

Metabolic and ionic responses of trout hepatocytes to anisosmotic exposure

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Summary

Trout hepatocytes exposed to hypo- or hyperosmotic conditions respond by swelling and shrinking, respectively, followed by regulatory volume changes that almost, although not completely, restore cell volume. These anisosmotic conditions have a significant impact on metabolic functions. In hyposmotic medium, oxygen consumption (\dot{V}_{O_2}) and glucose production rates were significantly reduced, whereas lactate accumulation was not significantly affected. By contrast, hyperosmotic conditions did not affect \dot{V}_{O_2} and lactate production but caused a sustained reduction in glucose production. Volume changes were also accompanied by alterations in intracellular free calcium ($[Ca^{2+}]_i$). At the cell population level, hyposmotic exposure evoked a moderate and slowly developing increase in $[Ca^{2+}]_i$, whereas hyperosmolarity caused a pronounced and sustained increase, which peaked at the time of maximum cell shrinkage but clearly exceeded a mere concentration effect due to volume

reduction. Responses of individual cells were highly variable in hyposmotic medium, with only 60% showing a clear increase in $[Ca^{2+}]_i$, while in hyperosmotic conditions all cells displayed elevated $[Ca^{2+}]_i$ levels. A decrease in intracellular pH (pHi) observed in hyposmotic medium was insensitive to EIPA, an inhibitor of Na^+/H^+ exchange, and SITS, an inhibitor of Cl^-/HCO_3^- exchange, but was prevented in Cl^- -free medium. In hyperosmotic medium, pHi increased. This alkalization did not occur under conditions of blocked Na^+/H^+ exchange and was significantly diminished upon inhibition of Cl^-/HCO_3^- exchange, suggesting an important role of these ion transporters in regulatory volume increase of trout hepatocytes.

Key words: rainbow trout, *Oncorhynchus mykiss*, hepatocyte, cell volume, oxygen consumption, intracellular free calcium, intracellular pH.

Introduction

In hepatocytes, as in most other cells, anisosmotic exposure evokes a change in cell volume, which in turn leads to the activation of regulatory mechanisms serving to re-establish near-isosmotic conditions. As some of these mechanisms involve a net transfer of acid–base equivalents across the cell membrane, volume regulation is often associated with changes in intracellular pH (pHi; Gleeson et al., 1990). In addition, alterations of cytosolic free calcium ($[Ca^{2+}]_i$) are often implicated in volume regulation, since, for example, studies with single neuroblastoma cells showed that an increase in $[Ca^{2+}]_i$ preceded the appearance of a regulatory volume decrease (RVD; Altamirano et al., 1998), whereas in gall bladder epithelial cells RVD was inhibited by agents that interfere with Ca^{2+} -dependent processes (Foskett and Spring, 1985).

Given the dependence of basal metabolic processes on the existence of a more or less tightly regulated intracellular milieu, changes of cell volume may also affect cellular metabolism. The latter aspect is best illustrated by the fact that,

in liver cells, the action of several hormones known to affect both metabolism and cell volume may to a large extent be mimicked by volume changes evoked by anisosmotic exposure in the absence of the hormones. This led Häussinger and Lang (1991) to propose that cell volume changes associated with the effect of hormones act as a second messenger in metabolism. Although interactive effects of cell volume and metabolism have also been documented in other cell types (Lang et al., 1998; Smets et al., 2002), most of these studies addressed cells of mammalian origin.

In fish, such studies have almost exclusively been conducted with red cells, revealing complex interactions between haemoglobin oxygen binding, acid–base regulation and ion transport (Nikinmaa, 1992; Ferguson and Boutilier, 1989; Roig et al., 1997). However, in these cells, metabolism is largely governed by their role in gas transfer and it therefore appears likely that a rather different relationship between metabolism and volume control will prevail in hepatocytes.

Given the role of the liver as a central metabolic organ and

the various differences in liver metabolism between teleosts and mammals that have already been documented (Walsh and Mommsen, 1992), we considered a study of the impact of anisotonic conditions on metabolic and ionic aspects in teleost hepatocytes of great interest, both in a general physiological context as well as from a comparative point of view.

Since, over the past decade, hepatocytes from the rainbow trout *Oncorhynchus mykiss* have been established as a model system for the investigation of teleostean cellular physiology (Canals et al., 1992; Furimsky et al., 2000; Krumschnabel et al., 1998; Mommsen et al., 1988; Schwarzbaum et al., 1996; Walsh, 1986), including the study of volume regulatory processes (Bianchini et al., 1988; Fossat et al., 1997; Michel et al., 1994), we chose to use these cells for our purpose. In the present study, we first examined how anisotonic exposure of trout hepatocytes affects their cell volume. We then investigated the impact of the volume changes associated with these conditions on both aerobic (oxygen consumption) and anaerobic (lactate production) metabolism and on the production of glucose, which is considered an important function of hepatic metabolism with regard to the maintenance of plasma glucose level.

In addition, we studied the consequences of anisotonic conditions on $[Ca^{2+}]_i$ and on pHi. As already noted above, various ion transporters involved in volume regulation are also of importance for the control of pHi. Hence, in order to gain some insight into the nature of these mechanisms, the impact of anisotonic conditions on pHi was further investigated both in complete and in ion-substituted media and in the presence of inhibitors of specific ion transporters.

Materials and methods

Materials

Collagenase (Type VIII), bovine serum albumin (BSA), nigericin, valinomycin, ionomycin and the transport inhibitors 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) and 4-acetamido-4'-isothio-cyanatostilbene-2,2'-disulfonic acid (SITS) were purchased from Sigma (Deisenhofen, Germany). Calcein, Fura 2 and 2',7'-bis-(2-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF), all as acetoxymethylesters (AM), were from Molecular Probes (Leiden, The Netherlands), and Cell-Tak was from BD Biosciences (Schwechat, Austria). Leibovitz L-15 medium was obtained from Invitrogen (formerly GIBCO BRL, Lofer, Austria). All other chemicals were of analytical grade and were purchased from local suppliers.

