EVIDENCE FOR THE ROLE OF A Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} COTRANSPORTER IN TROUT HEPATOCYTE pHi REGULATION

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Summary

The mechanisms of intracellular pH (pHi) regulation were examined in hepatocytes of the rainbow trout Oncorhynchus mykiss. pHi was monitored using the pH-sensitive fluorescent dye BCECF, and the effects of various media and pharmacological agents were examined for their influence on baseline pHi and recovery rates from acid and base loading. Rates of Na\textsuperscript{+} uptake were measured using \textsuperscript{22}Na, and changes in membrane potential were examined using the potentiometric fluorescent dye Oxonol VI. The rate of proton extrusion following acid loading was diminished by the blockade of either Na\textsuperscript{+}/H\textsuperscript{+} exchange (using amiloride) or anion transport (using DIDS). The removal of external HCO\textsubscript{3}\textsuperscript{−} and the abolition of outward K\textsuperscript{+} diffusion by the channel blocker Ba\textsuperscript{2+} also decreased the rate of proton extrusion following acid load. Depolarization of the cell membrane with 50 mmol l\textsuperscript{−1} K\textsuperscript{+}, however, did not affect pHi. The rate of recovery from base loading was significantly diminished by the blockade of anion transport, removal of external HCO\textsubscript{3}\textsuperscript{−} and, to a lesser extent, by blocking Na\textsuperscript{+}/H\textsuperscript{+} exchange. The blockade of K\textsuperscript{+} conductance had no effect. The decrease in Na\textsuperscript{+} uptake rate observed in the presence of the anion transport blocker DIDS and the DIDS-sensitive hyperpolarization of membrane potential during recovery from acid loading suggest that a Na\textsuperscript{+}-dependent electrogenic transport system is involved in the restoration of pHi after intracellular acidification. The effects on baseline pHi indicate that the different membrane exchangers are tonically active in the maintenance of steady-state pHi. This study confirms the roles of a Na\textsuperscript{+}/H\textsuperscript{+} exchanger and a Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger in the regulation of trout hepatocyte pHi and provides new evidence that a Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter contributes to pHi regulation.

Key words: rainbow trout, Oncorhynchus mykiss, hepatocyte, Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter, Na\textsuperscript{+}/H\textsuperscript{+} exchanger, Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger, intracellular pH, Na\textsuperscript{+} uptake, amiloride, DIDS.

Introduction

The regulation of intracellular pH (pHi) in eukaryotic cells is a concerted effort involving cytosolic buffers and different membrane transport proteins. The participation of the specific proteins in pHi regulation varies according to tissue and species. The Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE gene family) is presumed to be present in all eukaryotic cells and participates in acid extrusion by way of a 1:1 electroneutral exchange of intracellular H\textsuperscript{+} with extracellular Na\textsuperscript{+} (Noël and Pouyssegur, 1994; Wakabayashi et al., 1997; Yun et al., 1995). Several HCO\textsubscript{3}\textsuperscript{−}-dependent mechanisms are known to participate in pHi regulation, namely Na\textsuperscript{+}-dependent and Na\textsuperscript{+}-independent Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange and Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter (Madshus, 1988). The Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger (anion exchanger, AE, gene family) plays a role in recovery from intracellular alkalinization by removing HCO\textsubscript{3}\textsuperscript{−} from the cytoplasm in exchange for extracellular Cl\textsuperscript{−} in a 1:1 electroneutral fashion (Alper, 1994; Tonnesen et al., 1990). The role of the electrogenic Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter (NBC gene family) has been well documented in the mammalian kidney, where it participates in Na\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{−} reabsorption (Boron et al., 1997). The Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter can also behave indirectly as an acid extruder in some tissues (Dart and Vaughan-Jones, 1992; De la Rosa et al., 1999; Gleeson et al., 1989) as a result of the buffering of intracellular H\textsuperscript{+} by HCO\textsubscript{3}\textsuperscript{−} entering the cell. The recent cloning of the Na\textsuperscript{+}/HCO\textsubscript{3}\textsuperscript{−} cotransporter (Burnham et al., 1997, 1998; Romero et al., 1997, 1998) facilitates studies of the expression and regulation of this protein in kidney and other tissues.

