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Regulation of intracellular pH in anoxia-tolerant and anoxia-intolerant teleost hepatocytes

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Summary

Mechanisms of intracellular pH (pHi) regulation were investigated in anoxia-tolerant hepatocytes from goldfish *Carassius auratus*, and compared to the situation in the anoxia-intolerant hepatocytes from trout *Oncorhynchus mykiss*. Under normoxic conditions, the pHi of goldfish hepatocytes was regulated by a Na^+/H^+ exchanger and a Na^+-independent Cl^-/HCO_3^- exchanger, the latter being activated only after acidification of the cells. Mechanisms of acid secretion appear to be fuelled, at least in part, by lactate formation under fully aerobic conditions, as inhibition of glycolysis caused a drastic reduction of steady state proton release. In trout hepatocytes both a Na^+/H^+ exchanger and a Cl^-/HCO_3^- exchanger were found to be tonically active, as described previously. During chemical anoxia a constant pHi was maintained in goldfish hepatocytes, whereas it was reversibly reduced by 0.3 units in the trout cells. Under these conditions a reversible increase in the rate of acid secretion was induced in the cells from both species. In the goldfish cells this was based on a SITS-sensitive transporter, possibly involving export of lactate, with no contribution from Na^+/H^+ exchange. By contrast, in hepatocytes from trout, CN-induced acid secretion was dominated by the activity of the Na^+/H^+ exchanger. Brief exposure to extracellular acidosis had no dramatic effects on the energetics of hepatocytes from either species.

Key words: intracellular pH, goldfish, *Carassius auratus*, trout, *Oncorhynchus mykiss*, anoxia, acid secretion, Na^+/H^+ exchange, Cl^-/HCO_3^- exchange, lactate production, oxygen consumption.

Introduction

The goldfish hepatocyte has a remarkable degree of anoxia-tolerance, which is largely based on its ability to downregulate ATP production and ATP consumption coordinatedly during energy limiting conditions (Krumschnabel et al., 2000). This general reduction of ATP turnover (metabolic depression) is accompanied by a partial reduction of transmembrane K^+ fluxes and maintenance of intracellular free Ca^{2+} concentration at control levels, allowing the preservation of an intracellular milieu compatible with cell survival at reduced metabolic costs (Krumschnabel et al., 1996, 1997). A related feature of interest that has not yet been studied is the behaviour and regulation of intracellular pH (pHi) in these cells under anoxia. This appeared particularly interesting as metabolic depression has repeatedly been found to be accompanied by, or even induced by, a decrease of pHi and/or extracellular pH (pHe) (Hand and Gnaiger, 1988; Pörtner et al., 2000). In principle, there could be at least two scenarios occurring in anoxia-tolerant goldfish hepatocytes. In one, a decrease of pHi might occur, supporting anoxic survival by partially arresting ATP-consuming functions such as protein synthesis (Hand, 1997), while at the same time inhibiting degradative processes leading to cell injury (Gores et al., 1988). A potential drawback of this strategy, however, is that low pH might adversely affect glycolytic flux (Busa and Nuccitelli, 1984), which is the sole ATP-providing route under anoxia. In the other possibility, goldfish hepatocytes might be able to prevent alterations of pHi and thus maintain a constant intracellular milieu, by either increased buffering capacity, or by efficient acid release mechanisms being activated under anoxic conditions.

To gain insight into the above questions, the present study examined the mechanisms involved in pHi regulation in goldfish hepatocytes during both normoxia and chemical anoxia. In addition, the effects of extracellular acidosis on cell energetics were studied, since, as indicated above, low pHe is known to promote cell survival of anoxia-intolerant cells (Pentilla and Trump, 1974; Bonventre and Cheung, 1985; Gores et al., 1988), as well as to play a role in metabolic depression of anoxia-tolerant cells (Hand and Hardewig, 1996). Finally, since there are no comparable data available on these aspects of pH regulation in anoxia-intolerant teleost hepatocytes, the most relevant experiments were also conducted with trout hepatocytes.

Materials and methods

Materials

Collagenase (Type VIII), bovine serum albumin (BSA), 2′,7′-bis - (2-carboxypropyl)-5-(and -6) - carboxylfluorescein acetoxymethyl (BCPCF-AM), low-melting-point agarose gel, and inhibitors of transporters and metabolism were purchased from Sigma. All other chemicals were of analytical grade and were purchased from local suppliers.

Isolation of hepatocytes

Hepatocytes were isolated from goldfish *Carassius auratus* L. and trout *Oncorhynchus mykiss* Walbaum, using procedures and media as previously described (Krumschnabel et al., 1996). Except where otherwise noted, all incubations and measurements were conducted at the acclimation temperatures of the fish, which were 20 °C for goldfish and 15 °C for trout, respectively. Standard incubation salines consisted of (in mmol l^{-1}), for goldfish: 10 Hepes, 135 NaCl, 3.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 $NaHCO_3$, pH 7.6 at 20 °C, including 1 % bovine serum albumin (BSA); for trout: 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 1.5 $CaCl_2$, 5 glucose, and 1 % BSA, pH 7.6 at 20 °C. Cell viability as determined by Trypan Blue exclusion averaged >95 % and was maintained throughout the experiments described.

Measurement of intracellular pH

Intracellular pH (pHi) of hepatocytes was measured using the membrane-permeable fluorescent dye BCPCF-AM as described (Krumschnabel et al., 2001a). Briefly, hepatocytes (3×10^6 ml^{-1}) loaded with BCPCF-AM for 20 min at 20 °C (goldfish) or 15 °C (trout), were washed and suspended in BSA-free saline and placed in a thermostatted cuvette equipped with a stirring device. Fluorescence was measured with a Hitachi F-2000 Fluorescence Spectrophotometer with λ_{ex} set to 490 and 440 nm, alternating at 2 s intervals, and λ_{em} set to 535 nm. For each experiment a calibration was performed by permeabilising cells with 50 μg ml^{-1} digitonin, then adding known amounts of acid or base and taking repeated measurements of extracellular pH (pHe) with a capillary glass electrode (Fossat et al., 1997).

