J Comp Physiol B (2000) 170: 175–184

ORIGINAL PAPER

C. M. Wood · P. Pärt

Intracellular pH regulation and buffer capacity in CO_2/HCO_3^--buffered media in cultured epithelial cells from rainbow trout gills

Accepted: 30 November 1999

Abstract The influence of a CO_2/HCO_3^--buffered medium on intracellular pH regulation of gill pavement cells from freshwater rainbow trout was examined in monolayers grown in primary culture on glass coverslips; intracellular pH (pH_i) was monitored by continuous spectrofluorometric recording from cells loaded with 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxy-fluoroscein. When cells in HEPES-buffered medium at normal pH = 7.70 were transferred to normal CO_2/HCO_3^--buffered medium {$P_{CO_2} = 3.71$ mmHg, $[HCO_3^-] = 6.1$ mmol l^{-1}, extracellular pH (pH_e) = 7.70}, they exhibited a brief acidosis but subsequently regulated the same pHi (~7.41) as in HEPES. Buffer capacity (β) increased by the expected amount (5.5–8.0 slykes) based on intracellular $[HCO_3^-]$, and was unaffected by most drugs and treatments. However, after transfer to high $P_{CO_2} = 11.15$ mmHg, $[HCO_3^-] = 18.2$ mmol l^{-1} at the same $pH_e = 7.70$, the final regulated pHi was elevated (~7.53). The rate of correction of alkalosis caused by washout of this high P_{CO_2}, high-HCO_3^- medium was unaffected by removal of extracellular Cl^-. Removal of extracellular Na^+ lowered resting pH_i and greatly inhibited the rate of pH_i recovery from acidosis. Bafilomycin A_1 (3 μmol l^{-1}) had no effect on these responses. However amiloride (0.2 mmol l^{-1}) inhibited recovery from acidosis caused by washout of an ammonia prepulse, but did not affect resting pH_i, the latter differing from the response in HEPES where amiloride also lowered resting pH_i. Similarly 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, sodium salt (0.1 mmol l^{-1}) did not affect resting pH_i but slowed the rate of recovery from acidosis, though to a lesser extent than amiloride. Removal of extracellular Cl^- also slowed the rate of recovery but greatly increased β by an unknown mechanism; when this was taken into account, H^+ extrusion rate was unaffected. These results are consistent with the presence of Na^+-$(HCO_3^-)_N$ cotransport and/or Na^+-dependent HCO_3^-/Cl^- exchange, in addition to Na^+/H^+ exchange, as mechanisms contributing to "housekeeping" pH_i regulation in gill cells in CO_2/HCO_3^- media, whereas only Na^+/H^+ exchange is seen in HEPES. Both Na^+-independent Cl^-/HCO_3^- exchange and V-type H^+-ATPase mechanisms appear to be absent from these cells cultured in isotonic media.

Key words Fish gills · Cultured epithelial cells · Intracellular pH · Buffer capacity · Bicarbonate buffer

Abbreviations *BCECF* 2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxy-fluoroscein · *[^{14}C]DMO* carbon-14 labelled 5,5-dimethyl-2,4-oxazolidinedione · P_{CO_2} partial pressure of carbon dioxide · pH_e extracellular pH · *pHi* intracellular pH · *HEPES N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] · *SITS* 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, sodium salt

Introduction

Recently we have developed methods based on spectrofluorometry to measure intracellular pH (pH_i) and buffer capacity (β) in pavement cells from the gill epithelium of freshwater rainbow trout grown as a monolayer in primary culture (Pärt and Wood 1996). A Na^+/H^+-exchange mechanism was identified which contributed both to the regulation of resting pH_i and the rapid recovery from intracellular acidosis. In order to simplify the analysis, these experiments were performed in a medium nominally free of HCO_3^- and buffered with

Communicated by L. C.-H. Wang

C. M. Wood (✉) · P. Pärt[1]
Department of Biology,
McMaster University,
1280 Main Street West,
Hamilton, Ontario, Canada L8S 4K1
e-mail: woodcm@mcmail.cis.mcmaster.ca
Tel.: +1-905-525-9140 ext. 23537; Fax: +1-905-522-6066

Present address:
[1]European Commission,
Joint Research Centre, Environment Institute,
TP 460, I-21020 Ispra (VA), Italy

N-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] (HEPES) so as to minimize or eliminate any HCO_3^--dependent processes which might have masked the activity of the Na^+/H^+ antiport. Leguen et al. (1998) similarly reported the presence of Na^+/H^+ exchange in primary cultures of rainbow trout gill pavement cells grown and tested in HEPES-buffered, HCO_3^--free media.

The present study examines the more complex and natural situation where HCO_3^- and partial pressure of carbon dioxide (P_{CO_2}) are present in the medium at physiological levels, and HEPES is absent. There are several reasons why both qualitative and quantitative differences may be seen in the presence of HCO_3^- and absence of HEPES. Firstly, HCO_3^- is an important intracellular buffer and therefore we might expect the buffer capacity (β) of the cells to be greater (Roos and Boron 1981). Secondly, an additional complication here is the report by Walsh (1990) that HEPES may penetrate some fish cells. Assuming the same relative penetration of branchial cells by HEPES as reported by Walsh (1990) for hepatocytes (to about 10% of extracellular concentration), then the β value reported in our earlier work (Pärt and Wood 1996) would have been overestimated by about 5%. However, a third, more important concern is the potential interaction of HEPES with both intracellular and transport processes, such as the blockade of anion channels (e.g., Marshall and Hanrahan 1991). Fourthly, changes in P_{CO_2} are perhaps the commonest cause of acid-base disturbance in vivo (''respiratory acidosis'' and ''respiratory alkalosis''), and are ultimately compensated by HCO_3^- accumulation or loss (e.g., Claiborne 1998). Lastly, at least three different HCO_3^- transport mechanisms which may contribute to pH_i regulation have now been described in various other cell types of higher vertebrates (see Frelin et al. 1988; Madshus 1988; Ilundain 1992; Lubman and Crandall 1991, 1992; Krapf and Alpern 1993; Peral et al. 1995; Seki et al. 1996). These include electroneutral Na^+-independent Cl^-/HCO_3^- exchange (a usually acidifying antiport), electroneutral Na^+-coupled HCO_3^-/Cl^- exchange (a usually alkalinizing antiport), and electrogenic Na^+-$(HCO_3^-)_N$ co-transport (an alkalinizing or acidifying symport). In the nominal absence of CO_2/HCO_3^- in the medium, their potential contributions to pH_i regulation would be greatly reduced or eliminated. Indeed Thomas (1989) has counseled that ''he who works in HCO_3^--free media risks studying cellular and molecular pathology rather than physiology!''

The present study had several objectives. The first was to assess whether the resting levels of pH_i and intracellular β were the same in cultured gill pavement cells in CO_2/HCO_3^--buffered media as we and others have earlier described in HEPES-buffered media (Pärt and Wood 1996; Leguen et al. 1998). The second was to assess whether the contribution of Na^+/H^+ exchange to the regulation of resting pH_i and the recovery from ammonia prepulse acidosis was the same, and to detect the possible contributions of HCO_3^--dependent and Cl^--dependent processes. The third was to characterize the pH_i-regulatory response to CO_2-induced acidosis. Finally, we looked for the presence of processes such as HCO_3^-/Cl^- exchange which might contribute to recovery from intracellular alkalosis. There is a considerable body of circumstantial evidence that some type of Cl^-/HCO_3^- exchange in the gills of freshwater fish contributes to Cl^- uptake from the water and the coupled excretion of basic equivalents, but considerable uncertainty exists as to its exact cellular location or mechanism (reviewed by Goss et al. 1992, 1995; Kirschner 1997; Perry 1997; Claiborne 1998).

