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Ionic determinants of pH of acidic compartments under hypertonic conditions in trout hepatocytes

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SUMMARY

Exposure of trout hepatocytes to hypertonicity induced a decrease in acridine orange (AO) fluorescence, indicating a corresponding decrease in pH inside the lumen of acidic compartments (pH_L). Pre-exposure of cells to the specific V-ATPase inhibitor bafilomycin A1 (0.3 μmol l^{-1}) increased AO fluorescence – unmasking H^+ leaks under steady-state conditions – and partially removed the hypertonicity-induced pH_L decrease. The sustainability of the luminal acidification, but not the acidification itself, appeared to depend on a low K^+ and a high Cl^- conductance under hypertonic conditions. Increasing K^+ conductance using the specific ionophore valinomycin (10 μmol l^{-1}) or removal of extracellular Cl^- after an instant drop in AO fluorescence resulted in a reversal of luminal acidity. The alkalinization measured under hypertonic conditions in the absence of Cl^- was largely attenuated when cells were bathed in HCO_3^--free medium, signifying the possible presence of Cl^-/HCO_3^- exchange. Under steady-state conditions, while a slight and brief pH_L increase was measured upon exposure of cells to valinomycin, Cl^- removal, unexpectedly, induced a decrease in pH_L, indicating a role for extracellular Cl^- in limiting luminal acidification. This was confirmed by the substantial pH_L decrease measured upon exposure of cells to the anion exchanger inhibitor SITS (0.5 mmol l^{-1}). Furthermore, hypertonicity-induced acidification was still noticeable in the presence of SITS. On the other hand, the hypertonicity-induced acidification was significantly reduced in the absence of extracellular Na^+ or Ca^{2+}. However, BAPTA-AM induced an increase in steady-state pH_L that was independent of V-ATPase inhibition. Moreover, the BAPTA-induced alkalinization was still apparent after depletion of intracellular Ca^{2+} using the Ca^{2+} ionophore A23187 in Ca^{2+}-free medium. We conclude that pH_L of trout hepatocytes is sensitive to hypertonicity and ionic determinants of hypertonicity. Thus, changes in pH_L should be considered when studying pH adaptations to hypertonic stress.

Key words: trout hepatocyte, acidic compartments, V-ATPase, hypertonicity, acridine orange.

INTRODUCTION

Eukaryotes contain highly differentiated single-membrane organelles including Golgi apparatus, endosomes, secretory vesicles, lysosomes and synaptic vesicles. These organelles are characterized by an acidic pH in their lumen (pH_L), relative to the cytosol. The maintenance of pH_L represents a key requirement for many biological processes including membrane trafficking, protein degradation and coupled transport of small molecules (Forgac, 2007). pH_L is established by a proton-pumping vacuolar-type ATPase (V-ATPase) in combination with ion channels and transporters, whose varying distribution contributes to the organelle-specific luminal contents (Demaurex, 2002; Weisz, 2003). pH_L differs among various compartments with lysosomes being most acidic (pH ~4.6) (Kornfeld and Mellman, 1989). Recently, it has been postulated that proton movements across the acidic luminal membrane affect cytosolic pH (pH_i) (Nilsson et al., 2006).

With regard to pH_i, cells are endowed – at their plasma membrane – with proton pumps (H^+-ATPase), proton channels and ion transporters that drive H^+ or acid equivalents and HCO_3^- into and out of the cell (Puceat, 1999; Boron, 2004). The activities of these mechanisms are believed to regulate pH_i in a compensatory relationship with extracellular pH (pH_e) (Boron, 2004). On the other hand, there are also examples of intracellular pH changing without concomitant changes in extracellular pH, and *vice versa*. Glucose injection into human tumour cells substantially decreased pH_e while pH_i remained unchanged (Kozin et al., 2001). Similarly, pH_i of fish hepatocytes increased in response to Ca^{2+}-mobilizing agents with no change in pH_e (Ahmed and Pelster, 2007).

In addition to pH, cell hydration state (i.e. cell volume) represents another dynamic parameter of cellular homeostasis that changes within minutes in response to alterations in environmental conditions or hormonal stimulation. These changes in cell hydration act as a signal that modifies metabolism and gene expression due to complex alterations in protein phosphorylation (Lang et al., 1998; Haussinger and Schliess, 1999; Schliess and Haussinger, 2002). Interestingly, intracellular vesicular compartments have been reported to participate in cell volume-sensitive pathways, such as lysosomal proteolysis or the putative swelling-induced insertion/retrieval of bile acid-transporter molecules into/from the canalicular membrane in response to cell swelling/shrinkage (Schreiber et al., 1994). And recently, hypertonicity-induced endosomal acidification has been suggested to be an important upstream event signalling oxidative stress and triggering proapoptotic state in hepatocytes (Reinehr et al., 2006).

While the effect of cell volume changes on pH_i is well documented in different cell types (Lang et al., 1998; Wehner et al., 2003) including fish cells (Walsh, 1986; Fossat et al., 1997; Furimsky et al., 2000; Krumschnabel et al., 2003), studies concerning pH_L regulation in response to anisotonicity are rather

few and are restricted to mammalian cells. In rat liver parenchymal cells (Volkl et al., 1994; Schreiber and Haussinger, 1995) pH_L has been shown to decrease in response to hypertonicity and to increase following hypotonicity. On the other hand, pH_L of rat liver Kupffer cells appeared to be insensitive to anisotonicity (Schreiber et al., 1996). As pH_L changes induced by anisotonicity reflect movements of H^+ or acid equivalents between the cytosol and acidic luminal, it is reasonable to assume that such changes in pH_L might contribute to overall pH_i regulation. In fact, in a recent study on trout hepatocytes, the involvement of intracellular mechanisms in the regulation of pH_i under hypertonicity (Ahmed et al., 2006) was postulated.

Using acridine orange (AO) to probe pH_L, we investigated the possible involvement of V-ATPase activity in pH_L changes under hypertonicity. Furthermore, the contribution to pH_L regulation of ions typically accumulated in the cytosol following hypertonicity exposure was investigated.

MATERIALS AND METHODS

Chemicals

Collagenase (Type VIII), bovine serum albumin (BSA), fetal calf serum (FCS), acridine orange (AO), bafilomycin A1, amiloride, 4-acetamido-4′-isothiocyanato-stilbene-2,2′-disulphonic acid (SITS), A23187 and valinomycin were purchased from Sigma (Deisenhofen, Germany). Leibovitz L-15 medium was obtained from Invitrogen (Karlsruhe, Germany). All other chemicals were of analytical grade and were purchased from local suppliers.

