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Volumetric and ionic responses of goldfish hepatocytes to anisotonic exposure and energetic limitation

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Summary

The relationship between cell volume and K^+ transmembrane fluxes of goldfish (*Carassius auratus*) hepatocytes exposed to anisotonic conditions or energetic limitation was studied and compared with the response of hepatocytes from trout (*Oncorhynchus mykiss*) and rat (*Rattus rattus*). Cell volume was studied by video- and fluorescence microscopy, while K^+ fluxes were assessed by measuring unidirectional $^{86}Rb^+$ fluxes.

In trout and rat hepatocytes, hyposmotic (180 mosmol l^{-1}) exposure at pH 7.45 caused cell swelling followed by a regulatory volume decrease (RVD), a response reported to be mediated by net efflux of KCl and osmotically obliged water. By contrast, goldfish hepatocytes swelled but showed no RVD under these conditions. Although in goldfish hepatocytes a net ($^{86}Rb^+$)K^+ efflux could be activated by *N*-ethylmaleimide, this flux was not, or only partially, activated by hyposmotic swelling (120–180 mosmol l^{-1}).

Blockage of glycolysis by iodoacetic acid (IAA) did not alter cell volume in goldfish hepatocytes, whereas in the presence of cyanide (CN^-), an inhibitor of oxidative phosphorylation, or CN^- plus IAA (CN^-+IAA), cell volume decreased by 3–7%. Although in goldfish hepatocytes, energetic limitation had no effect on ($^{86}Rb^+$)K^+ efflux, ($^{86}Rb^+$)K^+ influx decreased by 57–66% in the presence of CN^- and CN^-+IAA but was not significantly altered by IAA alone. Intracellular K^+ loss after 20 min of exposure to CN^- and CN^-+IAA amounted to only 3% of the total intracellular K^+.

Collectively, these observations suggest that goldfish hepatocytes, unlike hepatocytes of anoxia-intolerant species, avoid a decoupling of transmembrane K^+ fluxes in response to an osmotic challenge. This may underlie both the inability of swollen cells to undergo RVD but also the capability of anoxic cells to maintain intracellular K^+ concentrations that are almost unaltered, thereby prolonging cell survival.

Key words: cell volume, goldfish, *Carassius auratus*, hepatocyte, trout, *Oncorhynchus mykiss*, rat, anoxia, K^+ flux, water transport.

Introduction

In hepatocytes of most vertebrate species, blockage of ATP production is followed by membrane depolarization, intracellular acidification, a rise of cytosolic Ca^{2+}, Mg^{2+} and Na^+, activation of degradative hydrolases, cell swelling, and K^+ loss (Lemasters et al., 1987; Herman et al., 1988; Carini et al., 1995). The cause underlying these alterations is the reduction of active ion transport, which is responsible for the maintenance of the gradients of most diffusible ions away from thermodynamic equilibrium, due to the lack of intracellular ATP.

In hepatocytes of the goldfish, a facultative anaerobe vertebrate, the above changes are slow to develop or do not occur at all. Specifically, during anoxia, goldfish hepatocytes display a set of dynamic metabolic responses that prolong cell survival, including: (1) reallocating metabolic energy to essential functions (Krumschnabel and Wieser, 1994); (2) avoiding the rise of cytosolic Ca^{2+} by a net extrusion of the cation (Krumschnabel et al., 1997) and (3) secreting protons that, together with a high cytosolic buffer capacity, allow cytosolic pH to be maintained (Krumschnabel et al., 2001a).

Although the principal mechanisms that allow goldfish hepatocytes to tolerate metabolic inhibition have thus been characterized, the potential osmotic disturbance associated with these conditions has not been studied so far. However, investigation of the osmotic responses is important because, in hepatocytes from anoxia-intolerant species, impairment of cell volume regulation associated with severe metabolic inhibition contributes significantly to the events leading to cell necrosis (Carini et al., 1999). Previous studies on volume regulation using mammalian hepatocytes (Häussinger and Lang, 1991; Wehner and Tinel, 2000) and fish hepatocytes (Bianchini et al., 1988, 1991) provided some insight into how vertebrate hepatocytes respond to an osmotic gradient in the absence or presence of

metabolic inhibitors. Under non-inhibited conditions, vertebrate hepatocytes challenged by either hyposmotic media or sodium-coupled amino acid uptake increase their volume, which is followed by a loss of K^+, Cl^- and water, resulting in a reduced cell volume [i.e. a regulatory volume decrease (RVD)].

A mismatch between intracellular and extracellular osmolarity may also develop during metabolic inhibition. For example, in rat hepatocytes, blockage of oxidative phosphorylation promotes an increase of intracellular Na^+, causing cell swelling (Carini et al., 1995). Furthermore, exposure of hepatocytes from rat (Anundi and de Groot, 1989), trout and goldfish (Krumschnabel et al., 1996) to hypoxia and anoxia enhances the glycolytic flux (a process termed the 'Pasteur effect'), thereby increasing the concentration of glycolytic intermediates. The degradation of glycogen to glucose phosphate and the anaerobic increase of glycolytic intermediates have been postulated, but not proven, to increase osmotically active substances in the cytosol, causing hepatocyte swelling (Wehner et al., 1992; Corassanti et al., 1990; Häussinger and Lang, 1991; Lang et al., 1998). At the same time, enhanced anaerobic metabolism is accompanied by an increased rate of export of organic osmolytes. For example, as reported for hepatocytes of trout and goldfish, chemical anoxia leads to an elevated transport of glucose and lactate out of the cell (Krumschnabel et al., 2001a), a response that might counteract the potential increases of intracellular osmolarity of energetically compromised cells.

In the present study, we examined how osmotic gradients affect cell volume and ($^{86}Rb^+$)K^+ transmembrane fluxes of goldfish hepatocytes, with particular focus on the osmotic effects of metabolic inhibition. The major aims of this investigation were to characterize the volumetric responses of these anoxia-tolerant cells under conditions of energetic steady state and energetic limitation and to compare these responses with those of anoxia-intolerant hepatocytes. Furthermore, as goldfish hepatocytes have an exceptional capability of maintaining ion homeostasis, we wanted to elucidate whether K^+ flux balance is also preserved in response to an osmotic challenge or whether, under these conditions, K^+ homeostasis is temporarily suspended in order to restore cell volume.

Materials and methods

Chemicals

Collagenase (Type IV for rat hepatocytes, Type VIII for trout and goldfish hepatocytes), L-alanine, aminooxyacetic acid (AOA), and poly-L-lysine were purchased from Sigma (St Louis, MO, USA). The acetoxymethyl (AM) ester of calcein was obtained from Molecular Probes (Eugene, OR, USA). $^{86}Rb^+$[RbCl] (1.48×10^8 Bq mg^{-1}; approximately 1.48×10^9 Bq ml^{-1}) was obtained from NEN Life Science Products (Boston, MA, USA). All other reagents were of analytical grade.