The effect of pHe on pHi was determined by incubation of BCPCF-loaded hepatocytes in standard saline, titrated to the specified pH with HCl or NaOH, until a constant fluorescent signal was obtained (usually within 5 min). Each measurement was subsequently calibrated as described above.

Transport mechanisms involved in pHi regulation under normoxia were evaluated by measuring rates of recovery of pHi in goldfish hepatocytes in response to acidification with 30 mmol l^{-1} sodium propionate. For discrimination between specific transporters, cells were pre-incubated for 20 min prior to acidification with either 1 mmol l^{-1} amiloride (an inhibitor of Na^+/H^+-exchange), 0.5 mmol l^{-1} 4-acetamido-4′-isothio-cyanatostilbene-2,2′-disulfonic acid (SITS, an inhibitor of Cl^-/HCO_3^- and $Na^+/Cl^-/HCO_3^-$ exchange), or in Na^+-free medium with Na^+ salts substituted by equimolar amounts of tetramethylammonium, or Cl^--free medium with Cl^- salts substituted by gluconate. Media containing amiloride or SITS were sonicated before use in order to bring all solids into solution. The potential impact of lactate production and/or export on pHi regulation was evaluated by inhibiting glycolytic flux with iodoacetic acid (IAA). IAA causes an immediate and complete blockage of lactate production in hepatocytes from goldfish and trout (Krumschnabel et al., 1994, 2001b). Rates of recovery of pHi after addition of sodium propionate were determined from the pHi change over the first 5 min after the lowest measured pHi was observed. To convert these rates (pH units min^{-1}) into rates of proton extrusion (mmol H^+ l^{-1} min^{-1}), the total apparent intracellular buffer capacity (β, mmol H^+ pH $unit^{-1}$ l^{-1}) was calculated from these experiments, applying the formula given in Furimsky et al. (2000). Acidification experiments were conducted for the goldfish cells only, since comparable data on trout hepatocytes have been published already (Walsh, 1986; Furimsky et al., 1999, 2000).

The impact of chemical anoxia on the pHi of hepatocytes was assessed as follows. BCPCF-loaded hepatocytes were incubated with 1 mmol l^{-1} NaCN in flasks wrapped in aluminium foil in a thermostatted water bath with constant shaking (120 r.p.m.). At various time points 1.5 ml samples of the suspension were removed for the measurement of pHi. Prior to transfer to the fluorescence cuvette, cells were briefly (5 s) centrifuged and suspended in fresh medium (including CN where appropriate) in order to prevent artefacts due to leakage of BCPCF. For washout of CN, 1.5 ml samples of cell suspension were diluted with 10 ml of fresh medium containing no CN and spun down at 60 ***g*** for 4 min. Cells were then suspended in fresh saline and left to recover for 30 min.

Note that CN will form HCN, a weak permeant acid, in solution. However, considering the intracellular buffer capacities of hepatocytes from goldfish (see Results) and trout (Furimsky et al., 2000), addition of 1 mmol l^{-1} NaCN will have only a minor, if any, effect on the pHi of the cells.

Proton secretion

Proton secretion of hepatocytes was estimated from the rate of acidification of external medium determined with a cytosensor microphysiometer (Molecular Devices) as described (Pelster, 1995), with modifications (Krumschnabel et al., 2001a). Briefly, hepatocytes (0.4×10^6 cells) were embedded in low-melting-point agarose gel (1.5 %) in polycarbonate capsules and loaded into the cytosensor chamber, where cells were constantly perfused by one of two media. An electromagnetic valve allowed a rapid switch between the perfusion media. The saline used was a low buffering capacity medium consisting of (in mmol l^{-1}): 138 NaCl, 5.1 KCl, 1.1 $CaCl_2$, 0.93 $MgSO_4$, 0.81 K_2HPO_4, 0.11 KH_2PO_4, titrated to pH 7.6. For experimental manipulations CN or transport inhibitors were added to this medium; for Na^+-free conditions NaCl was replaced by equimolar amounts of tetramethylammonium chloride. During a typical measuring cycle, cells were perfused with saline for a period of 90 s

followed by a 30 s flow-off period, during which acidic equivalents released by the hepatocytes acidified the measuring chamber. This signal was recorded *via* a light-addressable potentiometric sensor and the rate of acidification was derived from the slope of a line fitted to the sensor data. Since the complex geometry of the cytosensor chamber and the embedding procedure of the cells make it very difficult to assess the number of cells actually releasing acid equivalents into the measuring chamber, acidification rates are given as a percentage of the basal rate of proton release. For technical reasons, these experiments were conducted at 20 °C with hepatocytes from both goldfish and trout.

The impact of chemical anoxia and of transport inhibitors on the rate of proton secretion was assessed as follows. Cells were first left to recover from the embedding procedure for at least 30 min, after which time a constant rate of proton secretion had been established. Then hepatocytes were exposed to chemical anoxia for a period of 10 min, followed by a recovery phase of at least another 30 min. Finally the experimental chambers were perfused for 10 min each with medium containing the desired transport blocker, and subsequently with both the transport blocker and CN. In some experiments the order of exposure to chemical anoxia in the presence and absence of transport inhibitors was reversed, and it was seen that measurements were not affected by any time-dependent decay of the rate of proton secretion.