Materials and methods

Basic methods

Freshwater rainbow trout stocks, gill cell isolation methods, and culture techniques to obtain confluent pavement cell monolayers on glass coverslips were identical to those described in detail in the previous study (Pärt and Wood 1996). In brief, gill cells were grown in primary culture in flasks for 8–10 days, by which time only pavement cells persisted. They were then harvested by trypsination and replated on glass cover-slips for intracellular pH experiments 2–5 days later. For the latter, the cover-slip was mounted in a flow-through cell of a Perkin-Elmer LS-50 spectrofluorometer, and methods for direct continuous recording of pH_i by the BCECF [2′,7′-*bis*(2-carboxyethyl)-5(6)-carboxy-fluoroscein] technique (Rink et al. 1982) and calibration by the high-$[K^+]$-nigericin technique (Boyarsky et al. 1988) were identical to those of the previous study (Pärt and Wood 1996). The ammonia prepulse technique (Boron and De Weer 1976), the determination of intracellular buffer capacity (Roos and Boron 1981), and the statistical analysis and display of data also employed the same methods as outlined by Pärt and Wood (1996). Only details specific to the present study are given here.

Control of P_{CO_2}

Leibowitz L-15 medium (plus various additives) supplemented with 4 mmol l^{-1} $NaHCO_3$, and an atmosphere of 0.4–0.6% CO_2 (approximately 3.75 mmHg) in air were used throughout the 10- to 15-day period during which cells were cultured first in flasks and then on cover-slips in the incubator (19 ± 1 °C). The incubator (Forma Scientific, Marietta, Ohio, USA). was fitted with a CO_2-stat. In the pH_i experiments, precision CO_2/air mixtures were obtained by means of Wösthoff 301a-F gas mixing pumps (Bochum, Germany), and pHs were verified using Radiometer precision buffers and Radiometer GK2401C electrodes coupled to Radiometer pHm 84 Meters (Copenhagen, Denmark). Total CO_2 measurements, from which HCO_3^- concentrations could be calculated, were made with a Corning 965 total CO_2 analyzer (Essex, UK). Media were thoroughly gassed with the appropriate humidified mixture in a temperature-controlled manifold. From there, they were pumped directly via the Gilson Minipuls peristaltic pump (Villiers-Le-Bel, France) in a closed system to the thermostatted cuvette (19 ± 0.1 °C) in the Perkin-Elmer LS-50 spectrofluorometer (Beaconsfield, UK). Control P_{CO_2} was 3.71 mmHg, and an experimental high P_{CO_2} of 11.15 mmHg was also used. Measurements of media pH and total CO_2 in the manifold and the thermostatted cuvette were identical, indicating no loss of P_{CO_2} over the short transit distance. The glass cover-slip was mounted at a 45° angle against the excitation light beam by means of a custom-built holding device which incorporated inflow and outflow ports for the flow-through perfusion of the cuvette. Normal perfusion rate was 1 ml min^{-1}, but was increased to 30 ml min^{-1} for 60 s at times of solution changeover, which ensured 95% washout in 15 s, and complete renewal by 60 s.

Media and drugs

Medium 1

The control CO_2/HCO_3^--buffered medium used in pH_i trials was a modified Cortland salmonid saline (Wolf 1963) with the following composition (in mmol l^{-1}): NaCl 124.1, KCl 5.0, $CaCl_2 \cdot 2H_2O$ 1.6, $MgSO_4 \cdot 7H_2O$ 0.9, $NaH_2PO_4 \cdot H_2O$ 3.0, glucose 5.5. Prior to use, the medium was thoroughly gassed with a $P_{CO_2} = 3.71$ mmHg (in air) at the experimental temperature and then adjusted to pH 7.70 with $NaHCO_3$, resulting in a measured HCO_3^- concentration of 6.1 mmol l^{-1}.

Medium 2

The high CO_2/HCO_3^--buffered medium was made up identically but gassed with a $P_{CO_2} = 11.15$ mmHg (in air). After titration to pH 7.70 with $NaHCO_3$, the measured HCO_3^- concentration was 18.2 mmol l^{-1}.

Medium 3

The Na^+-free CO_2/HCO_3^--buffered medium was made up identically to medium 1, but with all sodium salts replaced with their choline counterparts, and pH adjusted to 7.70 with choline bicarbonate. Choline was used in accord with Graber et al. (1991), Lubman and Crandall (1992), and Peral et al. (1995).

Medium 4

The Cl^--free CO_2/HCO_3^- buffered medium was again made up identically to medium 1, but with all chloride salts replaced by their gluconate counterparts. In ammonia prepulse experiments in this medium, $(NH_4)_2SO_4$ was used instead of NH_4Cl.

Medium 5

The HEPES-buffered Cortland salmonid saline was nominally HCO_3^--free and gassed only with air; in practice, routine measurements with the Corning 965 analyzer indicated that HCO_3^- concentration was below the effective detection limit of 0.2 mmol l^{-1}. This medium had the following composition (in mmol l^{-1}): NaCl 133.0, KCl 5.0, $CaCl_2 \cdot 2H_2O$ 1.6, $MgSO_4 \cdot 7H_2O$ 0.9, $NaH_2PO_4 \cdot H_2O$ 3.0, glucose 5.5, HEPES 6.0. The pH was adjusted to 7.70 with NaOH.

Medium 6

The Cl^--free HEPES-buffered medium was made up identically to medium 5, but with all chloride salts replaced by their gluconate counterparts.

The origins of all chemicals and the preparation of SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, sodium salt), amiloride (hydrochloride), bafilomycin A_1, and nigericin (for calibration) were as described by Pärt and Wood (1996).

Results

When cultured epithelial cells from rainbow trout gills were transferred from HEPES-buffered media (nominally HCO_3^--free, $P_{CO_2} = 0.03$ mmHg) to control CO_2/HCO_3^- buffered media [$P_{CO_2} = 3.71$ mmHg, $HCO_3^- = 6.1$ mmol l^{-1} at unchanged extracellular pH (pH_e) = 7.70], there was a small initial drop in pH_i due to CO_2 entry (Fig. 1). However pH_i rapidly recovered and within 6–8 min had stabilized at a value (7.41 ± 0.03, $n = 20$) which was not significantly different from that in HEPES (7.42 ± 0.03, $n = 20$). However, in separate experiments, when the same transfer was made from HEPES to the high-CO_2/HCO_3^--buffered media ($P_{CO_2} = 11.15$ mmHg, $HCO_3^- = 18.2$ mmol l^{-1} at unchanged $pH_e = 7.70$), the initial acidification was greater, but pH_i recovered to a value (7.53 ± 0.02, $n = 19$) which was significantly higher

