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# Signalling pathways involved in hypertonicity- and acidification-induced activation of $Na^+/H^+$ exchange in trout hepatocytes

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## Summary

**In trout hepatocytes, hypertonicity and cytosolic acidification are known to stimulate $Na^+/H^+$ exchanger (NHE) activity, which contributes to recovery of cell volume and intracellular pH (pHi), respectively. The present study investigated the signalling mechanisms underlying NHE activation under these conditions. Exposing trout hepatocytes to cariporide, a specific inhibitor of NHE-1, decreased baseline pHi, completely blocked the hypertonicity-induced increase of pHi and reduced the hypertonicity-induced proton secretion by 80%. Changing extracellular pH (pHe) above and below normal values, and allowing cells to adjust pHi accordingly, significantly delayed alkalinization during hypertonic exposure, whereas following an acid load an enhanced pHi recovery with increasing pHe was seen. Chelating $Ca^{2+}$, and thereby preventing the hypertonicity-induced increase in intracellular $Ca^{2+}$ ($[Ca^{2+}]$i), significantly diminished hypertonic elevation of pHi, indicating that $Ca^{2+}$ signalling might be involved in NHE activation. A reduction in alkalinization and proton secretion was also observed in the presence of the protein kinase A (PKA) inhibitor H-89 or the calmodulin (CaM) inhibitor calmidazolium. A complete inhibition of hypertonic- and acidification-induced changes of pHi concurrent with an increase in hypertonically induced proton efflux was seen with the protein kinase C (PKC) inhibitor chelerythrine. Recovery of pHi following sodium propionate addition was reduced by more than 60% in the presence of cariporide, was sensitive to PKA inhibition, and tended to be reduced by CaM inhibition. In conclusion, we showed that NHE-1 is the main acid secretion mechanism during hypertonicity and recovery following acid loading. In addition, $Ca^{2+}$-, PKA- and CaM-dependent pathways are involved in NHE-1 activation for recovery of cell volume and pHi. On the other hand, PKC appeared to have an impact on NHE-independent pathways affecting intracellular acid–base homeostasis.**

Key words: trout hepatocyte, $Na^+/H^+$ exchange, intracellular pH, proton secretion, calcium, PKC, PKA, calmodulin.

## Introduction

The principal exchange proteins participating in the regulation of intracellular pH (pHi) in many cells are the $Na^+/H^+$ exchanger (NHE) that transports $H^+$ out of the cell, and the $Cl^-/HCO_3^-$ exchanger that transports basic ions. In addition to pHi regulation, NHE is involved in cell volume regulation (Wakabayashi et al., 1997b) and in many cells NHE-1, the most widely distributed, so-called house-keeping isoform, is stimulated by cell shrinkage, intracellular acidification and also by growth factors (Dunham et al., 2004). Various signalling pathways have been described that are involved in activation of this important exchange protein, including protein kinase C (PKC) (Wakabayashi et al., 1997b; Maly et al., 2002), protein kinase A (PKA) (Kandasamy et al., 1995), and $Ca^{2+}$/calmodulin ($Ca^{2+}$/CaM) (Zavoico et al., 1986; Wakabayashi et al., 1994). The long cytoplasmic C terminus of NHE has been shown to contain various subdomains that can be phosphorylated or bind to various regulatory proteins. By creating truncated mutants (Bianchini et al., 1995) these features could be confirmed at the molecular level (for reviews, see Ritter et al., 2001; Orlowski and Grinstein, 2004). In addition, one of the two proton-binding sites identified on the intracellular side of the NHE-1 protein appears not to be involved in the transport of protons, but, by allosterical modification of the protein, changes the transport properties of the exchanger (Zavoico et al., 1986; Putney et al., 2002).

