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Interdependence of Ca^{2+} and proton movements in trout hepatocytes

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Summary

This study was undertaken to investigate possible interrelationships between Ca^{2+} homeostasis and pH regulation in trout hepatocytes. Exposure of cells to Ca^{2+} mobilizing agents ionomycin (0.5 μmol l^{-1}) and thapsigargin (0.1 μmol l^{-1}) induced an increase in intracellular pH (pHi) that was dependent on Ca^{2+} influx from the extracellular medium as well as Ca^{2+} release from intracellular pools. Surprisingly, this increase in pHi and intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, was not accompanied by any change in proton secretion. By contrast, removal of extracellular Ca^{2+} (Ca^{2+}_e) using EGTA (0.5 mmol l^{-1}) briefly increased proton secretion rate with no apparent effect on pHi, while chelation of Ca^{2+}_i using BAPTA-AM (25 μmol l^{-1}) resulted in a drop in pHi and a sustained increase in proton secretion rate. $[Ca^{2+}]_i$ therefore affected intracellular proton distribution and/or proton production and also affected the distribution of protons across the cell membrane. Accordingly, changes in pHi were not always compensated for by proton secretion across the cell membrane.

Alteration in pH_e below and above normal values induced a slow, continuous increase in $[Ca^{2+}]_i$ with a tendency to stabilize upon exposure to high pH_e values. Rapid pHi increase induced by NH_4Cl was accompanied by an elevation in $[Ca^{2+}]_i$ from both extracellular and intracellular compartments. Ca^{2+}_e appeared to be involved in pHi regulation following NH_4Cl-induced alkalinization whereas neither removal of Ca^{2+}_e nor chelation of Ca^{2+}_i affected pHi recovery following Na-propionate exposure. Similarly, $[Ca^{2+}]_i$ increase induced by hypertonicity appeared to be a consequence of the changes in pHi as Na-free medium as well as cariporide diminished the hypertonicity-induced increase in $[Ca^{2+}]_i$. These results imply that a compensatory relationship between changes in pHi and proton secretion across cell plasma membrane is not always present. Consequently, calculating proton extrusion from buffering capacity and rate of pHi change cannot be taken as an absolute alternative for measuring proton secretion rate, at least in response to Ca^{2+} mobilizing agents.

Key words: trout hepatocyte, intracellular Ca^{2+}, intracellular pH, proton secretion, ionomycin, thapsigargin, BAPTA-AM, NH_4Cl, Na-propionate, hypertonicity.

Introduction

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is well known to be a major regulator of many intracellular events and a necessary component initializing various steps of activation in almost any cellular model. $[Ca^{2+}]_i$ is involved in key processes such as gene expression, exocytosis, contraction and cellular metabolism. Eukaryotic cells can increase $[Ca^{2+}]_i$ either by releasing compartmentalized Ca^{2+} from intracellular stores or by evoking Ca^{2+} influx into the cell from the extracellular space. In addition to an increase in $[Ca^{2+}]_i$, cell responses to stimulation by mitogens or hormones, as well as cell volume changes, often include rapid changes in intracellular pH (pHi) (Batlle et al., 1993; Martin-Requero et al., 1997; Krumschnabel et al., 2003). Typical Ca^{2+} transporting systems in plasma membranes have been reported to be Ca^{2+} channels, Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers (Carafoli, 1987). Similarly, a number of membrane transporters have been reported to be pH-regulating mechanisms, including Na^+/H^+ and Cl^-/HCO_3^-exchangers (Walsh, 1986; Fossat et al., 1997; Ahmed et al., 2006), Na^+/HCO_3^- cotransporter (Furimsky et al., 2000) and proton pump (Beyenbach and Wieczorek, 2006). A number of studies investigating the possible linkage between changes in $[Ca^{2+}]_i$ and pHi revealed that the nature of this linkage appears to be dependent on the cell type, as shown for example by analyzing the effect of changes in $[Ca^{2+}]_i$ on pHi. While an alkalinization induced by Ca^{2+} mobilizing agents has been reported to be dependent on extracellular Ca^{2+} (Ca^{2+}_e) in rat lymphocytes (Grinstein and Cohen, 1987), chicken granulose cells (Asem et al., 1992), rat hepatocytes (Anwer, 1993), cortical neurons (Ouyang et al., 1995), rabbit renal proximal tubules (Yamada et al., 1996) and human platelets (Poch et al., 1993), the absence of Ca^{2+}_e did not prevent that alkalinization in human lymphocytes (Cabado et al., 2000).

On the other hand, modifying pHi could also affect $[Ca^{2+}]_i$. In inner medullary collecting duct cells (IMCD) (Tsunoda, 1990; Slotki et al., 1993) and pancreatic acinar cells (Tsunoda, 1990), cytosolic acidification increases $[Ca^{2+}]_i$, but an increase in $[Ca^{2+}]_i$ has been observed in response to cytosolic alkalinization in lymphocytes (Grinstein and Goetz, 1985),

cultured smooth muscle cells (Siskind et al., 1989), lacrimal acinar cells (Yodozawa et al., 1997) and endothelial cells (Danthuluri et al., 1990).

Conflicting results have been reported with respect to a possible correlation between $[Ca^{2+}]_i$ and pHi under cell shrinkage stress. While the activity of pHi regulation mechanisms that accompany cell shrinkage has been reported to be regulated by $[Ca^{2+}]_i$ (Murao et al., 2005), other reports suggest, however, that an increase in $[Ca^{2+}]_i$ was the consequence of changes in pHi (Grinstein et al., 1985; Dascalu et al., 1992; Pedersen et al., 1996).

Several hypotheses have been outlined to account for the $[Ca^{2+}]_i$–pHi interrelationship, including Na^+/H^+ exchange (NHE) activity (Martin-Requero et al., 1997), Ca^{2+}/H^+ exchange activity (Schulz et al., 1989; Anwer, 1993; Daugirdas et al., 1995; Ouyang et al., 1995; Alfonso et al., 2005), competition for common intracellular buffer sites between Ca^{2+} and H^+, where an increase in $[Ca^{2+}]_i$ would cause a release of H^+ and *vice versa* (Grinstein et al., 1987), and H^+-sensitive Ca^{2+} channels as well as Ca^{2+}-sensitive H^+ channels (Dickens et al., 1990).

A recent study demonstrated that in trout hepatocytes hypertonic stress induces an intracellular alkalinization, a concomitant increase in the rate of proton secretion and an increase in $[Ca^{2+}]_i$ (Ebner et al., 2005). In addition, among other second messenger pathways, Ca^{2+} appears to be involved in the control of Na^+/H^+ exchange, providing another hint on a possible interdependence of Ca^{2+} and proton movements in these cells (Ahmed et al., 2006). A number of studies have investigated calcium–pH crosstalk, although most using mammalian cells, while studies on fish cells are lacking. Also, since metabolic activities are known to be regulated by calcium and affected by alteration in pHi, this makes hepatocytes a relevant model to investigate the possible link between pH and $[Ca^{2+}]_i$. Furthermore, in previous studies, membrane transport mechanisms were usually explored using different inhibitors and their activities were quantified by calculating proton extrusion from the rate of pHi increase or decrease and buffering power (which is calculated from measured pHi). This method assumes that changes in pHi result from, or are at least accompanied by, the transport of protons across the cell plasma membrane. To avoid this assumption we used a cytosensor microphysiometer in order to directly measure the acidification of the external medium by hepatocytes. We also attempted to identify the possible link between $[Ca^{2+}]_i$ and pHi in trout hepatocytes by separate and direct measurements of pHi, $[Ca^{2+}]_i$ and proton secretion rate. This was achieved by investigating the effect of manipulating $[Ca^{2+}]_i$ on pHi and of pHi alterations on $[Ca^{2+}]_i$ under steady-state condition and during hypertonic stress. Also included was the situation of pH recovery after an artificial intracellular acidification or alkalinization. We report that proton distribution across cell plasma membrane is not usually a compensation for changes in pHi and the involvement of intracellular mechanisms should be considered in future studies.