Previous studies have examined pHi regulation in teleost hepatocytes with varying results. In trout (Oncorhynchus mykiss) hepatocytes, a Na\textsuperscript{+}/H\textsuperscript{+} exchanger and a Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger are important in the regulation of pHi (Fossat et al., 1997; Walsh, 1986), whereas in the gulf toadfish (Opsanus beta), pHi regulation is achieved exclusively via modulation of Na\textsuperscript{+}/H\textsuperscript{+} exchange (Walsh, 1989). A more recent comparative study (Furimsky et al., 1999a) has demonstrated that, in addition to trout hepatocytes, those of black bullhead (Amiurus melas) and American eel (Anguilla rostrata) also rely on the Na\textsuperscript{+}/H\textsuperscript{+} exchanger and Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger for pHi regulation. In that same study, it was demonstrated that the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, though present in the eel, was not activated...
by a decrease in pH, as it is in other species. In rat hepatocytes, an electroneutral Na\(^+/\)HCO\(_3\)\(^-\) cotransporter participates together with the Na\(^+/\)H\(^+\) exchanger in net acid extrusion following a decrease in cytoplasmic pH (Gleeson et al., 1989). The activity of the Na\(^+/\)HCO\(_3\)\(^-\) cotransporter in rat hepatocytes has been linked to that of a K\(^+\) channel, where a decrease in pH leads to a decrease in K\(^+\) conductance, with the resulting membrane depolarization activating Na\(^+/\)HCO\(_3\)\(^-\) cotransport (Fitz et al., 1992).

In the few studies that have investigated pH regulation in teleost hepatocytes, no evidence has been offered regarding the possible role of a Na\(^+/\)HCO\(_3\)\(^-\) cotransporter. A recent study by Furimsky et al. (1999a), however, suggested that an additional Na\(^+\)-dependent ion-transport mechanism may be participating in proton extrusion following an acid load because removal of extracellular Na\(^+\) had a more pronounced effect on pH recovery from an acid load than did the Na\(^+/\)H\(^+\) exchanger blocker amiloride given alone.

The goal of the present study was to dissect the mechanisms of pH regulation in trout hepatocytes and specifically to provide evidence that a Na\(^+/\)HCO\(_3\)\(^-\) cotransporter is an integral component of this regulatory mechanism.

**Materials and methods**

**Experimental animals**

Rainbow trout *Oncorhynchus mykiss* (Walbaum) weighing 200–300 g were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada) and transported to the University of Ottawa in oxygenated water. Fish were maintained on a 12 h:12 h L:D photoperiod in fibreglass aquaria (water volume 500 l) supplied with flowing, aerated and dechlorinated City of Ottawa tap water. Trout were held at a water temperature of 15 °C and fed a commercial trout chow diet daily. Experiments were performed between July and September, following at least 2 months of acclimation to the holding facility.

**Cell isolation**

Trout were killed by a blow to the head, and hepatocytes were isolated according to the method of Mommsen et al. (1994). All solutions are modifications of the complete Hanks’ medium (in mmol l\(^{-1}\); NaCl, 136.9; KCl, 5.4; MgSO\(_4\), 0.8; NaHCO\(_3\), 5; Na\(_2\)HPO\(_4\), 0.33; KH\(_2\)PO\(_4\), 0.44; Hepes, 5; Na-Hepes, 5). The pH of all the experimental media was adjusted to 7.9 at room temperature (20 °C). A cannula was inserted into the hepatic portal vein, and blood was cleared from the liver by perfusion with a rinsing medium containing 1 mmol l\(^{-1}\) EGTA. The connection between the liver and the heart was cut to relieve vessel pressure during the initial stages of perfusion. Liver digestion was performed in situ using collagenase (Sigma type IV, 7.5 mg in 50 ml of Hanks’ medium). Liver digestion was indicated by a softening of the liver and dispersal of the tissue, at which time collagenase perfusion was terminated. The liver was then minced using a razor blade and filtered sequentially through 253 and 73 μm nylon mesh. The initial cell suspension was added to a 30 ml capacity centrifuge tube and washed by centrifugation (3 min at 1500 revs min\(^{-1}\) in a Sorvall RC centrifuge with SS-34 rotor) at 4 °C. Cells were washed repeatedly and finally resuspended and incubated in Hanks’ medium containing 1.5 mmol l\(^{-1}\) CaCl\(_2\), 3 mmol l\(^{-1}\) glucose, 2 mmol l\(^{-1}\) alanine, 2 mmol l\(^{-1}\) lactate and 1% (w/v) bovine serum albumin (BSA; ICN, clinical reagent grade, 98% fat free). Cells were counted using a Bright-Line haemocytometer (Spencer), and viabilities were determined using the Trypan Blue exclusion method (Mommsen et al., 1994). Cell populations in which viabilities were less than 70% post-isolation were discarded. Following the isolation procedure, cells were allowed to ‘rest’ for 2 h on ice before proceeding with the experiment. On the basis of a previous study by Furimsky et al. (1999b), hepatocytes isolated using this procedure maintained appropriate Na\(^+\) and Cl\(^-\) gradients.