Preparation of hepatocytes and cell culture

Hepatocytes were isolated from rainbow trout (*Oncorhynchus mykiss* Walbaum) acclimated to 15°C as previously described (Krumschnabel et al., 1996). Following isolation, hepatocytes were suspended in standard saline (for composition, see below) and were left to recover for one hour in a shaking water bath maintained at 19°C, which was also the temperature used in the experiments. Measurements of

metabolic parameters (oxygen consumption, lactate accumulation and glucose production) were then conducted with these freshly isolated cells. For the determination of intracellular free calcium and intracellular pH, cells (1×10^6 cells ml⁻¹) were suspended in Leibovitz L15 medium (0.95 mmol l⁻¹ CaCl₂, 5.33 mmol l⁻¹ KCl, 0.44 mmol l⁻¹ KH₂PO₄, 0.46 mmol l⁻¹ MgCl₂, 0.40 mmol l⁻¹ MgSO₄, 137.9 mmol l⁻¹ NaCl, 1.07 mmol l⁻¹ Na₂HPO₄, 4.99 mmol l⁻¹ galactose, 5 mmol l⁻¹ sodium pyruvate, and amino acids and vitamins according to the manufacturer's formulation) modified by addition of 10 mmol l⁻¹ Hepes, 5 mmol l⁻¹ NaHCO₃, 50 µg ml⁻¹ gentamycin and 100 µg ml⁻¹ kanamycin, pH titrated to 7.6. These cells were then plated on Cell-Tak (2 µg cm⁻² surface area)-coated glass cover slips and maintained in an incubator (19°C, 0.5% CO₂) overnight. Before use of the hepatocytes in ion measurements, the cultures were washed several times with fresh standard saline in order to remove non-adherent cells and debris. Both in freshly isolated cells and in cell cultures, viability, as determined from Trypan blue exclusion, was maintained at >95% throughout the experiments described.

Experimental media

The standard isosmotic incubation saline consisted of 10 mmol l⁻¹ Hepes, 136.9 mmol l⁻¹ NaCl, 5.4 mmol l⁻¹ KCl, 1 mmol l⁻¹ MgSO₄, 0.33 mmol l⁻¹ NaH₂PO₄, 0.44 mmol l⁻¹ KH₂PO₄, 5 mmol l⁻¹ NaHCO₃, 1.5 mmol l⁻¹ CaCl₂, 5 mmol l⁻¹ glucose, pH 7.6 at 19°C, and had an osmolarity of 284 mosmol l⁻¹. Hyposmotic conditions were created by exposing cells to a mixture of one volume of standard saline with an equal volume of the same medium lacking NaCl, yielding an osmolarity of 166 mosmol l⁻¹ and corresponding to 0.58 × isosmolarity. A mixture of equal volumes of standard saline with and without 400 mmol l⁻¹ sucrose served to establish hyperosmotic conditions. This medium had an osmolarity of 465 mosmol l⁻¹, equivalent to 1.6 × isosmolarity. Osmolarity of the media was measured by freezing point depression (Knaur Semi-Micro Osmometer, Berlin, Germany). For the measurement of the production of glucose and lactate, glucose was omitted from all media. Furthermore, as preliminary experiments showed that sucrose may be partially hydrolysed by the cells, resulting in a disturbing background signal in glucose measurements, sucrose was substituted by trehalose in these experiments. In ion substitution experiments, either Na⁺ salts or Cl⁻ salts were substituted by equimolar amounts of tetramethylammonium or gluconate, respectively.

Cell volume

Cell water volume was assessed by epifluorescence microscopy as described previously (Espelt et al., 2003). Briefly, hepatocytes were plated on glass cover slips that had been previously coated with 0.1% w/v poly-L-lysine. Each cover slip with attached cells was mounted in a chamber filled with isosmotic 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 for 45–60 min, and

the loading solution was washed out with isosmotic 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 and imaged with a 500 nm LP dichroic mirror and a 515 LP barrier filter. Normalised values of cell water volume (V_r) were computed from monitored changes in relative fluorescence (F_t/F_o) according to the following equation:

$$V_r = \frac{F_o/F_t - F_{\text{bkg}}}{1 - F_{\text{bkg}}},$$

where F_o is the fluorescence from a pinhole region of the cell equilibrated with isosmotic medium, F_t is the fluorescence of the same region of the cell exposed to an anisosmotic medium, and F_{bkg} is the background fluorescence.

A detailed description of the technique, its validation and corresponding computations can be found elsewhere (Alvarez-Leefmans et al., 1995; Altamirano et al., 1998).

Oxygen consumption

Rates of oxygen consumption (\dot{V}_{O_2}) were determined with a dual-chamber Cyclobios Oxygraph (Haller et al., 1994) as described previously (Krumschnabel et al., 1994). Before each measurement, two groups of cells from one preparation, at a density of 30×10^6 cells ml⁻¹, were incubated under either isosmotic or anisosmotic (hypo- or hyperosmotic) conditions for 30 min. Hepatocytes (5×10^6 cells ml⁻¹) were then injected into one of two measuring chambers containing the equivalent incubation medium, and oxygen consumption was followed over approximately 10 min. At the end of the experiment, cells were re-counted with a hemocytometer (Bürker-Türk, Friedrichsdorf, Germany).

Production of lactate and glucose

For the determination of the rates of lactate accumulation and glucose production, cells (10×10^6 cells ml⁻¹) were washed and subsequently incubated in glucose-free iso- or anisosmotic medium over a period of 60 min. At 0 min, 15 min, 30 min and 60 min, duplicate 50 μ l samples were removed, immediately precipitated with 10% ice-cold metaphosphoric acid and frozen for later measurement of glucose or lactate, using standard enzymatic NAD(P)H-coupled assays (Bergmeyer, 1984).