Materials and methods

Chemicals

Collagenase (Type VIII), bovine serum albumin (BSA), foetal calf serum (FCS), nigericin, valinomycin, ionomycin, thapsigargin and low-melting-point agarose gel were purchased from Sigma (Deisenhofen, Germany). NHE-1 inhibitor cariporide mesilate was kindly provided by Sanofi-Aventis Pharma (Vienna, Austria). Fura 2, 1,2-bis(*o*-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (acetoxymethyl ester) (BAPTA-AM) and 2′,7′-bis-(2-carboxypropyl)-5-(and-6)-carboxyfluorescein (BCPCF), all as acetoxymethylesters (AM), were purchased from Molecular Probes (Leiden, The Netherlands). Leibovitz L-15 medium was obtained from Invitrogen (Vienna, Austria). 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-litre 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 (Krumschnabel et al., 1996). 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 (1.5×10^6–2×10^6 cells ml^{-1}) were then suspended in Leibovitz L15 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 μg ml^{-1} gentamycin and 100 μg ml^{-1} kanamycin, pH titrated to 7.6. These cells were then plated on poly-L-lysine (5 μg ml^{-1})-coated glass cover slips and maintained in an incubator (19°C, 0.5% CO_2) overnight. For the determination of $[Ca^{2+}]_i$ or pHi and before loading the cells with the specific dye, cultures were washed several times with fresh standard saline in order to remove non-adherent cells and debris.

Experimental media

The standard saline used for measuring $[Ca^{2+}]_i$ and pHi consisted of 10 mmol l^{-1} Hepes, 136.9 mmol l^{-1} NaCl, 5.4 mmol l^{-1} KCl, 1 mmol l^{-1} $MgSO_4$, 0.33 mmol l^{-1} NaH_2PO_4, 0.44 mmol l^{-1} KH_2PO_4, 5 mmol l^{-1} $NaHCO_3$, 1.5 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} glucose, pH 7.6 at 19°C, and had an osmolarity of 284 mosmol l^{-1}. 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). NaCl was replaced with equimolar amounts of tetramethylammonium (TMA) in order to prepare Na^+-free isosmotic or Na^+-free hypertonic medium.

The standard isosmotic medium (low buffer capacity medium) used for measuring the H^+ release with the cytosensor microphysiometer (Molecular Devices, Munich, Germany) consisted of 138 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 0.81 mmol l^{-1} K_2HPO_4, 0.5 mmol l^{-1} $MgCl_2$, 0.11 mmol l^{-1} KH_2PO_4, 1.3 mmol l^{-1} $CaCl_2$, 5 mmol l^{-1} glucose) titrated to pH 7.6. For hyperosmotic conditions, 100 mmol l^{-1} NaCl was added to the same medium. Ca^{2+}-free medium for measurement of $[Ca^{2+}]_i$, pHi or proton secretion was prepared by replacing $CaCl_2$ with 0.5 mmol l^{-1} EGTA.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured in individual attached cells using the membrane-permeable Ca^{2+}-sensitive fluorescence dye Fura 2-AM. Cells, cultured as described above, were loaded with the dye for 1 h followed by two careful washes with standard saline, then the cover slips 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 340 nm and 380 nm, and emission was detected above 510 nm. The images were stored on a computer using the tillVISion software package (T.I.L.L. Photonics, Munich, Germany). Basal levels of $[Ca^{2+}]_i$ 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 of the saline covering the cells was exchanged for the same volume of Ca^{2+}-free or Na^+-free saline. 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 μmol l^{-1} of the calcium ionophore ionomycin. Applying these values and a dissociation constant (K_D) of 680 nmol l^{-1} (previously determined for our experimental set-up using a commercial calibration kit; Molecular Probes), absolute levels of $[Ca^{2+}]_i$ could be calculated using the formula given by (Grynkiewicz et al., 1985).

To investigate the effect of pH_e on $[Ca^{2+}]_i$, Fura 2-loaded cells were exposed, after establishing a baseline using standard medium of pH 7.6, to media of pH values 6.5, 6.8 and 8.2 for 30 min, followed by normal calibration.

To assess the effect of hyperosmotic challenge on $[Ca^{2+}]_i$ in the absence of extracellular Na^+, cells loaded with Fura 2-AM in standard saline were exposed for 5 min to Na^+-free saline followed by exposure to Na^+-free hyperosmotic medium. In order to obtain Ca^{2+}-depleted cells, hepatocytes were incubated in standard saline containing 25 μmol l^{-1} BAPTA-AM during Fura 2-loading, and these cells were then exposed to Ca^{2+}-free saline during measurements.

Intracellular pH measurement

pHi of individual hepatocytes was measured 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 μmol l^{-1}) and valinomycin (5 μmol l^{-1}), with a pH adjusted to 6.80, 7.20 or 7.60 (Pocock and Richards, 1992; Seo et al., 1994). When experimental media of pH values higher or lower than 7.6 were used, calibration media were adjusted to cover the range of pHi values determined.

Measurement of proton secretion

Proton secretion of hepatocytes was estimated from the rate of acidification of the external medium measured with a cytosensor microphysiometer as previously described (Pelster, 1995; Krumschnabel et al., 2001a). Hepatocytes (0.45×10^6 cells) were embedded in low-melting-point agarose gel (1.5%) on polycarbonate capsules, loaded into the cytosensor chamber, and superfused with low-buffer-capacity medium (given above). By the use of an electromagnetic valve, perfusion conditions could be rapidly switched from a control to a test solution. The perfusion cycle was set to 3 min, with 130 s of constant perfusion followed by a 40 s flow-off period. During the latter period, protons released by the hepatocytes acidify the measuring chamber and this signal is recorded *via* a light-addressable potentiometric sensor. From the slope of a line fitted to the sensor data, the rate of acidification was calculated. The following experimental protocol was used with all measurements. First, the cells were allowed to recover from embedding for at least 1 h, then a baseline of acid secretion was determined in freshly titrated saline. This was followed by switching to identical saline for the control cells and to the different test salines for treated cells.

Since both the geometry of the cytosensor chamber and the embedding procedure of the cells make it very difficult to determine the number of cells actually releasing acid equivalents into the measuring chamber, acidification rates were not given as H^+ s^{-1}, but the signal (μV s^{-1}) was converted to % of the basal rate of proton secretion measured under control conditions prior to the treatment.

Test compounds were made up in concentrated stock solutions dissolved in distilled water or dimethyl sulphoxide (DMSO) and were applied at the following final concentrations: ionomycin 0.5 μmol l^{-1} (1.5 mmol l^{-1} stock in DMSO), thapsigargin 0.1 μmol l^{-1} (1 mmol l^{-1} stock in DMSO), NH_4Cl 20 mmol l^{-1} (5 mol l^{-1} stock in H_2O), Na-propionate 30 mmol l^{-1} (3 mol l^{-1} stock in H_2O) and cariporide mesilate 10 μmol l^{-1} (2 mmol l^{-1} stock in H_2O). The final concentration of DMSO was always kept below 0.1%, a concentration that did not interfere with the measurements.

Statistics

Data are presented as means ± s.e.m. of N independent preparations. In experiments on cell cultures, data are shown as means ± 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

the appropriate *post-hoc* test, with a P value of <0.05 being considered as significant.