**Measurement of pH**

Cell suspensions at a concentration of 10\(^6\) cells ml\(^{-1}\) were used in experiments. Cells were washed and resuspended in Hanks’ medium (pH 7.9) containing 1.5 mmol l\(^{-1}\) CaCl\(_2\) and then incubated in the same solution containing 1 μmol l\(^{-1}\) (3 μl of 1 mmol l\(^{-1}\) stock dissolved in dimethylsulphoxide, DMSO) of the ester form of the pH-sensitive dye 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF-AM) for 20 min. Cells were then rinsed and resuspended in the experimental medium. All experimental media were modifications of the resuspension medium described above with the exception that BSA was omitted. Experimental media contained one of (or a combination of) the following: the Na\(^+/\)H\(^+\) exchanger blocker amiloride (1 mmol l\(^{-1}\)), the anion transporter blocker 4,4’-diisothiocyanostilbene-2,2’-disulphonic acid (DIDS) (0.5 mmol l\(^{-1}\)) or the K\(^+\) channel blocker Ba\(^{2+}\) (1 mmol l\(^{-1}\) BaCl\(_2\)) or they lacked NaHCO\(_3\). All blockers were dissolved in the experimental medium at the experimental concentrations, with sonication being necessary for amiloride and DIDS. Membrane depolarization was induced by washing cells into a medium in which 44 mmol l\(^{-1}\) NaCl was replaced with KCl to give a final [K\(^+\)] of 50 mmol l\(^{-1}\). BCECF-loaded cells were incubated in their respective experimental medium for 20 min prior to experimentation. All experiments were performed at room temperature (20 °C) using a fluorescence spectrophotometer (Photon Technology International, South Brunswick, NJ, USA). Cell suspensions (3 ml) were stirred continuously in a quartz cuvette during experimentation. Data were obtained as the ratio of the pH-sensitive excitation wavelength (490 nm) and the pH-insensitive excitation wavelength (440 nm) with the emission wavelength held at 535 nm. Baseline pH values were established prior to the acid or base insult (see Fig. 1). Acid load was induced by the addition of sodium propionate (150 μl of 1 mol l\(^{-1}\) stock; 50 mmol l\(^{-1}\) final concentration) directly to the cuvette containing the cell suspension, and base load was induced by the addition of NH\(_4\)Cl (60 μl of 1 mol l\(^{-1}\) stock; 20 mmol l\(^{-1}\) final concentration) to the cell.
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Recovery rates were measured over the initial 200 s after the lowest measurement of pHi following the acid load or the highest measurement of pHi following base load.

Standard curves were constructed using an in situ calibration method. Cells were first loaded with BCECF, washed and then resuspended in calibration media of varying pH. The calibration medium was the same as the resuspension medium, except that the concentrations of NaCl and KCl were reversed (NaCl, 5.4 mmol l\(^{-1}\); KCl, 136.9 mmol l\(^{-1}\)) to represent intracellular ion concentrations, there was no BSA in the medium and it contained 14 \(\mu\)mol l\(^{-1}\) of the K\(^+\)/H\(^+\) ionophore nigericin (stock dissolved in 95 % ethanol). Ratios obtained were representative of different intracellular pH values, and a standard curve was constructed using the appropriate software.