Animals

Goldfish *Carassius auratus* L. (10–30 g) were obtained commercially from local dealers in Buenos Aires. They were kept in 200 l tanks at 20°C. Rainbow trout *Oncorhynchus mykiss* Walbaum (150–250 g) were obtained from the Center of Aquaculture from the Universidad del Comahue (Bariloche, Argentina) and were maintained in 200 l tanks at 15°C. Fish were acclimated to the above-specified temperatures for at least two weeks before being used. Male Wistar rats *Rattus rattus* (200–300 g) were fasted for 12 h before being used.

Isolation of hepatocytes

Fish were killed by a blow to the head and transection of the spinal cord, whereas rats were anesthetized with pentobarbital (150 mg kg^{-1}, intraperitoneal). Hepatocytes of trout and rat were isolated by collagenase digestion methods, which required perfusion of the portal vein as described previously (Berry, 1974; Krumschnabel et al., 1996). The small size of goldfish, together with the diffuse distribution of hepatic tissues in cyprinids, prevented the use of the perfusion techniques for the isolation of hepatocytes. Therefore, goldfish hepatocytes were isolated by incubating fragments of liver tissue with a collagenase medium as described previously (Schwarzbaum et al., 1992; Krumschnabel et al., 1994).

Alternatively, in a few experiments shown in Fig. 2, goldfish hepatocytes were isolated by a similar procedure, except that collagenase medium was replaced by a collagenase-free medium containing EDTA, as described by Seddon and Prosser (1999).

After isolation, the cells were incubated in isotonic medium (see below) for 45 min in a shaking water bath at 20°C before use. The viability of isolated hepatocytes (>90%) was routinely assessed by Trypan blue exclusion (before the onset of each experiment) and retention of calcein fluorescence (at the end of each experiment).

Conversion factors in goldfish hepatocytes

The ratio of total protein content to cell number was 0.7±0.1 (mg protein 10^{-6} cells^{-1}) (N=13). Cell diameter assayed by videomicroscopy was 12.77±0.24 μm (147 cells from 10 independent preparations) and the percentage of dry mass was 25±1% (N=19). The yield of cells was $62.2\pm9.7\times10^6$ cells g fresh mass^{-1} (N=15). For each independent experiment, livers from 1–3 fish individuals were pooled.

Incubation protocols

Except where otherwise stated, cells from goldfish, rat and trout were incubated in media at 20°C (pH 7.45) with the following composition.

Isotonic control media:

medium A (goldfish): 10 mmol l^{-1} Hepes, 135.2 mmol l^{-1} NaCl, 3.8 mmol l^{-1} KCl, 1.3 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} KH_2PO_4, 1.2 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} $NaHCO_3$; osmolarity, 300 mosmol l^{-1};

medium B (trout): 10 mmol l^{-1} Hepes, 136.9 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 1.5 mmol l^{-1} $CaCl_2$, 0.33 mmol l^{-1} Na_2HPO_4, 0.44 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgSO_4$, 5 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} glucose; osmolarity, 292 mosmol l^{-1};

medium C (rat): 25 mmol l^{-1} Hepes, 120 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2.6 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} NaH_2PO_4, 0.5 mmol l^{-1} $MgSO_4$, 4.2 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} glucose, 0.2% bovine serum albumin (BSA); osmolarity, 265 mosmol l^{-1}.

Hypotonic media were prepared by mixing different amounts of isotonic media with medium D, which consisted of 10 mmol l^{-1} Hepes, 5 mmol l^{-1} KCl. Medium E (osmolarity, 503 mosmol l^{-1}) had the following composition: 10 mmol l^{-1} Hepes, 135.2 mmol l^{-1} NaCl, 3.8 mmol l^{-1} KCl, 1.3 mmol l^{-1} $CaCl_2$, 1.2 mmol l^{-1} KH_2PO_4, 1.2 mmol l^{-1} $MgSO_4$, 10 mmol l^{-1} $NaHCO_3$, 200 mmol l^{-1} sucrose.

Other test media for goldfish hepatocytes had the following composition:

medium F: medium A containing 10 mmol l^{-1} L-alanine plus 2.5 mmol l^{-1} AOA (ALA-AOA), an inhibitor of L-alanine conversion to pyruvate (Kristensen, 1986);

medium G: medium A containing 1 mmol l^{-1} ouabain (OB; a blocker of Na^+/K^+-ATPase activity);

hyposmotic media in the presence of 1 mmol l^{-1} *N*-ethylmaleimide (NEM), a thiol-alkylating agent known to activate KCl cotransport (Lauf, 1982);

iso- and hyposmotic media as described above, but at pH 7.8.

The osmolarity of all media was measured with a vapor pressure osmometer (5100 B; Wescor Inc., Logan, UT, USA).

Experiments on metabolic inhibition were performed by incubating hepatocytes in isotonic media A (goldfish) or C (rat) in the absence of inhibitors (control condition) or in the presence of 2 mmol l^{-1} sodium cyanide (CN^-), 0.5 mmol l^{-1} iodoacetic acid (IAA) or both (CN^-+IAA). In order to evaluate the nature of the K^+ channels potentially activated under chemical anoxia, goldfish hepatocytes were incubated in the presence of either 5 mmol l^{-1} $BaCl_2$, 1 mmol l^{-1} tetraethylammonium (both blockers of voltage-sensitive K^+ channels) or 1 mmol l^{-1} quinine (a blocker of Ca^{2+}-sensitive K^+ channels). Blockers were added 4 min before CN^- addition to block the specific K^+ channels before inhibition of mitochondrial activity.

Rates of ($^{86}Rb^+$)K^+ influx and efflux

Influx and efflux of K^+ were estimated using the radioactive isotope of Rb^+ ($^{86}Rb^+$), which acts as a K^+ analog (Krumschnabel et al., 1996). Pulse experiments were used to determine the initial rate of ion uptake at various times. Cells were incubated in iso- (medium A) and hypotonic (180 mosmol l^{-1}) media.

($^{86}Rb^+$)K^+ influx

Cells (12×10^6 cells ml^{-1}) were diluted 1:2 in medium A (isotonic condition) or hypotonic medium (180 mosmol l^{-1}), as required for each experiment. At 10 min, 20 min and 40 min, 3.5 ml of the cell suspension was transferred to 7 ml conical tubes containing 4.67×10^4 Bq mg^{-1} of $^{86}Rb^+$ (the dilution was negligible) and was gently agitated for 60 s. After 1 min, 4 min and 7 min of incubation, 500 µl duplicate samples of the cell suspension were transferred to 1.5 ml Eppendorf reaction tubes containing 500 µl of ice-cold 100 mmol l^{-1} $MgCl_2$ medium to stop the uptake. Cells were centrifuged for 4 s at 6700 ***g***; the supernatant was sucked out of the reaction tube, and external medium adhering to the cell pellet and the walls of the reaction tube was then diluted twice by carefully layering 1 ml of ice-cold $MgCl_2$ medium on top of the cells and removing it by aspiration. This procedure ensured a small background signal. The final cell pellet was vortexed with 1 ml of scintillation cocktail, and radioactivity was assessed by scintillation counting. 5 µl duplicate samples were used to determine specific radioactivity. Results were expressed as nmol $^{86}Rb^+$ 10^{-6} $cells^{-1}$ min^{-1}.