Preparation of cell cultures

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local hatchery and acclimated in 200 l aquaria with running water at 15°C. Fish were fed daily with trout pellets (AGRA TAGGER, Innsbruck, Austria) *ad libitum*. Hepatocytes were isolated following the collagenase digestion procedure described previously (Ahmed et al., 2006). In brief, fish were killed by a blow on the head, the liver was exposed, and the portal vein was cannulated. The liver was then perfused with Hepes-buffered saline to remove the blood, followed by perfusion with collagenase-containing saline (0.05% collagenase) until the tissue appeared soft and swollen. Subsequently, the liver was excised, cut into fine fragments with a pair of scissors and further incubated with collagenase-containing saline for a few minutes. The cells were finally filtered through two nylon screens (pore diameter 250 and 150 μm) and washed three times (60 ***g***, 4 min). After isolation, hepatocytes were left to recover in standard saline (see below) containing 1% BSA for 1 h in a shaking water bath thermostatically regulated to 19°C, which was also the temperature used during the experiments. Cell viability, as determined from Trypan Blue exclusion, was always >85%.

Hepatocytes (1.5×10^6 to 2×10^6 cells ml^{-1}) were then suspended in Leibovitz L-15 medium (0.95 $mmol\,l^{-1}$ $CaCl_2$, 5.33 $mmol\,l^{-1}$ KCl, 0.44 $mmol\,l^{-1}$ KH_2PO_4, 0.46 $mmol\,l^{-1}$ $MgCl_2$, 0.40 $mmol\,l^{-1}$ $MgSO_4$, 137.9 $mmol\,l^{-1}$ NaCl, 1.07 $mmol\,l^{-1}$ Na_2HPO_4, 4.99 $mmol\,l^{-1}$ galactose, 5 $mmol\,l^{-1}$ sodium pyruvate, amino acids and vitamins according to the manufacturer's formulation), modified by addition of 10 $mmol\,l^{-1}$ Hepes, 5 $mmol\,l^{-1}$ $NaHCO_3$, 50 $\mu g\,ml^{-1}$ gentamycin and 100 $\mu g\,ml^{-1}$ kanamycin, pH titrated to 7.6. These cells were then plated on poly-L-lysine (5 $\mu g\,ml^{-1}$)-coated glass coverslips and maintained in an incubator (19°C, 0.5% CO_2) overnight. Before the cells were loaded with AO, cultures were washed several times with fresh standard saline in order to remove non-adherent cells and debris.

Experimental media

The standard saline used consisted of (in $mmol\,l^{-1}$) 10 Hepes, 136.9 NaCl, 5.4 KCl, 1 $MgSO_4$, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 5 $NaHCO_3$, 1.5 $CaCl_2$ and 5 glucose, pH 7.6 at 19°C, and had an osmolarity of 284 $mosmol\,l^{-1}$. In ion substitution experiments, either Na^+ salts or Cl^- salts were replaced by equimolar amounts of tetramethylammonium (TMA) or gluconate, respectively. Replacement of $CaCl_2$ with 0.5 $mmol\,l^{-1}$ EGTA or omission of HCO_3^- was used to prepare Ca^{2+}-free or HCO_3^--free medium, respectively. To create hyperosmotic conditions, a mixture of one volume of standard saline with an equal volume of the same medium containing an additional 200 $mmol\,l^{-1}$ NaCl was used, yielding an osmolarity of 465 $mosmol\,l^{-1}$ (1.6×isosmolarity). Medium with 400 $mmol\,l^{-1}$ sucrose was used to attain the hyperosmotic condition when carrying out measurements using Cl^--free or Na^+-free medium.

Measurement of pH of acidic compartments (pH_L)

AO is a weakly basic dye that displays green fluorescence in the diluted monomeric form. As shown in Fig. 1, this dye accumulates in the lumen of acidic compartments, where it aggregates forming dimers, trimers and oligomers displaying a red fluorescence. Apparent pH_L was estimated in individual attached cells by following changes in the green fluorescence (Palmgren, 1991). Hepatocytes, cultured as mentioned above, were loaded with 5 $\mu mol\,l^{-1}$ AO for 10 min followed by two careful washes with standard saline, then the coverslips were mounted in a measuring chamber containing 1 ml saline and the chamber was fixed on the stage of an inverted Axiovert 100 epifluorescence microscope (Zeiss, Vienna, Austria) equipped with a ×40 ultraviolet objective. By means of a slow scan CCD video camera, fluorescence images were captured every 60 s, with excitation set to 493 nm, and emission was detected using a bandpass filter with a centre wavelength of 534 nm and an average bandwith of 30 nm (AHF analysentechnik AG, Tübingen, Germany). Using the tillVISion software package (T.I.L.L. Photonics, Munich, Germany), the field of measurement was chosen within one single cell and images were stored on a computer using the same software. Basal values of pH_L in standard saline were measured for at least 5 min before either half of the saline covering the cells was carefully exchanged for an equal volume of saline containing the desired compound(s), or all the covering saline was exchanged for the same volume of ion-free saline (Cl^- free, Na^+ free, etc.). The mean of the whole-cell AO fluorescence was calculated from the first five measurements or the five points before the medium was exchanged for hypertonic medium. These values were taken as 100% and results were expressed as a percentage of these mean values.

Tested compounds were made up in concentrated stock solutions dissolved in distilled water or DMSO and were applied at the following final concentrations: bafilomycin A1 0.3 $\mu mol\,l^{-1}$ (100 $\mu mol\,l^{-1}$ stock in DMSO), amiloride 100 $\mu mol\,l^{-1}$ (100 $mmol\,l^{-1}$ stock in DMSO), SITS 0.5 $mmol\,l^{-1}$ (200 $mmol\,l^{-1}$ stock in DMSO), BAPTA-AM 25 $\mu mol\,l^{-1}$ (25 $mmol\,l^{-1}$ stock in DMSO). The final concentration of DMSO was always kept below 0.3%, a concentration that did not interfere with the measurements.

Statistics

Data are presented as means ± s.e.m. of *N* individual cells. 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 the appropriate *post-hoc* test, with a *P*-value of <0.05 being considered as significant.