**Intracellular buffer capacity**

The total apparent intracellular buffer capacity was measured in hepatocytes incubated in either HCO\(_3^-\)-containing medium (5 mmol l\(^{-1}\) NaHCO\(_3\)) or HCO\(_3^-\)-free medium. Buffer capacities (\(\beta\)) were determined using the following equation:

\[
\beta = \frac{[\text{Propionate}][10(pH_{\text{min}} - pK_a)]}{[1 + 10(pH_{\text{input}} - pK_i)][pH_{\text{cyt}} - pH_{\text{min}}]},
\]

where [Propionate] is 50 mmol l\(^{-1}\), the pKa of propionate at room temperature is 4.87, pH\(_{\text{min}}\) is minimal pH\(_{\text{cyt}}\) following acid load, pH\(_{\text{input}}\) is the pH of the medium (7.9) and pH\(_{\text{cyt}}\) is baseline pHi prior to acid load.

The rate of influx or extrusion (\(J\)) of H\(^+\) equivalents were determined as:

\[
J = k\beta,
\]

where \(k\) is the initial rate of recovery (pH units min\(^{-1}\)) from an acid or base load and \(\beta\) is the apparent intracellular buffer capacity (mmol H\(^+\) pH unit\(^{-1}\) l\(^{-1}\)).

**Na\(^+\) flux studies**

The rate of Na\(^+\) uptake was measured at room temperature (20 °C) using \(^{22}\)Na as a tracer. Experiments were performed on 4 ml of cell suspensions at a cytocrill of approximately 3 %, representing approximately 15\(\times\)10\(^6\) cells ml\(^{-1}\). Cells were incubated in their respective media for 20 min and further exposed to ouabain (4 \(\mu\)l of 1 mol l\(^{-1}\) stock in DMSO; final concentration 1 mmol l\(^{-1}\)) for 20 min to block Na\(^+\)/K\(^+\)-ATPase activity. Uptake measurements were initiated with the addition of \(^{22}\)Na directly to the cell suspension. At different time intervals, 300 \(\mu\)l samples were taken in triplicate and added to microcentrifuge tubes (1.5 ml capacity) containing ice-cold stopping buffer (110 mmol l\(^{-1}\) MgCl\(_2\), 10 mmol l\(^{-1}\) imidazole, pH 7.90) and washed twice in this same medium. Cell pellets were lysed by the addition of 0.5 ml of 0.005 % Triton-X and deproteinized with 0.5 ml of 1 % trichloroacetic acid (TCA). Following centrifugation, the supernatant was collected into 25 ml capacity plastic scintillation vials. Radioactivity was measured in a liquid scintillant (Safety Solve high-flash-point scintillation cocktail, RPI, Mount Prospect, IL, USA) using a 2500 TR liquid scintillation analyzer (Packard). Total \(^{22}\)Na uptake was measured at 5 min intervals for 20 min following addition of the isotope.

**Membrane potential**

Changes in membrane potential were determined using the fluorescent potentiometric slow-response dye Oxonol VI (Molecular Probes, Eugene, OR, USA). Unlike BCECF, this dye is not contained exclusively within cells during experimentation, but is free to move between the medium and the cytoplasm with changes in membrane potential. Increases in membrane potential are indicated by an increase in fluorescence. A 3 \(\mu\)l volume of a concentrated stock solution of the dye (500 mmol l\(^{-1}\) in 95 % ethanol) was added to the cell suspension (3 ml at 10\(^6\) cells ml\(^{-1}\)) to give a final concentration of approximately 500 mmol l\(^{-1}\). The dye was allowed to equilibrate with the cells for 15 min prior to the beginning of the experiment. The excitation wavelength was 614 nm and the
emission wavelength 646 nm; fluorescence was measured as above. The fluorescence intensities varied slightly from experiment to experiment, so data are presented as relative fluorescence, with all values being divided by the baseline fluorescence values.

**Statistical analyses**

Data are presented as the mean ± the standard error of the mean (S.E.M.). Each trial represents an experiment performed on a single cell preparation from one individual fish. Statistical analyses were conducted using either a one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls test for the pairwise analysis of means or a two-way ANOVA with repeated measures using the Student–Newman–Keuls test for the pairwise analysis of means.

**Results**

**Steady-state pH**

The different experimental treatments had varying effects on baseline pH, suggesting that different membrane transporters are tonically active under resting conditions (Table 1). All experimental media, although different, were adjusted to the same pH of 7.9. Amiloride and DIDS caused a decrease in baseline pH that was exaggerated when both were in the medium together. The significant decrease in baseline pH observed when extracellular HCO₃⁻ was removed from the experimental medium was enhanced by the addition of amiloride to this same medium. In all cases, the addition of Ba²⁺ to the medium decreased baseline pH.