($^{86}Rb^+$)K^+ efflux

Hepatocytes were incubated in the presence of 6.47×10^4 ^{86}Bq Rb^+ ml^{-1} for 2.5 h. Subsequently, cells were diluted 1:2 in medium A or in hyposmotic medium, both containing 6.47×10^4 ^{86}Bq Rb^+ ml^{-1}. After 10 min, 20 min and 40 min of incubation, 1 ml of the cell suspension was transferred to 1.5 ml Eppendorf reaction tubes and centrifuged for 4 s at 6700 ***g***. The supernatant was sucked out of the reaction tube, and external medium adhering to the cell pellet and the walls of the reaction tube was then diluted twice by carefully layering 1 ml of either iso- or hypotonic medium on top of the cells and removing it by aspiration.

Following this, the cell pellet was resuspended in 1 ml of unlabeled medium A or hypotonic medium. From this cell suspension, 200 µl duplicate samples were withdrawn after 4 min and 8 min, and the pellets were separated from medium by rapid centrifugation as described for $^{86}Rb^+$ influx. An aliquot of the supernatant was removed, and radioactivity was determined by scintillation counting. Results were expressed as c.p.m. 10^{-6} $cells^{-1}$ min^{-1}.

Measurement of intracellular Na^+

200 µl aliquots of cell suspension (40×10^6 cells ml^{-1}) were layered on the top of 1 ml of phtalic/phtalate solution (40% phtalic acid plus 60% dibutyl-phthalate) and spun for 1 min at 6700 ***g***. After centrifugation, the phtalic/phtalate solution was removed by aspiration and the cell pellet diluted in 500 µl distilled water and sonicated for 30 min. In some experiments, aliquots of cell suspensions were preincubated in 100 mmol l^{-1} $MgCl_2$ (an Na^+-free medium) for 30 min. Cells were then spun for 4 s at 6700 ***g*** and resuspended in 100 mmol l^{-1} $MgCl_2$ before the onset of the experiment. The level of intracellular Na^+ was measured by flame photometry using an EEL Flame photometer (Evans Electroselenium Ltd, Halstead, UK).

Assessment of cell volume

Videomicroscopy

The size of hepatocytes was assessed by quantitative phase-contrast microscopy. Cells were loaded onto a 500 µl glass chamber (no adhesion medium was necessary because cells remained attached during measurements) and allowed to equilibrate for 20 min in a continuously superfused medium A

at 0.5 ml min^{-1}. At specific times following the start of the experiment, different media were superfused into the chamber at the same flow, according to the treatment.

Hepatocytes were viewed through phase-contrast optics (total magnification, 300×) on an inverted microscope (Olympus IMT-2) equipped with a 20×, N.A. 0.40 objective. Images were captured by means of a CCD camera (EDC-1000, Electrim Corp., Princeton, USA) operating at fixed gain. Images were recorded on a computer through data translation hardware boards and were processed by means of the Optimet program (Bioscan, Inc., Edmonds, USA). Cell volumes were estimated from diameters, assuming that the cells had spherical shape and that their volume changed by the same magnitude in all radial directions. In all cases, the diameters of the same cells were measured throughout the experiment and cell volumes were only computed for cells that remained alive during the whole experiment.

Results are presented as means ± S.E.M. of four independent experiments, using 13–15 cells per experiment. Volume data were expressed as total volume (V; in cm^3) or as relative cell volume (V_r), where $V_r=V_t/V_0$; V_t is the value of V at time t, and V_0 is the value of V at time 0. Under the experimental conditions applied in this study, this technique allowed changes in V of within 8% to be detected.

Epifluorescence microscopy

Hepatocytes were plated on 25 mm-diameter glass coverslips (Fischer Scientific, Pittsburg, PA, USA) that had been previously coated with 0.1% w/v poly-L-lysine. Each coverslip with attached cells was mounted in a chamber filled with isotonic medium and placed on the stage of a Nikon TE-200 epifluorescence inverted microscope. Hepatocytes were then loaded with 2 μmol l^{-1} of calcein-AM. Dye loading was monitored fluorometrically by sampling the signal of single cells every 180 s until fluorescence of the cells reached 5–10 times the autofluorescence level. The loading time was 45–60 min. The loading solution was then washed out with isotonic medium for at least 1 h before starting the experimental data acquisition. Experimental solutions were superfused at a rate of 2 ml min^{-1}.

Changes in cell water volume were inferred from readings of the fluorescence intensity recorded by exciting calcein through a 470 CWL excitation filter (Nikon Inc., Melville, USA) and were imaged with a 500 nm LP dichroic mirror (Nikon Inc.) and a 515 LP barrier filter (Nikon Inc.). Changes in fluorescence intensity due to changes in intracellular fluorophore concentration were recorded from a small region of dye-loaded cells using a customized microspectrophotometry system described in detail elsewhere (Alvarez-Leefmans et al., 1997). Values of V_r were computed from monitored changes in relative fluorescence (F_t/F_o), where F_o is the fluorescence from a pinhole region of the cell equilibrated with isotonic medium, and F_t is the fluorescence of the same region of the cell exposed to an anisotonic medium. This technique allows for continuous measurements of V_r changes to within 1% (Alvarez-Leefmans et al., 1995). A detailed description of the technique, its validation and corresponding computations can be found elsewhere (Alvarez-Leefmans et al., 1995; Altamirano et al., 1998).

Statistics

The effect of the different treatments on $^{86}Rb^+$ transmembrane fluxes was evaluated by one-way analysis of variance (ANOVA) followed by a Tukey–Kramer multiple comparisons test. $P\leq0.05$ was considered significant. Exponential fits for the calculation of V_m (maximal V) ± S.E. were calculated by non-linear regression.

Results

Volume changes in anisotonic media

Fig. 1 shows the time course of changes of V (cm^3) of goldfish hepatocytes exposed to (1) media of various nominal osmolarities ranging from 120 mosmol l^{-1} to 503 mosmol l^{-1} and (2) isosmotic medium with ALA-AOA. In isosmotic medium (approximately 300 mosmol l^{-1}), V remained constant over the entire period of observations. Upon exposure of cells to 120 mosmol l^{-1} (i.e. 60% hyposmotic) and 180 mosmol l^{-1} (i.e. 40% hyposmotic) media and to ALA-AOA, V increased and reached a steady level with no apparent signs of regulation. Plots of V *versus* time were reasonably well described by an exponential function with the parameters shown in Table 1.