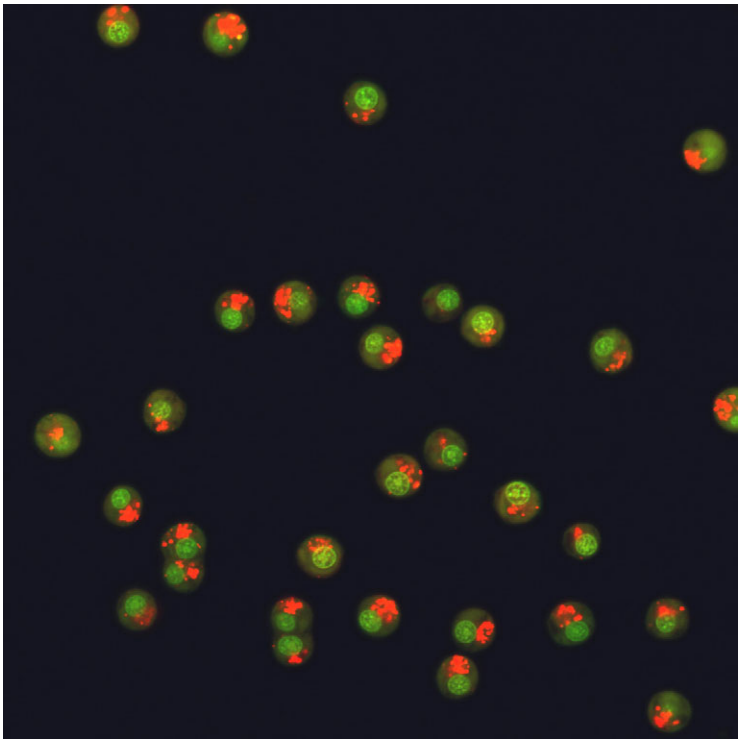

Fig. 1. Image of trout hepatocytes – using laser-scanning microscopy (LSM 510, Zeis) – pre-incubated with 5 μmol l^{-1} acridine orange (AO) for 10 min showing two distinct emission spectra: (1) green fluorescence corresponding to the monomeric AO emission, weak in the cytoplasm and bright in the nucleus, and (2) red fluorescence from separated or aggregated compartments in the cytoplasm, corresponding to aggregated AO emission. The red fluorescence indicates an accumulation of AO in the form of dimers and/or polymers due to the acidic pH inside the compartmental lumen. The LSM is equipped with a ×32 oil-immersion objective; 488 nm line of an argon laser was used for excitation; fluorescence emissions were simultaneously recorded in green (505–530 nm) and red (>650 nm) channels.

RESULTS

Involvement of V-ATPase in hypertonicity-induced pH_L changes

As depicted in Fig. 2, exposure of cells to hypertonicity induced a drop in AO fluorescence within the first 8 min, with the fluorescence change (ΔF) amounting to 56.1% of basal values. Thereafter, a stable signal was recorded until the hypertonic medium was removed. Following hypertonicity, isotonic medium is expected to act as hypotonic medium, supposedly increasing cell volume. Within this period, a slow increase in AO fluorescence was measured, shifting the hypertonicity-induced drop in AO fluorescence by 15.4% (ΔF). Thus, pH_L did not recover completely by the end of the exposure time.

Inhibition of V-ATPase using the specific inhibitor bafilomycin A1 resulted in an increase in basal AO fluorescence with ΔF amounting to 37.2% within 30 min. This increase in AO fluorescence corresponds to the amount of H^+ leaking out of acidic compartments. Normally, this leak is compensated by the activity of V-ATPase. Exposure of cells to hypertonicity in the continuous presence of bafilomycin A1 led to a drop in AO fluorescence (ΔF=30.9%) within the next 7 min. This was followed by a steady, slow increase, supposedly corresponding to the H^+ leak under these conditions. When isotonicity was re-introduced, an immediate drop in AO fluorescence (ΔF=13%) was recorded. AO fluorescence stabilized afterwards with a tendency to decrease by the end of the experimental period.

K^+ involvement

K^+ is a major constituent of the cytosol and accordingly its role as a counterion compensating for the electrogenic transport of H^+ by V-ATPase is expected. In the next set of experiments we tested the involvement of K^+ conductance in maintaining pH_L under steady-state and hypertonic conditions using the K^+ ionophore valinomycin. Fig. 3 shows that exposure of trout hepatocytes to 10 μmol l^{-1} valinomycin induced a slight increase in AO fluorescence (ΔF=8.9%) that was sustained for only 5 min, apparently due to the transport of K^+ from the cytosol to acidic compartments increasing resistance against V-ATPase activity and favouring H^+ leak. This was followed by a steady decrease in AO fluorescence (ΔF=16.5% below basal values) until the end of the exposure period. Thereafter, and upon exposure of cells to hypertonicity, a brief (2 min) drop in AO fluorescence was measured (ΔF=17.3%) followed by a fluorescence increase stabilizing at a near steady-state value (4% below basal values). In the continuous presence of valinomycin, this was reversed upon returning to isotonicity, with AO fluorescence decreasing to 34.1% below basal values by the end of the exposure period. Cytosolic [K^+] is known to increase and decrease following exposure to hypertonicity and hypotonicity, respectively. Accordingly, changes in AO fluorescence in response to hypertonicity and the subsequent re-introduction of isotonicity in the presence of valinomycin reflect changes in the [K^+] gradient across acidic luminal membranes. The absence of these changes in the absence of valinomycin points to a low conductivity of K^+ across acidic luminal membranes.

Cl^- involvement

We next studied the possible involvement of Cl^- in the hypertonicity-induced pH_L decrease by either removal of extracellular Cl^- or inhibition of anion transport with the specific inhibitor SITS. Unexpectedly, as shown in Fig. 4, removal of extracellular Cl^- induced a significant decrease in pH_L, measured as a drop in AO fluorescence (ΔF=16.8% in 5 min) followed by a steady decrease until the end of 30 min. When this was followed by exposure to hypertonic conditions in the absence of Cl^-, a further drop in AO fluorescence (ΔF=20.3% for 1 min) was measured with a subsequent continuous fluorescence increase, overshooting basal values (6.8% above initial values). This pH_L increase was reversed upon returning to isotonic conditions (34.8% below initial values). The effect of Cl^- removal on basal values was partly due to whole-media exchange around the cells because in a control experiment the

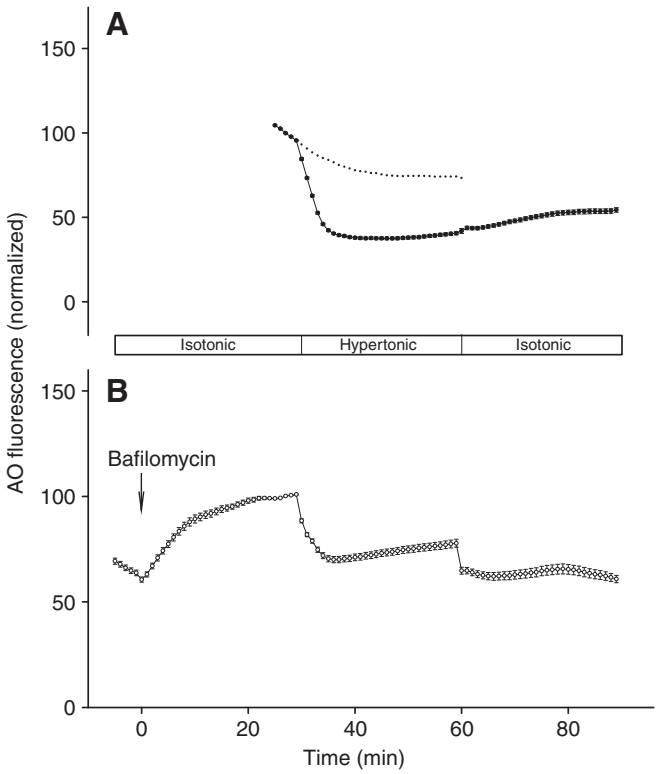

Fig. 2. Changes in AO fluorescence in response to (A) exposure of cells to hypertonicity (1.6×isosmolarity) followed by restoration of isotonicity and (B) exposure of cells to bafilomycin A1 (0.3 μmol l^{-1}) followed by the same exposure conditions as in A in the continuous presence of bafilomycin A1. Data are means ± s.e.m. of 64–66 cells from three to four independent preparations. Data were normalized to the mean calculated from five points preceding the hypertonicity exposure. Dotted line corresponds to normal quenching of AO (three independent preparations, 54 cells, data normalized to the mean calculated from the first five points).