Lactate production and export

Cells were incubated in a thermostatted water bath with constant shaking and duplicate samples were removed as indicated under Results. Samples were immediately centrifuged at 6000 ***g*** for 5 s, supernatants and cell pellets were separated, and both sub-samples were precipitated with ice-cold 10 % metaphosphoric acid. [Lactate] was determined using a standard fluorimetric method. Lactate found in the supernatants is described as 'lactate export', whereas the sum of lactate exported and lactate measured in cell pellets is referred to as 'lactate production'.

Other methods

Descriptions of the methods employed for the determination of oxygen consumption by use of a Cyclobios Oxygraph (Haller et al., 1994) and of cellular ATP contents with the luciferase/luciferin method have been given before (Brown, 1982; Krumschnabel et al., 1994).

Statistics

Data are presented as means ± S.E.M. of the number (N) of independent preparations. Statistical differences were evaluated applying analysis of variance (ANOVA) followed by appropriate post-tests indicated in the text. For statistical evaluation of the cytosensor data, the rates in the absence of any inhibitor and the mean rates determined over each 10 min experimental period were compared. $P<0.05$ was considered to be significant.

Results

Effect of pHe on pHi

Fig. 1 shows pHi as a function of pHe in hepatocytes from goldfish and trout. In goldfish hepatocytes, a non-linear increase in pHi with pHe was observed: in the lower range (pHe 6.8–7.4), experimental points were close to the line for pHi=pHe, whereas at higher pHe values, pHi became less sensitive to pHe. Intracellular pH values of trout hepatocytes varied roughly linearly with pHe (r^2=0.8; Fig. 1, continuous line). This pattern is similar to one previously reported (Walsh, 1986), validating the use of BCPCF as an indicator of pHi in the cells from both species as well as the method of calibration applied in the present study.

Regulation of steady state pHi

The nature of the transport mechanisms involved in the maintenance of steady state pHi was deduced from the effect of transport blockers and metabolic inhibitors on the rate of

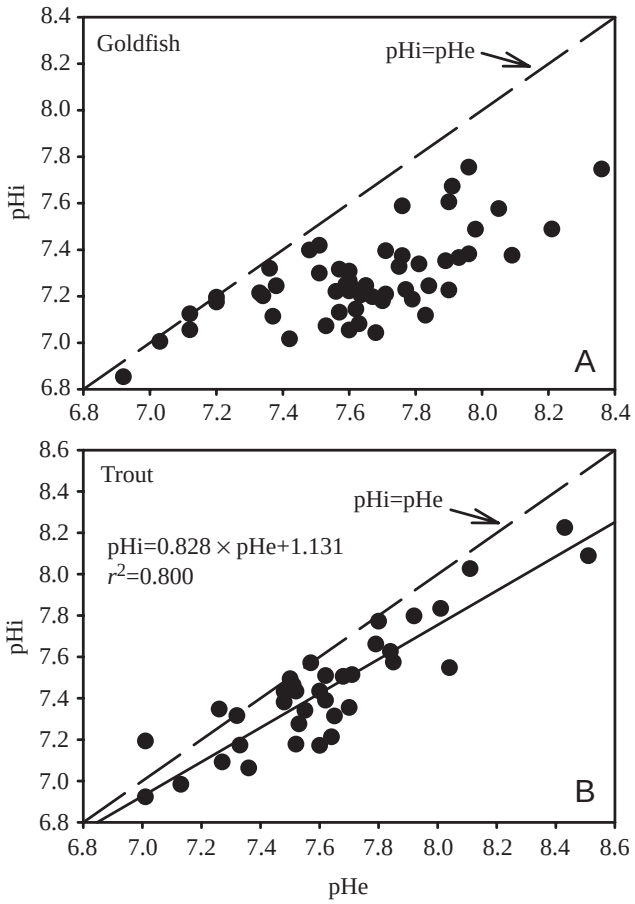

Fig. 1. Intracellular pH (pHi) as a function of extracellular pH (pHe) in goldfish (A) and trout (B) hepatocytes. BCPCF-loaded hepatocytes (3×10^6 ml^{-1}) were incubated in standard saline, titrated to the specified pH with HCl or NaOH, until a constant fluorescent signal was obtained. Each measurement was subsequently calibrated as described in Materials and methods. Data are from 21 (goldfish) and 15 (trout) hepatocyte preparations. The linear regression analysis equation is shown for trout hepatocytes (continuous line).

Table 1. *Effect of metabolic and transport inhibitors on steady state acid secretion of hepatocytes from goldfish and trout*

	Secretion (% of control rate)	
Inhibitor	Goldfish	Trout
Amiloride	92±3 (9)*	80±3 (11)**
Na^+-free	86±4 (8)*	85±3 (7)*
SITS	101±4 (19)	88±3 (14)**
Amiloride + SITS	80±4 (9)**	70±4 (7)**
Iodoacetic acid	58±3 (12)**	99±3 (5)

Values are means ± S.E.M. of the number of experiments (in parentheses).

*P<0.05, **P<0.01 compared to basal rate; statistics were performed on non-normalized data.

proton secretion, determined with the cytosensor microphysiometer. As shown in Table 1, in goldfish hepatocytes, amiloride and exposure to Na^+-free saline reduced the rate of proton secretion by 8 % and 14 %, respectively. SITS did not affect proton secretion, whereas the simultaneous presence of both amiloride and SITS caused a significant decrease of 20 % (see also Fig. 5A). The most pronounced effect, however, was a 42 % reduction in acid release seen upon addition of the glycolytic inhibitor IAA.