Results

Effect of Ca^{2+}-mobilizing agents on cell pH

Source of the increase in $[Ca^{2+}]_i$

The Ca^{2+} ionophore ionomycin and the endoplasmic ATPase inhibitor thapsigargin were used to induce an increase in $[Ca^{2+}]_i$ while studying the concurrent effect on pHi. As a first step, the origin of the $[Ca^{2+}]_i$ increase induced by 0.5 μmol l^{-1} ionomycin and 0.1 μmol l^{-1} thapsigargin was determined. As shown in Fig. 1A, incubation of cells with 0.5 μmol l^{-1} ionomycin elicited a rapid and significant increase in $[Ca^{2+}]_i$ from a basal value of 70.3±5.2 nmol l^{-1} to a peak of 1016±68 nmol l^{-1} within 4 min, after which $[Ca^{2+}]_i$ started to decline, reaching a value of 510.6±84.1 nmol l^{-1} by the end of the experiment. In the absence of Ca^{2+}_e, hepatocytes showed a basal $[Ca^{2+}]_i$ of 65.3±3.8 nmol l^{-1} that increased upon addition of 0.5 μmol l^{-1} ionomycin to a maximum of 354.8±36 nmol l^{-1} within 7 min. Thereafter, $[Ca^{2+}]_i$ declined and reached baseline values after 12 min, supposedly due to an enhanced Ca^{2+} extrusion across the plasma membrane and/or an enhanced Ca^{2+} reuptake by intracellular stores. Similarly, exposure of cells to 0.1 μmol l^{-1} thapsigargin induced a significant increase in $[Ca^{2+}]_i$ from a basal value of 100±10.1 nmol l^{-1} to a maximum of 639.8±45.2 nmol l^{-1} within 11 min, followed by a slow decrease, reaching a value of 366.8±49.1 nmol l^{-1} by the end of the experiment (Fig. 1B). In Ca^{2+}-free medium, 0.1 μmol l^{-1} thapsigargin induced a slow increase in $[Ca^{2+}]_i$ from a basal value of 68.3±6.1 nmol l^{-1} to a maximum of 258.5±21 within 11 min, then declined to a value of 84.4±14 nmol l^{-1} by the end of the experiment.

Effect of mobilizing Ca^{2+} on pHi

Recognizing that Ca^{2+} influx as well as Ca^{2+} release from intracellular stores contributed to ionomycin- and thapsigargin-induced $[Ca^{2+}]_i$ increase, the effect of both Ca^{2+} mobilizing agents on pHi was investigated. Fig. 2 demonstrates that exposure of cells to 0.5 μmol l^{-1} ionomycin or 0.1 μmol l^{-1} thapsigargin induced a brief acidification followed by a sustained alkalinization in the absence as well as in the presence of Ca^{2+}_e. In Ca^{2+}-containing medium, pHi increased upon addition of 0.5 μmol l^{-1} ionomycin and 0.1 μmol l^{-1} thapsigargin, from basal values of 7.16±0.03 and 7.02±0.03 to values of 7.56±0.03 and 7.55±0.04 within 20 min, respectively. Thereafter, pHi remained elevated at that level until the end of the experiment. In the absence of Ca^{2+}_e, basal pHi values of 7.00±0.06 and 7.00±0.02 were measured, which increased upon addition of 0.5 μmol l^{-1} ionomycin or 0.1 μmol l^{-1} thapsigargin to values of 7.31±0.05 and 7.32±0.04 within 15 min and 10 min, respectively. After reaching the peak of the alkalinization, the

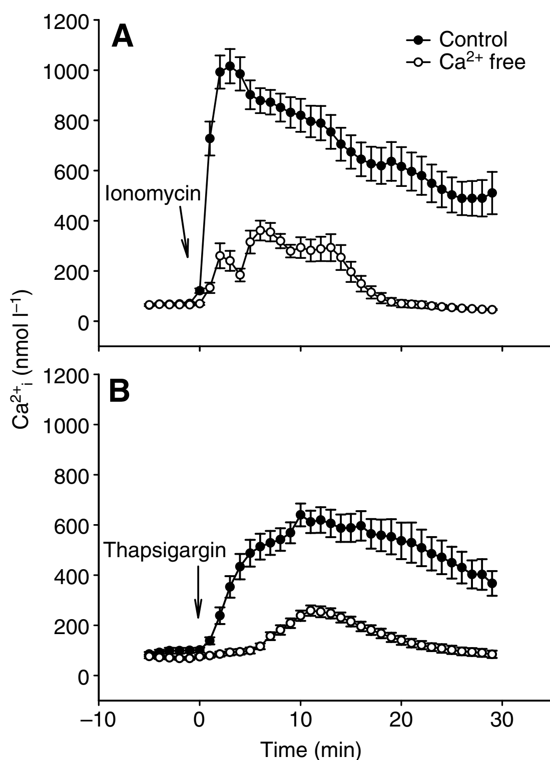

Fig. 1. Changes in $[Ca^{2+}]_i$ of trout hepatocytes following addition of 0.5 μmol l^{-1} ionomycin (A) or 0.1 μmol l^{-1} thapsigargin (B) (at the time indicated by the arrows) in the absence and presence of Ca^{2+}_e. Data are means ± s.e.m. of 37–44 cells from four independent preparations in A and of 20–32 cells from three independent preparations in B.

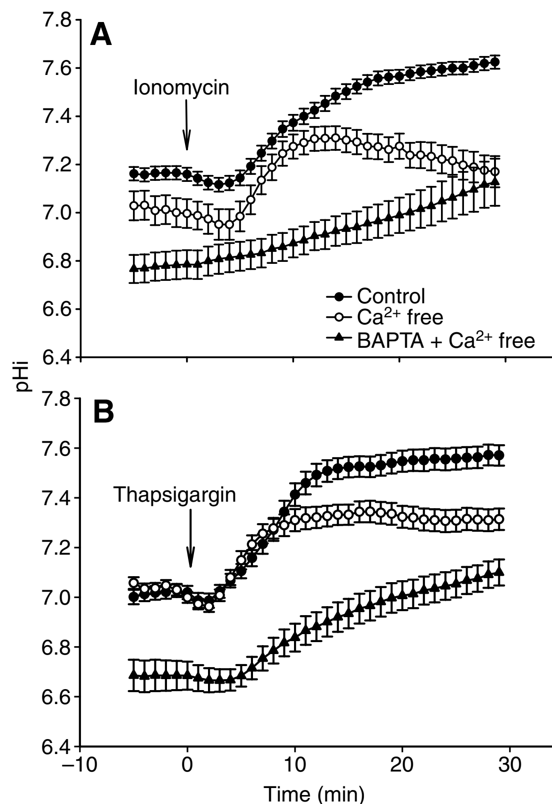

Fig. 2. Changes in pHi of trout hepatocytes following addition of 0.5 μmol l^{-1} ionomycin (A) or 0.1 μmol l^{-1} thapsigargin (B) (at the time indicated by the arrows) using Ca^{2+}-containing medium, Ca^{2+}-free medium and Ca^{2+}-free medium following incubation of cells with the intracellular Ca^{2+} chelating agent BAPTA-AM (25 μmol l^{-1}). Data are means ± s.e.m. of 19–128 cells from 3–16 independent preparations in A and of 28–39 cells from 3–4 independent preparations in B.

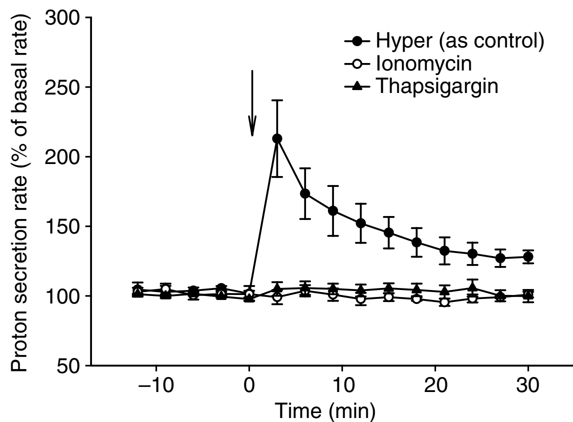

Fig. 3. Changes in proton secretion rate of trout hepatocytes following addition (at the time indicated by the arrow) of 0.5 μmol l^{-1} ionomycin, 0.1 μmol l^{-1} thapsigargin or hypertonic medium (1.6× isosmolarity). Data are means ± s.e.m. from three independent preparations.

pHi stabilized at that level in the presence of thapsigargin, while a slow pHi decrease was observed in the presence of ionomycin, reaching a value of 7.17±0.06 by the end of the experiment.

Hepatocytes incubated in Ca^{2+}-containing solution with the Ca^{2+}_i chelating agent BAPTA-AM (25 μmol l^{-1}) showed considerably lower basal pHi values (6.79±0.06 and 6.78±0.08) before addition of 0.5 μmol l^{-1} ionoymcin or 0.1 μmol l^{-1} thapsigargin, respectively. While a slow and constant pHi increase was measured in response to ionomycin exposure, reaching a value of 7.13±0.1, upon treatment of cells with 0.1 μmol l^{-1} thapsigargin a more rapid alkalinization was observed, especially during the first 10 min of the incubation.

