the latter case, the time before fluorescence measurements began was typically 10–15 s. This is considered the 'zero time' point.

Measurement of membrane potential

Membrane potential was measured from the degree of accumulation of $S^{14}CN^-$, as previously applied to rat hepatocytes (Hoek *et al.* 1980). Toadfish hepatocytes were incubated in 0.36 μCi ml^{-1} $S^{14}CN^-$ (ICN Radiochemicals, Irvine, CA) and samples were centrifuged through bromododecane, as previously applied to measurements of intracellular compounds in fish hepatocytes (Walsh, 1986, 1987). Values were corrected for a small extracellular fluid content (i.e. less than 15 % of total water) by incubation of hepatocytes with [^{14}C]polyethylene glycol (New England Nuclear, Boston, MA). Corrections for intracellular binding of $S^{14}CN^-$ by hepatocytes was made by abolishing the membrane potential by incubation of cells in K^+-rich medium (as in BCECF calibrations) with 10 μg ml^{-1} of the potassium ionophore valinomycin (1 μl of a stock solution of 10 mg ml^{-1} 75 % dimethyl formamide/25 % ethanol was added per ml of cell suspension).

Chemicals, calculations and statistical analyses

All biochemicals were purchased from Sigma Chemical Co. (St Louis, MO) unless noted, and 3-mercaptopicolinic acid was a generous gift from Smith, Kline & French (Philadelphia, PA). All other chemicals were reagent grade. Buffering capacity was calculated according to Boron (1977) using the change in intracellular ammonia concentration calculated from known extracellular concentrations (30 mmol l^{-1} initial to 0.2 mmol l^{-1} final, measured by the method of Solorzano, 1969) and the appropriate values of pKa and NH_3 solubility (Boutilier *et al.* 1984). Rates of acid extrusion were calculated by multiplying the change in pHi per minute by the total intracellular buffering capacity (β=2.3[HCO_3^-]+β_a: Burton, 1978). Carbon dioxide tension (P_{CO_2}) and intracellular and extracellular [HCO_3^-] were calculated using the appropriate values for pKa and CO_2 solubility (Boutilier *et al.* 1984) and measured values of pHe, pHi and C_{CO_2}. Membrane potential was calculated using the intracellular to extracellular ratio of $S^{14}CN^-$, corrected for extracellular space, and the Nernst equation as in Hoek *et al.* (1980). Intracellular pH measured by the BCECF method was calculated according to the following equation:

$$\mathrm{pHi} = \frac{\left\{ \dfrac{F_{490/518} - \text{autofluorescence}_{490} - \text{leaked BCECF}_{490}}{F_{440/518} - \text{autofluorescence}_{440} - \text{leaked BCECF}_{440}} + (\text{intercept of standard curve}) \right\}}{(\text{slope of standard curve})}$$

All statistical tests were performed according to Zar (1974).

Results

BCECF methodology

The BCECF-fluorescence method (Rink *et al.* 1982) for measurement of pHi was successfully applied to toadfish hepatocytes using only a few modifications.