Intracellular free calcium

The effect of anisosmotic exposure on intracellular free calcium concentration ($[Ca^{2+}]_i$) was assessed in individual attached cells, cultured as described above. Hepatocytes were loaded with the Ca^{2+} -sensitive fluorescence dye Fura 2-AM and were mounted on the stage of an inverted fluorescence microscope as described in detail before (Krumschnabel et al., 2001a). Fluorescence images were captured every 30 s, with excitation set to 340 nm and 380 nm, and emission was measured above 510 nm. Basal levels of $[Ca^{2+}]_i$ in standard saline were measured for at least 5 min before half of the saline

covering the cells was exchanged for an equal volume of hypo- or hyperosmotic stock and measurements were continued for at least another 30 min. At the end of each experiment, a calibration was performed by determination of a maximum fluorescence ratio, obtained after addition of 4.5 mmol l⁻¹ $CaCl_2$, and a minimum ratio, obtained after adding 20 mmol l⁻¹ EGTA, both in the presence of 7.2 μ mol l⁻¹ of the calcium ionophore ionomycin. Applying these values and a dissociation constant (K_D) value of 680 nmol l⁻¹, determined for our experimental set-up by use of a commercial calibration kit (Molecular Probes), absolute levels of $[Ca^{2+}]_i$ could be calculated using the formula given by Grynkiewicz et al. (1985).

Intracellular pH

Intracellular pH (pHi) of individual hepatocytes was determined in cells loaded with the pH-sensitive fluorescence dye BCPCF-AM, applying the same microscopic set-up and experimental protocol as above. Excitation was set to 490 nm and 440 nm, and emission was again recorded above 510 nm. Calibrations were performed by replacing the experimental medium with high K^+ saline, where the concentrations of NaCl and KCl were reversed, containing the cation ionophores nigericin (10 μ mol l⁻¹) and valinomycin (5 μ mol l⁻¹) with a pH adjusted to 6.80, 7.20 or 7.60 (Pocock and Richards, 1992; Seo et al., 1994).

Statistics

Data are presented as means \pm S.E.M. of N independent preparations. In experiments on cell cultures, data are shown as means \pm S.E.M. of n individual cells. In this case, at least three independent cultures from three different preparations were used. Differences between treatments were evaluated with Student's t -test or analysis of variance (ANOVA) followed by Tukey's *post-hoc* test, with a P value of <0.05 being considered as significant.

Results

Cell volume changes during anisosmotic exposure

In a first series of experiments, the effect of anisosmotic conditions on hepatocyte volume was investigated. As depicted in Fig. 1A, exposure of trout hepatocytes to hyposmotic medium ($0.58 \times$ isosmolarity) evoked a rapid swelling of the cells, which peaked at $167 \pm 2\%$ of the isosmotic cell volume after 8 min of exposure. This was followed by an exponential shrinking phase, and cell volume reached a plateau at $114 \pm 2\%$ of the isosmotic volume after approximately 40 min. Re-exposure to isosmotic medium at this point resulted in a rapid cell shrinkage to $76 \pm 2\%$ of the control volume and a subsequent phase of volume increase. Hyperosmotic conditions ($1.6 \times$ isosmolarity) elicited a rapid decrease in cell volume to a minimum of $77 \pm 1\%$ of the control value after 8 min, with a subsequent near-linear increase to $93 \pm 1\%$ after approximately 40 min of exposure (Fig. 1B). Re-establishing isosmotic conditions caused the hepatocytes to swell to

111±1% of the control volume and this was again followed by a volume decrease.

Effects of anisotonic exposure on cellular metabolism

As the two Oxygraph chambers allowed only comparisons of two treatments at a time, independent controls were measured for both hypo- and hyperosmotically treated cells. Controls and hyposmotically exposed cells respired at a rate of 0.88 ± 0.06 nmol 10^{-6} cells min^{-1} and 0.71 ± 0.05 nmol 10^{-6} cells min^{-1} , respectively, which was significantly different ($P<0.05$). Hepatocytes serving as controls for hyperosmotic conditions had a \dot{V}_{O_2} of 0.76 ± 0.05 nmol 10^{-6} cells min^{-1} , which was not significantly different from the rate of 0.78 ± 0.04 nmol 10^{-6} cells min^{-1} observed in hyperosmotically treated cells. In Fig. 2, these data are