Effect of mobilizing Ca^{2+} on proton secretion

In order to test the hypothesis that the increase in pHi in response to mobilizing Ca^{2+} by ionomycin or thapsigargin was due to or accompanied by proton movements across the cell plasma membrane, proton secretion rate was measured after exposure of cells to the Ca^{2+} mobilizing agents. As shown in Fig. 3, neither ionomycin (0.5 μmol l^{-1}) nor thapsigargin (0.1 μmol l^{-1}) had a noticeable effect on the rate of proton secretion, implying that none of the hydrogen-secreting mechanisms was involved in the ionomycin- or thapsigargin-induced pHi increase. As a control, hepatocytes exposed to hypertonic medium (1.6× isosmolarity) showed a significant increase in proton secretion from a basal rate of 102±5.4% to a peak of 213±27%, followed by slow recovery, reaching a rate of 128±4.6% of the basal rate by the end of the experiment.

Effect of $[Ca^{2+}]_i$ decrease on cell pH

Effect of exposure to Ca^{2+}-free medium and BAPTA-AM on $[Ca^{2+}]_i$

As shown in Fig. 4A, removal of Ca^{2+}_e by incubation with 0.5 mmol l^{-1} EGTA instantly increased $[Ca^{2+}]_i$ from 72±3.64 nmol l^{-1} to 131.8±10.5 nmol l^{-1}, and $[Ca^{2+}]_i$ then returned to the basal level (73±3.72 nmol l^{-1}) within 3 min. This may reflect a mechanical stimulus due to exchanging the whole medium covering the cells with Ca^{2+}-free saline. A control experiment in which the whole standard saline was exchanged for the same volume of standard saline indeed showed that a sham change of the solution caused a similar change in intracellular Ca^{2+} concentration. $[Ca^{2+}]_i$ stabilized during the next 7 min before the $[Ca^{2+}]_i$ increased again, reaching a maximum value of 102±8.94 nmol l^{-1}, and remained increased around that level. Addition of 25 μmol l^{-1} BAPTA-AM significantly diminished $[Ca^{2+}]_i$ from a value of 82±4.94 nmol l^{-1} to a value of 27±3.4 nmol l^{-1} within 15 min. On the other hand, exposure of cells to 25 μmol l^{-1} of BAPTA-AM in the presence of Ca^{2+}_e significantly decreased the $[Ca^{2+}]_i$ from 73.6±4.96 nmol l^{-1} to 41.9±5.83 nmol l^{-1} within 15 min.

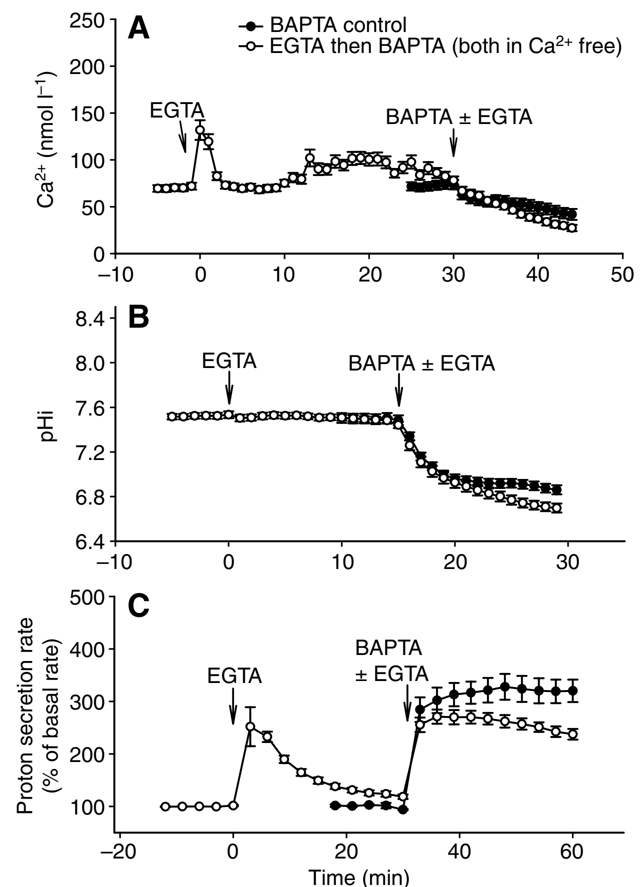

Fig. 4. Changes in $[Ca^{2+}]_i$ (A), pHi (B) and proton secretion rate (C) of trout hepatocytes upon exposure of cells to Ca^{2+}-free medium containing 0.5 mmol l^{-1} EGTA followed by addition of 25 μmol l^{-1} of the intracellular Ca^{2+} chelating agent BAPTA-AM. The effect of exposure to 25 μmol l^{-1} BAPTA-AM in the presence of Ca^{2+}_e is depicted as BAPTA control in the three experiments. Data are means ± s.e.m. of 27–38 cells from 3–4 independent preparations in A and B and from three independent preparations in C.

Effect of exposure to Ca^{2+}-free medium and BAPTA-AM on pHi

Exposure of cells to Ca^{2+}-free medium showed no obvious effect on hepatocyte pHi for a period of 15 min (Fig. 4B). Upon addition of 25 μmol l^{-1} BAPTA-AM in the presence of EGTA, pHi significantly decreased from a value of 7.48±0.03 to a value of 6.7±0.04 within 15 min. In the presence of Ca^{2+}_e, BAPTA-AM significantly decreased pHi from a value of 7.5±0.04 to a

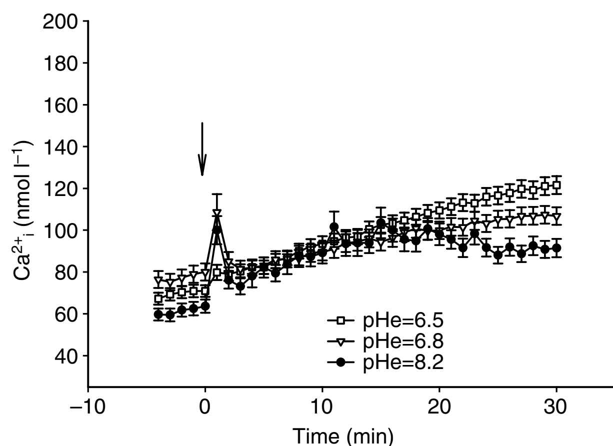

Fig. 5. Effect of exposure of trout hepatocytes to different pH_e (at the time indicated by the arrow) on $[Ca^{2+}]_i$. Data are means ± s.e.m. of 43–60 cells from 6–7 independent preparations.

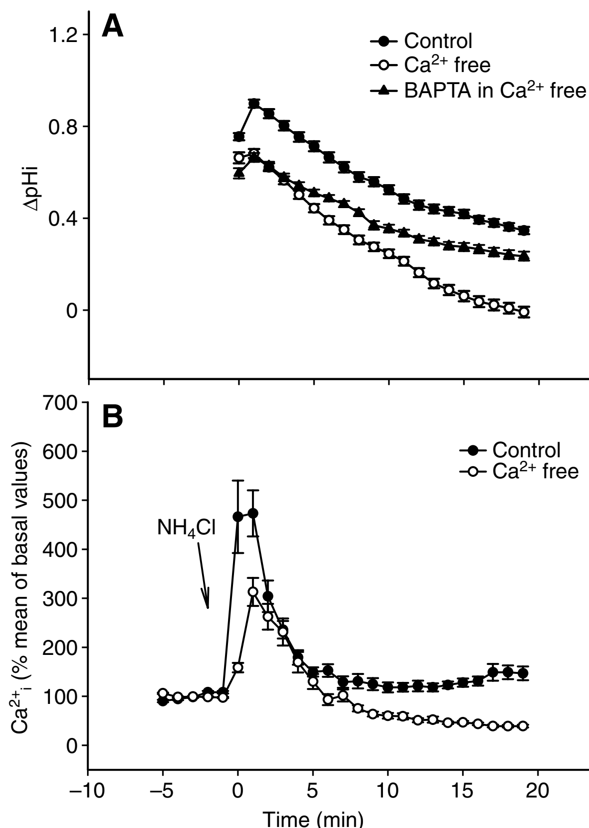

Fig. 6. Changes in pHi (expressed as ΔpHi) (A) and in $[Ca^{2+}]_i$ (expressed as % mean of basal values) (B) of trout hepatocytes following addition of 20 mmol l^{-1} NH_4Cl. pHi measurements were performed using Ca^{2+}-containing medium, Ca^{2+}-free medium and Ca^{2+}-free medium after incubation of cells with BAPTA-AM, while $[Ca^{2+}]_i$ measurements were performed using Ca^{2+}-containing and Ca^{2+}-free media. Data are means ± s.e.m. of 45–58 cells in A and 24–38 cells in B from three independent preparations.

value of 6.86±0.04 by the end of the experimental time. The time-dependent pHi decrease in response to EGTA + BAPTA exposure was statistically different from that with BAPTA only (one-phase exponential decay curve fitting; data not shown).