The receptor-mediated pathways activating NHE have been relatively extensively studied, whereas the 'receptor-independent' activation of NHE by osmotic stress or by intracellular acidification is less well characterized (Putney et al., 2002). In neutrophils, a stimulation of NHE activity was observed in response to an isosmotic decrease in cell volume, suggesting that changes in cell size may be recorded (Krump et al., 1997). Direct phosphorylation of NHE as well as MAP

kinase signalling have also been reported to be involved in the activation of NHE (Grinstein et al., 1992; Moor and Fliegel, 1999), and contradicting results exist concerning the involvement of $Ca^{2+}$ ions (Grinstein et al., 1985b; Mitsuhashi and Ives, 1988; Murao et al., 2005). In Ehrlich Ascites tumour cells PKC appears to be involved in the hyperosmotic activation of NHE (Pedersen et al., 2002). Overall, however, intracellular signalling pathways involved in the regulatory volume increase (RVI) response following hyperosmotic exposure remain enigmatic. To make things more complicated, in many fish red cells (Brauner et al., 2002) as well as hepatocytes (Tuominen et al., 2003) activation of NHE during hyperosmotic stress (red cells) or following acidification (hepatocytes) is inhibited by oxygen, i.e. the NHE is more strongly activated under hypoxic conditions. In addition, in fish red cells a catecholamine-responsive NHE isoform has been described. This isoform, β-NHE, shows some similarity to NHE-1, but it was found to differ considerably from mammalian NHE-1 isoform in many aspects (Malapert et al., 1997; Pedersen and Cala, 2004).

Despite these apparent peculiarities of fish cell NHE, most of the previous investigations focused on red cells, and almost nothing is known regarding the regulation of NHE in other cells from fish. An interesting model system to investigate this are trout hepatocytes, since in these cells both RVI following cell shrinkage (Ebner et al., 2005) and the associated intracellular alkalinization (Fossat et al., 1997; Krumschnabel et al., 2003), as well as pHi recovery after acidification (Walsh, 1986; Tuominen et al., 2003) are to a large extent dependent on this ion exchanger, suggesting an important role for NHE in cell function. Furthermore, changes in cell volume as well as pHi may exert strong effects on hepatocyte metabolism (Walsh and Mommsen, 1992; Haussinger and Schliess, 1995) and therefore control of this important transporter is clearly crucial for these cells.

Thus, in order to shed light on the signalling pathways leading to NHE activation in trout hepatocytes, specifically focusing on NHE-1, the present study aimed to elucidate the possible role of altered extra- and intracellular pH, $Ca^{2+}$, PKC, PKA and CaM in cell signalling upon induction of cell shrinkage with hypertonic medium or intracellular acidification by the weak acid, sodium propionate. Our data showed that NHE-1 is the main exchanger responsible for pHi changes during hypertonic challenge and, together with SITS-sensitive sodium-dependent $Cl^{-}/HCO_3^{-}$ exchange it is responsible for pHi recovery after acidification of the cells. Absence of $Ca^{2+}$ and changing the extracellular pH to levels above or below the normal values significantly reduced the hypertonicity-induced increase of pHi. By contrast, pHi recovery from sodium propionate loading increased with increasing medium pH. Both hypertonicity- and acidification-induced NHE-1 activity were reduced in the presence of inhibitors of PKA and CaM. Chelerythrine, a PKC inhibitor, also inhibited the acidification-, as well as the hypertonicity-induced NHE activity.

## Material and methods

### Chemicals

Collagenase (type VIII), bovine serum albumin (BSA), foetal calf serum (FCS), nigericin, valinomycin, ionomycin, propionic acid (sodium salt), low-melting-point agarose gel, 4-acetamido-4′-isothio-cyanatostilbene-2,2′-disulfonic acid (SITS), chelerythrine chloride, *N*-2-P-bromocinnamylaminoethyl-5-isoquinolinesulfoniamide (H-89) and calmidazolium chloride were purchased from Sigma (Deisenhofen, Germany); the NHE-1 inhibitor cariporide mesilate was kindly provided by Sanofi-Aventis Pharma (Vienna, Austria). Fura 2, BAPTA 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.

### Experimental animal and hepatocytes isolation

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local hatchery and were acclimated in 200 l aquaria with running water at 15°C. Fish were fed daily with trout pellets (EWOS Aquaculture International, Austria) *ad libitum*. Hepatocytes were isolated using the collagenase digestion procedure described previously (Krumschnabel et al., 1996). Briefly, 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 a Hepes-buffered saline to remove the blood, followed by perfusion with a collagenase-containing saline (0.05% collagenase) until the tissue appeared soft and swollen. Subsequently, the liver was excised, cut into fine fragments with 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 thermostated to 19°C, which was also the temperature used during the experiments. Cells viability, as determined from Trypan Blue exclusion, was always >85%.