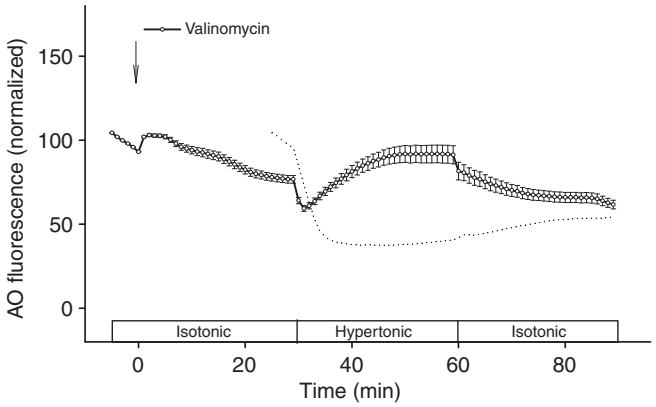

Fig. 3. Changes in AO fluorescence following exposure of cells to valinomycin (10 μmol l^{-1}) under steady-state conditions followed by exposure to hypertonicity then restoration of isotonic conditions, in the continuous presence of valinomycin. Data are means ± s.e.m. of 67 cells from three independent preparations. Data were normalized to the mean calculated from the first five points. For comparison, the control (Fig. 2A) is shown as a dotted line.

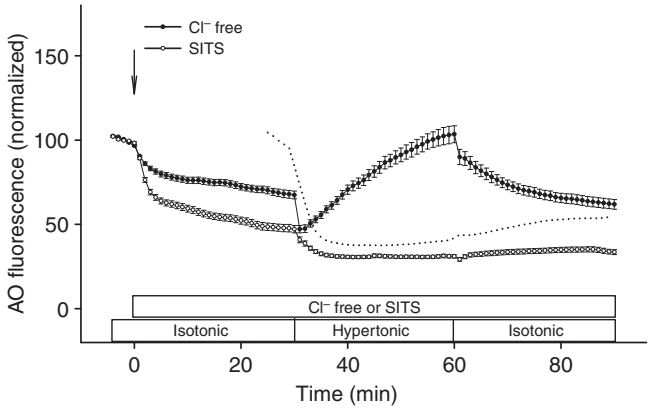

Fig. 4. Changes in AO fluorescence following exposure of cells to Cl^--free medium or SITS (0.5 mmol l^{-1}) under steady-state conditions followed by exposure to hypertonicity then restoration of isotonic conditions, in the continuous absence of Cl^- or presence of SITS. Data are means ± s.e.m. of 34–38 cells from three independent preparations. Data were normalized as in Fig. 3. For comparison, the control (Fig. 2A) is shown as a dotted line.

exchange of all, but not half, of the standard saline for the same medium induced a decrease in AO fluorescence with ΔF amounting to 8% in 5 min, possibly due to mechanical disturbance. However, the decrease was significantly different from that of control, which was confirmed by the effect of the anion inhibitor SITS. Exposure of hepatocytes to 0.5 mmol l^{-1} SITS induced a significant drop in AO fluorescence (ΔF=51.2% in 30 min) which, upon exposure to hypertonicity, decreased further (ΔF=15.8% in 7 min) before the signal stabilized. Replacing the hypertonic medium with isotonic medium in the presence of SITS had no obvious effect on the AO fluorescence. Data measured in the absence of Cl^- indicated that the hypertonicity-induced acidity was dependent on the Cl^- gradient across vesicular membranes. The still noticeable acidity in the presence of SITS under hypertonic conditions suggested, however, that Cl^- transport was not involved. Together, these data indicate that the sustainability of acidification – not acidification itself – was dependent on the presence of a Cl^- gradient across the luminal membrane. Furthermore, Cl^- conductance appeared to be high compared with that of K^+.

HCO_3^- involvement

The effect of anisotonicity on pH_L in the absence of extracellular Cl^- prompted us to investigate a possible role for HCO_3^- in pH_L regulation under hypertonicity challenge. As shown in Fig. 5, removal of extracellular HCO_3^- induced a decrease in AO fluorescence (ΔF=11.1% within 5 min). This small (3.1% lower than that induced by total exchange of the medium, see above), but significant, change indicated a slight effect of HCO_3^- transport on pH_L regulation under steady-state conditions. Moreover, the absence of extracellular HCO_3^- affected neither the pattern nor the degree of pH_L changes induced by hypertonicity and the subsequent return to isotonicity. On the other hand, HCO_3^- omission along with replacing Cl^- with gluconate showed a substantial drop in steady-state pH_L (ΔF=37.1% by the end of 30 min) indicating that HCO_3^- removal enhanced the acidification induced by Cl^- removal. Under hypertonicity, the initial response in the absence of both Cl^- and HCO_3^- was similar to that seen in the absence of Cl^- only, i.e. a 1 min drop in AO fluorescence (ΔF=16.3%). Nonetheless, the subsequent alkalinization seen in the absence of Cl^- was substantially attenuated in the absence of both Cl^- and HCO_3^- (ΔF=46.2% below compared with 6.8% above basal values in the absence of Cl^- and HCO_3^- or Cl^- only,

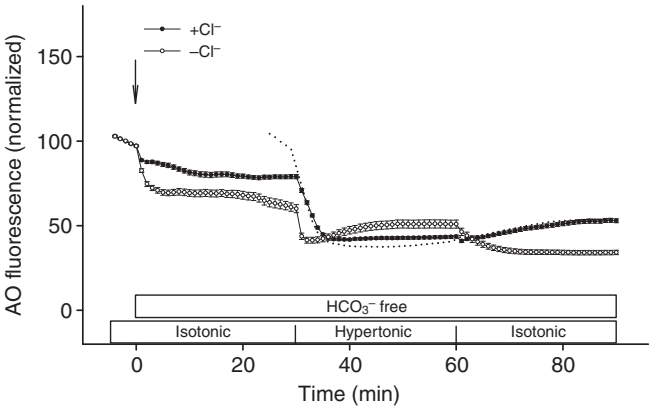

Fig. 5. Changes in AO fluorescence following exposure of cells to Cl^--free medium in the presence or absence of HCO_3^- under steady-state conditions followed by exposure to hypertonicity then restoration of isotonic conditions, in the continuous absence of Cl^- or both Cl^- and HCO_3^-. Data are means ± s.e.m. of 38–55 cells from three independent preparations. Data were normalized as in Fig. 3. For comparison, the control (Fig. 2A) is shown as a dotted line.