Effect of exposure to Ca^{2+}-free medium and BAPTA-AM on proton secretion rate

Measurement of proton secretion using the cytosensor microphysiometer (Fig. 4C) showed that exposure of cells to Ca^{2+}-free medium significantly elevated proton secretion from a basal rate of 101±1.5% to a rate of 252±37% within 3 min, followed by a slow decrease, reaching a rate of 118±4.3% after 30 min. Thereafter, and upon addition of 25 μmol l^{-1} BAPTA-AM, proton secretion rate significantly increased to 255±14% within 3 min, followed by a further increase, reaching a value of 270.9±13.4%. After a transient stabilization at this level, a slow decrease in the proton secretion rate was observed, reaching a value of 237.4±10.2% by the end of the experimental time. Exposure of cells to BAPTA-AM in the presence of Ca^{2+}_e significantly elevated the proton secretion rate from a basal value of 93.7±1.2% to 284.3±23.5% in 3 min, followed by a further, albeit slow, increase, reaching a peak of 327.7±24.5% within the next 15 min and stabilizing at this level until the end of the experiment.

Effect of manipulating pH_e on $[Ca^{2+}]_i$

To further investigate the interaction between pHi and $[Ca^{2+}]_i$, we attempted to indirectly induce changes in pHi by exposing hepatocytes to standard media with pH values above (8.2) or below (6.8 and 6.5) the normal pH value of 7.6. As shown in Fig. 5, measured basal $[Ca^{2+}]_i$ values were 70.9±2.8 nmol l^{-1} and 80±4.1 nmol l^{-1}. Upon exposure of cells to media of pH values 6.5 and 6.8, $[Ca^{2+}]_i$ increased slowly to reach a value of 121.6±4.3 nmol l^{-1} and 106.8±4.2 nmol l^{-1}, respectively, by the end of the experiment. Exposure of cells to a medium of pH 8.2 also induced a slow increase in $[Ca^{2+}]_i$ from the basal value of 63.7±3.2 nmol l^{-1} to a maximum value of 103.5±7.3 nmol l^{-1} within 15 min. This was followed by a slow decrease, establishing a new basal $[Ca^{2+}]_i$ level around 91.4±4.4 nmol l^{-1} by the end of the experiment. In the three treatments, and due to mechanical disturbance resulting from medium change (see above), a transient increase in $[Ca^{2+}]_i$ was measured directly following exchange of the whole medium around the cells for standard medium with the desired pH value.

Effect of manipulating pHi on $[Ca^{2+}]_i$

Effect of NH_4Cl-induced pHi increase on $[Ca^{2+}]_i$

In the following set of experiments we attempted to investigate the effect of an imposed pHi increase on $[Ca^{2+}]_i$. Exposure of cells in Ca^{2+}-containing medium, in Ca^{2+}-free medium and in Ca^{2+}-free medium following incubation of cells with 25 μmol l^{-1} BAPTA to 20 mmol l^{-1} NH_4Cl induced an immediate increase in pHi from basal values of 7.22±0.01, 7.45±0.03 and 7.13±0.03, respectively, to peak values of 8.13±0.02, 8.13±0.01 and 7.80±0.03, respectively, within 2 min, followed by a slow pHi recovery reaching 7.57±0.01, 7.44±0.02 and 7.37±0.02 by the end of the experimental time. Presented as ΔpH (for better comparison), Fig. 6A shows that the presence of Ca^{2+}_e enhanced the NH_4Cl-induced increase in pH_i, and incubation of cells in the absence of Ca^{2+}_e resulted in

a significantly faster pHi recovery, which was prevented in cells in which $[Ca^{2+}]_i$ was also removed.

As a next step, the effect of 20 mmol l^{-1} NH_4Cl-induced alkalinization on $[Ca^{2+}]_i$ was investigated. An instantaneous increase was measured in $[Ca^{2+}]_i$ from basal values of 43.1±3.5 and 68.2±5 nmol l^{-1} to peak values of 210.3±22.4 and 199.7±15.2 nmol l^{-1} in the presence and absence of $Ca^{2+}{}_e$, respectively. In both treatments, the increase in $[Ca^{2+}]_i$ was followed by a rapid decrease, establishing a new steady state at a slightly higher level (52.6±4.9 nmol l^{-1}) in the presence of $Ca^{2+}{}_e$ and at a considerably lower level (28±2.8 nmol l^{-1}) in Ca^{2+}-free medium. For better comparison, data are presented as percentage mean of basal values (Fig. 6B).

Effect of Na-propionate-induced pHi decrease on $[Ca^{2+}]_i$

Next, changes in $[Ca^{2+}]_i$ following addition of the weak acid Na-propionate (to induce a drop in pHi) were examined. As depicted in Fig. 7A, addition of 30 mmol l^{-1} Na-propionate to cells in Ca^{2+}-containing medium, in Ca^{2+}-free medium and in Ca^{2+}-free medium following incubation of cells with 25 μmol l^{-1} BAPTA induced an instant decrease in pHi from basal values of 7.05±0.01, 6.98±0.03 and 6.57±0.03, respectively, to values of 6.63±0.02, 6.57±0.03 and 6.3±0.04, respectively, which was followed by a pHi recovery towards baseline. Data presented as ΔpH (Fig. 7A) (for better comparison) showed no significant difference in the rate of recovery between the three treatments.

The effect of Na-propionate on $[Ca^{2+}]_i$ was then elucidated (presented as percentage of basal values) (Fig. 7B). In the presence of $Ca^{2+}{}_e$, an increase in $[Ca^{2+}]_i$, after a gap of 5 min, was measured upon addition of 30 mmol l^{-1} Na-propionate, starting from a basal value of 100.6±5.6 nmol l^{-1}, reaching a value of 146.7±12.8 nmol l^{-1} by the end of the experiment. On the other hand, in Ca^{2+}-free medium, $[Ca^{2+}]_i$ increased from a basal value of 101.2±6.2 nmol l^{-1} to a peak value of 134.7±10 nmol l^{-1} within 8 min, followed by a continuous decrease in $[Ca^{2+}]_i$, reaching a value of 90.9±5 nmol l^{-1} by the end of the experiment. From these experiments, it can be concluded that the increase in $[Ca^{2+}]_i$ has an extracellular, as well as an intracellular, origin. Also, this increase appears to be a result, not a cause, of the decrease in pHi induced by Na-propionate.