In trout hepatocytes, amiloride, SITS and both inhibitors in combination significantly reduced proton secretion to 80, 88 and 70 % of the control rate, respectively (Table 1; see also Fig. 5B for the effect of amiloride). Omission of Na^+ from the perfusate reduced proton secretion to 85 % (Table 1). Addition of IAA had no effect on proton secretion in the trout cells.

Mechanisms of pHi regulation of goldfish hepatocytes after acidification with sodium propionate

Addition of sodium propionate to suspensions of goldfish hepatocytes produced a rapid intracellular acidification, followed by a gradual recovery of pHi towards the initial value over the next 15 min (Fig. 2A). Inhibition of transport mechanisms by, for example, replacement of Cl^- in the medium, resulted in a significant alteration of the rate of pHi recovery (Fig. 2B). Using the mean value of 34.1±4.0 mmol l^{-1} pH $unit^{-1}$ (N=27) for total apparent intracellular buffering capacity, β, derived from these experiments, rates of pHi recovery were converted to rates of proton extrusion (mmol H^+ l^{-1} min^{-1}) under the various conditions studied (Fig. 3). In the presence of 1 mmol l^{-1} of the Na^+/H^+-exchanger blocker amiloride and in saline containing a low level of Na^+ (the presence of 30 mmol l^{-1} Na^+ being due to the addition of sodium propionate) the rate of proton extrusion after acidification was decreased by approximately 70 %. The anion exchanger blocker SITS (0.5 mmol l^{-1}), and the replacement of

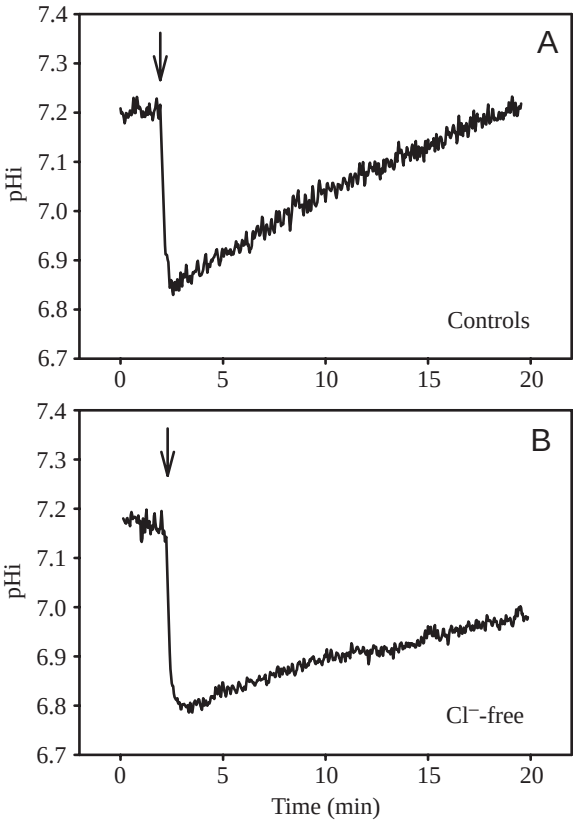

Fig. 2. The effect of 30 mmol l^{-1} sodium propionate on pHi in goldfish hepatocytes incubated in standard saline (A, Controls) and in nominally Cl^--free medium, Cl^- being replaced by gluconate (B, Cl^--free). Arrows indicate addition of sodium propionate.

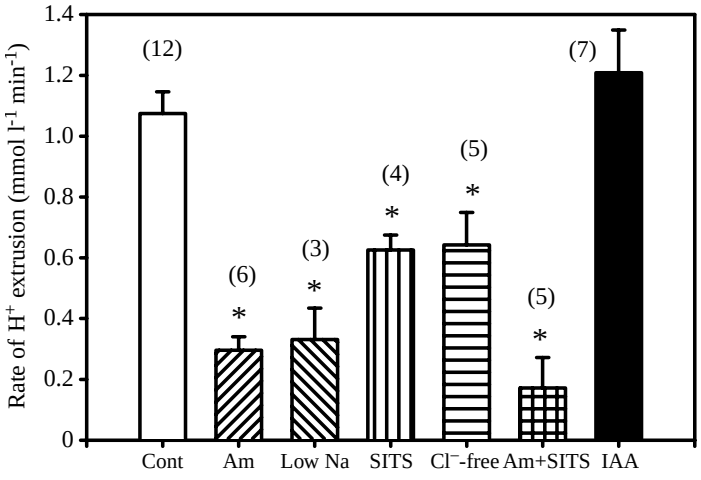

Fig. 3. Rates of proton extrusion by goldfish hepatocytes in response to acidification with 30 mmol l^{-1} sodium propionate (see Fig. 2) in standard saline (Cont), with 1 mmol l^{-1} amiloride (Am), in low Na^+ medium (low Na), with 0.5 mmol l^{-1} of the anion exchanger blocker SITS (SITS), in Cl^--free medium (Cl^--free), with both amiloride and SITS (Am+SITS), and with 0.5 mmol l^{-1} iodoacetic acid (IAA). Rates shown were determined from the change of pHi over the first 5 min after the lowest measured pHi was observed. Values are means + S.E.M. of the number of hepatocyte preparations, given in parentheses. * indicates a significant difference from controls and IAA-treated cells (P<0.05, one-way ANOVA followed by Tukey's procedure).