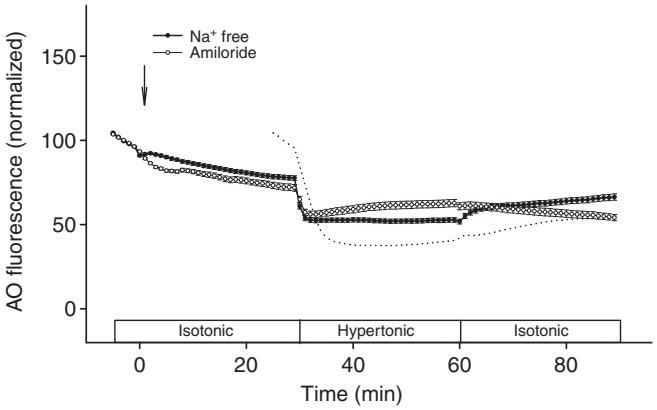

Fig. 6. Changes in AO fluorescence following exposure of cells to Na^+-free medium or amiloride (100 μmol l^{-1}) under steady-state conditions followed by exposure to hypertonicity then restoration of isotonic conditions, in the continuous absence of Na^+ or presence of amiloride. Data are means ± s.e.m. of 34–41 cells from three independent preparations. Data were normalized as in Fig. 3. For comparison, the control (Fig. 2A) is shown as a dotted line.

respectively). Returning to isotonicity resulted in a decrease in AO fluorescence (ΔF=11.5%) within 10 min, after which a constant signal was measured until the end of the exposure time. These data suggest a possible inward HCO_3^- transport parallel and additive to Cl^- transport under steady-state conditions. That is, the removal of each ion alone led to an acidification while removal of both led to a more severe acidification. Under hypertonic conditions, and following the one-point pH_L drop, the alkalinization was induced by removal of extracellular Cl^-, presumably due to Cl^- transport out of the acidic lumen. The dependence of such alkalinization on the presence of extracellular HCO_3^- suggests the presence of Cl^-/HCO_3^- exchange activated by disturbing the Cl^- gradient across luminal membranes. While exchanging Cl^- for HCO_3^- will not affect membrane potential, the addition of HCO_3^- to the acidic lumen should result in the consumption of H^+ (translocated by V-ATPase) forming CO_2 and water. The return to isotonicity switched off this mechanism and accordingly unmasked the V-ATPase activity, measured as a decrease in AO fluorescence.

Na^+ involvement

The contribution of Na^+ transport mechanisms to pH_L regulation was then investigated. As shown in Fig. 6, removal of extracellular Na^+ showed no effect on steady-state pH_L, while the hypertonicity-induced acidification was attenuated. A drop in AO fluorescence (ΔF=23.8%) was measured upon exposure of cells to hypertonic medium, which was followed by a stable signal until the end of the 30 min exposure. Exchanging the hypertonic medium for an isotonic one in the continuous absence of extracellular Na^+ induced a slow and sustained fluorescence increase (ΔF=13.6%). To confirm these results, we further studied the effect of inhibiting Na^+/H^+ exchange (NHE) on the hypertonicity-induced pH_L decrease. NHE inhibition using the non-specific inhibitor amiloride (100 μmol l^{-1}) decreased steady-state AO fluorescence with ΔF amounting to 13.2% within 5 min, followed by the usual steady decrease seen in controls. Again, exposure to hypertonicity induced a drop in AO fluorescence (ΔF=14.5%) within 2 min, followed by steady, slow alkalinization raising AO fluorescence (ΔF=5.5%) by the end of the test period. Returning to isotonicity, in the presence of amiloride, arrested the increase in AO fluorescence and, instead, a slow decrease (ΔF=8.6%) was measured until the end of the exposure period. The reduced acidification in response to hypertonicity in this set of experiments clearly indicated a contribution of Na^+-dependent mechanisms in pH_L regulation under hypotonic conditions. The difference between the effect of Na^+-free medium and amiloride might be due to the presence of different Na^+ transport systems with different sensitivity to amiloride.

Ca^{2+} involvement

We have shown recently that removal of extracellular Ca^{2+} or chelation of intracellular Ca^{2+} affects pH_i under steady-state conditions (Ahmed and Pelster, 2007) and during hypertonicity challenge (Ahmed et al., 2006). Thus, in the next set of experiments, we attempted to investigate possible changes in pH_L in response to the above-mentioned conditions. As shown in Fig. 7, removal of extracellular Ca^{2+} induced a decrease in AO fluorescence (ΔF=11.7% within 5 min), which decreased further upon exposure of cells to hypertonicity, with ΔF amounting to 20.1% within 30 min. Restoring isotonic conditions resulted in a slight increase in AO fluorescence (ΔF=6.7% within 7 min) and a stable signal was recorded until the end of the exposure period. Considering the reduction of the hypertonicity-induced acidification in the absence of extracellular Ca^{2+}, we attempted to investigate the effect of chelating intracellular Ca^{2+} on the hypertonicity-induced pH_L decrease. As shown in Fig. 8A, exposure of cells to BAPTA-AM (25 μmol l^{-1}) alone induced a continuous increase in AO fluorescence (ΔF=94.8% within 30 min). Pre-incubation of cells in Ca^{2+}-free medium induced a decrease in AO fluorescence (ΔF=11.3% within 5 min) that, upon the addition of BAPTA-AM, in the continuous absence of extracellular Ca^{2+}, increased with ΔF amounting to 69.7% by the end of the exposure time. The difference between the rate of increase in the presence and absence of extracellular Ca^{2+} was not significant, as calculated following curve-fitting data to one-phase exponential association (GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, CA, USA).

To test the effect of intracellular Ca^{2+} chelation on the hypertonicity-induced pH_L decrease, we chose to incubate cells with BAPTA-AM in standard saline for 1 h; thereafter, cells were washed

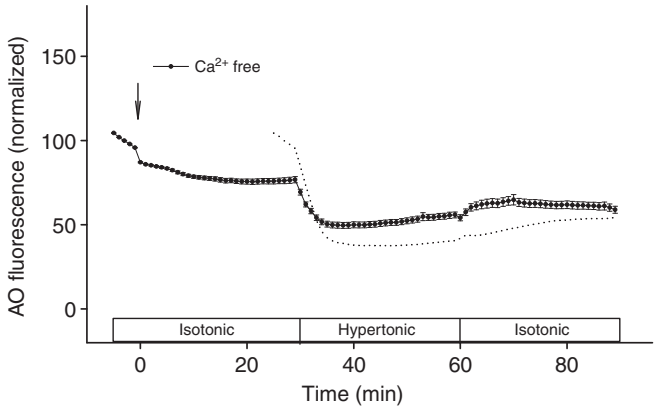

Fig. 7. Changes in AO fluorescence following exposure of cells to Ca^{2+}-free medium under steady-state conditions followed by exposure to hypertonicity then restoration of isotonic conditions, in the continuous absence of Ca^{2+}. Data are means ± s.e.m. of 65 cells from four independent preparations. Data were normalized as in Fig. 3. For comparison, the control (Fig. 2A) is shown as a dotted line.