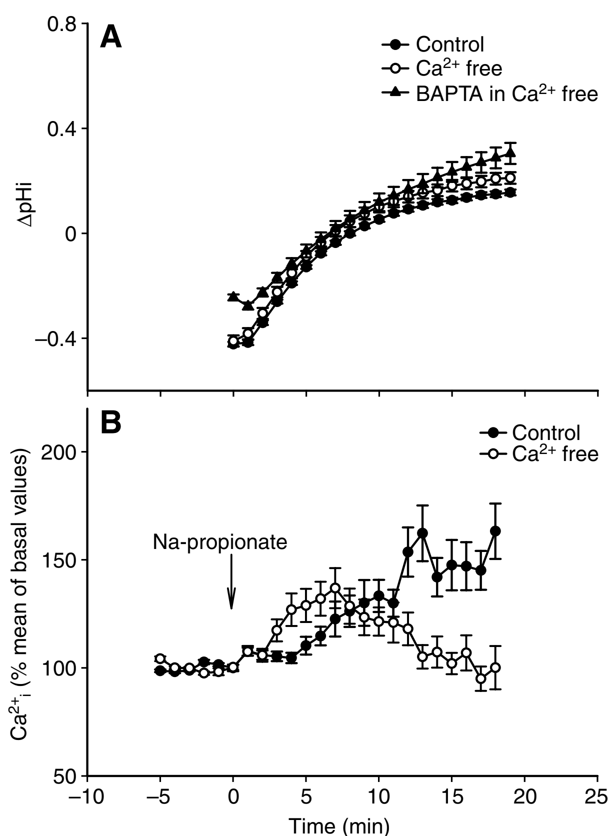

Fig. 7. Changes in pHi (expressed as ΔpHi) (A) and in $[Ca^{2+}]_i$ (expressed as % mean of basal values) (B) of trout hepatocytes following addition of 30 mmol l^{-1} Na-propionate. pHi measurements were performed using Ca^{2+}-containing medium, Ca^{2+}-free medium and Ca^{2+}-free medium after incubation of cells with BAPTA-AM, while $[Ca^{2+}]_i$ measurements were performed using Ca^{2+}-containing and Ca^{2+}-free media. Data are means ± s.e.m. of 33–51 cells from three independent preparations in A and 36–43 cells from four independent preparations in B.

pHi against $[Ca^{2+}]_i$ under hypertonicity conditions

Cell volume change, as well as other stimuli, has been reported to induce rapid changes in pHi and $[Ca^{2+}]_i$ in various cell types. Among these stimuli, cell volume has received more attention in our lab. In this section, and after investigating the effect of manipulating $[Ca^{2+}]_i$ on pHi and of manipulating pHi on $[Ca^{2+}]_i$, we attempted to investigate the link between pHi and $[Ca^{2+}]_i$ in response to hypertonicity as an independent stimulus. In many cell types, shrinkage-induced alkalinization is known to be a result of NHE activation. In our previous work (Ahmed et al., 2006), we have shown that trout hepatocytes responded to hypertonicity by an increase in $[Ca^{2+}]_i$ and a cariporide (a NHE-1 specific inhibitor)-sensitive alkalinization. In addition, a complete blockage of the hypertonicity-induced increase in $[Ca^{2+}]_i$, *via* removal of $Ca^{2+}{}_e$ along with chelation of $Ca^{2+}{}_i$, did not block, but attenuated, the hypertonicity-induced increase in pHi (expressed as hypertonicity (cell shrinkage)-induced NHE-1 activity). We then tested the possibility that the hypertonicity-induced increase in $[Ca^{2+}]_i$ might be a result of the concurrent pHi increase. As shown in Fig. 8A, exposure of cells to hypertonicity increased $[Ca^{2+}]_i$ from a basal value of 55.7±2.9 nmol l^{-1} to a peak of 118.4±5.7 nmol l^{-1} within 6 min and remained around 99.3±5.6 nm until the end of the experiment. Inhibition of NHE-1 by cariporide (5 μmol l^{-1}) completely abolishes the hypertonicity induced alkalinization, as shown by Ahmed et al. (Ahmed et al., 2006). Addition of cariporide caused a slight $[Ca^{2+}]_i$ increase from a basal value of 48.5±3.5 nmol l^{-1} to a new steady state around 58±3.7 nmol l^{-1}. Upon exposure of these cells to hypertonic medium containing cariporide, the hypertonicity-induced $[Ca^{2+}]_i$ increase was almost abolished, and $[Ca^{2+}]_i$ increased slowly, reaching a value of 90±6.2 nmol l^{-1} by the end of the experiment. In an attempt to block all Na^+/H^+ exchange completely, cells were exposed to Na^+-free saline, which elicited a rapid and transient increase in $[Ca^{2+}]_i$ from a basal value of 62±2.7 nmol l^{-1} to a value of 102.6±6.9 nmol l^{-1}, apparently due to mechanical disturbances caused by exchanging the whole medium covering the cells (see

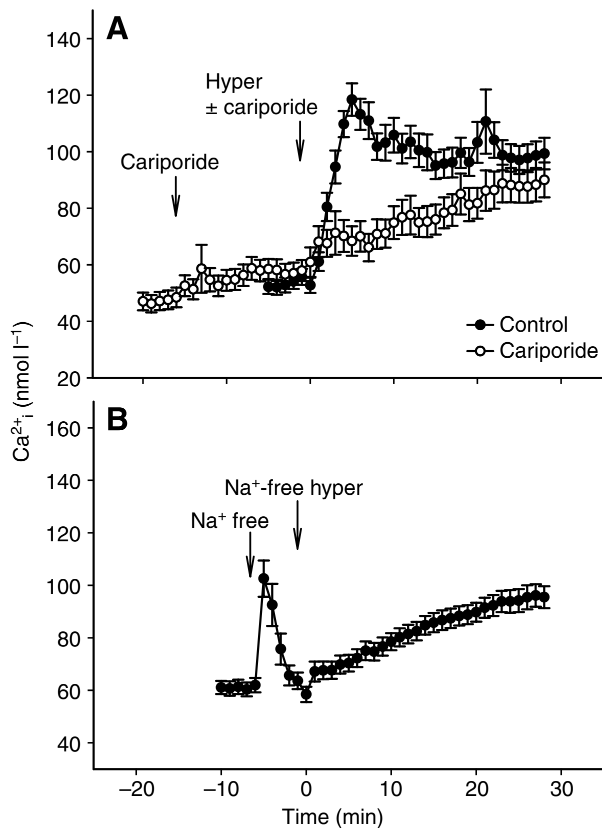

Fig. 8. Effect of 0.5 μmol l^{-1} cariporide (A) or removal of extracellular Na^+ (B) on the hypertonicity-induced $[Ca^{2+}]_i$ increase. Data are means ± s.e.m. of 35–76 cells from 3–7 independent preparations.

above). Subsequent exposure of these cells to Na^+-free hypertonic medium similarly resulted in a significantly reduced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ gradually increased, reaching a value of 95.5±4.2 nmol l^{-1} by the end of the experiment (Fig. 8B).

Discussion

pHi changes in response to $[Ca^{2+}]_i$ changes

We have shown in the present study that exposure of trout hepatocytes to either ionomycin or thapsigargin lead to an increase in $[Ca^{2+}]_i$ *via* influx across the plasma membrane and mobilization from intracellular stores. The ionomycin-induced increase in $[Ca^{2+}]_i$ was considerably faster and larger than that induced by thapsigargin, apparently due to the different mechanisms underlying the $[Ca^{2+}]_i$ increase; the ionophore ionomycin induces a rapid Ca^{2+} flux through the membranes along a concentration gradient, while the inhibitor of SERCA (sarco/endoplasmic reticulum calcium ATPase), thapsigargin, is preventing Ca^{2+} re-uptake after passive leakage and therefore results in a much slower increase in $[Ca^{2+}]_i$. In parallel to the increase in $[Ca^{2+}]_i$, ionomycin and thapsigargin induced a biphasic response in pHi, a brief acidification followed by a sustained alkalinization. Similar observations have been reported for other cell lines in response to agents known to raise $[Ca^{2+}]_i$, such as ionomycin (Grinstein and Cohen, 1987; Slotki et al., 1989; Slotki et al., 1993) or the hormones bradykinin (Slotki et al., 1993; Pedersen et al., 1998), thrombin (Zavoico et al., 1986; Pedersen et al., 1998), angiotensin II and arginine vasopressin (Ganz et al., 1988).