### Hepatocyte preparation

For the determination of intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]i$) and intracellular pH (pHi), hepatocytes ($1.5\times10^{6}$ to $2\times10^{6}$ cells $ml^{-1}$) were 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, and 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 coverslips and maintained in an incubator (19°C, 0.5% $CO_2$) overnight. Before loading the cells with the specific dye for the determination of $[Ca^{2+}]i$ or pHi,

the cultures were washed several times with fresh standard saline in order to remove non-adherent cells and debris.

### *Experimental media*

The standard isosmotic saline used for measuring $[Ca^{2+}]$i and pHi 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 $CaCl_2$, 5 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).

The standard isosmotic medium (low buffer capacity medium) used for measuring the $H^+$ release with the Cytosensor microphysiometer consisted of (in mmol $l^{-1}$) 138 NaCl, 5 KCl, 0.81 $K_2HPO_4$, 0.5 $MgCl_2$, 0.11 $KH_2PO_4$, 1.3 $CaCl_2$, 5 glucose, titrated to pH 7.6. For hyperosmotic conditions, an additional 100 mmol $l^{-1}$ NaCl was added to the same medium.

### *Intracellular free $Ca^{2+}$ measurement*

Intracellular free $Ca^{2+}$ ($[Ca^{2+}]$i) of hepatocytes 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 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 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). Basal levels of $[Ca^{2+}]$i in standard saline were measured for at least 5 min before half of the saline covering the cells was carefully exchanged for an equal volume of hyperosmotic stock and measurements were continued for at least another 30 min. In control experiments, in which half of the bathing medium was exchanged for the same volume of isotonic standard medium, no effect on $[Ca^{2+}]$i (or pHi, see below) was noted, ruling out the possibility that detected changes were due to mechanical stress created by fluid exchange. 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 setup by the use of a commercial calibration kit (Molecular Probes, Leiden, The Netherlands), absolute levels of $[Ca^{2+}]$i could be calculated using the formula given by (Grynkiewicz et al., 1985).

To assess the effect of hyperosmotic challenge on $[Ca^{2+}]$i in the absence of extracellular $Ca^{2+}$ ($Ca^{2+}$e), cells, loaded with Fura 2-AM in standard saline, were incubated with $Ca^{2+}$-free saline and, after determination of a baseline during the initial 5 min the cells were exposed to $Ca^{2+}$-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*

Intracellular pH of individual hepatocytes was measured in cells loaded with the pH-sensitive fluorescent dye BCPCF-AM, applying the same microscope setup 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 than 7.6 were used, calibration media were adjusted to cover the range of pHi values determined.

To assess the effect the extracellular pH (pHe) on pHi during hyperosmotic challenge, cultured cells were incubated with BCPCF-AM for 30 min using standard saline titrated to the desired pH. All other media used during the course of the experiment were also titrated to the same pH.

Recovery of pHi from an acid load was assessed by adding 30 mmol $l^{-1}$ of the weak acid sodium propionate and following changes of pHi for 15 min before performing calibration.

To determine the effect of the presence of different inhibitors on both pHi and $H^+$ secretion in response to hyperosmotic challenge or to an acid load, the cells – after stabilisation – were exposed to the individual or to the combined inhibitors (concentrations are given below) and were then left for several minutes before exchanging for hyperosmotic medium or addition of sodium propionate.

The rate of alkalinization after hypertonic exposure or acid loading was calculated from the linear portion of the increase of pHi after addition of hypertonic saline or sodium propionate (pH units $min^{-1}$), respectively, and multiplication of this slope by the total apparent intracellular buffering capacity (β) determined from the extent of acidification induced after addition of a known amount sodium propionate, following the equation:

$$\beta = \{[\text{propionate}]10(\text{pHmin}-\text{p}K_a)\} / [1+10(\text{pHinput}-\text{p}K_a)](\text{pHcyto}-\text{pHmin}) ,$$

where [propionate] is 30 mmol $l^{-1}$, the p$K_a$ of propionate at room temperature is 4.87, pHmin is minimal pHcyto following acid load, pHinput is the pH of the medium (7.6) and pHcyto is baseline pHi prior to acid load (Furimsky et al., 2000).