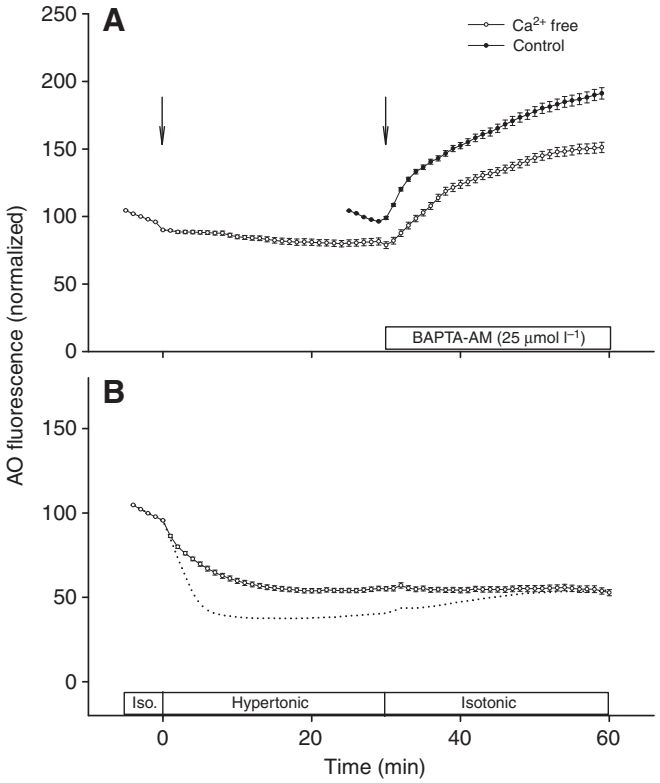

Fig. 8. Changes in AO fluorescence following (A) exposure of cells to BAPTA-AM (25 μmol l^{-1}) in the presence and absence of extracellular Ca^{2+} and (B) exposure of cells (pre-incubated with 25 μmol l^{-1} BAPTA-AM for 1 h) to hypertonicity followed by restoration of isotonic conditions, in the continuous absence of extracellular Ca^{2+}. Data are means ± s.e.m. of 61–126 cells from four to five independent preparations. Data were normalized as in Fig. 3. For comparison, the control (Fig. 2A) is shown as a dotted line in B.

twice with Ca^{2+}-free standard saline to remove BAPTA-AM from the incubation medium. This was followed by incubation with AO in Ca^{2+}-free medium and subsequent measurements were carried out in Ca^{2+}-free medium. As shown in Fig. 8B, exposure of these cells to hypertonicity resulted in a decrease in AO fluorescence at a significantly slower rate compared with that of the hypertonicity control (data were curve fitted to one-phase exponential decay using GraphPad Prism, same version as above) with ΔF amounting to 40% within 30 min. Re-introducing isotonicity resulted in no change in AO fluorescence.

Swelling of cells was observed following incubation with BAPTA-AM; therefore, we tested whether the alkalinization induced by BAPTA-AM was the consequence of a swelling effect. We exposed the cells to hypertonic medium for 30 min, inducing the usual sustained decrease in AO fluorescence. As shown in Fig. 9A, subsequent addition of BAPTA-AM induced a continuous alkalinization with ΔF amounting to 47.2% within 30 min. At the end of the exposure time bafilomycin A1 was added to test whether BAPTA-induced alkalinization was due to V-ATPase inhibition. Under hypertonic conditions and in the continuous presence of BAPTA-AM, bafilomycin A1 induced a further increase in AO fluorescence with ΔF amounting to 93.7% within 30 min. We repeated the same experiment, reversing the order of BAPTA-AM and bafilomycin A1 addition. As shown in Fig. 9A, following hypertonicity-induced pH_L acidification, bafilomycin A1 addition increased AO fluorescence within the next 30 min (ΔF=57.6%). Subsequent addition of BAPTA-AM induced a second increase in AO fluorescence (ΔF=67.6%). These data imply that the BAPTA-induced alkalinization of acidic compartments was not due to V-ATPase inhibition. Adding to the complexity, exposure of cells to 0.5 mmol l^{-1} of $ZnCl_2$ [to inhibit H^+ leak channels (Schapiro and Grinstein, 2000)] had no effect on the BAPTA-induced alkalinization of pH_L (data not shown).

We next tested whether BAPTA-induced alkalinization was totally related to Ca^{2+} decrease in the cytosol and supposedly inside acidic compartments. To answer this, we exposed cells to the Ca^{2+} ionophore A23187 (2 μmol l^{-1}) in Ca^{2+}-free medium for 30 min before exposure to BAPTA-AM. A23187 is known to bind Ca^{2+} at neutral and acidic pH values (Liu and Hermann, 1978). Accordingly, incubation of cells with A23187 in Ca^{2+}-free medium would cause a depletion of Ca^{2+} stores including acidic compartments. As shown in Fig. 9B, exposure of hepatocytes to A23187 induced a decrease in AO fluorescence (ΔF=31.1% within 7 min and stabilized thereafter) possibly through exchanging luminal Ca^{2+} for cytosolic H^+. Addition of BAPTA-AM, in the continuous presence of A23187 and absence of extracellular Ca^{2+}, still induced an increase in AO fluorescence (ΔF=47.9%). These data indicate that the BAPTA-induced alkalinization was not totally related to the decrease in Ca^{2+} in the cytosol or inside acidic compartments.