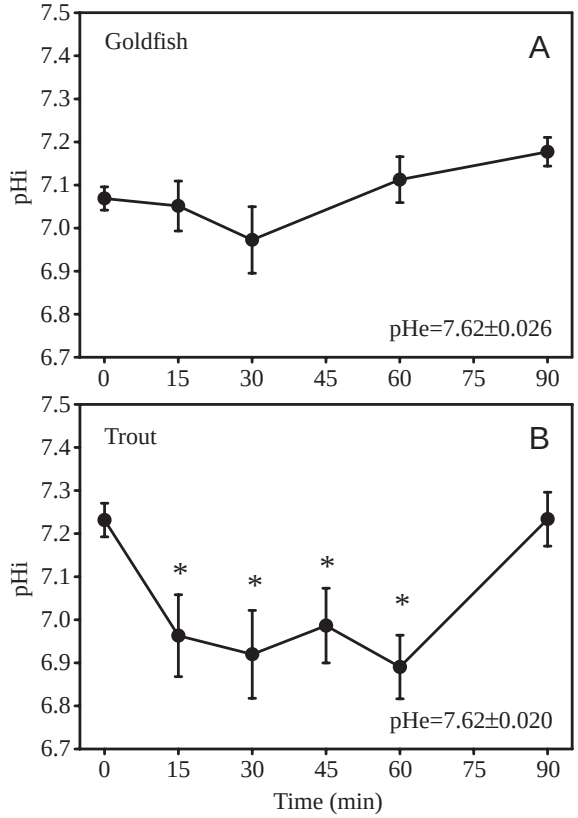

Fig. 4. Intracellular pH (pHi) during chemical anoxia, created by addition of 1 mmol l^{-1} CN at time zero, and after washout of CN at 60 min, in hepatocytes from goldfish (A) and trout (B). Values are means ± S.E.M. of 4 (goldfish, 3 values at 15 min) and 6 (trout) hepatocyte preparations. * indicates a significant difference compared to the initial value (repeated-measures ANOVA followed by Tukey's procedure).

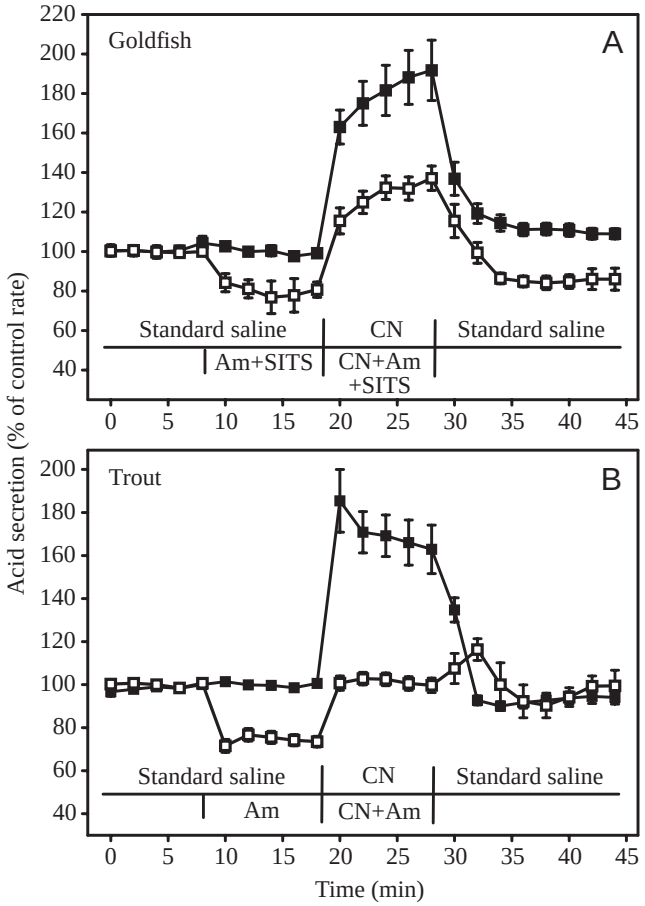

Fig. 5. Effect of chemical anoxia and transport inhibitors on acid secretion (% of control rate) by goldfish (A) and trout (B) hepatocytes, as estimated from the rate of acidification of the external medium measured with a cytosensor microphysiometer. At the times indicated, the perfusion medium was changed. Closed symbols, cells subjected to CN-treatment only; open symbols, cells treated with CN and transport inhibitors. Values are means ± S.E.M. of 9 (goldfish) and 7 (trout) experiments. For abbreviations, see legend to Fig. 3.

Cl^- by gluconate in the experimental saline, reduced proton extrusion by 42 % and 40 %, respectively. The combined presence of both amiloride and SITS reduced proton removal by approximately 85 %, which was not significantly different from the inhibition seen with either inhibitor alone. Inhibition of glycolysis with IAA had no effect on the rate of pHi recovery after cellular acidification.

pHi regulation under chemical anoxia

In goldfish hepatocytes the pHi did not change significantly during a 60 min period of chemical anoxia (Fig. 4). By contrast, the pHi in trout cells decreased by approx. 0.3 units under these conditions, and this decrease was fully reversible within 30 min after CN washout. Acid secretion in goldfish hepatocytes, as estimated with a cytosensor microphysiometer, showed an immediate increase in response to chemical anoxia and a slightly slower decrease to baseline values after restoration of control conditions (Fig. 5A). Na^+/H^+ exchange did not appear to be involved in this response, since neither amiloride nor Na^+-free conditions had any appreciable effect on proton secretion (Table 2). In contrast, SITS as well as SITS+amiloride (Fig. 5A) significantly diminished the CN-induced increase in proton secretion (Table 2). Export of lactate, either by lactate-H^+ cotransport or by non-ionic diffusion, which could serve as a means of acid extrusion, was indeed observed in the cells from goldfish (Fig. 6), rendering intracellular accumulation of this compound negligible. As inhibition of lactate production by IAA not only prevented the CN-induced increase in proton secretion but even decreased it below the basal rate seen in controls (Tables 1, 2), an attempt was made to block lactate transport across the cell membrane. The most common inhibitor of lactate transport, α-cyano-4-hydroxycinnamate, could not be applied, as it interfered with both proton secretion measurements (owing to its buffering capacity) and with lactate determination (owing to its absorption and fluorescence characteristics). However, 0.5 mmol l^{-1} quercetin, an inhibitor of the monocarboxylate transporter in red blood cells (Poole and Halestrap, 1993) and glial cells (Volk et al., 1997), significantly reduced lactate