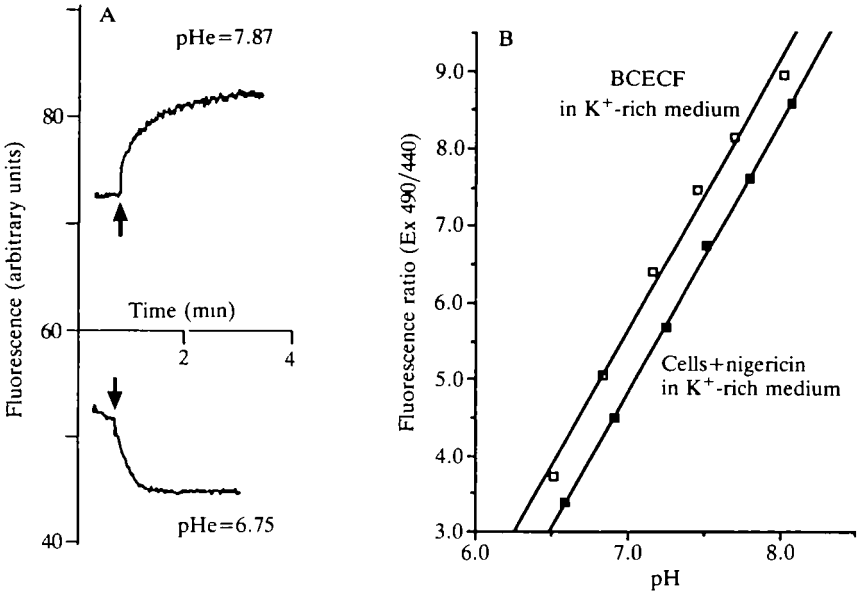

Fig. 1. (A) Time course of nigericin (added at arrow) equilibration of pHi with pHe in K^+-rich medium in toadfish hepatocytes. (B) pH calibration curve of fluorescence ratio $F_{490/518}/F_{440/518}$ *vs* pH of medium. The equation for BCECF in medium (□) is: ratio=−19.0593+3.5283pH, r=0.99; and for cells equilibrated with nigericin and K^+-rich medium (■) it is: ratio=−19.9651+3.5422pH, r=0.99).

Optimal incubation time for toadfish hepatocytes with BCECF-AM was slightly longer than with mammalian cell types. Also, since toadfish hepatocytes showed some fluorescence without the dye, the excitation and emission wavelengths were chosen to minimize this interference. However, since this fluorescence by the hepatocytes could not be completely eliminated in the useful wavelength range of the dye, this endogenous fluorescence (usually less than 15 % of the signal caused by the dye at 490 nm) was routinely subtracted from the signal. By employing these modifications, the calibration lines for the dye *in situ* and free in solution agreed well (Fig. 1). Although the slopes of the two lines agreed precisely, the lines were slightly offset (Fig. 1); this has been observed previously in other cell types and is attributed to the slight modification of the fluorescence characteristics of BCECF by binding to intracellular components (Rink *et al.* 1982).

Measurements of pHi by BCECF compared reasonably well with measurements by the DMO method, except that pHi measured by BCECF changed less at low and high extremes of pHe (Fig. 2).

Ionic mechanisms of pHi regulation

In preliminary experiments, toadfish hepatocytes were subjected to pHi perturbations by a variety of non-physiological means (30 mmol l^{-1} DMO, sodium

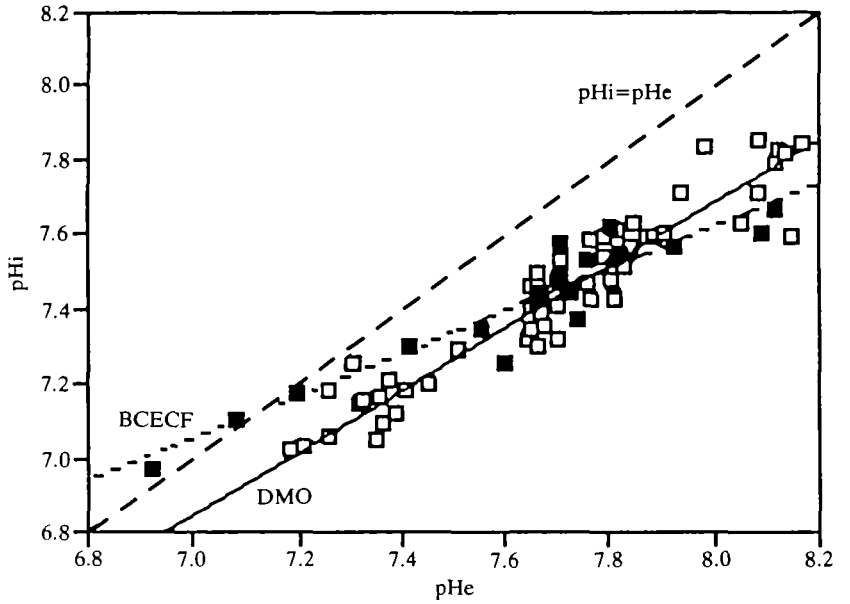

Fig. 2. Intracellular pH *vs* extracellular pH in toadfish hepatocytes as determined by the DMO method (□, solid line) (pHi=1.1976+0.8104pHe, r=0.95) and by the BCECF method (■, dotted line) (pHi=3.037+0.5727pHe, r=0.95). Line for equivalence (pHi=pHe, dashed line) is included as a reference. pHe was varied by HCl titration, and media were equilibrated with 0.25 % CO_2.