DISCUSSION

We have shown that exposure of trout hepatocytes to hypertonic conditions induced a decrease in pH_L that was almost irreversible upon the restoration of isotonicity. Bafilomycin A1 reduced the hypertonicity-induced pH_L decrease by about 50%. Previous studies in rat hepatocytes (Schreiber et al., 1994; Reinehr et al., 2006) have shown similar pH_L acidification in response to exposure to hypertonic conditions. This acidification was also sensitive to V-ATPase inhibition by bafilomycin A1 (Reinehr et al., 2006), but not by concanamycin A (Schreiber et al., 1996). In the study of Reinehr and colleagues, the effect of hypertonicity on pH_L was removed at a bafilomycin A1 concentration of only 0.1 μmol l^{-1} (Reinehr et al., 2006). In our study we used a three times higher

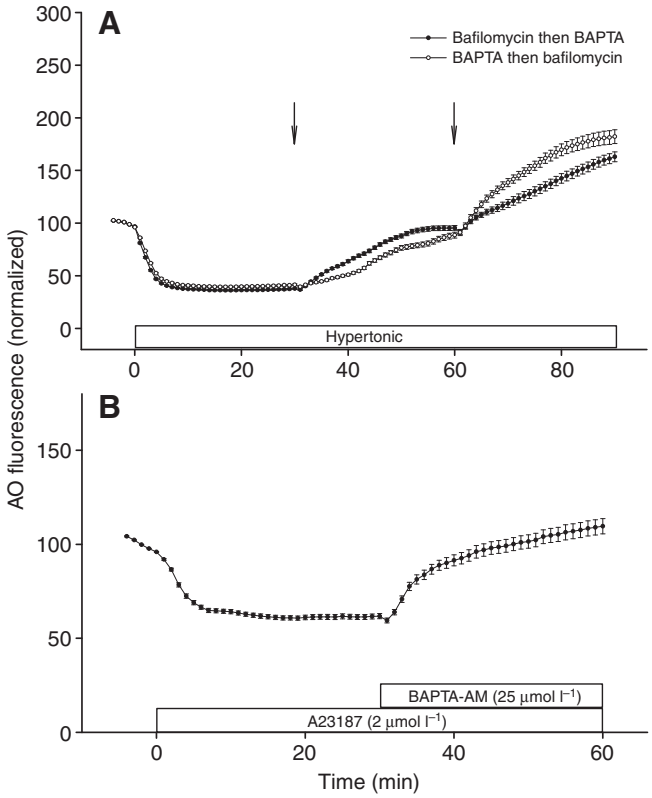

Fig. 9. (A) Effect of sequential addition of bafilomycin A1 (0.3 μmol l^{-1}) and BAPTA-AM (25 μmol l^{-1}) on the hypertonicity-induced changes in AO fluorescence. Data are means ± s.e.m. of 56–68 cells from three independent preparations. Data were normalized as in Fig. 3. (B) Changes in AO fluorescence following exposure of cells to A23187 (2 μmol l^{-1}) in Ca^{2+}-free medium followed by BAPTA-AM (25 μmol l^{-1}), in the continuous presence of A23187 and absence of extracellular Ca^{2+}. Data are means ± s.e.m. of 90 cells from five independent preparations. Data were normalized as in Fig. 3.

concentration (0.3 μmol l^{-1}), but pH_L acidification was only inhibited by about 50%. We therefore conclude that in trout hepatocytes pH_L changes under hypertonic conditions are dependent on V-ATPase-dependent and V-ATPase-independent pathways. pH_L acidification was fully and rapidly reversible upon re-exposure of rat cells to isotonic conditions (Schreiber et al., 1994). This indicates that pH_L sensitivity to anisotonicity might be dependent on the cell type. It is widely acknowledged that pH_L is maintained by a balance between intra-luminal V-ATPase proton pumping into and intrinsic proton leak out of acidic compartments (Grabe and Oster, 2001; Demaurex, 2002; Paroutis et al., 2004). In our study, the absence of complete recovery upon the restoration of isotonic conditions seems to be a consequence of hypertonicity-induced inhibition of H^+ leak pathways. This inhibition is obvious when comparing the effect of the H^+ leak in the presence of bafilomycin A1 before and after exposure to hypertonicity. While a pH_L increase was observed upon exposure of cells to bafilomycin A1, following a transient exposure to hypertonicity this pH_L increase was completely absent. The instant drop in pH_L following the restoration of isotonicity might be a result of a mechanical effect (due to exchanging all of the medium around the cells) exaggerated by the reduced H^+ leak. The absence of this effect in other experiments might indicate a low compartmental sensitivity for such a mechanical effect at low pH_L values.

Counterion transport

Proton pumping into acidic compartments is expected to build up an inside-positive electrical gradient ($\Delta\psi$) that will antagonize further inward H^+ transport while promoting the outward H^+ leak. It was postulated (Al-Awqati et al., 1992) that movement of compensating charges (counterion transport) can alter $\Delta\psi$ and, consequently, change the balance between H^+ pumping and H^+ leak. Accordingly, pH_L regulation will be dependent on the permeability of vesicular membranes to counterions. Because of the high concentration of Cl^- and K^+ and their high conductive permeability across other cellular membranes, both ions were considered to be the main counterions possibly affecting $\Delta\psi$.

K^+ involvement

Under hypertonicity, the expected cell shrinkage is known to activate plasma membrane transport mechanisms that accumulate K^+ in the cytosol (Lang, 2007). Accordingly, the reversal of hypertonicity-induced acidification in the presence of valinomycin indicated a low K^+ conductance across vesicular membranes. This also signifies that it is the reduction in H^+ leak, rather than the activation of H^+ pumping, that is responsible for the sustained acidification. These data are consistent with previous reports (Wu et al., 2000; Wu et al., 2001) in which the difference in the degree of acidification among acidic compartments has been reported to be partly a consequence of a reduction in H^+ leak. Under steady-state conditions, K^+ conductance has been reported to be high in Chinese hamster ovary cells (Demaurex et al., 1998), HeLa cells (Wu et al., 2000) and AtT-20 cells (Wu et al., 2001) because valinomycin did not affect pH_L. Our results showed a slight increase in pH_L in response to valinomycin, which might indicate that under steady-state conditions K^+ conductance is also low in trout hepatocytes. In vesicles [K^+] has been reported to be close (slightly lower) to that of the cytosol (Schapiro and Grinstein, 2000) and a similar gradient could be responsible for the small and brief pH_L increase induced by valinomycin in the present study.

Cl^- involvement

Inwardly directed Cl^- currents are believed to play an essential role in regulating the pH_L by dissipating $\Delta\psi$ (Al-Awqati, 1995; Futai et al., 1998; Li and Weinman, 2002; Faundez and Hartzell, 2004). This was supported by the observation that removal of extracellular Cl^- induced an increase in pH_L in human fibroblasts (Seksek et al., 1995) and in HeLa cells (Llopis et al., 1998). Also, radioactive chloride was taken up into vacuolar membrane vesicles of yeast upon ATP hydrolysis in a protonophore-sensitive manner (Wada et al., 1992). However, other reports showed no effect of Cl^- removal, and it was concluded that Cl^- is not essential in maintaining steady-state pH_L (Schreiber et al., 1996; Wu et al., 2000; Wu et al., 2001). Under hypertonic conditions, removal of extracellular Cl^- or inhibition of Cl^- transport using DIDS has been reported to eliminate the pH_L acidification induced by hypertonicity in rat hepatocytes (Schreiber et al., 1996; Reinehr et al., 2006). In our study, hypertonicity-induced acidification was not inhibited by inhibition of Cl^- uptake using Cl^--free medium or the anion inhibitor SITS. However, sustaining the acidification appeared to depend on the presence of a [Cl^-] gradient favouring Cl^- transport from the cytosol to acidic compartments. Interestingly, the alkalinization of vesicles in the absence of Cl^- was substantially reduced upon removal of extracellular HCO_3^-, indicating the presence of Cl^--dependent HCO_3^- transport across the membrane of acidic compartments. In a study on acidic vesicles of amoeba (Giglione and Gross, 1995), HCO_3^- transport into acidic vesicles was reported. Inside the vesicle, according to that study, HCO_3^- would