Table 2. *Acid secretion by hepatocytes from goldfish and trout under chemical anoxia in the absence and presence of metabolic and transport inhibitors*

	Secretion (% of control rate)	
Treatment	Goldfish	Trout
CN	151±7 (9)	171±5 (7)
CN + Amiloride	137±10 (9)	101±3 (7)**
CN	171±3 (4)	142±8 (3)
CN + Na^+-free	177±5 (4)	103±6 (3)*
CN	166±4 (11)	146±5 (9)
CN + SITS	143±4 (11)**	145±4 (9)
CN	180±6 (9)	147±7 (7)
CN + Amiloride + SITS	128±4 (9)**	98±6 (7)**
CN	132±5 (12)	157±5 (5)
CN + IAA	52±5 (12)**	83±4 (5)*

Values are means ± S.E.M. of the number of experiments (in parentheses).

*P<0.05, **P<0.01 compared to the rate in the presence of CN only.

export, although this coincided with an overall decrease in lactate production (Fig. 6). Unexpectedly quercetin induced a significant transient increase in proton secretion while at the same time it appeared to dampen, though not eliminate, a subsequent CN-induced increase in acid release (Fig. 7).

In trout hepatocytes, chemical anoxia induced an increase in the rate of proton secretion similar to that observed in the goldfish cells (Table 2, Fig. 5B). However, in contrast to the situation in goldfish cells, amiloride, both alone and together with SITS (Fig. 5B), as well as exposure to Na^+-free

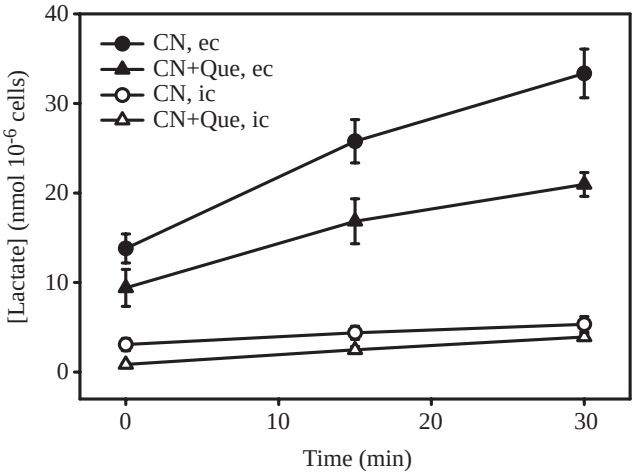

Fig. 6. Production and export of lactate by goldfish hepatocytes exposed to chemical anoxia in the presence or absence of 0.5 mmol l^{-1} quercetin (Que). Ic, intracellular [lactate]; ec, extracellular [lactate]. Values are means ± S.E.M. of 6 (CN) and 3 (CN+Que) hepatocyte preparations.

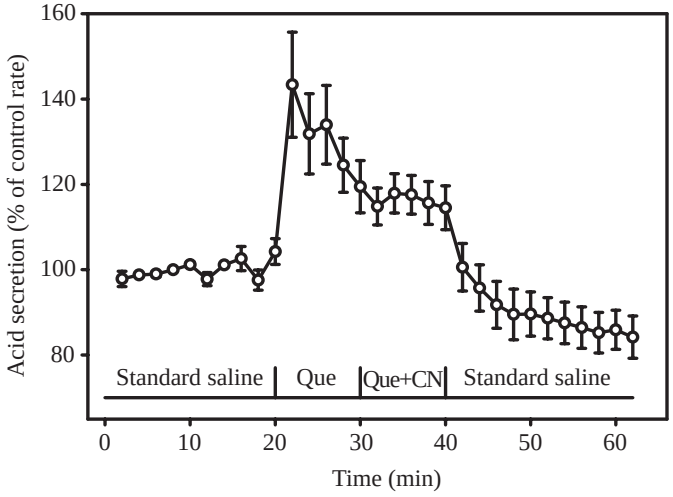

Fig. 7. Effect of 0.5 mmol l^{-1} quercetin (Que) on acid secretion determined with a cytosensor microphysiometer of goldfish hepatocytes in the absence and presence of CN. Perfusion medium was changed as indicated in the figure. Values (% of control rate) are means ± S.E.M. of 8 experiments.

conditions, totally prevented a CN-induced increase in proton secretion above the control rate, whereas SITS alone had no effect at all (Table 2). The simultaneous application of CN and IAA caused a decrease in proton secretion to 83 % of the control rate (Table 2).

Effect of extracellular acidosis on cell energetics

The effect of extracellular acidosis on cell energetics was evaluated in hepatocytes after a 15 min period of incubation in saline titrated to pH 7.6 or 6.6, followed by measurements of various parameters at the same pH. As summarised in Table 3, neither normoxic rates of ATP production, estimated from oxygen consumption, nor anoxic ATP generation, determined as lactate formation during a 30 min period of chemical anoxia, were significantly affected by extracellular acidosis in hepatocytes from goldfish and trout. [ATP], which remained constant in anoxic goldfish hepatocytes, was elevated at pH 6.6 as compared to 7.6 (significant after 30 min of chemical anoxia). In trout hepatocytes cellular [ATP] decreased during anoxia at pH 7.6, whereas it remained unaltered at pH 6.6. However, this was obviously because of the lower initial [ATP] of hepatocytes at pH 6.6.