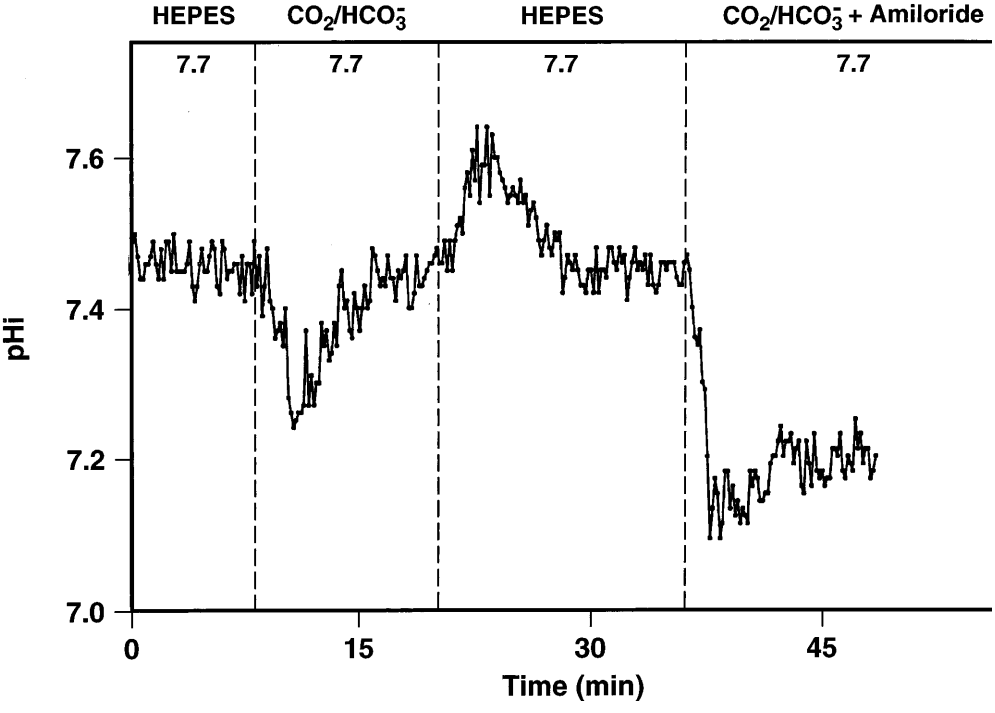

Fig. 1 Representative original recording of intracellular pH (pH_i) showing the acidification and subsequent recovery to the same pH_i which occurs when cultured gill cells are transferred from a HEPES-buffered medium *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] (pH = 7.70) to a CO_2/HCO_3^--buffered medium (pH = 7.70, partial pressure of carbon dioxide (P_{CO_2}) = 3.71 mmHg, $[HCO_3^-] = 6.1$ mmol l^{-1}), the alkalinization and subsequent recovery to the same pH_i which occurs when transferred back to HEPES, and the substantially greater acidosis and inhibited rate of recovery which occurs when transferred to the same CO_2/HCO_3^- medium containing 0.2 mmol l^{-1} amiloride

Table 1 The influence of various treatments upon resting intracellular pH (pH_i) in gill cells incubated in CO_2/HCO_3^--buffered medium at control partial pressure of carbon dioxide (P_{CO_2}) = 3.71 mmHg and $[HCO_3^-]$ = 6.1 mmol l^{-1}. Means ± 1 SEM (*SITS* 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid, sodium salt)

	Control	Treatment	*n*
Na^+-free	7.39 ± 0.09	7.17 ± 0.11*	5
Amiloride (0.2 mmol l^{-1})	7.45 ± 0.04	7.37 ± 0.10	5
Cl^--free	7.52 ± 0.03	7.59 ± 0.03	6
SITS (0.1 mmol l^{-1})	7.52 ± 0.03	7.50 ± 0.02	4
Bafilomycin A_1 (3 μmol l^{-1})	7.53 ± 0.05	7.49 ± 0.06	6

* Indicates significance difference ($P < 0.05$) from the paired control value

than that in HEPES (7.42 ± 0.02, $n = 19$). An example can be seen in the first portion of Fig. 3, although that experiment had another objective (see below). Thus, the cells regulate the same pH_i at normal physiological levels of P_{CO_2} and HCO_3^- as they do in HEPES, but not when P_{CO_2} and HCO_3^- are elevated to rather abnormal levels representative of compensated hypercapnia.

Further experiments concentrated on responses under normal P_{CO_2} and HCO_3^- conditions. Resting pH_i was significantly reduced by the withdrawal of Na^+ but not by the application of the Na^+/H^+ exchange blocker amiloride (0.2 mmol l^{-1}; Table 1). However, resting pH_i was not altered by application of the vacuolar-type H^+-ATPase blocker bafilomycin A_1 (3 μmol l^{-1}), withdrawal of Cl^-, or application of the anion exchange blocker SITS (0.1 mmol l^{-1}; Table 1). Overall, these results were similar to those seen previously in HEPES-buffered media (Pärt and Wood 1996), apart from the fact that pH_i did not fall significantly with amiloride in CO_2/HCO_3^--buffered media. They suggest that regulation of resting pH_i under physiological levels of P_{CO_2} and HCO_3^- is Na^+-dependent, anion-independent, and not associated with vacuolar ATPase activity.

In view of the equivocal effect of amiloride and the apparent absence of anion-dependent mechanisms (despite the presence of HCO_3^-) at resting pH_i, we tested whether their presence could be seen under conditions of mild CO_2-induced acidification. The protocol illustrated in the latter part of Fig. 1 was employed – in the presence of various experimental treatments. The degree of initial acidification ΔpH_i) was significantly increased approximately two-fold in the presence of amiloride (0.2 mmol l^{-1}), and the rate of pH_i recovery was reduced by over 80% (Fig. 1, Table 2). In contrast, SITS (0.1 mmol l^{-1}) and bafilomycin A_1 (3 μmol l^{-1}) had no effect on the response (Table 2). However, withdrawal of Cl^- reduced the degree of initial acidification by about 50% without significantly altering the pH_i recovery rate (Table 2). These results indicate that Na^+/H^+ exchange is very important in correcting small deviations in pH_i in normal CO_2/HCO_3^--buffered media, and that neither V-type ATPase-dependent nor anion-dependent mechanisms are involved, though the reduced degree of acidification in Cl^--free media complicates the latter interpretation.

The ammonia prepulse technique was employed to examine the dynamics of pH_i regulation under normal CO_2/HCO_3^- conditions in response to a larger deviation in pH_i, to allow direct comparison with regulation in HEPES-buffered media, and to provide estimates of intracellular buffer capacity. The protocol illustrated in the example of Fig. 2 was utilized, with the pulse being given first under a treatment condition (in this case control), and then repeated with the treatment plus the added presence of amiloride (0.2 mmol l^{-1}) during the washout. The latter served to reveal the possible effects of anion-dependent mechanisms once the contribution of the Na^+/H^+ exchanger had been largely removed.