**Intracellular buffer capacity**

The total apparent intracellular buffer capacity (β) was determined in hepatocytes under control conditions (medium containing 5 mmol l⁻¹ NaHCO₃) and in HCO₃⁻-free conditions. The buffer capacity in HCO₃⁻-containing media was 45.3±3.2 mmol H⁺ pH unit⁻¹ l⁻¹. The buffer capacity in HCO₃⁻-free medium was 32.7±1.5 mmol H⁺ pH unit⁻¹ l⁻¹. These values were considered when determining the influx or extrusion of H⁺ equivalents in the different experimental media.

**Recovery from acid loading**

The mechanism of pH recovery from an acid load involves a variety of membrane exchangers whose roles can be dissected pharmacologically (see Fig. 1A). The rate of proton extrusion following acid loading was decreased by the Na⁺/H⁺ exchanger blocker amiloride (1 mmol l⁻¹), as well as by DIDS (0.5 mmol l⁻¹), a compound that inhibits the activity of both the Cl⁻/HCO₃⁻ exchanger and the Na⁺/HCO₃⁻ cotransporter (Fig. 2). When amiloride and DIDS were combined in the experimental medium, the rate of proton extrusion following acid loading was decreased even further. Removal of HCO₃⁻ from the extracellular medium diminished the rate of proton extrusion following acid loading (Fig. 3). The K⁺ channel blocker Ba²⁺ also decreased proton extrusion, but to a lesser extent. When Ba²⁺ was added to the HCO₃⁻-free medium, the rate of proton extrusion did not differ from that in the HCO₃⁻-free medium alone. However, the addition of amiloride to the

<table>
<thead>
<tr>
<th>pHi</th>
<th>ΔpHi (acid)</th>
<th>% Recovery (acid)</th>
<th>ΔpHi (alkaline)</th>
<th>% Recovery (alkaline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.71±0.01</td>
<td>0.29±0.02</td>
<td>97.8±5.1</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>Amiloride (1 mmol l⁻¹)</td>
<td>7.62±0.01*</td>
<td>0.29±0.01</td>
<td>63.2±7.6*</td>
<td>0.39±0.02</td>
</tr>
<tr>
<td>DIDS (0.5 mmol l⁻¹)</td>
<td>7.61±0.02*</td>
<td>0.25±0.02</td>
<td>66.5±3.0*</td>
<td>0.43±0.02</td>
</tr>
<tr>
<td>Amiloride (1 mmol l⁻¹)+DIDS (0.5 mmol l⁻¹)</td>
<td>7.43±0.02*</td>
<td>0.22±0.02*</td>
<td>43.2±8.4*</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>Control</td>
<td>7.72±0.01</td>
<td>0.29±0.01</td>
<td>95.0±5.3</td>
<td>0.38±0.03</td>
</tr>
<tr>
<td>HCO₃⁻-free</td>
<td>7.67±0.01*</td>
<td>0.37±0.02*</td>
<td>77.5±4.0*</td>
<td>0.37±0.02</td>
</tr>
<tr>
<td>HCO₃⁻-free+Amiloride (1 mmol l⁻¹)</td>
<td>7.63±0.01*</td>
<td>0.35±0.02*</td>
<td>50.8±5.1*</td>
<td>0.35±0.02</td>
</tr>
<tr>
<td>Ba²⁺ (1 mmol l⁻¹)</td>
<td>7.62±0.02*</td>
<td>0.28±0.01</td>
<td>88.5±5.7</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>HCO₃⁻-free+Ba²⁺ (1 mmol l⁻¹)</td>
<td>7.64±0.01*</td>
<td>0.37±0.03*</td>
<td>67.5±6.1*</td>
<td>0.34±0.02</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.

The relative recoveries (% recovery back to baseline pH) were determined 8 min following intracellular acidification or alkalization.

* indicates a significant difference from respective control values.
Intracellular pH regulation in trout hepatocytes

HCO₃⁻-free medium decreased the rate of recovery significantly (P<0.05) compared with those same treatments (Fig. 3). The relative recoveries to baseline pHi at 8 min (the time at which full recovery usually occurred following an acid load under control conditions) showed that, with the exception of Ba²⁺ (1 mmol l⁻¹), the inhibitory effects of the different treatments were consistent over time (Table 1).