Inhibitors were made up in concentrated stock solutions dissolved in distilled water or DMSO and were applied at the following final concentrations: cariporide mesilate 10 μmol $l^{-1}$

(2 mmol $l^{-1}$ stock in $H_2O$), SITS 0.5 mmol $l^{-1}$ (100 mmol $l^{-1}$ stock in DMSO), chelerythrine 1 and 5 μmol $l^{-1}$ (2.5 mmol $l^{-1}$ stock in $H_2O$), calmidazolium 5 μmol $l^{-1}$ (2.5 mmol $l^{-1}$ stock in DMSO) and H-89 1 μmol $l^{-1}$ (1 mmol $l^{-1}$ stock in DMSO). The final concentration of DMSO was always kept below 0.5%, a concentration that did not interfere with the measurements.

### *Measurement of proton release*

Proton secretion of the hepatocytes was estimated from the rate of acidification of external medium measured with a cytosensor microphysiometer (Molecular Devices, Munich, Germany) as previously described (Pelster, 1995; Krumschnabel et al., 2001a). Hepatocytes ($0.45\times10^6$ 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 and this was followed by switch 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 the percentage of the basal rate of proton secretion, measured under control conditions prior to the treatment.

### *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 Tukey's *post-hoc* test, with a $P$ value of <0.05 being considered as significant.

## Results

### *Effects of hypertonicity*

#### *Changes in pHi during hypertonic challenge and the effect of cariporide*

The effect of hypertonic challenge on both pHi and proton secretion in trout hepatocytes is depicted in Fig. 1A and B. As can be seen, hypertonic stress induced an increase in pHi from a basal value of 7.39±0.026 to a peak value of 8.00±0.019 (means ± s.e.m.) which was attained after approx. 10 min and remained fairly constant thereafter (Fig. 1A). The rate of proton secretion showed a sharp increase upon hypertonic exposure, reaching 256±7% of the basal value after 3 min, and subsequently started to decline until it stabilized after 24 min at around 151±5% of the basal rate (Fig. 1B).

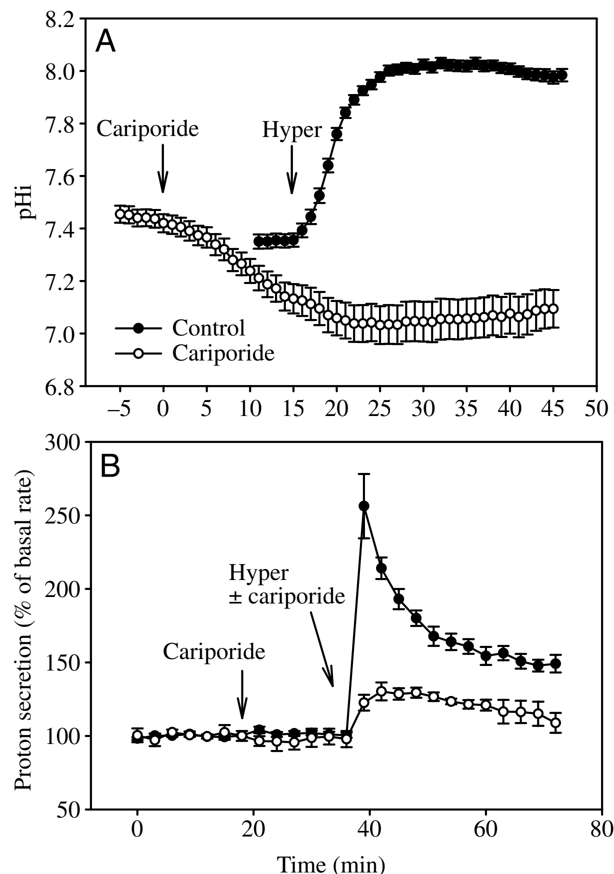


Fig. 1. Changes in pHi (A) and in proton secretion rate (B) of trout hepatocytes upon exposing cells to hypertonicity (1.6× isosmolarity) in the presence and absence of 10 μmol $l^{-1}$ cariporide. Values are means ± s.e.m. of 34–42 cells from three or four independent preparations in A and from 4–7 independent preparations in B.