The ionomycin-induced increase in $[Ca^{2+}]_i$ in the presence, as well as the absence, of Ca^{2+}_e caused a significant alkalinization of the cells. This suggests that the main cause of ionomycin-induced alkalinization was Ca^{2+} release from intracellular stores. The significantly lower pHi of hepatocytes in the absence of Ca^{2+}_e, however, revealed that after the release from internal stores Ca^{2+} influx was responsible for the sustained alkalinization observed between 15 and 30 min of the incubation. Nevertheless, $[Ca^{2+}]_i$ already decreased at this time, indicating that it was not the absolute level of $[Ca^{2+}]_i$ that triggered the increase in pHi. The absence of the steep slope of alkalinization in the absence of an increase in $[Ca^{2+}]_i$ due to the lack of Ca^{2+} clearly demonstrated that it was the increase in Ca^{2+}_i that triggered the alkalinization rather than an unspecific side effect of ionomycin.

To explore the underlying mechanisms for a similar alkalinization, various inhibitors of the acid-transporting mechanisms have often been used, and the amount of protons transferred across the cell membrane has been calculated from buffer capacity and the change in pHi. In our study, we directly measured the rate of proton secretion in response to Ca^{2+} mobilization using a cytosensor microphysiometer. The results revealed that the changes in pHi could not be explained by a transfer of protons through the cell membrane, as ionomycin and thapsigargin caused a significant alkalinization of the cells but proton secretion was not affected. By contrast, the hypertonicity-induced increase in pHi, which is comparable to the ionomycin-induced increase in pHi, was highly sensitive to cariporide (NHE-1 inhibitor) and amiloride (general NHE inhibitor) (Ebner et al., 2005; Ahmed et al., 2006). Accordingly, the hypertonicity-induced alkalinization was indeed brought about by the activation of proton transport proteins in the cell membrane, but the alkalinization induced by Ca^{2+} mobilization in the present study appears to be due to an intracellular sequestration of protons or to a significant reduction in the rate of cellular proton production. With respect to proton buffering, the increase in $[Ca^{2+}]_i$ would cause competition with H^+ on protein buffering sites, resulting in a release, not a sequestration, of protons (Grinstein et al., 1987; Dickens et al., 1989). Also, proton sequestration would be very limited as protons cannot accumulate indefinitely. In addition, although a presence of Ca^{2+}/H^+ exchange in the intracellular Ca^{2+} pool has been reported (Schulz et al., 1989), this Ca^{2+}/H^+ exchange typically operates as a reuptake of Ca^{2+} from the cytosol into Ca^{2+} stores in exchange for H^+, and only a reversal of this exchanger can increase both pHi and $[Ca^{2+}]_i$. Finally, a decrease in metabolic acid production can be the reason for the alkalinization. The absence of stimulation of proton secretion across the plasma membrane indicates that acid production can be measured (unmasked) only by inhibiting the intracellular H^+-removing mechanisms, which is not possible without affecting membrane transports across the plasma membrane. Importantly, and regardless of the intracellular mechanism(s) by which Ca^{2+} mobilizing agents induced the observed increase in pHi, the new steady-state pHi had no effect on the proton secretion rate, as shown in Fig. 3. Accordingly, the mobilization of Ca^{2+} modified the proton distribution across the cell membrane. This

observation is not in line with previous studies on other cells in which an alkalinization induced by Ca^{2+} mobilizing was reported to be due to the activation of NHE (Poch et al., 1993; Martin-Requero et al., 1997) or Ca^{2+}/H^{+} exchange (Schulz et al., 1989; Anwer, 1993; Daugirdas et al., 1995; Ouyang et al., 1995; Yamada et al., 1996; Alfonso et al., 2005) across the cell plasma membrane.

Similarly, inducing an increase in $[Ca^{2+}]_i$ by inhibition of SERCA Ca^{2+}-ATPase confirmed the intracellular link between $[Ca^{2+}]_i$ and pHi, although the effect of thapsigargin on pHi did not completely mimic the effect of ionomycin. Removal of Ca^{2+}_e together with chelation of $[Ca^{2+}]_i$ did not prevent, but attenuated, the thapsigargin-induced alkalinization, while in the presence of ionomycin the alkalinization was almost abolished. This indicates that the response to thapsigargin is not completely due to Ca^{2+} changes, and contributions from other mechanisms cannot be excluded. Finally, our preliminary work using Acridine Orange could not support a role for the V-ATPase in the ionomycin/thapsigargin-induced pH changes; however, experiments are being undertaken to fully exclude or confirm any possible involvement of V-ATPase.

In several cell lines, resting $[Ca^{2+}]_i$ has been reported to be maintained by Ca^{2+} influx based on the observation that removal of Ca^{2+}_e induces a fall in $[Ca^{2+}]_i$. While this $[Ca^{2+}]_i$ decrease was associated with a fall in pHi in avian heart fibroblast cells (Dickens et al., 1990), no effect on pHi was recorded in IMCD cells (Slotki et al., 1989), human epidermoid A-431 cells (Kiang, 1991) or cortical neurons (Ouyang et al., 1995). In the present study, removal of Ca^{2+}_e showed no apparent effect on pHi while the concurrent effect on $[Ca^{2+}]_i$ was surprising: although basal $[Ca^{2+}]_i$ was unchanged for a period of 10 min, the continued absence of Ca^{2+}_e resulted in a sustained, albeit small, increase in $[Ca^{2+}]_i$, which can only be explained by a release from intracellular Ca^{2+} stores. This $[Ca^{2+}]_i$ increase in the presence of a concentration gradient that favours Ca^{2+} efflux indicates an inhibitory effect on Ca^{2+} efflux pathways. On the other hand, while the EGTA-induced increase in proton secretion rate indicated a removal of H^{+} from the intracellular fluid, this was not accompanied by a change in pHi. A compensatory intracellular production of H^{+}, independent of changes in $[Ca^{2+}]_i$, was the most likely explanation for this observation.

By contrast, chelation of $[Ca^{2+}]_i$ using BAPTA resulted in a fall in $[Ca^{2+}]_i$ and pHi (Dickens et al., 1990; Kiang, 1991). Attempting to explain the link between such an increase in $[H^{+}]$ in response to a decrease in $[Ca^{2+}]_i$ induced by BAPTA, Dickens et al. proposed the existence of a Ca^{2+} channel and a H^{+} channel (Dickens et al., 1990). According to this hypothesis, a fall in $[Ca^{2+}]_i$ would increase the conductance of the H^{+} channel, allowing an influx of H^{+} down its electrochemical gradient, leading to an acidification; a fall in $[H^{+}]$, in turn, is supposed to enhance Ca^{2+} influx, leading to $[Ca^{2+}]_i$ increase. Conversely, the Ca^{2+} channel would be blocked by an increase in $[H^{+}]$, and the H^{+} channel would be blocked by an increase in $[Ca^{2+}]_i$. The simultaneous fall in $[Ca^{2+}]_i$ and pHi observed following exposure of cells to BAPTA is in agreement with the results of Dickens et al. (Dickens et al., 1990) and Kiang (Kiang, 1991). However, the concomitant increase in proton secretion rate is not in line with the hypothesis that the decrease in $[Ca^{2+}]_i$ stimulates an influx of protons along the electrochemical gradient. It is also unlikely that pHi changes resulted from competition between Ca^{2+} and H^{+} for common buffering sites (Grinstein et al., 1987; Dickens et al., 1989), given the fact that decreasing $[Ca^{2+}]_i$ would open up more H^{+} binding sites and consequently decrease $[H^{+}]$. The concurrent decrease in $[Ca^{2+}]_i$ and pHi in response to BAPTA exposure rules out a possible activity for intracellular Ca^{2+}/H^{+} exchange, while a contribution, however small, of such a mechanism across the plasma membrane can be deduced because, in the absence of intracellular Ca^{2+} (BAPTA), proton secretion rate (Fig. 4C) was slightly lower and pHi was slightly higher (Fig. 4B) in the absence than in the presence of Ca^{2+}_e. Furthermore, although we used nominally HCO_3^--free medium in our measurements, atmospheric CO_2 is expected to create some HCO_3^- in the medium and so the possible involvement of Na^{+}/HCO_3^- exchange was tested. The presence of the Na^{+}/HCO_3^- exchanger inhibitor DIDS had no effect on the BAPTA-induced proton secretion rate (data not shown). This rules out the possibility that the extracellular acidification was due to HCO_3^- influx. Consequently, the intracellular acidification combined with an increase in proton secretion suggest that a significant stimulation of proton production occurred under these conditions. Preliminary results using Acridine Orange showed that BAPTA induces an alkalinization of intracellular acidic stores. This might explain, at least in part, the effect of BAPTA on pHi and proton secretion rate.