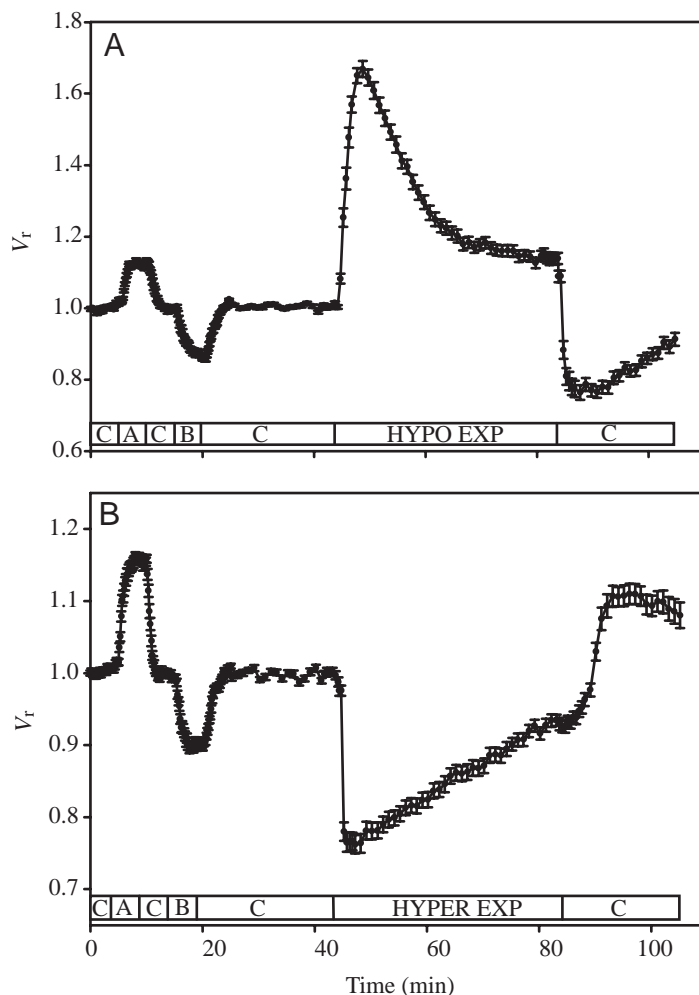


Fig. 1. Changes of relative cell water volume (V_r) of trout hepatocytes exposed to (A) hyposmotic medium or (B) hyperosmotic medium, followed by re-exposure to isosmotic conditions. C, A and B denote isosmotic control saline, hyposmotic calibration saline (264 mosmol l^{-1}) and hyperosmotic calibration saline (308 mosmol l^{-1}), respectively. HYPO EXP (166 mosmol l^{-1}) and HYPER EXP (465 mosmol l^{-1}) indicate duration of experimental hypo- and hyperosmotic exposure. Values are means \pm s.e.m. of 60 cells from four independent preparations.

summarised in a normalised form so as to allow a better comparison between treatments.

Rates of lactate accumulation were rather variable, both in isosmotic and anisotonic conditions (Fig. 3). Hence, despite large differences in the means, hyposmotic exposure did not result in a significant change in lactate production as compared with controls. Similarly, although under hyperosmotic conditions lactate production appeared to be reduced throughout the entire experiment, this decrease was not significant.

Compared with isosmotic controls, the rate of glucose production was significantly diminished by approximately 38% between 0 min and 15 min of hyposmotic incubation but was no longer different from the control values during the rest of the experiment (Fig. 4). In hyperosmotic medium, a significant reduction of glucose production was seen throughout the entire period investigated, the reduction amounting to 40–55% of control values.

Effects of anisotonic exposure on intracellular free calcium

Both hypo- and hyperosmotic conditions elicited significant changes in $[\text{Ca}^{2+}]_i$ in most hepatocytes, which, however, showed high variability between different cells. As demonstrated by the examples depicted in Fig. 5, individual responses to hyposmotic exposure ranged from an early drop in $[\text{Ca}^{2+}]_i$, which was absent in some cells, followed by a sustained increase (Fig. 5A; approximately 36% of the cells), through a series of oscillatory increases (Fig. 5B; 26%), to comparatively slight fluctuations around the baseline value of $[\text{Ca}^{2+}]_i$ (Fig. 5C; 38%). Upon hyperosmotic exposure, cells responded with an early pronounced increase in $[\text{Ca}^{2+}]_i$ followed by smaller (Fig. 5D; 49%) or larger (Fig. 5E; 16%) oscillatory

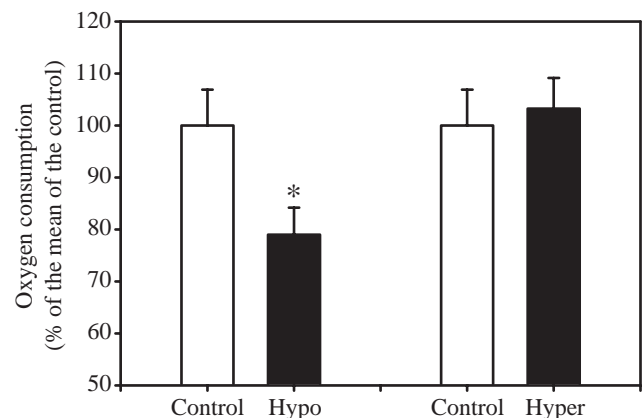


Fig. 2. Rates of oxygen consumption of trout hepatocytes after 30 min of incubation under isosmotic (control; 284 mosmol l^{-1}), hyposmotic (hypo; 166 mosmol l^{-1}) and hyperosmotic (hyper; 465 mosmol l^{-1}) conditions. Data are expressed as % of the mean of the control and are presented as means \pm s.e.m. of 12 (hyposmotic exposure) and 11 (hyperosmotic exposure) paired experiments. * $P<0.05$ compared with controls.

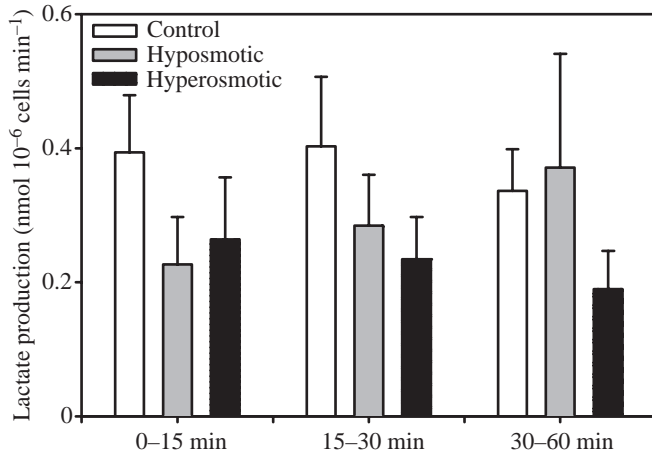


Fig. 3. Rates of lactate accumulation of cells incubated under iso- (284 mosmol l⁻¹), hypo- (166 mosmol l⁻¹) or hyperosmotic conditions (465 mosmol l⁻¹). Rates were calculated from the difference in lactate concentration at the onset and the end of each period, divided by the duration of the period. Data are means + S.E.M. of 11 experiments.

increases, or by a single and sustained increase of [Ca²⁺]_i (Fig. 5F; 35%). The mean response of all cells examined yielded a slow progressive increase of [Ca²⁺]_i under hypotonic conditions, whereas in hypertonic medium a faster increase was observed, which peaked at 10 min of exposure and persisted thereafter (Fig. 6).