Discussion

pHi regulation under normoxia

In hepatocytes from goldfish and trout, pHi is a function of pHe within the broad range of values tested (pHe 6.8–8.4). However, whereas the pHi-pHe relationship is roughly linear in the case of the trout (Fig. 1B), in goldfish hepatocytes (Fig. 1A) pHi is more sensitive to pHe in the more acidic part of the range tested (pHe 6.8–7.4).

Independently of this finding, data shown in Table 1, Fig. 2 and Fig. 3 unequivocally show that pHi of goldfish hepatocytes

Table 3. *Effect of extracellular acidosis on parameters of cell energetics of hepatocytes from goldfish and trout*

	Goldfish		Trout	
	pHe=7.6	pHe=6.6	pHe=7.6	pHe=6.6
$\dot{V}_{O_2}$	0.63±0.11	0.61±0.09	0.86±0.11	0.74±0.08
Lactate production	0.26±0.11	0.30±0.13	0.40±0.10	0.30±0.06
$[ATP]_{t=0}$ (normoxic)	3.96±0.84	5.79±0.95	3.04±0.71	2.50±0.44
$[ATP]_{t=30}$ (anoxic)	3.47±0.67	5.87±0.99*	2.31±0.49‡	2.35±0.42

Values for rates of oxygen consumption ($\dot{V}_{O_2}$) and lactate production are nmol 10^{-6} cells min^{-1}.

Normoxic rates of ATP production (nmol 10^{-6} cells) were estimated from oxygen consumption at time zero of anoxia.

Anoxic rates of ATP production were determined as lactate formed during a 30 min period of chemical anoxia (see Materials and methods for details).

Values are means ± S.E.M. of 6–7 (goldfish) and 4–6 (trout) paired experiments.

*P<0.05 compared to pHe=7.6; ‡P<0.05 compared to value at time zero.

is actively regulated both under steady state conditions and when an acid load is imposed on the cells. An analysis of the mechanisms involved in pHi regulation showed that, as in other species, a Na^+/H^+ exchanger is present in goldfish hepatocytes. This conclusion is supported by (1) the decrease of steady state proton secretion in the presence of amiloride and the absence of extracellular Na^+ (Table 1) and (2) the observation that pHi recovery was significantly impaired both by amiloride and by incubation of cells in low Na^+ medium (Figs 2, 3). Another ion transporter present in goldfish hepatocytes seems to be the Cl^-/HCO_3^- exchanger or the $Na^+/Cl^-/HCO_3^-$ transport system. While inhibition of this transporter did not affect maintenance of pHi under control conditions (Table 1), both SITS and incubation with Cl^--free medium decreased the rate of pHi recovery after acidification with sodium propionate (Fig. 3).

As shown in Table 1, glycolytic activity seems to be an important source of acidic equivalents in hepatocytes from goldfish but not in those from trout. The fact that lactate is produced under fully aerobic conditions in hepatocytes from both species and, furthermore, that this is more pronounced in goldfish than in trout hepatocytes, has been observed before (Krumschnabel et al., 2000, 2001b).

There are several possible mechanisms by which glycolysis affects pHi regulation. Firstly, lactic acid produced under aerobic conditions may dissociate in the cells and thereby fuel other pHi regulatory processes transporting protons out of the cell. This would be like the situation found in isolated eel gas gland cells, where protons produced by glucose metabolism were found to be released by, among others, amiloride- and DIDS-sensitive transporters (Pelster, 1995). Furthermore, such a link might underlie the unexpected transitory increase in acid secretion (Fig. 7) upon partial inhibition of lactate release with quercetin (Fig. 6), in that intracellular accumulation of lactic acid could have triggered a compensatory increase in proton-extruding transporters, resulting in a temporary overshoot of acid secretion. Secondly, lactate may be directly transported out of the cells, either by non-ionic diffusion or by lactate-H^+ co-transport *via* a monocarboxylate transporter. The latter transport mechanism was found to be particularly adapted to the export of lactate in highly glycolytic cells (Dimmer et al., 2000). Moreover, a contribution of lactate export to the maintenance of steady state pHi has previously been described in rat glial cells (Volk et al., 1997) and in active skeletal muscle (Juel, 1996). A third possibility to account for the effect of IAA on proton release would be that total cell metabolism was reduced upon inhibition of glycolysis. In fact, in a recent study we found that oxygen consumption decreased by approximately 12 % after 30 min of incubation with IAA (Krumschnabel et al., 2001b). A combination of the above effects will probably prove to be responsible for the decreased rate of proton release.

It is important to note that however large the contribution of lactate production and/or export may be to steady state acid release, the observation that recovery from an acid load was not affected by IAA (Fig. 3) suggests that it is not directly involved in the regulation of pHi after an acidic challenge.

Note, however, that in goldfish hepatocytes the combination of amiloride and SITS reduced proton secretion by more than the sum of inhibition seen with each transport inhibitor alone (Table 1, Table 2), which may indicate that upon blockage of one transport mechanism a compensatory increase in the activity of other transporters is induced. The present data should therefore only be taken as an estimate for the importance of any given mechanism for regulating pHi, as they may not fully reflect its actual importance under non-inhibited conditions.