**Recovery from base loading**

The rate of influx of proton equivalents following base loading was decreased by the Na⁺/H⁺ exchange blocker amiloride and to a greater extent by the anion transport blocker DIDS (Fig. 4). Recovery from base loading was also dependent on the presence of extracellular HCO₃⁻, as shown by the decrease in recovery in its absence (Fig. 5). The rate of proton influx in HCO₃⁻-free medium that contained amiloride or Ba²⁺ did not differ from that in HCO₃⁻-free medium alone. The K⁺ channel blocker Ba²⁺ had no effect on the rate of recovery in normal medium. The relative recoveries 8 min following the base load were consistent with the inhibitory effects observed initially (Table 1). The degree of intracellular alkalization was similar in all treatments.

**Na⁺ fluxes**

The rate of unidirectional Na⁺ uptake into hepatocytes was calculated from the regression of total Na⁺ uptake at each time point from 5 to 20 min. The rate of Na⁺ uptake was unstable during the initial 5 min assessment period but remained constant in the subsequent 15 min period. The activity was not assumed to be zero at time zero because trapped extracellular fluids in the cell pellets or non-specific binding could increase baseline levels of ²²Na. Indeed, the regression analyses demonstrated a positive y-intercept. Thus, the rates of Na⁺ uptake were significantly decreased in hepatocytes in the presence of amiloride or DIDS (Fig. 6).

**Membrane depolarization**

The relationship between a HCO₃⁻-dependent transport protein and a K⁺ channel was investigated by observing the direct effects of cell depolarization on pHi. When cells were depolarized by the addition of 50 mmol l⁻¹ K⁺ to the extracellular medium, no effect on pHi was observed in either the normal or HCO₃⁻-free medium. The mean change in baseline pHi (ΔpHi) following cell depolarization in normal medium was −0.015±0.02, whereas that in HCO₃⁻-free medium was 0.001±0.01 (N=5).

**Membrane potential**

A slight decrease in fluorescence was observed immediately
after the addition of sodium propionate. This immediate decrease can be explained by dilution of the dye signal and does not reflect a change in membrane potential. The addition of an equal volume of saline (150 μl) to the medium had no effect other than decreasing the baseline fluorescence (by approximately 5%). During the recovery phase from acid loading, the hepatocyte membrane experienced an initial slight depolarization and then a hyperpolarization (the potential became more negative) that was diminished when DIDS was present in the medium (Fig. 7).

Discussion

The participation of Na+/H+ exchange and Na+/HCO3− cotransport in pH regulation has been reported in several mammalian cell types including hepatocytes (De la Rosa et al., 1999; Gleeson et al., 1989; Kikeri et al., 1990). Prior to the present study, the role of a Na+/HCO3− cotransporter in pH regulation in fish hepatocytes had not been examined specifically. Thus, the generally accepted model of pH regulation in fish hepatocytes (Walsh and Mommsen, 1992) incorporates only Na+/H+ and Cl-/HCO3− exchangers. Evidence from the present study, however, suggests that, in trout, the Na+/HCO3− cotransporter may play a significant role in H+ extrusion that is complementary to that of the Na+/H+ exchanger. Plasma HCO3− levels are low in teleosts (approximately 5–10 mmol l⁻¹) compared with those in mammals (approximately 25 mmol l⁻¹). Nevertheless, extracellular HCO3−, albeit at low concentrations, still appears to play a significant role in the ability of trout hepatocytes to regulate pH. Despite the low levels in fish plasma, HCO3−-dependent acid extrusion is not unheard of in fish tissues because evidence does exist for the role of a Na+-dependent Cl-/HCO3− exchanger in Atlantic cod (Gadus morhua) red blood cells (Berenbrink and Bridges, 1994) and in trout and carp (Cyprinus carpio) thrombocytes (Nikinmaa et al., 1999).