Effects of anisosmotic exposure on intracellular pH

The impact of hypo- and hypertonic conditions on pHi of trout hepatocytes is depicted in Fig. 7. Hypotonic medium caused a decrease in pHi of 0.2 units within 5–10 min to a lower steady level, and this decrease was fully reversible within 5 min after re-exposure to isotonic conditions. In

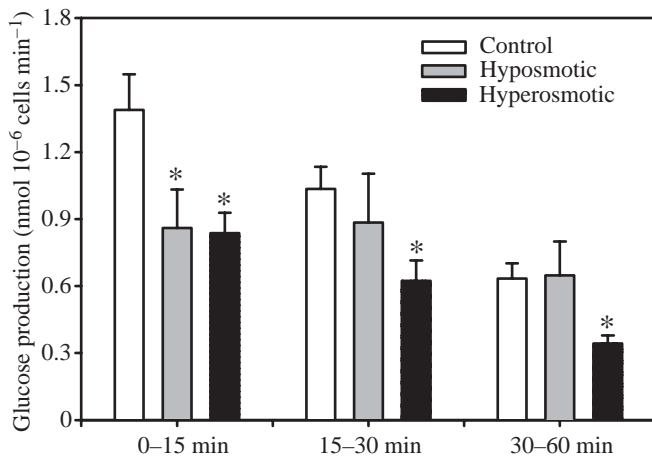


Fig. 4. Rates of glucose production of cells incubated under iso-, hypo- or hypertonic conditions. Medium osmolarities and calculation of rates were as in Fig. 3. Data are means + S.E.M. of 15 (controls), 9 (hypotonic) and 10 (hypertonic) experiments. **P* < 0.05 compared with controls.

hypertonic medium, pHi showed an increase of 0.4 units, which was completed after approximately 15 min, and re-exposure to isotonic medium caused a decrease of pHi within 15 min to a value not significantly different from the initial pHi.

In order to gain some insight into the mechanisms involved in these pHi responses, the effects of anisosmotic conditions on pHi were then repeated in both complete and ion-substituted media and in the presence of ion transport inhibitors. Examples of these experiments are shown in Fig. 8; a summary of the data is given in Table 1.

As shown in Table 1, omission of Na⁺ from the incubation medium resulted in a significant decrease of pHi by 0.10 units. Subsequent exposure to hypotonic Na⁺-free medium caused a further significant decrease by 0.08 pH units. Although this value tended to be lower than the decrease of 0.19 pH units evoked by hypotonic medium in the presence of Na⁺, this difference was not significant. A similar picture was seen with EIPA, the inhibitor of Na⁺/H⁺ exchange, which produced a decrease of pHi by 0.22 units under isotonic conditions and a further acidification by 0.20 units in hypotonic medium. Exposure to Cl⁻-free conditions resulted in an alkalinization of

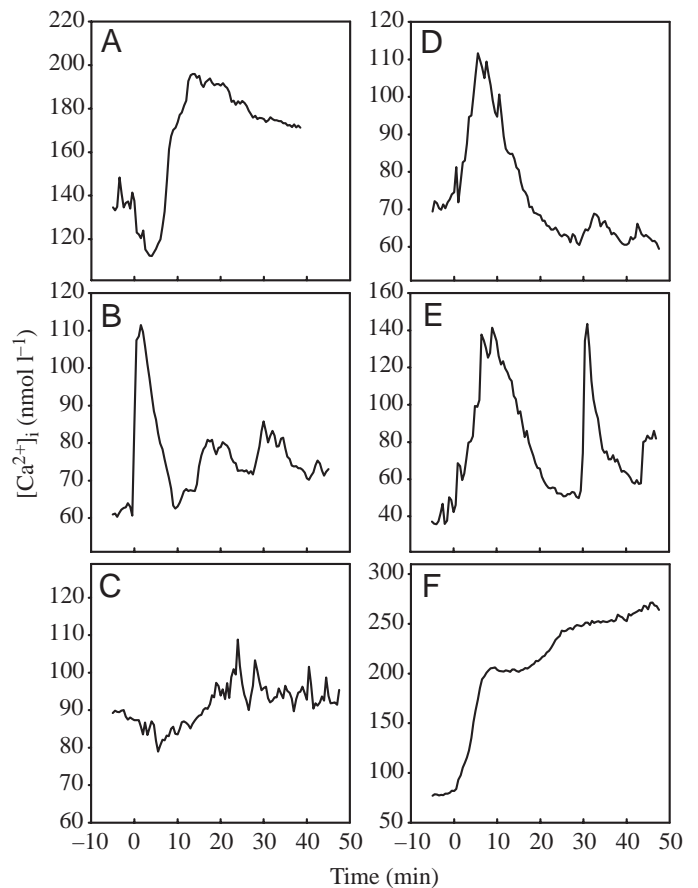


Fig. 5. Alterations of intracellular free calcium ([Ca²⁺]_i) of individual hepatocytes induced by hypotonic (A–C) or hypertonic (D–F) conditions. Anisosmotic conditions were established at time zero and were maintained throughout the experiment.

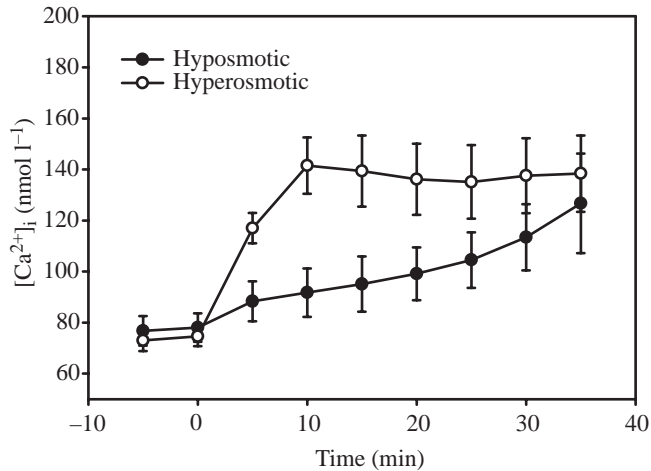


Fig. 6. Intracellular free calcium ($[Ca^{2+}]_i$) of hepatocytes under hyposmotic and hyperosmotic conditions established at time zero. Data are means \pm S.E.M. of 61 (hyposmotic) and 49 (hyperosmotic) cells.

pHi by 0.37 units, but subsequently imposed hyposmotic conditions caused no further significant change of pHi. SITS, the inhibitor of both Na^+ -dependent and Na^+ -independent Cl^-/HCO_3^- exchange, caused a significant decrease of pHi by 0.27 units; hyposmotic exposure with SITS significantly decreased pHi further by 0.10 units.