$[Ca^{2+}]_i$ changes in response to pH_e changes

Based on previous studies in trout hepatocytes (Walsh, 1986; Krumschnabel et al., 2001b) reporting that pHi is determined to a large extent by pH_e exhibiting a direct linear relationship over a broad range of pH values, we further investigated the possible correlation between steady-state pHi and steady-state $[Ca^{2+}]_i$ in trout hepatocytes by changing pH_e while monitoring $[Ca^{2+}]_i$. Our data imply a link between steady-state $[Ca^{2+}]_i$ and pHi in trout hepatocytes, given that a continuous increase in $[Ca^{2+}]_i$ accompanies cellular regulation mechanisms to adjust pHi in response to changes in pH_e. At alkaline pH values, intracellular $[Ca^{2+}]$ was adjusted to lower values, while acidic extracellular pH values resulted in a slight increase in $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ changes in response to pHi changes

Our data demonstrate that the rapid pHi increase upon application of the weak base NH_4Cl resulted in a transient increase in $[Ca^{2+}]_i$, both in the presence and absence of Ca^{2+}_e, which is consistent with observations in smooth muscle cells (Siskind et al., 1989), rat pheochromocytoma cells (Dickens et al., 1989), bovine lactotrophs (Zorec et al., 1993), rat lacrimal (Yodozawa et al., 1997) and pancreatic acinar cells (Speake and Elliott, 1998) but not with those in rat lymphocytes (Grinstein and Goetz, 1985), where the NH_4Cl-induced increase in $[Ca^{2+}]_i$ was absent in the absence of Ca^{2+}_e. The short delay, together with the reduction in increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium, indicated that the increase in $[Ca^{2+}]_i$ was dependent on extracellular as well as intracellular Ca^{2+}. Furthermore, entrance of Ca^{2+} from the outside may have preceded release from intracellular stores. However, this Ca^{2+} entrance was not a prerequisite for the release of Ca^{2+} from intracellular stores, as

has been reported in rat lymphocytes, where the absence of Ca^{2+}_e prevented the increase in $[Ca^{2+}]_i$ (Grinstein and Goetz, 1985). Less than 5 min after reaching maximum $[Ca^{2+}]_i$, Ca^{2+} concentrations returned to control levels, both in the presence and absence of Ca^{2+}_e.

The pHi recovery profile appeared to be more complete in the absence of Ca^{2+}_e. This suggests a link between Ca^{2+}_e and the removal of H^+. Presence of Ca^{2+} in the extracellular space slowed down proton uptake and also increased the extent of the initial alkalinization. If we assume the presence of a Ca^{2+}/H^+ exchanger in the cell membrane, this could be explained by a reversal of the Ca^{2+}/H^+ activity due to a reversal of the $[Ca^{2+}]$ gradient across the cell membrane in the absence of Ca^{2+} in the extracellular space. Supporting this assumption, chelation of Ca^{2+}_i and removal of Ca^{2+}_e inhibited this mechanism, resulting in a slower pHi recovery (Fig. 6A). Looking at the peak of pHi increase in response to NH_4Cl exposure, the difference between the control treatment, on the one hand, and the absence of Ca^{2+}_e or both Ca^{2+}_e and chelation of Ca^{2+}_i, on the other hand, indicated that the initial alkalinization appeared to be independent of both extracellular and intracellular Ca^{2+} changes.

A rapid cell acidification is usually achieved by exposure of cells to weak acids or by the ammonium pulse technique, in which cells are transiently incubated with a weak base, and removal of this base induces a rapid acidification. With respect to the effect of this acidification on $[Ca^{2+}]_i$, conflicting results have been reported. A simultaneous transient increase in $[Ca^{2+}]_i$ both in the absence and presence of Ca^{2+}_e was recorded in rat pheochromocytoma cells (Dickens et al., 1989) and vascular smooth muscle (Dickens et al., 1989; Batlle et al., 1993) while no effect on $[Ca^{2+}]_i$ has been reported in toxoplasma gondii tachyzoites (Moreno and Zhong, 1996) and rat pancreatic acinar cells (Speake and Elliott, 1998). In the present study, exposure of trout hepatocytes to the weak acid Na-propionate induced a slow increase in $[Ca^{2+}]_i$. This response was not as instantaneous as the concomitant acidification but was somewhat delayed, which indicates that the alkalinization response of the cell could be the reason for these changes. Also, a recovery from the $[Ca^{2+}]_i$ increase to basal values was only observed in the absence of Ca^{2+}_e, indicating that after an initial lag phase the acidification induced a continuous influx of Ca^{2+}, through the cell membrane, possibly due to the activities of a pHi-regulating mechanism. Looking at the range of $[Ca^{2+}]_i$ increase following NH_4Cl-induced alkalinization compared with that induced by Na-propionate acidification, it could be concluded that $[Ca^{2+}]_i$ is more sensitive to alkalinization than to acidification.

pHi and $[Ca^{2+}]_i$ under hypertonicity conditions

Our approach to investigate the link between cellular pH and Ca^{2+} in previous sections was to manipulate cellular pH while measuring $[Ca^{2+}]_i$ and *vice versa*. In this section, we chose to track pH–Ca^{2+} interaction in response to hypertonicity as an independent stimulus. In trout hepatocytes as well as in many other cell types (Cossins and Gibson, 1997), NHE is known to be responsible for pHi regulation under steady-state conditions and in response to hypertonic stress. Unselective inhibition of NHE isoforms using Na^+-free medium (Krumschnabel et al., 2003) as well as selective inhibition of NHE-1 using the specific inhibitor cariporide (Ahmed et al., 2006) caused an acidification of the cell and completely blocked the hypertonicity-induced pHi increase. In the present work, pre-treatment of cells with cariporide removed the peak of hypertonicity-induced increase in $[Ca^{2+}]_i$ (Fig. 8A). This clearly demonstrated that the hypertonicity-induced increase in $[Ca^{2+}]_i$ was the consequence of the alkalinization, brought about by activation of NHE-1. The slow steady increase in $[Ca^{2+}]_i$ observed under these conditions can hardly be due to the activity of other NHE isoforms since a similar increase in $[Ca^{2+}]_i$ was measured while using Na^+-free medium (Fig. 8B). Inhibition of NHE results in an acidification of the cell, and a low intracellular pH induced a slow increase $[Ca^{2+}]_i$ (see Fig. 5). These results indicate that the main increase in $[Ca^{2+}]_i$ during hypertonicity is due to activation of NHE, which is consistent with the results in other cell lines (Mitsuhashi and Ives, 1988; Dascalu et al., 1992; Pedersen et al., 1996). However, the observation that removal of Ca^{2+}_e, as well as removal of Ca^{2+}_e along with chelation of Ca^{2+}_i, attenuated the hypertonicity-induced increase in pHi (Ahmed et al., 2006) indicates a positive feedback between pHi increase and $[Ca^{2+}]_i$, so that an increase in pHi due to hypertonicity-induced activity of NHE causes a rise in $[Ca^{2+}]_i$, which might in turn augment the pHi increase.

In summary, we report in the present study that increasing $[Ca^{2+}]_i$ using ionomycin or thapsigargin, removal of Ca^{2+}_e with the use of EGTA or buffering of $[Ca^{2+}]_i$ using BAPTA lead to changes in pHi and proton secretion in a way that cannot be explained by assuming a transfer of protons across the cell membrane. Instead, intracellular sequestration of protons and a change in metabolic proton production are more likely to be involved, and therefore a change in $[Ca^{2+}]_i$ may modify the relation between pHi and pHe. Ca^{2+} also appeared to be involved in pHi regulation following rapid alkalinization by NH_4Cl, while an increase in $[Ca^{2+}]_i$ following hypertonic stress or rapid acidification by Na-propionate appeared to be a consequence of the activity of pHi regulation mechanisms.

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