In the presence of the specific NHE-1 inhibitor cariporide, basal pHi decreased from 7.42±0.03 to 7.13±0.05 within 15 min. This decrease in pHi continued even after exposing the cells to hypertonic medium, and reached a value of 7.03±0.07 within the next 10 min where it stabilized (Fig. 1A). On measuring the rate of proton secretion, we observed that cariporide exerted a rather minute effect on the basal value, which transiently declined by about 3%. Upon exposing the cells to hypertonic medium in the presence of the inhibitor, proton secretion increased to only 130% of the basal rate, which was 21% of the hypertonicity-induced rate change in the absence of the inhibitor. Subsequently the rate started to decrease slowly until the end of the experiment (Fig. 1B).

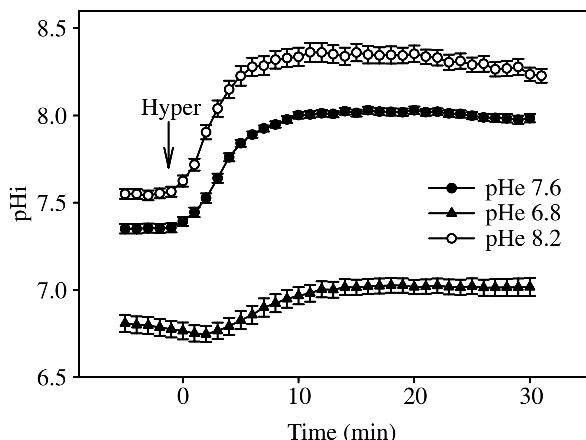


Fig. 2. Effect of incubation at different pHe (6.8, 7.6 and 8.2) on the hypertonicity-induced changes in pHi. Values are means ± s.e.m. of 17–42 cells from three independent preparations.

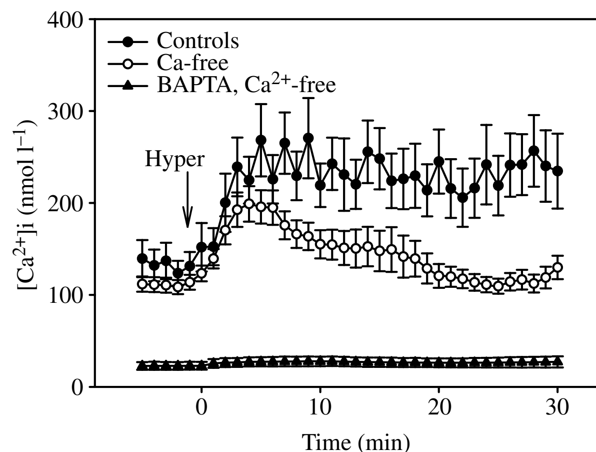


Fig. 3. Changes in $[Ca^{2+}]$i upon hypertonic challenge using (a) $Ca^{2+}$-containing medium (control), (b) $Ca^{2+}$-free medium and (c) $Ca^{2+}$-free medium along with preincubation of cells with the intracellular $Ca^{2+}$-chelating agent BAPTA-AM. Values are means ± s.e.m. of 21–34 cells from 3–5 independent preparations.

*pH-dependence of the hypertonic alkalinization*

Media with pH adjusted to 6.8, 7.6 and 8.2 were used to study the effect of pHe on changes of pHi during hypertonic challenge. After 30 min of incubation in these media, hepatocytes had a basal pHi of 6.77±0.05 (pHe 6.8), 7.36±0.03 (pHe 7.6) and 7.56±0.03 (pHe 8.2). As shown in Fig. 2, in spite of these different baseline levels, hypertonicity induced an increase in pHi regardless of the altered pHe, with the highest points being attained after about 10 min in all experiments, when pHi remained more or less stable until the end of the measuring period. In order to compare the rates of alkalinization following hypertonic exposure, calculated from the linear part of each curve, we determined the intracellular buffering capacities of the hepatocytes maintained at different pHe values. These buffering capacities, which were derived from experiments similar to those shown in Fig. 9, amounted to 28.06±1.23 (pHe 6.8), 14.13±1.03 (pHe 7.6) and 14.14±1.74 (pHe 8.2) mmol $H^+$ pH $unit^{-1}$ $l^{-1}$ (25-97 cells from four to six preparations). Using these values, we obtained rates of alkalinization, which were significantly reduced at low pH (0.99±0.07 mmol $H^+$ $l^{-1}$ $min^{-1}$, $n$=17 cells) and in the high pH saline (1.14±0.09 mmol $H^+$ $l^{-1}$ $min^{-1}$, $n$=35 cells) when compared to standard medium (1.51±0.07 mmol $H^+$ $l^{-1}$ $min^{-1}$, $n$=42 cells).