In the experimental series investigating the effect of hyperosmotic medium, Na^+ -free conditions elicited a decrease in pHi of 0.24 units. Subsequent exposure to hyperosmotic conditions caused virtually no change in pHi, whereas an increase of 0.42 pH units was observed in the presence of Na^+ . Similar to the effect of Na^+ -free medium, EIPA caused a significant intracellular acidification, whereas no further

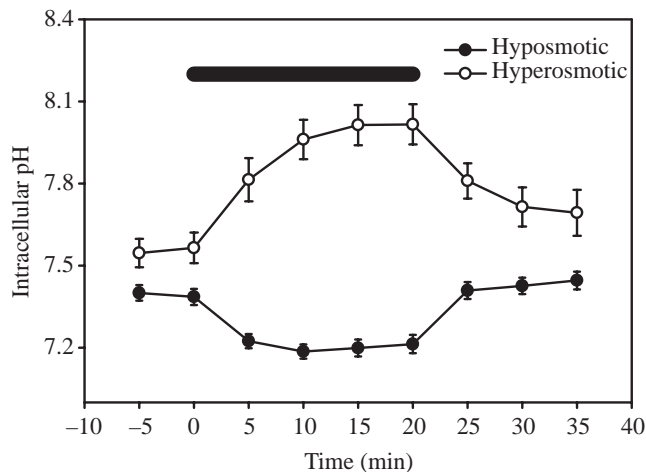


Fig. 7. Changes of intracellular pH of hepatocytes in response to hyposmotic and hyperosmotic conditions and after re-establishing isosmotic conditions. The duration of anisotonic exposure is indicated by the black bar. Data are means \pm S.E.M. of 54 (hyposmotic) and 18 (hyperosmotic) cells.

change of pHi was seen under hyperosmotic conditions in the presence of EIPA. By contrast, the increase of pHi evoked in Cl^- -free medium was followed by further significant alkalinization of 0.25 pH units in hyperosmotic medium. This increase, however, was significantly smaller than the one measured in Cl^- -containing medium. SITS decreased pHi by 0.10 units. Exposure to hyperosmotic medium in the presence of SITS elicited an increase of pHi by 0.18 units, which was significantly different from that seen in its absence.

Discussion

Volume changes and metabolism

In line with previous investigations (Bianchini et al., 1988; Espelt et al., 2003), hyposmotic exposure of trout hepatocytes caused cell swelling followed by a regulatory volume decrease (RVD; Fig. 1A). After 40 min of hyposmotic exposure, cell volume attained an apparent new steady state at approximately 14% above the original isosmotic volume. Re-introduction of isosmotic medium produced rapid cell shrinkage, which is in agreement with previous observations on hepatocytes from trout (Fossat et al., 1997) and rat (Corasanti et al., 1990)

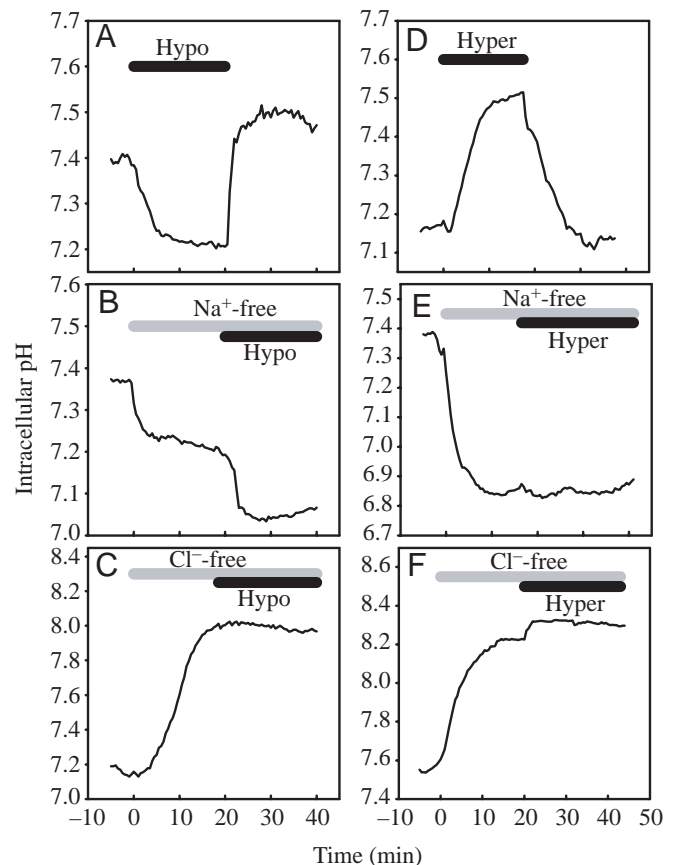


Fig. 8. Changes of intracellular pH of individual hepatocytes induced by hyposmotic (A–C) or hyperosmotic (D–F) conditions in complete and in ion-substituted media. The presence of ion-substituted and anisotonic media is indicated by grey and black bars, respectively.