The situation in trout hepatocytes seems to be less complicated, as no such potentially compensatory phenomena were observed. Thus, in agreement with previously published findings (Walsh, 1986; Furimsky et al., 2000), both amiloride- and SITS-sensitive transporters were found to be tonically active in these cells, with the combined inhibitory action of both agents roughly adding up to the effects noted with each inhibitor alone (Table 1).

pHi regulation under chemical anoxia in goldfish hepatocytes

The observation that pHi is maintained at a relatively constant level in goldfish hepatocytes during chemical anoxia is in line with the capability of these cells to preserve

homeostasis of other ions such as K^+ and Ca^{2+} under these conditions (Krumschnabel et al., 1996, 1997). Compared to other cells, goldfish hepatocytes show only a moderate decrease in cellular [ATP] in the presence of CN (Krumschnabel et al., 1994; Dorigatti et al., 1996) or no decrease at all (Krumschnabel et al., 1997) (present study, Table 3). Therefore, one important source of proton production in anoxia-sensitive cells, the net hydrolysis of ATP (Busa and Nuccitelli, 1984; Pörtner, 1987), is low or absent. Nevertheless, as indicated by the significant increase in proton release from the cells (Table 2) at constant pHi (Fig. 4), an increased production of acidic equivalents must have been induced by chemical anoxia. This may be due to lactate production, the rate of which increases under these conditions. With respect to the transporters involved in acid removal, a switch in the relative importance of different mechanisms must have occurred, compared to the normoxic situation. Na^+/H^+ exchange, although of considerable importance under normoxia (Table 1, Fig. 3), did apparently not contribute to the maintenance of pHi during chemical anoxia in goldfish hepatocytes, since neither amiloride nor Na^+-free conditions significantly affected the rate of proton release evoked by CN (Table 2). This is consistent with the idea that this transporter is not fully activated as long as pHi does not decrease (Renner et al., 1989). By contrast, while the SITS-sensitive transporter was apparently silent under normoxia (Table 1), SITS did cause a significant reduction in proton release under chemical anoxia (Table 2). The lack of effect of Na^+-free conditions suggests that this transporter is a Na^+-independent Cl^-/HCO_3^- exchanger. However, it should be kept in mind that SITS may also inhibit the monocarboxylate transporter and thus lactate release from the cells (Poole and Halestrap, 1993), although our data are somewhat ambiguous concerning the role of lactate metabolism and/or release in terms of pHi regulation. The drastic reduction of proton release in the presence of IAA, both with and without CN (Table 2), indicates that protons generated in concert with the formation of lactate play a decisive role in the regulation of proton homeostasis. However, our attempt to clarify the role of lactate release in this context failed insofaras partial inhibition of lactate release by quercetin (Fig. 6) also caused a decrease in overall lactate production, possibly by a feedback inhibition of glycolytic activity. Hence, the relatively smaller increase in CN-induced acid secretion in the presence of quercetin (Fig. 7) cannot be fully attributed to the reduction of lactate release.

pHi regulation under chemical anoxia in trout hepatocytes

The decrease of pHi induced by chemical anoxia in trout hepatocytes, as well as its reversibility upon washout of CN, agrees with observations on other anoxia-sensitive cells (Schoenecker and Weinman, 1994; Ruß et al., 1996). This intracellular acidification occurred despite a significant increase in the rate of acid secretion, the magnitude of which was similar to that seen in goldfish hepatocytes (Table 2). However, the mechanisms involved in acid removal under chemical anoxia differed from that observed in the goldfish cells. In contrast to goldfish hepatocytes a Cl^-/HCO_3^- exchange mechanism was apparently not operative in anoxic trout hepatocytes, as SITS had no effect on CN-induced acid secretion (Table 2). Furthermore, we found that Na^+/H^+ exchange was the predominant mechanism of acid removal, with amiloride and exposure to Na^+-free saline totally preventing an increase in the rate of proton secretion above baseline levels (Fig. 5, Table 2). The increase in acid release from the diminished rate obtained in the presence of amiloride to that seen when both amiloride and CN were present was also SITS-insensitive (Table 2). We again, therefore, suspect lactate release to be the responsible mechanism. This is in line with our observation that trout hepatocytes, like the goldfish cells, did not accumulate lactate within the cytosol, but completely released it into the incubation medium (data not shown).

The predominant role of Na^+/H^+ exchange as a proton release mechanism contrasts with findings in rat hepatocytes where the Na^+/H^+ exchanger was inhibited under chemical anoxia, the resulting acidification prolonging cell survival (Schoenecker and Weinman, 1994). Whether this reflects different mechanisms controlling the activity of this ion transporter in both cell types remains unresolved.

Extracellular acidosis and cell energetics

Although extracellular acidosis is involved in the induction of metabolic depression in other anoxia-tolerant organisms (Hand and Hardewig, 1996; Reipschläger and Pörtner, 1996), it appeared to have no effect on the energetics of goldfish hepatocytes. Thus neither oxygen consumption nor rates of lactate production, estimates of normoxic and anoxic rates of ATP production, were affected at low, as compared to normal, pHe (Table 3). On the other hand, the elevated level of cellular ATP after 30 min of chemical anoxia at pHe 6.6 suggests that ATP-consuming processes may be partially inhibited at low pHe, as described for mitochondrial protein synthesis in the anoxia-tolerant brine shrimp embryo (Kwast and Hand, 1996). Although at present we have no data to substantiate this speculation, we believe that this might occur in the liver *in situ*, and thus contribute to metabolic depression, as plasma pH decreases during anoxia in the closely related crucian carp (Van den Thillart and Van Waarde, 1991).

In trout hepatocytes, incubation at low pHe had no significant effect on any parameter of cell energetics studied here. Although it seemed that low pHe helped to preserve ATP levels during chemical anoxia, we believe this to be accidental, as initial ATP levels of hepatocytes at pH 6.6 were already slightly lower than at pH 7.6 (Table 3).

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