The response to ammonia prepulse (30 mmol l^{-1}, 6 min; Fig. 2) under normal CO_2/HCO_3^- conditions was almost identical to that reported previously in HEPES-buffered media (Pärt and Wood 1996). The pulse exposure produced an abrupt rise in pH_i ($\Delta pH_i = +0.6$–0.8 units) followed by a gradual fall. When ammonia was washed away, the cells immediately became very acidic ($\Delta pH_i = -0.6$ to 0.8 units relative to the original resting value), followed by a rapid recovery. The recovery rate was much more rapid than seen earlier with transfer to CO_2/HCO_3^--buffered media (cf. Tables 2 and 3). When the protocol was repeated on the same cells with the addition of 0.2 mmol l^{-1} amiloride to the washout medium, the extent of acidosis was not significantly altered, but the pH_i recovery rate was reduced by over 80% (Table 3). Again these results were similar to those seen previously in HEPES media, indicating that Na^+/H^+ exchange is the major mechanism responsible for recovery in severe acidosis, even in the presence of physiological levels of HCO_3^-.

However, unique to the CO_2/HCO_3^--buffered media, SITS (0.1 mmol l^{-1}) also substantially and significantly reduced the rate of pH_i recovery by about 65%, without significantly altering the extent of initial acidosis (Table 3). The addition of amiloride in the presence of

Table 2 The influence of various treatments upon the degree of acidification and the rate of pH_i recovery after transfer of gill cells from HEPES-buffered medium *N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid] to P_{CO_2}/HCO_3^--buffered medium at control P_{CO_2} = 3.71 mmHg and $[HCO_3^-]$ = 6.1 mmol l^{-1}. Means ± SEM. Amiloride and bafilomycin tests were also performed with paired internal controls (e.g., Fig. 1), with the same statistical results

	ΔpH_i	pH_i recovery rate (pH units min^{-1})	*n*
Control	−0.218 ± 0.025	0.068 ± 0.010	5
Amiloride (0.2 mmol l^{-1})	−0.394 ± 0.061*	0.010 ± 0.004*	15
Cl^--free	−0.098 ± 0.010*	0.067 ± 0.011	6
SITS (0.1 mmol l^{-1})	−0.160 ± 0.015	0.085 ± 0.008	4
Bafilomycin A_1 (3 μmol l^{-1})	−0.251 ± 0.025	0.073 ± 0.014	6

* Indicates significant difference ($P < 0.05$) from the control value

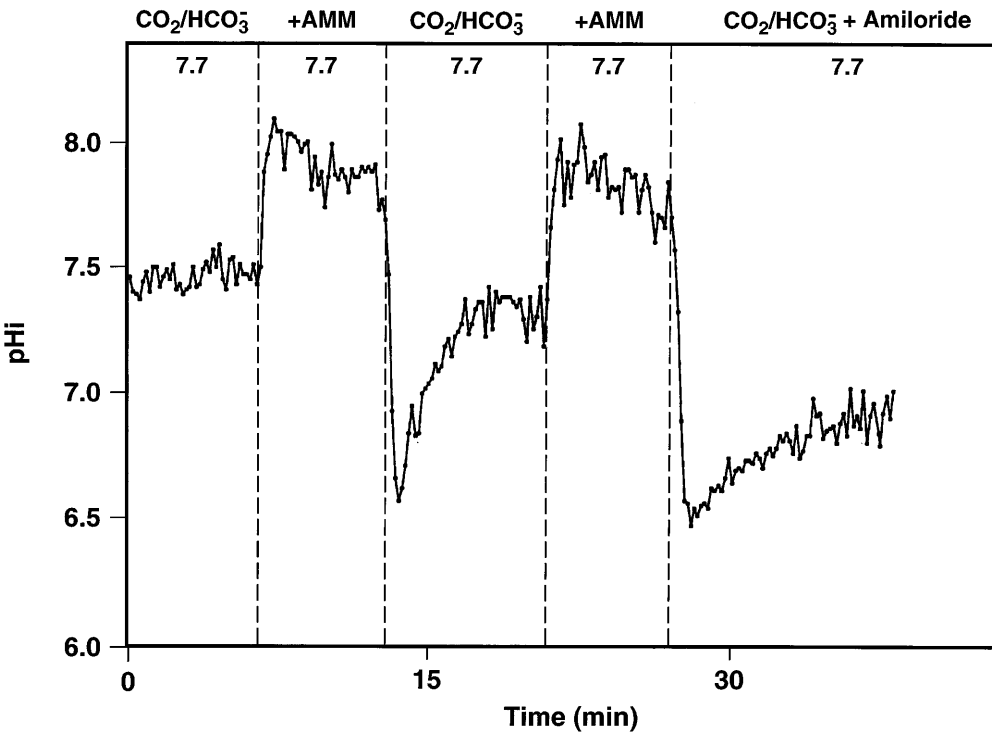

Fig. 2 Representative original recording of pH_i responses to ammonia "prepulses" (6 min exposure to 30 mmol l^{-1} NH_4Cl, followed by washout) of cultured gill cells kept in a CO_2/HCO_3^--buffered medium (pH = 7.70, P_{CO_2} = 3.71 mmHg, $[HCO_3^-]$ = 6.1 mmol l^{-1}) throughout the experiment. Note the rapid alkalinization which occurs immediately upon exposure to ammonia, the subsequent slow correction, the immediate acidification which occurs upon washout, and the rapid subsequent recovery. After the second ammonia exposure, the presence of amiloride (0.2 mmol l^{-1}) greatly inhibited the rate of recovery

SITS further reduced the rate of pH_i recovery (a significant effect) to a value which was lower (88% inhibition; not significant) than that seen with amiloride alone (Table 3). Note that the low *n* number may have contributed to the lack of significance.

In initial trials in Cl^--free media, an ammonia prepulse of 30 mmol l^{-1} (15 mmol l^{-1} $(NH_4)_2SO_4$) produced an acidification of substantially smaller magnitude than that seen with the other treatments. The effect was reminiscent of the reduced acidification associated with Cl^--free media in the transfer to CO_2/HCO_3^- experiments (Table 2). Therefore, in order to compare pH_i recovery rates at the same degree of acidosis, an ammonia prepulse of 60 mmol l^{-1} (30 mmol l^{-1} $(NH_4)_2SO_4$) was used. Under these conditions, Cl^--free media reduced the pH_i recovery rate significantly below that seen in control media, and the addition of amiloride in the continued absence of Cl^- further reduced the recovery rate (Table 3). Indeed, the value with amiloride plus Cl^--free (94% inhibition) was significantly below that seen with amiloride alone.

The pH_i responses to both the onset and the washout of the ammonia prepulse provided estimates of the buffer capacity (β) of the cells over somewhat different ranges of pH_i (onset = 7.4–8.2; washout = 7.9–6.5 – see Roos and Boron 1981; Pärt and Wood 1996). In the case of CO_2/HCO_3^--buffered media, the β values will include both bicarbonate and non-bicarbonate components. Under control conditions, β values were significantly greater by 5.5–8.0 slykes in CO_2/HCO_3^--buffered media than in HEPES-buffered media (Table 4). β values were also considerably greater in the higher pH_i range (pulse onset) than in the lower range (pulse washout), a difference expected due to the higher HCO_3^- contribution at higher pH_i. This difference, while still significant, was greatly reduced in the nominal absence of HCO_3^- (i.e.,