The specific roles of ion transporters such as the Na+/HCO3− cotransporter and the Cl-/HCO3− exchanger are often examined pharmacologically using stilbene derivatives such as 4-acetamido-4′-isothio-cyanato-stilbene-2,2′-disulfonic acid (SITS) and DIDS. These compounds are similar in structure, and both are potent inhibitors of anion transport. Binding of stilbene derivatives with the Na+/HCO3− cotransporter and Cl-/HCO3− exchanger is believed to involve an interaction with consensus sites on the transporters (Bartel et al., 1989; Okubo et al., 1994; Romero et al., 1998). In the study of Walsh (1986), addition of the anion transport blocker SITS increased baseline pH in trout hepatocytes, but did not influence the rate of recovery from acid or base loading. Similarly, SITS did not significantly affect acid extrusion in toadfish (Opsanus beta) hepatocytes after acid loading (Walsh, 1989). In a previous study performed in our laboratory, SITS did not affect baseline pH or recovery rates from acid or base loading in three teleost species (trout, eel, bullhead; M. Furimsky, unpublished results). In the present study, DIDS caused a decrease in baseline pH, significant decreases in pH recovery rates following acid and base loading (Table 1; Figs 2, 4) and decreased Na+ uptake rates (Fig. 6). The different results obtained using SITS versus DIDS are unexplained but may reflect their different chemical properties influencing binding interactions with the transporters on the teleost hepatocyte. Although it had no effect in the fish model, SITS did significantly inhibit recovery from an acid load, induced by gassing with 10% CO2 in rat hepatocytes (Fitz et al., 1992).

The pH recovery rate from acid loading in trout hepatocytes is amiloride-sensitive, indicating that the Na+/H+ exchanger is active in acid extrusion as it is in most cells types. Similar results were observed in hepatocytes of the gulf toadfish.
(Walsh, 1989) and black bullhead (Furimsky et al., 1999a). However, pH recovery was not totally abolished during blockade of the Na+/H⁺ exchanger, suggesting that another transporter may be involved in H⁺ extrusion. The study of Furimsky et al. (1999a) showed that a V-type H⁺-ATPase was not involved in recovery from acid loading, but suggested the contribution of another Na⁺-dependent mechanism because Na⁺ removal caused a 75% decrease in recovery rate compared with a 50% decrease in the presence of amiloride. Walsh (1986) did not observe such a difference in the rate of pH recovery between Na⁺-free medium and medium containing amiloride. The discrepancies between the two studies may be related to the different methods used to measure pH, namely the BCECF method (present study) that measures exclusively cytoplasmic pH compared with the DMO method (Walsh, 1986) that measures the pH of all intracellular compartments.

During blockade of anion transport using DIDS, an increase in baseline pH would be expected if DIDS were acting exclusively upon a Cl⁻/HCO₃⁻ exchanger as a result of accumulation of intracellular HCO₃⁻. Indeed, such an increase in baseline pH was observed previously after the removal of extracellular Cl⁻ (Furimsky et al., 1999a). The decrease in baseline pH and the inhibitory effects on proton extrusion rate observed in the present study indicate that a Na⁺/HCO₃⁻ cotransporter is present and does contribute to pH regulation during steady-state conditions and in response to acid loading (Table 1; Fig. 2). The importance of HCO₃⁻ in hepatocyte pH regulation is not restricted to its role as a significant intracellular buffer; it is also required as an anion to drive Na⁺/HCO₃⁻ cotransport. The reduction in the rate of proton extrusion observed following acid loading in HCO₃⁻-free medium is similar to that observed with DIDS, providing further evidence for the involvement of a Na⁺/HCO₃⁻ cotransporter (Fig. 3). The amount of intracellular HCO₃⁻ being removed to the medium as a result of cell metabolism may be sufficient to allow for some transport activity to occur. In mammals, in which the extracellular HCO₃⁻ concentration is much higher than in fish, the effect of removal from the medium has a more pronounced effect: it significantly decreases baseline pH and recovery from acid loading (Gleeson et al., 1989). Studies on perfused rat liver, however, have provided evidence that metabolic HCO₃⁻ is participating in pH regulation to a certain extent, since H⁺ efflux in a HCO₃⁻-free medium is decreased even further when carbonic anhydrase is inhibited by acetazolamide (Vidal et al., 1998).