acetate or NH_4Cl, and 50 mmol l^{-1} $NaHCO_3$/5 % CO_2). DMO, sodium acetate and CO_2 all produced acidifications of pHi, as did an ammonium chloride prepulse followed by washout (see De Weer, 1978). However, the NH_4Cl prepulse method gave the most consistent acidifications and rates of recovery, so it was employed in further experiments. Toadfish hepatocytes responded to NH_4Cl with an initial alkalinization followed by a slow return to normal values by about 30 min (Fig. 3A). When cells were centrifuged and resuspended in normal medium (final measured NH_3 values were always less than 0.2 mmol l^{-1}), there was a distinct acidification of pHi followed by a slow recovery (Fig. 3B) and recovery was complete by 30–45 min. Rates of acid extrusion for the first 10 min of this recovery period (Table 1) compare reasonably with rates of acid extrusion for other tissues (Nuccitelli and Deamer, 1982). To determine which membrane transport system(s) was responsible for this recovery, hepatocytes were incubated with known inhibitors of acid–base transporters during recovery from acidification by NH_4Cl prepulse. SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid) had no effect on recovery (Table 1). However, amiloride substantially increased the extent of the initial acidification (Fig. 3B) and significantly slowed the rate of recovery from acidification (Fig. 3B; Table 1). Similar results were obtained when extracellular [Na^+] was lowered by substituting *N*-methyl-D-glucamine (Table 1).

Buffering capacity, membrane potential and regulation of pHi under simulated physiological conditions

Non-bicarbonate buffering capacity in toadfish hepatocytes was calculated by using the zero time pHi values for amiloride recovery and 30 min pHi values for NH_4Cl prepulse and found to be -42.80 ± 6.18 mmol pH unit^{-1} l^{-1} (N=7). Membrane potential in toadfish hepatocytes was calculated from corrected distribution

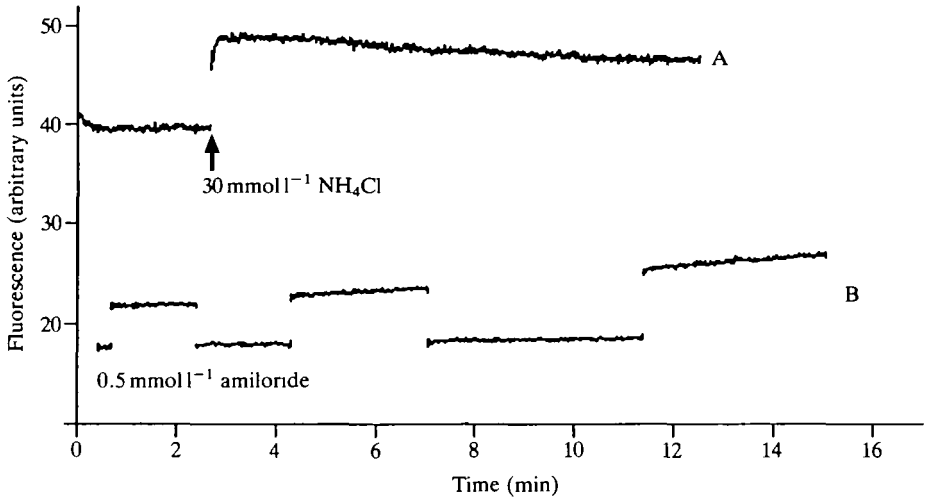

Fig. 3. (A) Time course of alkalinization and initial recovery of pHi by NH_4Cl prepulse (added at arrow). NH_4Cl prepulse normally lasted for 30 min; the record is truncated for illustrative purposes. (B) Time course of recovery from acidification of pHi by NH_4Cl prepulse (30 min) and subsequent washout (by centrifugation and resuspension) in normal medium (upper trace) and normal medium plus 0.5 mmol l^{-1} amiloride (lower trace). These two traces are from simultaneous experiments with hepatocytes from the same fish (also the same fish as in Fig. 3A) and the discontinuity of the traces is due to switching of the cuvette changer. Time zero in this case represents the moment that normal medium (with or without amiloride) was added to the cell pellet.