Table 3 The influence of various treatments upon the degree of acidification (relative to original resting pH_i) and the rate of pH_i recovery in gill cells after washout of a 6-min ammonia prepulse. Means ± 1 SEM. An ammonia pulse of 30 mmol l^{-1} was used throughout, except in the case of Cl^--free tests, where 60 mmol l^{-1} was employed to achieve a comparable degree of acidification. All experiments were performed in CO_2/HCO_3^--buffered media at normal P_{CO_2} = 3.71 mmHg and $[HCO_3^-]$ = 6.1 mmol l^{-1}, except in the case of HEPES (nominally HCO_3^- free, P_{CO_2} = 0.03 mmHg)

	n	Treatment		Treatment plus amiloride (0.2 mmol l^{-1})	
		ΔpH_i	pH_i recovery rate (pH units min^{-1})	ΔpH_i	pH_i recovery rate (pH units min^{-1})
Control	5	−0.770 ± 0.039	0.288 ± 0.021	−0.792 ± 0.035	0.050 ± 0.007$^+$
SITS (0.1 mmol l^{-1})	4	−0.540 ± 0.075	0.081 ± 0.004*	−0.612 ± 0.080	0.035 ± 0.003$^+$
Cl^--free	6	−0.820 ± 0.066	0.149 ± 0.028*	−0.722 ± 0.059	0.018 ± 0.003$^{+,*}$
HEPES (from Pärt and Wood 1996)	5	−0.630 ± 0.085	0.250 ± 0.030	−0.700 ± 0.078	0.045 ± 0.010$^+$

* Indicates significant difference ($P < 0.05$) from corresponding control value; $^+$ from corresponding treatment value

Table 4 The influence of various treatments upon the apparent intracellular buffer capacity (β) of gill cells as estimated from the pH_i response to the onset and washout of an ammonia prepulse. Means ± 1 SEM. An ammonia pulse of 30 mmol l^{-1} was used throughout, except in the case of Cl^--free tests, where 60 mmol l^{-1} was employed to achieve comparable pH_i disturbances. All experiments were performed in CO_2/HCO^-_3-buffered media at normal P_{CO_2} = 3.71 mmHg and $[HCO^-_3]$ = 6.1 mmol l^{-1}, except in the case of HEPES (nominally HCO^-_3 free, P_{CO_2} = 0.03 mmHg)

	n	β (slykes)	
		Pulse onset (pH_i range = 7.4–8.2)	Pulse washout (pH_i range = 7.9–6.5)
Control	5	22.72 ± 0.92	15.63 ± 1.04**
SITS (0.1 mmol l^{-1})	4	19.22 ± 2.09	15.86 ± 1.73**
Cl^--free	6	84.23 ± 10.86*	55.48 ± 7.98*·**
HEPES (from Pärt and Wood 1996)	5	14.64 ± 0.65*	12.16 ± 0.55*·**

* Indicates significant difference ($P < 0.05$) from corresponding control value

** Indicates significant difference ($P < 0.05$) from corresponding "pulse onset" value

HEPES; Table 4). SITS (0.1 mmol l^{-1}) had no significant effect on the β values, but three- to-four-fold greater estimates were obtained in the presence of Cl^--free media (Table 4).

It must be noted that these very high β estimates under Cl^--free conditions were obtained using an ammonia prepulse of 60 mmol l^{-1} rather than 30 mmol l^{-1}. In preliminary experiments with a 30 mmol l^{-1} ammonia prepulse in Cl^--free media, the β values (33–37 slykes) were still elevated relative to those in normal media (16–23 slykes), but the difference was not as great as with 60 mmol l^{-1}. We therefore suspected that at 60 mmol l^{-1} total ammonia, β was overestimated due to incomplete equilibration of NH_3 across the cell membranes and/or some pathological effect. High-P_{CO_2} washout experiments provided another opportunity to calculate β values (over the upper pH_i range only), but without the possible complications associated with high ammonia levels. In paired trials ($n = 7$), these yielded values of 25.53 ± 2.24 slykes under control conditions (very similar to the control estimates in Table 4) and 33.19 ± 2.77 slykes in Cl^--free media, a significantly higher value. These results confirm that in the presence of CO_2/HCO^-_3, the absence of Cl^- really does elevate intracellular β. Furthermore, since only non-HCO^-_3 buffer capacity is measured in a CO_2 washout experiment, this result demonstrates that at least a portion of the elevated β seen in Cl^--free media is due to elevated non-HCO^-_3 buffer capacity, and cannot be explained solely by elevated intracellular $[HCO^-_3]$.

In Fig. 1, it can be seen that once resting pH_i was re-established after transfer from HEPES to control CO_2/HCO^-_3 media, transfer back to HEPES induced a mild alkalosis due to the rapid washout of CO_2, leaving behind an excess of HCO^-_3. We exploited this approach to evaluate whether a Cl^--dependent mechanism was involved in the recovery from alkalosis. A high-P_{CO_2} (11.15 mmHg), high-HCO^-_3 (18.2 mmol l^{-1}) medium was employed to achieve a pH_i elevation of significant

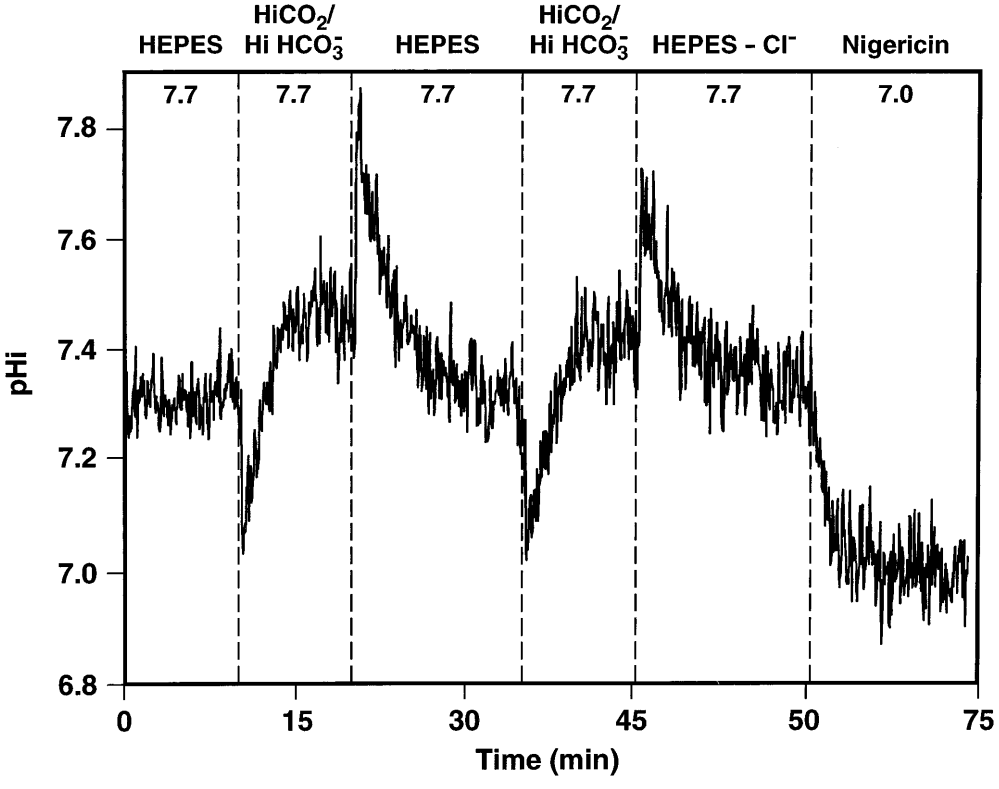

Fig. 3 Representative original recording of pH_i showing the acidification and subsequent recovery to a new higher pH_i which occurs when cultured gill cells are transferred from a HEPES-buffered medium (pH = 7.70) to a high-CO_2/high-HCO^-_3-buffered medium (pH = 7.70, P_{CO_2} = 11.15 mmHg, $[HCO^-_3]$ = 18.2 mmol l^{-1}), the alkalinization and subsequent recovery to the original pH_i which occurs when transferred back to HEPES. In the second cycle, the same protocol was followed, but the HEPES medium lacked Cl^-, resulting in a smaller alkalinization, but unchanged rate of correction. At the end of the experiment, calibration with a 140 mmol l^{-1} K^+ buffer containing 0.010 mmol l^{-1} nigericin (pH = 7.00) is shown