The absence of RVD in hyposmotic medium led us to perform a series of experiments to check the validity of this result as follows.

(1) Goldfish hepatocytes that had been isolated in collagenase-free medium were exposed to 180 mosmol l^{-1} medium (Fig. 2A), showing a volumetric response similar to that of hepatocytes isolated with collagenase and, importantly, no RVD response.

(2) As a comparison, rat and trout hepatocytes were exposed to iso- and hypotonic media under conditions identical to those used for goldfish hepatocytes. In the cells from both species, exposure to 180 mosmol l^{-1} media induced an increase of V_r to

Table 1. *Results of fitting an exponential function of the form* $V=V_0+(V_m-V_0)(1-e^{-kt})$ *to experimental data of Fig. 1*

Treatment	V_m (10^{-9} cm^3)	$t_{1/2}$ (min)
120 mosmol l^{-1}	3.46±0.24	7.54±1.29
180 mosmol l^{-1}	2.00±0.12	5.44±1.60
180 mosmol l^{-1} (CFM)	2.03±0.18	7.92±4.05
ALA-AOA	1.63±0.08	11.93±7.07
503 mosmol l^{-1}	0.90±0.07	8.41±1.54

Values of parameters ± S.E. (standard error of the fit).

V_0 and V_m denote initial and maximal/minimal total volume (V), respectively. The value of $t_{1/2}$ (the time necessary to achieve 50% of V_m) was calculated as follows: $t_{1/2}=\ln2/k$, where k denotes a constant. ALA-AOA denotes an isosmotic medium in the presence of 10 mmol l^{-1} L-alanine plus 2.5 mmol l^{-1} aminooxyacetic acid. CFM denotes experiments with hepatocytes that had been isolated in collagenase-free medium (see Materials and methods).

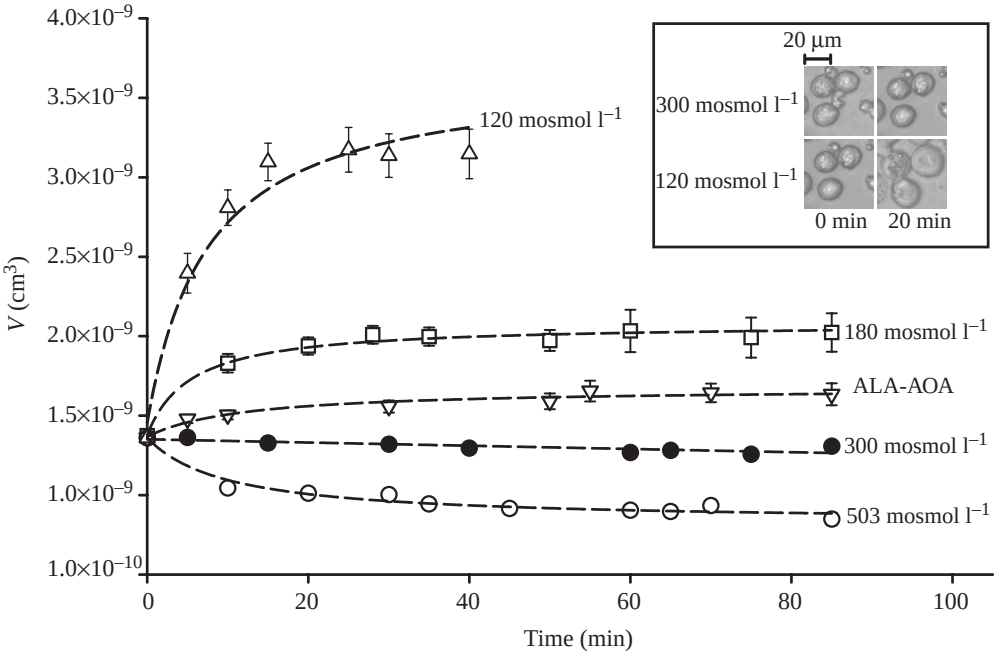

Fig. 1. Time course of total volume (V) change in goldfish hepatocytes, assessed by quantitative phase-contrast microscopy. Cells were exposed to media of the various osmolarities indicated at pH 7.45 and to L-alanine, aminooxyacetic acid (ALA-AOA). Results are means ± S.E.M. (N=4; in certain points, the error bars lie within the symbol). Lines represent the exponential fit to experimental data with parameters of best fit given in Table 1. The inset shows phase-contrast micrographs of goldfish hepatocytes in 300 mosmol l^{-1} (top panels) and 120 mosmol l^{-1} media (bottom panels).

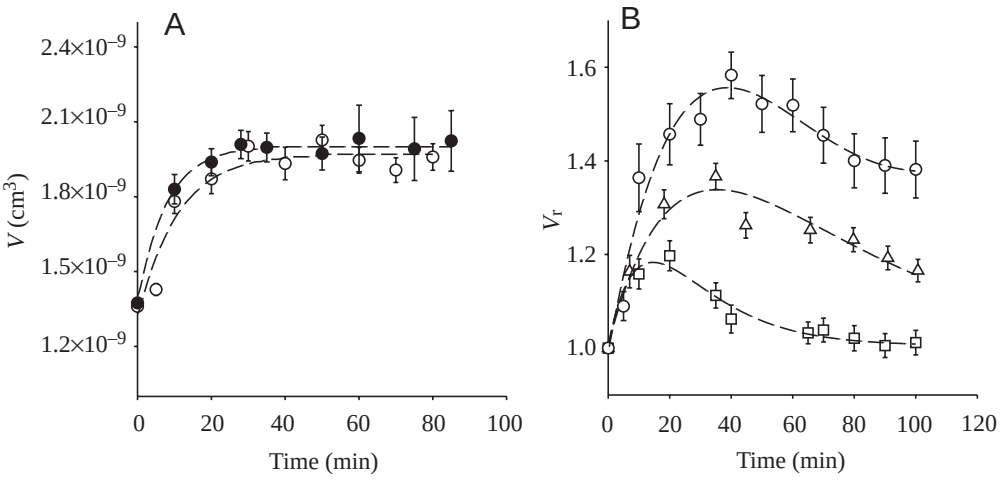

Fig. 2. Time course of volume changes in hepatocytes from goldfish, trout and rat, assessed by quantitative phase-contrast microscopy. (A) Total volume (V) changes of hepatocytes from goldfish exposed to 180 mosmol l^{-1} medium at pH 7.45. Hepatocytes were isolated in collagenase-containing medium (filled circles) or in collagenase-free medium (open circles). (B) Relative volume (V_r) changes of hepatocytes of rat (triangles) and trout (squares) exposed to 180 mosmol l^{-1} medium at pH 7.45 and of goldfish hepatocytes (circles) exposed to 180 mosmol l^{-1} at pH 7.8. Values are means ± S.E.M. (N=4). Lines represent the empirical fit to data, with values of the parameters given in Table 1 and in the Results section.

a maximum, followed by a volume decrease towards initial values (Fig. 2B). An exponential function of the form $V_r=V_{r0}+Ate^{-nt}$ was fitted to the experimental data, yielding maximal V_r values of 1.34 (rat) and 1.18 (trout) when t=1/n (V_{r0} is the value of V_r at time 0, whereas A and n denote constants).