Table 1. Effects of anisosmotic conditions, ion substitution and transport inhibitors on intracellular pH (pHi) of trout hepatocytes

Treatment	Baseline	Before	Δ pHi	After 15 min	Δ pHi
Hyposmotic (n=54)		7.39±0.03		7.20±0.03 [†]	-0.19±0.02
Na ⁺ -free (n=20)	7.43±0.07	7.33±0.08*	-0.10±0.03	7.25±0.10 [†]	-0.08±0.04
EIPA (n=35)	7.44±0.02	7.21±0.03*	-0.22±0.03	7.01±0.03 [†]	-0.20±0.03
Cl ⁻ -free (n=31)	7.48±0.07	7.86±0.08*	0.37±0.05	7.93±0.08	0.08±0.05 [‡]
SITS (n=75)	7.48±0.02	7.21±0.02*	-0.27±0.03	7.12±0.02 [†]	-0.10±0.02
Hyperosmotic (n=67)		7.50±0.04		7.93±0.04 [†]	0.42±0.02
Na ⁺ -free (n=39)	7.23±0.04	6.98±0.06*	-0.24±0.03	6.96±0.09	-0.02±0.04 [‡]
EIPA (n=33)	7.30±0.04	6.91±0.02*	-0.39±0.04	6.91±0.03	0.00±0.03 [‡]
Cl ⁻ -free (n=27)	7.47±0.04	7.88±0.06*	0.41±0.03	8.13±0.06 [†]	0.25±0.04 [‡]
SITS (n=77)	7.43±0.02	7.34±0.03*	-0.10±0.02	7.51±0.03 [†]	0.18±0.02 [‡]

Baseline, before and after 15 min denote initial pHi, pHi before anisosmotic exposure and pHi after 15 min of anisosmotic exposure, respectively. Δ pHi indicates change of pHi induced by exposure to transport inhibitors, ion-substituted or anisosmotic medium. Data are means \pm S.E.M. of *n* cells. Ion transport inhibitors EIPA and SITS were applied at concentrations of 100 μ mol l⁻¹ and 500 μ mol l⁻¹, respectively. **P*<0.05, significantly different from baseline pHi; [†]*P*<0.05, significantly different from pHi before anisosmotic exposure; [‡]*P*<0.05, significantly different from Δ pHi in controls.

subjected to this condition (referred to as pseudo-hypertonic stress). The finding that hyposmotic cell volume recovery remained incomplete is of interest inasmuch as this residual volume change has been found to influence the activity of various metabolic functions in liver cells (Häussinger and Lang, 1991). In fact, in mammals it has been postulated that a 'swollen state' may act as a signaling mechanism (Lang et al., 1998), which would slow down metabolism, particularly if hypotonic stress is transient in nature or when the osmotic transmembrane gradient does not threaten cell lysis (Strange et al., 1996). In agreement with this idea, we observed a significant decrease of the rate of oxidative metabolism in trout hepatocytes, as estimated by \dot{V}_{O_2} measurements (Fig. 2). This response is species specific, since in goldfish (*Carassius auratus*) hepatocytes hyposmotic exposure caused an increase in \dot{V}_{O_2} (C. Manzel and G. Krumschnabel, unpublished observations).

Almost a mirror image to that described above was observed regarding volume changes of trout hepatocytes in hyperosmotic medium. That is, cells first shrank and then exerted a regulatory volume increase (RVI), which almost, although not completely, restored cell volume within the time investigated (Fig. 1B). This is remarkable, since in many cells, including rat hepatocytes, RVI is rather slow (Wehner and Tinel, 2000) or virtually absent (Corasanti et al., 1990) unless the cells are subjected to pseudo-hypertonic conditions as mentioned above. In contrast to hyposmotic medium, hyperosmolarity left \dot{V}_{O_2} of trout hepatocytes unaltered, despite the fact that the cells were still in a shrunken state at the time respiratory rate was assessed (Fig. 2). Similar observations have been reported for kidney cortex (Gyory et al., 1981) and L-929 cells (Clegg and Gordon, 1985), in which hyperosmotic conditions were also without effect on \dot{V}_{O_2} . It has been hypothesised by Clegg and Gordon (1985) that this reflects the highly structured organisation of cellular metabolism, which may render endogenous respiration of the cells largely

independent of metabolite concentration in the aqueous cell compartments.

Somewhat different responses to hypo- and hyperosmotic exposure were also seen in the experiments examining the rates of lactate accumulation and glucose production. In hyposmotic medium, lactate production was not significantly altered, although the lack of significance may in part be due to the large variability observed (Fig. 3). At the same time, glucose production was initially significantly diminished but returned to control levels thereafter (Fig. 4). By contrast, while hyperosmotic conditions also had no effect on the rate of lactate production, glucose production was significantly decreased throughout the entire experiment.

In rat hepatocytes, alterations in glycogen synthesis and glycolytic activity were found to be related to the decrease in pHi associated with hyposmotic conditions (Peak et al., 1992). However, although intracellular acidification was also seen in hyposmotically induced trout hepatocytes (Fig. 7), we recently observed that both \dot{V}_{O_2} and lactate production were largely unaffected by a decrease in extracellular pH by as much as one pH unit (Krumschnabel et al., 2001b). As such a decrease in extracellular pH is accompanied by a pronounced acidification of pHi in trout hepatocytes (Walsh, 1986; Krumschnabel et al., 2001b), the change in pHi does not appear to be directly involved in the modification of glucose metabolism in these cells.

As noted before, the lack of an effect of hyperosmolarity on \dot{V}_{O_2} has been previously described in other cells, but the unaltered rate of lactate production and the decreased cellular output of glucose under these conditions contradict earlier observations on perfused rat liver (Lang et al., 1989) and L-929 cells (Clegg et al., 1990), where glycolytic flux was enhanced. Furthermore, in rat hepatocytes, alkalization *per se* stimulated glycolysis (Peak et al., 1992), which was apparently not the case in the present study. Thus, in summary,

both hypo- and hyperosmotic conditions tended to cause a decrease of metabolic functions in trout hepatocytes, which is in contrast to the generally opposing effects observed in mammalian cells (Häussinger et al., 1994).