Table 5 The influence of Cl^--free medium upon the degree of alkalinization and the rate of pH_i recovery after transfer of gill cells from high-CO_2/HCO_3^- medium at P_{CO_2} = 11.15 mmHg, HCO_3^- = 18.2 mmol l^{-1}, to HEPES-buffered medium. Means ± 1 SEM (n = 7)

	ΔpH_i	pH_i recovery rate (pH units min^{-1})
Control	+0.417 ± 0.033	0.109 ± 0.012
Cl^--free	+0.308 ± 0.030*	0.109 ± 0.009

* Indicates significant difference ($P < 0.05$) from the control value

size (Fig. 3). After stabilization of pH_i in this medium, exposure of the gill cells to HEPES (P_{CO_2} = 0.03 mmHg, nominally HCO_3^--free) raised pH_i to 7.8–8.0, followed by a rapid recovery to the original resting pH_i. When the washout was performed in Cl^--free HEPES, the degree of alkalinization was significantly attenuated (Table 5), in accord with earlier results where Cl^--free conditions increased apparent β (e.g., Tables 2, 4). However, Cl^--free conditions had absolutely no effect on the rate of pH_i recovery. The clear conclusion is that a mechanism dependent upon external Cl^-, such as Cl^-/HCO_3^- exchange, plays no role in the recovery from intracellular alkalosis.

Discussion

To our knowledge, this is the first study to evaluate the mechanisms of acid-base regulation in fish gill cells in a CO_2/HCO_3^--buffered medium. The most important new finding of this study, revealed by growing and testing the branchial cells in a medium which closely duplicates the in vivo conditions of trout blood, is the presence of a Na^+-dependent, HCO_3^--dependent, SITS-sensitive alkalinizing mechanism. This mechanism definitely contributes to recovery from acidification, and may also participate in the maintenance of resting pH_i. Previously, using cultured branchial cells in HEPES-buffered medium, we and others had found only the presence of a Na^+/H^+ exchanger to maintain resting pH_i and recover from acidification (Pärt and Wood 1996; Leguen et al. 1998). Na^+/H^+ exchange mechanisms appear to be almost universally present in animal cells (Frelin et al. 1988; Ilundain 1992), and have been found in other isolated fish cells such as hepatocytes (Walsh 1986) and erythrocytes (Nikinmaa 1990). This same Na^+/H^+ exchanger is clearly present and important in gill cells in a CO_2/HCO_3^--buffered medium, but it is not the only mechanism. Other aspects of pH_i regulation under physiological conditions of P_{CO_2} and HCO_3^- are somewhat similar to those previously described in HEPES-buffered, HCO_3^--free media. Thus, at pH_e 7.70, the regulated pH_i is identical (~7.41) in the two media, and the same as the value measured in vivo in freshwater rainbow trout (Wood and LeMoigne 1991). Intracellular buffer capacity is similar, but elevated by about the expected amount by the contribution of intracellular HCO_3^- (see below). Furthermore, a V-type ATPase (e.g., H^+-ATPase) does not appear to involved in pH_i regulation in either medium, despite the presence of CO_2 to "fuel" a proton-pump in the CO_2/HCO_3^--buffered medium.

Particularly noteworthy of the differences between cells in the two media is the fact that in CO_2/HCO_3^--buffered media, amiloride did not affect resting pH_i, whereas Na^+ removal induced intracellular acidosis (Table 1). In contrast, in HEPES-buffered media, both treatments induced acidosis, but Na^+ removal was more effective (Pärt and Wood 1996). These differences might be dismissed as a statistical aberration, were it not for the fact that Pärt and Bergstrom (1995), using a different pH_i measurement method ([carbon-14 labelled 5,5-dimethyl-2,4-oxazolidinedione, [^{14}C]DMO), static rather than dynamic measurements] and gill cells from a different stock of trout (Sweden versus Canada) reported an identical phenomenon in preliminary experiments – a lack of amiloride effect in CO_2/HCO_3^-, the presence of an amiloride effect (acidosis) in HEPES, and the presence of a Na^+-removal effect (acidosis) in both media. Calculations based on the buffer capacity (β) measurements of Table 4 indicate that the difference in amiloride effect on resting pH_i between the two media cannot be explained by differences in β. The obvious conclusion is that in the presence of CO_2/HCO_3^-, a mechanism dependent on extracellular Na^+ is activated to help regulate pH_i when the Na^+/H^+ exchanger is disabled. In the absence of CO_2/HCO_3^-, such a mechanism does not occur. This evidence and this conclusion are both very similar to those of Little et al. (1995), Faff et al. (1996), and Faber et al. (1998) on three mammalian cultured cell preparations: rat aortic smooth muscle cells, bovine aortic epithelial cells, and mouse brain microglial cells, respectively.

Possible mechanisms responsible for this phenomenon would be either a Na^+-$(HCO_3^-)_N$ co-transport mechanism or a Na^+-coupled HCO_3^-/Cl^- exchanger (or both) operating to transport HCO_3^- inwardly (Frelin et al. 1988; Boron and Boulpaep 1989; Kahn et al. 1990; Krapf and Alpern 1993; Seki et al. 1996; Faber et al. 1998). The higher pH_i regulated in high-P_{CO_2}/high-HCO_3^- media (at unchanged pH_e; Fig. 3) might also reflect such mechanisms. Experimentally, these mechanisms are difficult to distinguish, as discussed further below. Both of these mechanisms are generally considered to be sensitive to stilbene derivatives. However, the lack of an effect of SITS on resting pH_i (Table 1) does not argue against the presence of such a HCO_3^--dependent transporter; indeed, it is the expected result if such mechanisms are silent at rest when the Na^+/H^+ exchanger is operating normally.