(3) To evaluate the possibility that the occurrence of RVD in goldfish hepatocytes is activated only at a more alkaline extracellular pH, cells were exposed to iso- and hypotonic (180 mosmol l^{-1}) media at pH 7.8. Under this condition, exposure to 180 mosmol l^{-1} induced an increase of V_r to a maximum at 1.58±0.05, followed by a volume decrease of approximately 13% (V_r=1.38±0.06) of isosmotic values (Fig. 2B).

Volume changes under conditions of energetic limitation

As preliminary experiments indicated that changes in cell volume during metabolic inhibition may be very small and thus lie within the error of the videomicroscopy technique, we decided to measure the effect of metabolic inhibition on V_r by using quantitative epifluorescence microscopy. Results of these experiments are shown in Figs 3, 4.

In goldfish hepatocytes, addition of IAA had no significant effect on V_r (Fig. 3A). In the presence of CN^-, V_r decreased by 3.2±0.51% (t=34 min; P<0.01, N=4), with subsequent recovery towards initial values (Fig. 4A). A similar pattern of shrinking followed by an increase in V_r, and even cell swelling, was observed in the presence of both CN^- and IAA, although initial V_r shrinkage was more pronounced

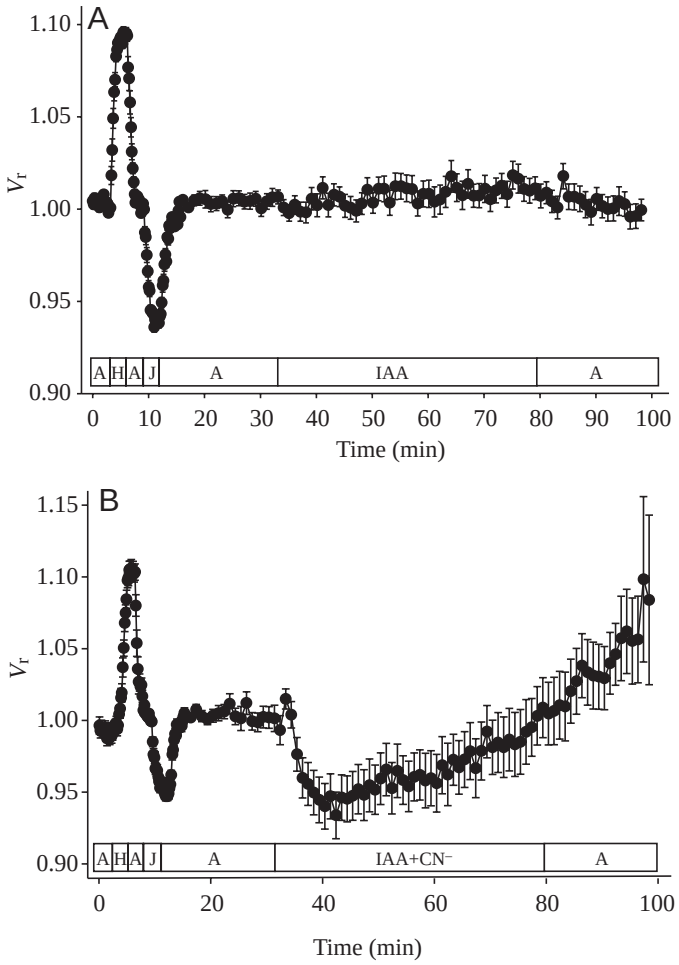

Fig. 3. Relative volume (V_r) *versus* time in goldfish hepatocytes incubated in control medium A or in medium with (A) iodoacetic acid (IAA) or (B) cyanide plus iodoacetic acid (CN^-+IAA). Calibration was performed with anisosmotic media of 264 mosmol l^{-1} (medium H) and 308 mosmol l^{-1} (medium J). Results are means ± S.E.M. (N=4).

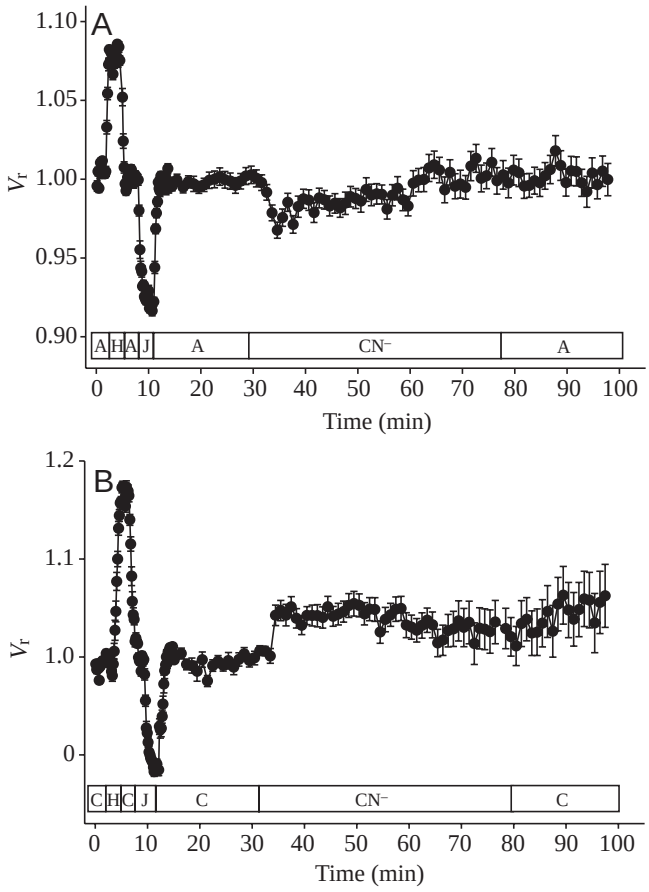

Fig. 4. Relative volume (V_r) *versus* time in hepatocytes from (A) goldfish and (B) rat incubated in control media (denoted as A for goldfish cells and C for rat cells) or in medium with cyanide (CN^-). Calibration was performed with anisosmotic media as follows: (A) 264 mosmol l^{-1} (medium H) and 308 mosmol l^{-1} (medium J); (B) 225 mosmol l^{-1} (medium H) and 293 mosmol l^{-1} (medium J). Results are means ± S.E.M. (N=4).