combine with translocated H^+, preventing the formation of a large chemical gradient. Apart from this study, and while anion exchangers have already been detected in acidic compartments (Holappa et al., 2001; Holappa and Kellokumpu, 2003), evidence for a contribution of HCO_3^- to organelle pH regulation is lacking (Paroutis et al., 2004). Actually, in the present study, removal of extracellular HCO_3^- appeared to have a slight effect on steady-state pH_L while no effect on the hypertonicity-induced acidification or the subsequent restoration of isotonicity was seen. These data might suggest that HCO_3^- transport is not active under normal conditions and that it can be activated upon reversing the Cl^- gradient across the luminal membranes.

To our surprise, under steady-state conditions, Cl^- removal significantly decreased pH_L. A total medium exchange appeared to contribute to this effect. Nevertheless, the comparatively pronounced pH_L decrease observed upon exposure of cells to the anion inhibitor SITS (and DIDS, not shown) confirmed the unexpected results of Cl^- removal. These observations cannot be explained according to the above-mentioned charge compensation model. If this effect is due to an inhibition of charge compensation, the only explanation would be to assume the existence of a continuous outward Cl^- current increasing the inside positive potential of the acidic lumen and accordingly limiting acidification by H^+ pumping. Inhibition of this current with SITS should lead to dissipation of $\Delta\psi$ and enhance acidification. However, a continuous outward current cannot exist without assuming an inward current (of the same magnitude) from the cytosol in order to replace Cl^-. In addition, removal of extracellular Cl^- should activate a possible outward current leading to pH_L alkalinization instead of the measured acidification in this study. Furthermore, inhibition of SITS-sensitive HCO_3^- uptake is not likely, as HCO_3^- removal had only a very minor effect on steady-state pH_L. Thus, the contribution of Cl^- to the control of steady-state pH_L remains elusive.

Involvement of Na^+/H^+ exchange

The role of plasma membrane NHEs in pH_i regulation is well established both under steady-state conditions and following hypertonicity (Lang, 2007). In trout hepatocytes, exposure of cells to NHE-specific inhibitors (Ahmed et al., 2006) or removal of extracellular Na^+ (Krumschnabel et al., 2003) decreased steady-state pH_i and abolished the hypertonicity-induced pH_i increase. Intracellularly, NHE isoforms have been reported to reside in the membranes of acidic compartments (Numata and Orlowski, 2001; Brett et al., 2002; Nakamura et al., 2005); however, an involvement of NHE activity in maintaining pH_L has not been detected (Demaurex et al., 1998; Llopis et al., 1998; Schapiro and Grinstein, 2000). In the present study removal of extracellular Na^+ showed no effect on basal pH_L. Yet, the slight acidification induced by amiloride might indicate a minor participation of an amiloride-sensitive H^+ leak mechanism. On the other hand, the hypertonicity-induced pH_L decrease was attenuated in the absence of extracellular Na^+ or the presence of amiloride. This indicates a contribution from Na^+-dependent mechanisms in the acidification of acidic compartments in response to hypertonic conditions. Following the instant pH_L drop induced by hypertonicity, the sustained acidification measured in the absence of extracellular Na^+ compared with the slow recovery in the presence of amiloride points to the possible activity of non-NHE Na^+-sensitive mechanisms regulating pH_L under hypertonic conditions. A similar conclusion could be drawn from the difference in the pH_L response pattern upon the reintroduction of isotonicity in the absence of extracellular Na^+ compared with the presence of amiloride.

Involvement of Ca^{2+}

Acidic organelles are known to operate as Ca^{2+}-storage sites in many cells (Pozzan et al., 1994; Srinivas et al., 2002). This Ca^{2+} accumulation is attained either directly by the activities of Ca^{2+}-ATPases (Missiaen et al., 2004) or indirectly through exchanging Ca^{2+} for H^+ driven by the proton gradient generated by V-ATPase (Dunn et al., 1994; Christensen et al., 2002). In the present study, steady-state pH_L was slightly decreased upon removal of extracellular Ca^{2+}. In our previous work (Ahmed and Pelster, 2007), this treatment was not accompanied by a change in cytosolic $[Ca^{2+}]$ for a period of 10 min, except for an initial brief (but high) increase in $[Ca^{2+}]_i$ supposedly due to a mechanical effect. If Ca^{2+} release from acidic compartments is contributing to this $[Ca^{2+}]_i$ increase, then it is possible that Ca^{2+}–H^+ exchange is involved. The existence of this exchange is supported by the pH_L acidification upon release of Ca^{2+} from acidic stores using A23187. Under hypertonic conditions, Ca^{2+} influx from the extracellular medium as well as Ca^{2+} release from cellular stores is the source of the measured increase in $[Ca^{2+}]_i$ (Ahmed et al., 2006).

Interestingly, BAPTA exposure induced an increase in pH_L. This alkalinization of acidic compartments might explain the BAPTA-induced pH_i decrease measured in these cells (Ahmed and Pelster, 2007). Sequentially adding bafilomycin A1 and BAPTA in a different order suggested that BAPTA increased pH_L through activating H^+ leak pathways. While the presence of a H^+ leak pathway (voltage-gated H^+ channel) sensitive to micromolar concentrations of Zn^+ has been reported in acidic compartments of Chinese hamster ovary cells (Demaurex et al., 1998), in our study even higher concentrations (up to 0.5 mmol l^{-1}) did not change the pH_L alkalinization induced by BAPTA (data not shown). In addition, the effect of BAPTA was not abolished by the absence of HCO_3^- or inhibition of anion exchange by SITS (data not shown), and it was independent of the presence of Ca^{2+}. Therefore the H^+ leak activation appeared to be a direct effect of BAPTA, independent of $[Ca^{2+}]$ changes.

In summary, we have shown in this work that acidic compartments of trout hepatocytes are highly sensitive to hypertonic conditions with a high dependence on low K^+ and high Cl^- conductance. Manipulations known to affect intracellular pH-dependent ion transporters under hypertonic conditions have been shown to also affect pH_L. Hypertonicity-induced acidification of pH_L was clearly dependent on V-ATPase activity, but V-ATPase-independent pathways like NHE activity or the extent of the proton leak, for example, are involved as well. Interestingly, BAPTA-AM appeared to induce H^+ leak out of acidic compartments independent (at least partly) of its effect as an intracellular Ca^{2+} chelator. We believe that pH_L has to be considered as an important factor in the overall regulation of cellular pH.

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