The strongest evidence in favor of this explanation was seen in the case of recovery from profound acidosis induced by the washout of an ammonia prepulse. Under these circumstances, SITS alone significantly inhibited the rate of recovery from acidosis, though not to the same extent as amiloride (Table 3). Almost certainly this

SITS effect was exerted on one or both of the HCO_3^--dependent mechanisms outlined above, and was not due to blockade of Na^+-independent Cl^-/HCO_3^- exchange because (1) the latter mechanism is usually acidifying, not alkalinizing, and (2) even during correction of alkalosis, such a mechanism is not seen (see below). Note that Cl^- removal also significantly reduced the rate of pH_i correction from acidosis (Table 3). At first glance, this would seem to favor the participation of a Na^+-coupled HCO_3^-/Cl^- exchanger rather than a Na^+-$(HCO_3^-)_N$ co-transporter, but the situation is not that simple. Firstly (as argued below), once the change in intracellular buffer capacity caused by this treatment is taken into account, the *apparent* reduction disappears or becomes a slight increase in terms of net H^+ export rate ($\beta \times$ pH units min^{-1}). Secondly, it must be remembered that the Cl^--site on this exchanger is on the intracellular side, not the extracellular side. Thus, as discussed by many workers (e.g., Boyarsky et al. 1988; Frelin et al. 1988; Boron and Boulpaep 1989), the interpretation of Cl^--removal experiments is problematic and time dependent; depending on exact conditions, one might expect first an acceleration of pH_i correction accompanying an improved Cl^- gradient, changing to no effect and finally an inhibition later on (e.g., Kahn et al. 1990) as intracellular Cl^- stores are depleted. Electrical measurements alone provide an infallible diagnosis; only the Na^+-$(HCO_3^-)_N$ co-transporter is electrogenic (Boron and Boulpaep 1989).

Furthermore, the *apparent* lack of difference in pH_i correction rates between cells in CO_2/HCO_3^- versus HEPES (Table 3) does not signify the absence of HCO_3^- dependency. Again, once the higher intracellular buffer capacity (β) of cells in CO_2/HCO_3^- media is taken into account (Table 4), the H^+ export rates ($\beta \times$ pH units min^{-1}) are significantly lower by 33% in the nominal absence of HCO_3^- (3.04 ± 0.36 versus 4.50 ± 0.33 mmol l^{-1} ICF min^{-1}, $n = 5$). Blockade of a Na^+-$(HCO_3^-)_N$ co-transporter and/or a Na^+-coupled HCO_3^-/Cl^- exchanger which are activated to import HCO_3^- during acidosis are both reasonable explanations for the effects of SITS and HCO_3^- removal.

Recovery from alkalosis caused by CO_2 washout (e.g., Fig. 3) was not the primary focus of this study, but was rather done to rule in (or out) the presence of Na^+-independent Cl^-/HCO_3^- exchange using conditions (alkalosis, cells pre-incubated in CO_2/HCO_3^-) which should favor its expression. The clear absence of an inhibitory effect of Cl^- removal on the rate of pH_i correction under these circumstances (Table 5) argues strongly that this mechanism is absent. A variety of other possible mechanisms may be involved in the correction of intracellular alkalosis, e.g., HCO_3^- efflux via a now outwardly directed Na^+-$(HCO_3^-)_N$ co-transporter, via a Na^+-coupled HCO_3^-/Cl^- exchanger (though this directionality would be unusual), via HCO_3^- channels, or via reversal of Na^+/H^+ exchange. All these are worthy of investigation in future studies.

The elevation in intracellular buffer capacity (β) in CO_2/HCO_3^--buffered media relative to HEPES-buffered media (by 5.5–8.0 slykes; Table 4) was exactly as expected from theory (Roos and Boron 1981). Assuming P_{CO_2} equilibration (3.71 mmHg) across the cell membrane, intracellular $[HCO_3^-]$ at resting $pH_i = 7.41$ would be about 3.1 mmol l^{-1}; therefore intracellular β should be $2.3 \times 3.1 = 7.1$ slykes above the value in HEPES, which was the observed result (Table 4). However, the most surprising finding of the present study was the effect of Cl^--removal in elevating intracellular β. As outlined in the Results, the exact size of that elevation is probably over-estimated by the values obtained (with 60 mmol l^{-1} ammonia) in Table 4, but the phenomenon was certainly real. One might expect a small increase in total β if Cl^--removal "trapped" additional HCO_3^- generated by metabolism inside the cell. However, the P_{CO_2}-washout experiments demonstrated a significant increase in β from 25.5 slykes to 33 slykes, and by definition, this must have been an elevation in *non-HCO_3^-* β because CO_2 titration measures only *non-HCO_3^-* β (Roos and Boron 1981). If we assume conservatively that total β was raised to about 30 slykes in Cl^--free media, then this would bring the rate of H^+ extrusion during acidosis up to about 4.50 mmol l^{-1} ICF min^{-1} (i.e., 0.149 pH units $min^{-1} \times 30$ slykes), identical to the value calculated earlier for the control treatment in CO_2/HCO_3^- media, again indicating the absence of a Na^+-independent Cl^-/HCO_3^- exchange mechanism. A similar calculation would also argue that HCO_3^- extrusion during the correction of alkalosis might actually be accelerated by Cl^--removal (Table 5), but the mechanism is unclear.

At present, we have no explanation why Cl^--removal should cause a sudden increase in intracellular β of cultured gill epithelial cells. Measurements of BCECF fluorescence at the isobestic wavelength indicated no change in dye concentration, so the phenomenon was not associated with cellular dehydration. This sort of observation is unusual but not unprecedented. For example, Kayser et al. (1992) measured a 20% elevation of intracellular β under Cl^--free conditions in cultured human thyroid epithelial cells. Graber et al. (1991) reported that removal of extracellular Na^+ caused a threefold elevation of intracellular β in cultured opossum kidney (OK) cells, measured using similar ammonia prepulse methods. However, they could not detect the elevation by direct titration of cell lysates, suggesting that it was a metabolic or membrane transport phenomenon. For example, it is possible that removal of a key ion affects certain enzyme activities, such that metabolism produces fewer protons (or consumes more) or membrane permeability to H^+ is altered. Clearly, these ideas should be tested in future experiments.

Bafilomycin A_1 (3 μmol l^{-1}), at a concentration higher than that which causes substantial inhibition of Na^+ uptake by trout gills in vivo (Bury and Wood 1999), had no effect on pH_i regulation of cultured gill cells in CO_2/HCO_3^--buffered media (Tables 1, 2). This is

in accord with earlier results in HEPES-buffered media (Pärt and Wood 1996), and shows that the apparent absence of a V-type ATPase/Na^+-channel mechanism for pH_i regulation was not due to an absence of CO_2 needed to "fuel" the H^+-pump (cf. Lin and Randall 1995). When this conclusion is combined with the conclusion that Na^+-independent Cl^-/HCO_3^- exchange is also absent from cultured gill cells, it is apparent that the two mechanisms (Lin and Randall 1995; Kirschner 1997; Perry 1997) thought to be principally responsible for active Na^+ uptake (against H^+ excretion) and Cl^- uptake (against HCO_3^- excretion) by rainbow trout gills in vivo are missing from cultured gill cells in vitro. It could be argued that this is because the cultured gill cells are pavement cells, not chloride cells. However, we believe the more important explanation is that the gill cells are cultured and tested in isotonic media representative of blood plasma, and are not apically exposed to freshwater. Therefore, the apical mechanisms normally present in vivo may not be expressed under the present culture conditions. It is probable that the Na^+/H^+ exchange, Na^+-$(HCO_3^-)^N$ co-transport, and/or Na^+-coupled HCO_3^-/Cl^- exchangers identified in these cells are basolateral mechanisms responsible for normal "housekeeping " of pH_i. Quite possibly, the basolateral transporters have redistributed throughout the apical membranes during culture because the cells may not be polarized. Potentially important interactions which may occur between apical and basolateral acid-base transport mechanisms (e.g., Seki et al. 1996) would not be seen. Therefore, the next step is to examine acid-base, Na^+ and Cl^- exchanges in polarized cultures of gill epithelia on filters where apical freshwater and basolateral "plasma" exposure can be maintained (cf. Wood and Pärt 1997; Wood et al. 1998).