(6.6±1.6% compared with controls at 42 min; Fig. 3B; P<0.01, N=4).

Comparative experiments with rat hepatocytes incubated with CN^- showed that these cells increased in V_r by 5.1±1.1% over control values without any regulation being detected (Fig. 4B; P<0.01, N=4).

Unidirectional ($^{86}Rb^+$)K^+ fluxes in goldfish hepatocytes

Using $^{86}Rb^+$ as a K^+ analog, we determined the impact of the different inhibitory treatments on the transmembrane influx and efflux of K^+ in goldfish hepatocytes.

Anisotonic media

Because in many cell types K^+ efflux is an early event mediating the RVD (Häussinger et al., 1994; Fugelli et al., 1995), we tested whether ($^{86}Rb^+$)K^+ transmembrane fluxes decouple during the first 40 min of incubation under hyposmotic conditions. Results in Figs 5, 6 show that the magnitude of ($^{86}Rb^+$)K^+ efflux, as well as that of the influx, remained constant in isosmotic as well as in hyposmotic (i.e. 180 mosmol l^{-1}) media. In separate experiments, swelling of hepatocytes was induced by exposure to 180 mosmol l^{-1} medium for 30 min and then 1 mmol l^{-1} NEM, an activator of Cl^--dependent K^+ transport in fish cells was added (Bianchini et al., 1988; Jensen, 1994; Bogdanova and Nikinmaa, 2001). In this condition, ($^{86}Rb^+$)K^+ efflux increased approximately 3.9-fold (386±44%, N=4) with respect to control values (Fig. 7).

As a slight RVD had been observed in hyposmotic medium at pH 7.8 (Fig. 2B), we performed another series of ($^{86}Rb^+$)K^+ flux experiments as described above but with media adjusted to this higher pH value. Again, ($^{86}Rb^+$)K^+ efflux and influx remained constant in isosmotic media. In 180 mosmol l^{-1} medium, however, although ($^{86}Rb^+$)K^+ influx remained constant (0.30±0.04 nmol 10^{-6} cells^{-1} min^{-1} in controls *versus* 0.36±0.03 nmol 10^{-6} cells^{-1} min^{-1} in hypotonically treated cells, N=4), efflux increased significantly to 152±13% (N=4) of the isosmotic control values (Fig. 7).

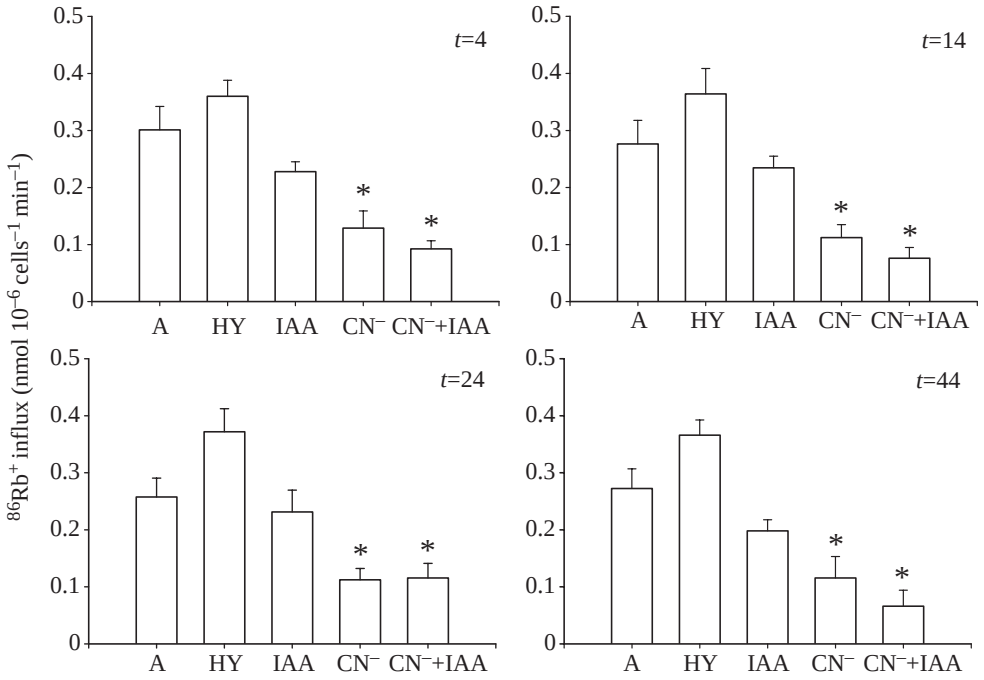

Fig. 5. Rates of $^{86}Rb^+$ influx in goldfish hepatocytes during 44 min of exposure to control medium (A), 180 mosmol l^{-1} medium (HY), isosmotic media with cyanide (CN^-), iodoacetic acid (IAA) and cyanide plus iodoacetic acid (CN^-+IAA). Fluxes were measured at 4 min, 14 min, 24 min and 44 min after the start of the experiment. Results are means + S.E.M. (N=4). An asterisk indicates P<0.05 with respect to isosmotic control values.