Table 1. *Rates of acid extrusion during initial 10 min of intracellular pH recovery from NH_4Cl prepulse in toadfish hepatocytes*

Treatment	Acid extrusion rate (mmol l^{-1} min^{-1})
Normal recovery	1.15±0.07 (7)
Amiloride recovery (0.5 mmol l^{-1})	0.44±0.07 (7)*
Low-$[Na^+]_e$ recovery	0.55±0.17 (4)*
Normal recovery	1.28±0.15 (4)
SITS recovery (0.5 mmol l^{-1})	0.96±0.15 (4)

Values are means±S.E.M.

*Significantly different from corresponding normal recovery by paired Student's t-test, $P<0.05$.

ratios of $S^{14}CN^-$ and found to be -33.67 ± 3.07 mV ($N=3$). This value did not vary substantially with changes in pHe or with pHi perturbations, although the binding correction factor did increase with decreasing pHe (results not shown). When toadfish hepatocytes were exposed to a physiological range of extracellular variables that change during strenuous exercise *in vivo* (P. J. Walsh, unpublished data), only changes in C_{CO_2} and pHe had significant effects on pHi (Table 2). Some of the variables had no effect on pHi (i.e. elevated [glucose], [epinephrine], [norepinephrine]), and when hepatocytes were exposed to these changes in the presence of amiloride to block Na^+/H^+ exchange, changes in pHi were similar to those that occurred under normal circumstances (i.e. a rapid acidification of about 0.1–0.15 units with little further change; Table 2). The changes in pHi caused by elevated C_{CO_2} were relatively small (approx. -0.2 units), and these changes were corrected rapidly (within 10 min). Exposure of cells to pHe values lower than normal led to a readjustment of pHi to a lower value (Fig. 2; Table 2), and these adjustments were almost complete within 30 s and complete by 5 min. (Kinetics are not shown for these very rapid changes. One limitation of the BCECF method, which applies to fish hepatocyte suspensions but not to immobilized cells, is that some pHi adjustments can occur in the brief time it takes to resuspend cells in a new medium and return them to the fluorometer.)

An increase in [lactate] to levels similar to post-exercise levels had no significant effect on pHi (Table 3). Since lactate is a moderately strong acid (pKa=4) this lack of effect was interesting and further experiments were designed to determine the mechanistic basis of this response. When Na^+/H^+ exchange was inhibited by amiloride, in addition to the initial acidification noted under normal conditions (Table 3), upon addition of lactate there was a slow but steady further acidification of pHi, indicating that lactate uptake and/or metabolism created an intracellular acid load. To determine if transport, metabolism or both processes caused the acid load, further experiments were conducted. When lactate metabolism was inhibited by 1 mmol l^{-1} each of 3-mercaptopicolinic acid (3-MPA) and aminooxyacetic acid (AOA) (Walsh, 1987), pHi increased, and it continued to increase during the 30 min exposure to lactate (Table 3), indicating that lactate metabolism, not lactate transport, was responsible for generating the acid load.

Discussion

Intracellular pH values obtained by the BCECF method for toadfish hepatocytes *in vitro* at normal ('resting') pHe (7.70) (Fig. 2) agree well with *in vivo* pHi values for fish liver obtained by the DMO method (Walsh and Moon, 1982; Milligan and Wood, 1986, 1987; Perry *et al.* 1988). At resting pHe, pHi determinations in toadfish hepatocytes *in vitro* by the BCECF and DMO methods agree closely (Fig. 2). However, as pHe becomes very alkaline or acidic, intracellular pH values measured by the BCECF method appear to be more 'narrowly regulated' than those measured by the DMO (Fig. 2). In previous studies using BCECF, it was demonstrated that this marker is confined primarily

Table 2. *Effect of physiological variables on intracellular pH in toadfish hepatocytes*

Treatment	Intracellular pH			
	Without amiloride		With 0.5 mmol l^{-1} amiloride†	
	0 min§	30 min	0 min§	30 min
Normal medium‡	7.432±0.010	7.395±0.035	7.323±0.026	7.313±0.032
High C_{CO_2} (9.5 mmol l^{-1})	7.226±0.031*	7.401±0.034	7.090±0.013*	7.164±0.020*
Low pH medium (7.00)	7.102±0.064*	7.028±0.031*	7.029±0.056*	6.961±0.035*
High [glucose] (3.5 mmol l^{-1})	7.370±0.038	7.448±0.027	7.233±0.073	7.282±0.045
High [epinephrine] (25 nmol l^{-1})	7.323±0.021	7.428±0.070	7.222±0.046	7.319±0.052
High [norepinephrine] (25 nmol l^{-1})	7.374±0.021	7.399±0.080	7.253±0.045	7.298±0.047

Values are means±S.E.M. (N=3).