Acknowledgements We thank Rod Rhem for excellent technical assistance, and Dr. Lisle Gibbs and Dr. Colin Nurse for the loan of equipment. Supported by grants from NSERC (CMW), NSERC Collaborative Program (CMW and PP, Dr. D.G. McDonald, P.I.), the Swedish Environmental Protection Agency (PP), the C.F. Lundstrom Foundation (PP), and an NSERC Foreign Researcher Award (PP). All experimental procedures complied with current animal care regulations in Canada.

References

Boron WF, Boulpaep EL (1989) The electrogenic Na^+/HCO_3^- cotransporter. Kidney Int 36: 392–402

Boron WF, Weer P de (1976) Intracellular pH transients in squid giant axon caused by CO_2, NH_3, and metabolic inhibitors. J Gen Physiol 67: 91–112

Boyarsky G, Ganz MB, Sterzel RB, Boron WF (1988) pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^-. Am J Physiol 255: C844–C856

Bury N, Wood CM (1999) The mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+-channel. Am J Physiol 277: R1385–R1391

Claiborne JB (1998) Acid-base regulation. In: Evans DH (ed) The physiology of fishes, 2nd edn. CRC Press, Bethesda, pp 577–622

Faber S, Lang H-J, Hock FJ, Scholkens BA, Mutschler E (1998) Intracellular pH regulation in bovine aortic endothelial cells: evidence of both Na^+/H^+ exchange and Na^+-dependent Cl^-/HCO_3^- exchange. Cell Physiol Biochem 8: 202–211

Faff L, Ohlemeyer C, Kettenham H (1996) Intracellular pH regulation in cultured microglial cells from mouse brain. J Neurosci Res 46: 294–304

Frelin C, Vigne P, Ladoux A, Lazdunski M (1988) The regulation of intracellular pH in cells from vertebrates. Eur J Biochem 174: 3–14

Goss GG, Perry SF, Wood CM, Laurent PL (1992) Mechanisms of ion and acid-base regulation at the gills of freshwater fish. J Exp Zool 263: 143–159

Goss GG, Perry SF, Laurent PL (1995) Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. In: Wood CM, Shuttleworth TJ (eds) Fish physiology, vol 14. Cellular and molecular approaches to fish ionic regulation. Academic Press, San Diego, pp 257–284

Graber M, DiPaola J, Hsiang F, Barry C, Pastoriza E (1991) Intracellular pH in the OK cell. I. Identification of H^+ conductance and observations on buffering capacity. Am J Physiol 261: C1143–C1153

Ilundain A (1992) Intracellular pH regulation in intestinal and renal epithelial cells. Comp Biochem Physiol 101A: 413–424

Kahn AM, Cragoe EJ, Allen JC, Halligan RD, Shelat H (1990) Na^+-H^+ and Na^+-dependent Cl^-/HCO_3^- exchange control pH_i in vascular smooth muscle. Am J Physiol 259: C134–C143

Kayser L, Hoyer PE, Perrild H, Wood AM, Robertson WR (1992) Intracellular pH regulation in human thyrocytes: evidence of both Na^+/H^+ and Na^+-dependent Cl^-/HCO_3^- exchange. J Endocrinol 135: 391–401

Kirschner LB (1997) Extrarenal mechanisms in hydromineral and acid-base regulation in aquatic vertebrates. In: Dantzler WH (ed) The handbook of physiology. American Physiological Society, Bethesda, pp 577–622

Krapf R, Alpern RJ (1993) Cell pH and transepithelial H^+/HCO_3^- transport in the renal proximal tubule. J Membr Biol 131: 1–10

Leguen I, Pisam M, Bidet M, Tauc M, Poujeol P (1998) pH_i regulation and ultrastructural analysis in cultured gill cells from freshwater or seawater-adapted trout. Fish Physiol Biochem 18: 297–309

Lin H, Randall DJ (1995) Proton pumps in fish gills. In: Wood CM, Shuttleworth TJ (eds) Fish physiology, vol 14. Cellular and molecular approaches to fish ionic regulation. Academic Press, San Diego, pp 229–255

Little PJ, Neylon CB, Farrelly CA, Weissberg PL, Cragoe EJ Jr, Bobik A (1995) Intracellular pH in vascular smooth muscle: regulation by sodium-hydrogen exchange and multiple sodium-dependent HCO_3^- mechanisms. Cardiovasc Res 29: 239–246

Lubman RL, Crandall ED (1991) Na^+-HCO_3^- symport modulates intracellular pH in alveolar epithelial cells. Am J Physiol 260: L555–L561

Lubman RL, Crandall ED (1992) Regulation of intracellular pH in alveolar epithelial cells. Am J Physiol 262: L1–L14

Madshus IH (1988) Regulation of intracellular pH in eukaryotic cells. Biochem J 250: 1–8

Marshall WS, Hanrahan JW (1991) Anion channels in the apical membrane of mammalian corneal epithelium primary cultures. Invest Opthamol Vis Sci 32: 1562–1568

Nikinmaa M (1990) Vertebrate red blood cells: adaptations of function to respiratory requirements. Springer, Berlin Heidelberg New York

Pärt P, Bergstrom E (1995) Primary cultures of teleost branchial epithelial cells. In: Wood CM, Shuttleworth TJ (eds) Fish physiology, vol 14. Cellular and molecular approaches to fish ionic regulation. Academic Press, San Diego, pp 207–227

Pärt P, Wood CM (1996) Na^+/H^+ exchange in cultured epithelial cells from fish gills. J Comp Physiol B 166: 37–45

Peral MJ, Calonge ML, Ilundain AA (1995) Na^+-HCO_3^- cotransporter and intracellular pH regulation in chicken enterocytes. Eur J Physiol 430: 612–616

Perry SF (1997) The chloride cell: structure and function in the gills of freshwater fishes. Annu Rev Physiol 59: 325–347

Rink TJ, Tsien RY, Pozzan T (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. J Cell Biol 95: 189–196

Roos A, Boron WF (1981) Intracellular pH. Physiol Rev 61: 296–434

Seki G, Coppola S, Yoshitomi K, Burckhardt B-C, Samarija I, Muller-Burger S, Fromter E (1996) On the mechanism of bicarbonate exit from renal proximal tubular cells. Kidney Int 49: 1671–1677

Thomas R (1989) Bicarbonate and pH_i response. Nature (Lond) 337: 601

Walsh PJ (1986) Ionic requirements for intracellular pH regulation in rainbow trout hepatocytes. Am J Physiol 250: R24–R29

Walsh PJ (1990) Fish hepatocytes accumulate HEPES: a potential source of error in studies of intracellular pH regulation. J Exp Biol 148: 495–499

Wolf K (1963) Physiological salines for freshwater teleosts. Prog Fish Cult 25: 135–140

Wood CM, LeMoigne J (1991) Intracellular acid-base responses to environmental hyperoxia and normoxic recovery in rainbow trout. Respir Physiol 86: 91–113

Wood CM, Pärt P (1997) Cultured branchial epithelia from freshwater fish gills. J Exp Biol 200: 1047–1059

Wood CM, Gilmour KM, Pärt P (1998) Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol 119A: 87–96