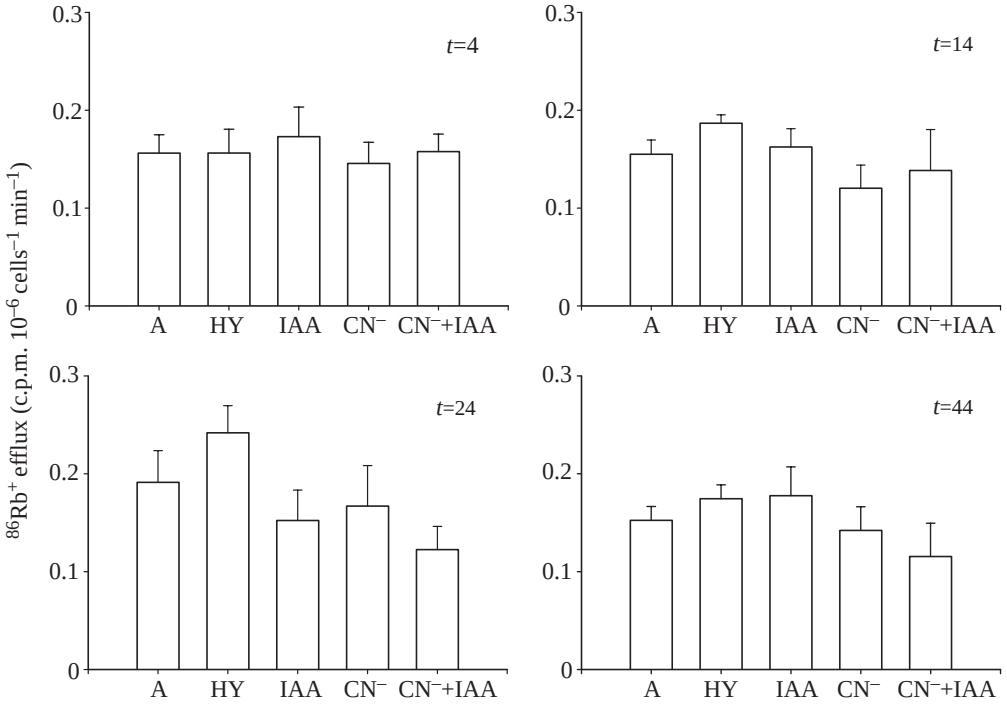

Fig. 6. Rates of $^{86}Rb^+$ efflux in goldfish hepatocytes during 44 min of exposure to control medium (A), 180 mosmol l^{-1} medium (HY), isosmotic media with cyanide (CN^-), iodoacetic acid (IAA), and cyanide plus iodoacetic acid (CN^-+IAA). Fluxes were measured at 4 min, 14 min, 24 min and 44 min after the start of the experiment. Results are means + S.E.M. (N=4).

Metabolic inhibition

Incubation in the presence of IAA did not significantly affect $^{86}Rb^+$ influx. In the presence of both CN^- and CN^-+IAA, however, $^{86}Rb^+$ influx decreased acutely, with flux values reaching 47±11% (CN^-) and 34±5% (CN^-+IAA) of control values (Fig. 5) after 4 min of incubation.

Metabolic inhibition by either CN^-, IAA or CN^-+IAA did not significantly (P>0.05, N=4) affect $^{86}Rb^+$ efflux (Fig. 6). In addition, ($^{86}Rb^+$)K^+ efflux under chemical anoxia was not altered in goldfish hepatocytes preincubated in the presence of either 5 mmol l^{-1} $BaCl_2$, 1 mmol l^{-1} tetraethylammonium (both blockers of voltage-sensitive K^+ channels) or 1 mmol l^{-1} quinine (a blocker of Ca^{2+}-sensitive K^+ channels) (P>0.75, N=4; data not shown).

Intracellular Na^+

Exposure of goldfish hepatocytes to CN^- for 40 min produced no significant change in the level of intracellular Na^+

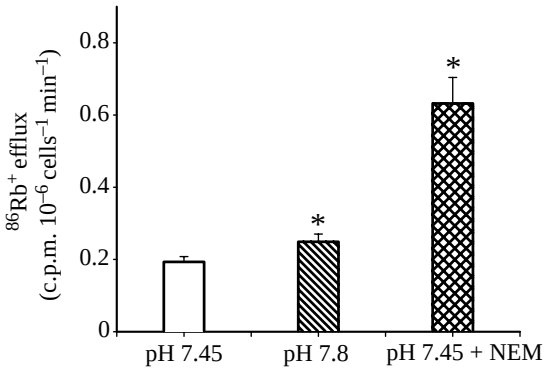

Fig. 7. Rates of $^{86}Rb^+$ efflux in goldfish hepatocytes exposed to 180 mosmol l^{-1} medium and pH 7.45, pH 7.8 or pH 7.45 + *N*-ethylmaleimide (NEM). Results are means + S.E.M. (*N*=4). An asterisk indicates $P<0.05$ with respect to isosmotic values.

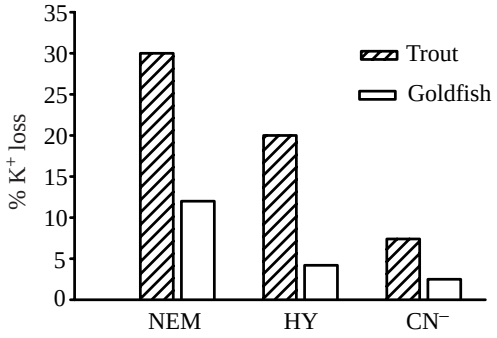

Fig. 9. Loss of intracellular K^+ (as a percentage of control values) in trout and goldfish hepatocytes exposed to *N*-ethylmaleimide (NEM), hyposmotic medium (HY) and cyanide (CN^-). For trout hepatocytes, loss of intracellular K^+ was derived from data of Bianchini et al. (1988, 1991) and Krumschnabel et al. (1996).

(Fig. 8), whereas in the presence of 1 mmol l^{-1} ouabain a significant increase in Na^+ content was noted after this time of incubation (Fig. 8). Furthermore, in cells preincubated for 30 min in the presence of 100 mmol l^{-1} $MgCl_2$ (an isosmotic Na^+-free medium), Na^+ content was approximately 16% of control values and showed no significant change over 40 min.

Discussion

The results in this study show, for the first time, the volumetric response of goldfish hepatocytes exposed to transmembrane osmotic gradients generated by changes in extracellular as well as intracellular osmolarity.

In hyperosmotic medium (503 mosmol l^{-1}), goldfish hepatocytes shrank to a constant volume without showing regulatory volume increase, a feature also observed in hepatocytes from other vertebrate species (Corassanti et al., 1990). On the other hand, in hypotonic media (including isosmotic medium with L-alanine), cells increased their volume

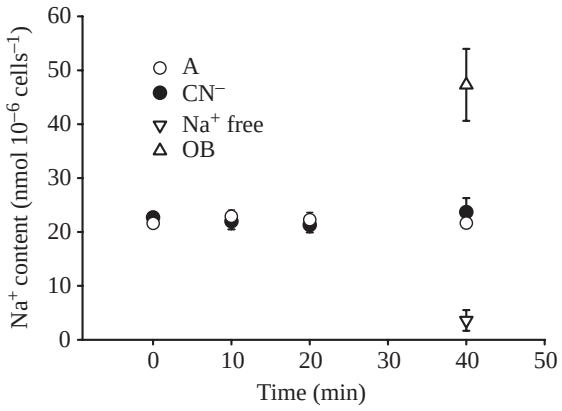

Fig. 8. Intracellular Na^+ (nmol 10^{-6} cells^{-1}) *versus* time in hepatocytes of goldfish exposed to control medium (A), cyanide (CN^-), ouabain (OB) and isotonic 100 mmol l^{-1} $MgCl_2$ (Na^+-free medium). Results are means ± S.E.M. (*N*=4).

following an exponential time course (Fig. 1). In hepatocytes, as well as other cells of most vertebrates, swelling triggers a volume regulatory response that is largely mediated by an increased plasma membrane conductance for K^+ and Cl^- (Bakker-Grunwald, 1983; Wehner et al., 1992; Wang et al., 1996; Roe et al., 2001). However, unlike hepatocytes of trout and rat, experiments using videomicroscopy (Figs 1, 2) and fluorescence microscopy (not shown) showed that goldfish hepatocytes displayed no (pH 7.45) or only a slight (pH 7.8) RVD. As hypotonic swelling was not accompanied by an increase in K^+ efflux (Fig. 6), the almost complete absence of RVD might have been the consequence of transporters of K^+ and/or Cl^- being inactivated. Incubation of hypotonically swollen cells in the presence of NEM showed that ($^{86}Rb^+$)K^+ efflux can in fact be increased almost 4-fold in goldfish cells (Fig. 7). Thus, although a mechanism promoting efflux of water appears to be present, it was seemingly not activated under the conditions applied. Because Jensen (1994) showed that in red cells of carp (a species belonging to the same family as the goldfish) ($^{86}Rb^+$)K^+ efflux is activated as extracellular pH is increased from 7.6 to 7.8, we tested whether the same is true for goldfish hepatocytes. Our results showed that activation of both ($^{86}Rb^+$)K^+ efflux as well as of a minor RVD indeed occurred, but the relative change of both parameters was much lower than in cells of rat and trout.