Cell volume and $[Ca^{2+}]_i$

Although alterations of $[Ca^{2+}]_i$ have often been found to be associated with cell volume changes in mammalian cells (Hoffmann and Dunham, 1995), and recently also in teleost cells (Leguen and Prunet, 2001), the role of $[Ca^{2+}]_i$ in the volume regulatory responses of cells appears to be rather variable. In fish, a strict dependence of RVD on $[Ca^{2+}]_i$ has been reported for goldfish renal proximal tubule cells (Terreros and Kanli, 1992), whereas no requirement for Ca^{2+} has been documented in trout tubule cells (Kanli and Norderhus, 1998). In the present study, the importance of $[Ca^{2+}]_i$ changes for RVD and RVI has not been specifically addressed, but our results clearly showed that anisosmotic conditions evoke alterations of $[Ca^{2+}]_i$ in trout hepatocytes (Figs 5, 6). In hyposmotic medium, these $[Ca^{2+}]_i$ responses were not reflected in dramatic changes of the mean $[Ca^{2+}]_i$ levels but were clearly visible at the individual cell level. The reason underlying the heterogeneity of cellular responses is not clear, but a remarkable variability at the cell level has also been reported for other cells (Jorgensen et al., 1997). As in this previous study, the alterations of $[Ca^{2+}]_i$ did not seem to be closely related to the changes of cell volume in the trout hepatocytes. Furthermore, in previous investigations it has been shown that the increase of K^+ efflux induced by hyposmotic conditions was not altered by the Ca^{2+} ionophore A23187 or inhibitors of Ca^{2+} -dependent K^+ channels (Bianchini et al., 1988), whereas a significant reduction of RVD and of taurine release was seen in the absence of extracellular Ca^{2+} (Michel et al., 1994). The role of $[Ca^{2+}]_i$ in the RVD response of trout hepatocytes therefore remains ambiguous at present.

Changes of $[Ca^{2+}]_i$ under hyperosmotic conditions have received comparatively little attention, but an increase (frog skeletal muscle; Chawla et al., 2001), no change (Ehrlich ascites tumour cells; Pedersen et al., 1998) and even a decrease of $[Ca^{2+}]_i$ have been reported (rabbit proximal tubular cells; Raat et al., 1995). In trout hepatocytes, hyperosmolarity unequivocally caused an increase of $[Ca^{2+}]_i$ that was apparent both at the population and the single cell level (Figs 5, 6). Importantly, this elevation was seen in all cells studied, and the occurrence of the initial Ca^{2+} peak coincided with the time of maximum cell shrinkage. Furthermore, the absolute increase to nearly twice the resting level clearly exceeded a mere concentration effect due to volume reduction, which would have accounted for an increase of about 25%. Although the importance of these changes for RVI remains to be elucidated, we believe that the sum of these findings is suggestive of their physiological significance.

Volume regulatory mechanisms and their impact on pHi

The pHi regulatory mechanisms of trout hepatocytes have been repeatedly studied before (Walsh, 1986; Furimsky et al.,

2000; Krumschnabel et al., 2001b). Our current data, collected as a corollary during the ion substitution and transport inhibitor experiments, largely corroborate these studies, and therefore the interested reader should refer to these reports for a detailed analysis on this topic.

Available reports on the transport mechanisms involved in RVD of trout hepatocytes indicate that, upon hyposmotic swelling, KCl cotransport and taurine release are the main routes mediating the loss of osmolytes (Bianchini et al., 1988, 1991; Michel et al., 1994). In principle, neither of these transport pathways should directly modify pHi, but our observations here show that a significant and reversible acidification was induced by hyposmolarity (Fig. 7). Although similar observations have been reported for many other cells, the reasons underlying this decrease in pHi appear to be largely unresolved (Jakab et al., 2002). Our present data indicate that, in the trout cells, Na^+/H^+ exchange was not responsible for the acidification, as neither Na^+ -free conditions nor EIPA altered the decrease of pHi (Table 1). Similarly, Cl^-/HCO_3^- exchange was apparently not involved, since SITS did not affect the hyposmotic pHi change. Interestingly, however, the omission of Cl^- from the incubation medium prevented the hyposmotic acidification. A tentative explanation for this is that, under these conditions, RVD, and in turn also the pHi changes associated with it, might be blocked, since it was shown that both the increases in K^+ permeability (Bianchini et al., 1988) and in taurine release (Michel et al., 1994) were diminished in Cl^- -free media. A partial inhibition of RVD has also been reported for trout renal tubules in the presence of either Cl^- channel blockers or an inhibitor of KCl cotransport (Kanli and Norderhus, 1998).

The RVI response of many cells includes activation of Na^+/H^+ exchange and parallel Cl^-/HCO_3^- exchange, causing the net uptake of NaCl followed by water (Hoffmann and Dunham, 1995). The same is obviously true for trout hepatocytes, since in the present and a previous study (Fossat et al., 1997) hyperosmotic shrinkage induced an increase in pHi that was sensitive to inhibitors of Na^+/H^+ exchange and Na^+ -free conditions, as well as to an inhibitor of Cl^-/HCO_3^- exchange or Cl^- -free conditions. Furthermore, Fossat et al. (1997) directly demonstrated an amiloride- and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)-sensitive increase in Na^+ and Cl^- uptake, respectively, in hyperosmotic medium. But while inhibition of Na^+/H^+ exchange completely abolished the pHi changes, this was not the case with inhibition of Cl^-/HCO_3^- exchange, which only diminished the extent of alkalization. This suggests that, as in other cells (Bevensee et al., 1999; Cabado et al., 2000), Na^+/H^+ exchange is the main mechanism involved in alkalization in hyperosmotic medium. In addition, despite significantly different effects on baseline pHi of the removal of extracellular Cl^- and the presence of SITS, the alkalization induced by hyperosmolarity was similar in magnitude. Thus, although the activity of the Na^+/H^+ exchanger is known to be dependent on pHi in isosmotic conditions (Fossat et al., 1997), this may be less pronounced in hyperosmotic conditions. Furthermore, in

several cell types, e.g. rat lymphocytes (Grinstein et al., 1985) and renal mesangial cells (Bevensee et al., 1999), the pHi dependence of Na⁺/H⁺ exchange was found to be alkali shifted in hyperosmotic medium, allowing sustained acid extrusion at increased pHi.

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