* Significantly different from corresponding value in normal medium, $P<0.05$; paired Students' t-test.

† All amiloride values are significantly different from corresponding value without amiloride, $P<0.05$.

‡ Normal medium=0.4 mmol l^{-1} lactate, 1.3 mmol l^{-1} glucose, pH 7.70, C_{CO_2}=2.5 mmol l^{-1}, no epinephrine or norepinephrine. Changes from normal medium are given in parentheses.

§ '0 min' represents the first measurement obtained approx. 15 s after exposure to new conditions by resuspension in new medium.

Table 3. *Effects of elevated lactate (5 mmol l^{-1}) on intracellular pH in toadfish hepatocytes*

Treatment	Intracellular pH 0 min‡	30 min
High [lactate] medium (0.5 mmol l^{-1})	7.406±0.035	7.450±0.027
+Amiloride (0.5 mmol l^{-1})	7.307±0.018*	7.154±0.054†
+3-MPA/AOA (1 mmol l^{-1})	7.537±0.047*	7.608±0.066†

Values are means±S.E.M. (N=6).

3-MPA=3-mercaptopicolinic acid; AOA=amino-oxyacetic acid.

*,† denote significantly different from each other and corresponding high [lactate] medium value, $P<0.05$, by model III, two-way ANOVA.

‡'0 min' represents the first measurement obtained approx. 15 s after exposure to new conditions by resuspension in new medium.

to the cytoplasmic compartment (Rink *et al.* 1982), whereas DMO yields an average pHi including most, if not all, of the intracellular compartments (Hinke and Menard, 1978). Therefore, it is likely that the differences in pHi values between the two methods at low and high pHe (Fig. 2) can be attributed to the differential regulation of the cytoplasmic *vs* 'other' compartments. Despite the close tracking of average (DMO) pHi with pHe in hepatocytes from toadfish (Fig. 2) and other fish (*Anguilla rostrata*, Walsh and Moon, 1983; *Salmo gairdneri*, Walsh, 1986), pHi is a regulated variable in fish hepatocytes. Most values of pHi fall below the line of equality for pHi and pHe (Fig. 2; Walsh and Moon, 1983; Walsh, 1986). Furthermore, in the present study I determined the membrane potential in toadfish hepatocytes to be −33.76±3.07 mV (see Results), a value in close agreement with previous studies of skate hepatocytes (Wondergem *et al.* 1985) and rat hepatocytes (Hoek *et al.* 1980). This measurement allows the estimate (using the Nernst equation; Eckert and Randall, 1983) that pHi would be approximately 0.6 units below pHe if protons were in electrochemical equilibrium. Since this difference is never observed in toadfish hepatocytes incubated under physiological conditions (Tables 2, 3; Fig. 2), we can conclude that pHi is truly regulated in toadfish hepatocytes and probably in fish hepatocytes in general.

Further evidence that pHi is regulated in fish hepatocytes comes from studies of pHi perturbations by a variety of non-physiological means. pHi is rapidly corrected from acid loading by Na^+/H^+ exchange in rainbow trout hepatocytes (Walsh, 1986). Recovery from acid loading by toadfish hepatocytes is also inhibited by amiloride and low extracellular [Na^+] (Table 1), suggesting that toadfish hepatocytes also possess a Na^+/H^+ exchanger. Interestingly, rainbow trout (Walsh, 1986) and skate (Hugentobler *et al.* 1987) hepatocytes possess SITS-sensitive anion exchanger (i.e. a Cl^-/HCO_3^- exchanger that may also

transport SO_4^{2-}), and inhibition of this exchanger with SITS (or removal of Cl^-) in rainbow trout hepatocytes elevates (DMO) pHi by 0.15 units, regardless of pHe (Walsh, 1986). However, SITS had no effect on pHi or on pHi recovery in toadfish hepatocytes (Table 1). Whether this difference is due to methodological biases, or represents a real species difference, deserves further attention.