In rat hepatocytes, the Na⁺/HCO₃⁻ cotransporter is believed to be linked to a pH-sensitive K⁺ channel (Fitz et al., 1992). In the present study, the blockade of K⁺ channels on trout hepatocytes had a similar effect on reducing the rate of proton extrusion following an acid load as the removal of extracellular HCO₃⁻, whereas addition of Ba²⁺ and removal of HCO₃⁻ had no cumulative effects on recovery. These data provide evidence that the presumptive Na⁺/HCO₃⁻ cotransporter on trout hepatocytes, as in rat, is linked to K⁺ conductance. The model of Na⁺/HCO₃⁻ cotransporter activation proposed by Fitz et al. (1992) was developed by demonstrating that blockade of K⁺ channels with Ba²⁺ decreased the rate of recovery from acid loading and that cell depolarization caused an increase in pH that did not occur when extracellular HCO₃⁻ was removed. Such a direct link could not be established in our study, suggesting that the effects of K⁺ channel blockade on baseline pH and recovery from an acid load may occur by a different mechanism.

Of the different HCO₃⁻ transporters, Na⁺-dependent and Na⁺-independent Cl⁻/HCO₃⁻ exchangers are electroneutral, whereas the Na⁺/HCO₃⁻ cotransporter can have a Na⁺:HCO₃⁻ stoichiometry of 1:3, 1:2 or 1:1, depending on tissue (Boron et al., 1997). In the proximal tubule of the mammalian kidney, a 1Na⁺:3HCO₃⁻ cotransporter is crucial in HCO₃⁻ reabsorption from the lumen. The stoichiometry of Na⁺/HCO₃⁻ cotransport is not clear when it is participating in acid extrusion, but it is suggested to be either electroneutral or 1:2 (Boron et al., 1997). Although we were not specifically examining the stoichiometry of the putative cotransporter, it was of interest to determine whether recovery from acid loading was an electrogenic process. If recovery from acid loading was occurring only by way of a Na⁺/H⁺ exchanger, no change in membrane potential would be expected. However, hyperpolarization (a more negative membrane potential) should occur during the activation of an electronegative Na⁺/HCO₃⁻ cotransporter. The results of the present study using the potentiometric dye Oxonol VI indicated that, during the time of recovery following acid loading, the membrane potential did indeed decrease and that this decrease was blocked by DIDS (Fig. 6).

In response to intracellular base loading induced by NH₄Cl, normal pH recovery principally involves the Cl⁻/HCO₃⁻ exchanger, but may also involve, to a lesser extent, the Na⁺/H⁺ exchanger and the Na⁺/HCO₃⁻ cotransporter (Fig. 3). The decrease in proton influx rate observed in the presence of amiloride following a base load suggests that, under control conditions, the protons being retained when the Na⁺/H⁺ exchanger turns off contribute to buffering intracellular base. The fact that membrane transporters are tonically active means that they can contribute to pH regulation not only by increasing their activity but also by decreasing their activity under certain conditions. When both DIDS and amiloride were in the medium, recovery rates did not differ from that when DIDS was in the medium alone, suggesting that the Cl⁻/HCO₃⁻ exchanger is dominant over the Na⁺/H⁺ exchanger in its role in base extrusion. Extracellular HCO₃⁻, as in recovery from an acid load, is also important in recovery from a base load (Fig. 4). When intracellular HCO₃⁻ levels are diminished as a result of washing the cells into a HCO₃⁻-free medium, this has two consequences on the cell’s ability to regulate pH: not only is the apparent intracellular buffer capacity decreased, but Cl⁻/HCO₃⁻ exchange is also inhibited by the very low concentrations of intracellular HCO₃⁻ needed to drive the transporter. Blocking K⁺ channels with Ba²⁺ had no effect on recovery from a base load.

The interplay and cooperation that exist between different membrane ion transporters (pumps, channels) have been well
documented in different experimental models. Thus, the fact that a Na\(^+\)/HCO\(_3^-\) cotransporter appears to participate in pH\(_i\) regulation in the trout hepatocyte, together with a Na\(^+\)/H\(^+\) exchanger and a Cl\(^-\)/HCO\(_3^-\) exchanger, is not surprising. However, while it appears that the model of pH\(_i\) regulation in the trout hepatocyte may be more similar to the mammalian model than previously believed, significant differences still remain, namely the degree of reliance on extracellular HCO\(_3^-\) and the relationship that exists between Na\(^+\)/HCO\(_3^-\) cotransport and the activity of a K\(^+\) channel. Different organisms have evolved to live in different environments, and the mechanisms by which different tissues deal with environmental changes have evolved accordingly.

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