Another situation prone to result in a net K^+ flux out of the cells can occur as a consequence of ATP depletion caused by metabolic inhibition (Wang et al., 1996). Accordingly, results of the present study showed that, in goldfish hepatocytes, CN^- as well as CN^-+IAA lead to a 60–67% reduction in ($^{86}Rb^+$)K^+ influx (95–99% of which is driven by Na^+/K^+-ATPase activity; see Krumschnabel et al., 1996). As no concurrent reduction of K^+ efflux was observed in the present study, a significant net K^+ efflux developed as a consequence of Na^+/K^+-ATPase inhibition.

We therefore evaluated how the ensuing K^+ flux imbalance would alter cell volume. Using a fluorometric technique to detect small changes in cell volume, we verified that rat hepatocytes

swelled by approximately 5.1% in the presence of cyanide. This increase agrees with previous reports showing swelling in anoxic hepatocytes of anoxia-intolerant species, including rat (Carini et al., 1999) and trout (Krumschnabel et al., 1998). Moreover, incubation of rat hepatocytes with IAA and CN^- caused V_r to increase by more than 20% (Gores et al., 1989).

By contrast, goldfish hepatocytes showed the opposite response, as CN^- alone or in combination with IAA led to an acute decrease in V_r by 3% and 7%, respectively (Figs 3, 4), followed by a subsequent volume increase to (CN^-) or even above (CN^-+IAA) initial values. Thus, in goldfish hepatocytes, changes in cell volume paralleled changes in intracellular K^+. However, although ($^{86}Rb^+$)K^+ influx decreased to 30–40% of control values in less than 4 min, the magnitude of net K^+ efflux was relatively small, as was the magnitude of volume decrease.

In trout hepatocytes, by comparison, a somewhat different situation has been described. Firstly, during hypotonic swelling and RVD, K^+ efflux was 6–7 times higher than the influx (see Bianchini et al., 1988) (in goldfish cells at pH 7.8, this ratio was only 1.5), allowing complete volume recovery in approximately 40 min (Fig. 2B). In addition, during acute chemical anoxia, K^+ efflux was more than three times higher than the corresponding influx and could be blocked 55% by $BaCl_2$, an inhibitor of voltage-sensitive K^+ channels (Krumschnabel et al., 1996, 1998). This suggests that a significant part of anoxic K^+ leakage is due to membrane depolarization.

In goldfish cells, on the other hand, ($^{86}Rb^+$)K^+ efflux was insensitive to either $BaCl_2$ or TEA, a result that, together with the lower K^+ influx decay, could explain the lower degree of decoupling of K^+ transmembrane fluxes. Similar to trout hepatocytes, quinine, a known inhibitor of Ca^{2+}-sensitive K^+ channels, did not prevent the CN^--induced K^+ loss. Thus, in contrast to the case of rat hepatic and hepatoma cells (Wang et al., 1996; Roe et al., 2001), cytosolic calcium is not implicated in the decoupling of K^+ transmembrane fluxes.

In Fig. 9, we used the results on ($^{86}Rb^+$)K^+ unidirectional fluxes of this and previous studies to calculate the relative K^+ loss under various conditions. It can be seen that, in trout hepatocytes, the relative loss of intracellular K^+ after 20 min of incubation with NEM, hyposmotic medium and cyanide amounts to 30%, 20% and 7%, respectively, whereas in the goldfish the same treatments yield 12%, 4% and 2.5% K^+ loss.

The goldfish cells therefore appear to have the capability to maintain intracellular K^+ relatively constant, which might underlie both the inability of hypotonically swollen goldfish hepatocytes to undergo RVD and, at the same time, prevent the swelling of anoxic cells.

What factors other than K^+ fluxes can affect cell volume during metabolic inhibition in goldfish hepatocytes? One could argue that, as chemical anoxia in goldfish hepatocytes leads to an enhanced production of lactate immediately followed by lactate export (Krumschnabel et al., 2001a), the loss of this organic osmolyte may trigger the observed cell shrinkage. However, this hypothesis has to be discarded as cell shrinkage is also observed in the presence of both IAA and CN^-, a condition where lactate production is fully inhibited (Krumschnabel et al., 2001b).

In hepatocytes of anoxia-intolerant species, excessive accumulation of lactic acid is one of the main factors responsible for cytosolic acidification. Accordingly, it has been demonstrated that in hepatocytes of the rat (Carini et al., 1995), as well as in those of the trout (Krumschnabel et al., 2001a), chemical anoxia leads to impairment of Na^+/K^+-ATPase activity and cytosolic acidosis followed by the activation of the Na^+/H^+ exchanger. This results in net Na^+ influx, cell swelling and progressive loss of cell viability. In line with this, in rat hepatocytes, cytoprotection was achieved by substituting NaCl with choline chloride or by preventing sodium accumulation with glycine (Carini et al., 1999).

In the anoxic goldfish cells, the situation is different. Firstly, the magnitude of the anoxia-induced decrease of Na^+/K^+-ATPase activity is lower than in trout hepatocytes, with no increase in intracellular Na^+ (Fig. 8). Secondly, anoxic goldfish hepatocytes do not activate Na^+/H^+ exchange, and intracellular pH does not decrease, which may be attributed to a high buffering capacity together with proton secretion sensitive to SITS (4-acetamido-4-isothio-cyanostilbene-2,2-disulfonic acid) (Krumschnabel et al., 2001a). Thus, in goldfish hepatocytes under metabolic inhibition, increases in cell volume due to sodium overload are prevented.

Altogether, these findings point to an outstanding capability of goldfish cells to maintain ionic gradients under a variety of conditions that are considered a severe challenge to ion homeostasis. While this is of great advantage during anoxic periods, as it allows the prevention or reduction of the transitory loss of intracellular K^+ under conditions of energetic limitation, at the same time it prevents K^+ being used as an osmolyte for RVD, thereby limiting the capability to regulate cell volume under certain conditions.

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