Despite considerable potential to recover from acid loading by Na^+/H^+ exchange, it appears that this system has only a modest role in determining pHi under resting or basal conditions. When Na^+/H^+ exchange is inhibited by 0.5 mmol l^{-1} amiloride, pHi is lowered by only about 0.1 unit and, at least during a 30 min incubation, pHi did not continue to drift downwards (Table 2). This observation suggests that under control conditions, proton leakage and metabolic acid loading are slow, and/or that intracellular buffers are capable of stabilizing pHi. In this respect, non-bicarbonate intracellular buffering capacity in toadfish hepatocytes is substantial, as measured by NH_4Cl prepulse and washout (-42.80 ± 6.18 mmol pH unit^{-1} l^{-1}, see Results), and agrees well with previous measurements on liver tissue by HCl and CO_2 titrations (Heisler, 1986; Milligan and Wood, 1986, 1987). Total intracellular buffering capacity would be the sum of non-bicarbonate buffering capacity and $2.3\times[HCO_3^-]$ (Burton, 1978) or about $-43+(-6)=-49$ mmol pH units^{-1} l^{-1} under physiological conditions.

The importance of the Na^+/H^+ exchanger to pHi regulation increases under simulated exercise stress in toadfish hepatocytes. This exchanger is responsible for recovery from increased P_{CO_2} (respiratory acidosis), and for maintenance of pHi (albeit at lower values than resting pHi) at decreased pHe and constant P_{CO_2} (metabolic acidosis), in that inhibition with 0.5 mmol l^{-1} amiloride prevents recovery or maintenance of pHi (Table 2). In addition, this sytem is important in protecting the cells from acidosis generated by lactate metabolism. When hepatocytes were exposed to elevated lactate levels typical of peak values in toadfish blood following exercise (5 mmol l^{-1}, P. J. Walsh, unpublished data), there was no effect on pHi (Table 3). However, when Na^+/H^+ exchange was inhibited, in addition to the immediate depression of pHi noted under resting conditions, pHi continued to drift downwards (Table 3). This acidification by lactate (which is normally compensated for by Na^+/H^+ exchange) could be the result of transport of lactate into the cell, metabolism of lactate, or a combination of the two processes. When metabolism of lactate is inhibited at elevated [lactate], there is an initial increase in pHi, followed by a further increase (Table 3), suggesting that lactate metabolism at elevated [lactate] is responsible for the acidification. Since lactate transport is by passive diffusion in toadfish hepatocytes, and continues to some degree even when metabolism is inhibited (Walsh, 1987), these data also suggest that transport of lactate *per se* does not acidify the cell.

In two *in vivo* studies of the effects of strenuous exercise on intracellular acid–base status in fish, pHi of liver was largely unaffected during the initial large extracellular acidosis (rainbow trout, Milligan and Wood, 1986; starry flounder, Milligan and Wood, 1987). The results of the present study with toadfish hepatocytes and a previous study with rainbow trout hepatocytes indicate that this

in vivo pHi homeostasis is largely the result of intracellular buffering capacity and a Na^+/H^+ exchange system. Interestingly, in both the starry flounder and the rainbow trout, hepatic pHi steadily increases (by approx. 0.5 and 0.2 units, respectively) during the later stages of recovery (Milligan and Wood, 1986, 1987). Despite the examination of several relevant variables, the present *in vitro* study did not provide a mechanistic insight into the *in vivo* increase in pHi associated with long-term recovery from exercise. At least in short-term exposures, none of the post-exercise variables caused an increase in hepatocyte pHi (Tables 2, 3). Thus, the observed increase in hepatic pHi *in vivo* may be due to longer exposure to these factors, or to some other parameter not investigated. Experiments with hepatocytes in long-term culture may offer mechanistic insights into this interesting *in vivo* phenomenon. Alternatively, an increase in hepatic pHi during recovery from exercise may not be a general phenomenon in fish, and the presence or absence of this phenomenon should be examined in the toadfish.

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