*Intracellular $Ca^{2+}$ during hypertonic exposure*

As shown in Fig. 3, exposing cells to hypertonicity induced an increase in $[Ca^{2+}]$i from 131±16 nmol $l^{-1}$ to a maximum of 268±39 nmol $l^{-1}$ after approximately 6 min, a level where it remained until the end of the experiment. In the absence of $Ca^{2+}$e, the cells responded with a similar initial increase in $[Ca^{2+}]$i, from 114±8 nmol $l^{-1}$ to a maximum of 199±19 nmol $l^{-1}$ within 5 min, but subsequently $[Ca^{2+}]$i started to decline to reach the baseline value at the end of the experiment. Chelating intracellular free $Ca^{2+}$ using 25 μmol $l^{-1}$ BAPTA-AM along with $Ca^{2+}$-free medium lowered basal $[Ca^{2+}]$i to 23±4.3 nmol $l^{-1}$ and completely abolished any alteration in $[Ca^{2+}]$i upon hypertonic exposure.

*Effect of manipulating $[Ca^{2+}]$i on pHi changes during hypertonic exposure*

In order to elucidate whether there is a link between the hypertonicity-induced changes in $[Ca^{2+}]$i and pHi, alterations in pHi were measured during hypertonic challenge in control saline, in $Ca^{2+}$-free medium and in $Ca^{2+}$-free medium after pre-treating cells with BAPTA-AM. Fig. 4 shows that, in the absence of $Ca^{2+}$e, pHi increased in a similar, but slightly delayed fashion when compared to controls. Expressed as initial rate of proton secretion, the alkalinization in control medium and $Ca^{2+}$-free medium amounted to 1.55±0.08 and 0.98±0.07 mmol $H^+$ $l^{-1}$ $min^{-1}$, respectively, which were significantly different ($P$<0.05). Chelating $[Ca^{2+}]$i with BAPTA-AM caused a dramatic decrease in pHi under isotonic conditions which was not affected further by subsequent addition of $Ca^{2+}$-free medium (Fig. 4, inset). When cells, pre-treated in this manner, were exposed to hypertonicity, pHi increased with the corresponding rate of proton secretion amounting to 1.14±0.12 mmol $H^+$ $l^{-1}$ $min^{-1}$ ($P$<0.05 compared to controls), but did not attain a new steady state over the period investigated.

*The involvement of phosphorylation pathways in the hypertonicity-induced NHE-1 activation*

Next, we examined the potential involvement of PKC, PKA and CaM in the activation of NHE-1 by investigating the effect of their specific inhibitors on pHi changes and proton secretion during hypertonic challenge. As shown in Fig. 5A, exposure of cells to 1 and 5 μmol $l^{-1}$ chelerythrine, a PKC inhibitor, induced a transient decrease in pHi from basal values of

7.15±0.03 and 7.19±0.03 to values of 7.08±0.03 and 6.96±0.05 followed by slow recovery establishing new steady-state pHi values of 7.14±0.05 and 7.01±0.04, respectively. The hypertonicity-induced increase in pHi was completely blocked by 5 μmol $l^{-1}$ of the inhibitor and attenuated by a chelerythrine concentration of 1 μmol $l^{-1}$. Proton secretion rate calculated from these data amounted to 0.67±0.1 mmol $H^+$ $l^{-1}$ $min^{-1}$. In control hepatocytes from the same preparations exposure to hypertonicity resulted in a similar pattern of alkalinization as observed before (Fig. 5A).

Fig. 5B shows the impact of chelerythrine on the rate of proton secretion. As can be seen, when given alone, 5 μmol $l^{-1}$ of the inhibitor induced an increase in the proton secretion rate to 120±6.8% of the basal rate within 18 min and also increased the hypertonicity-induced proton secretion rate to 242±23% within 6 min, which remained until the end of the experiment. In contrast, control hepatocytes measured in parallel showed an increase of proton secretion to a maximum of 187±19% followed by a slow decrease reaching 117±4% by the end of the experimental time (Fig. 5B).

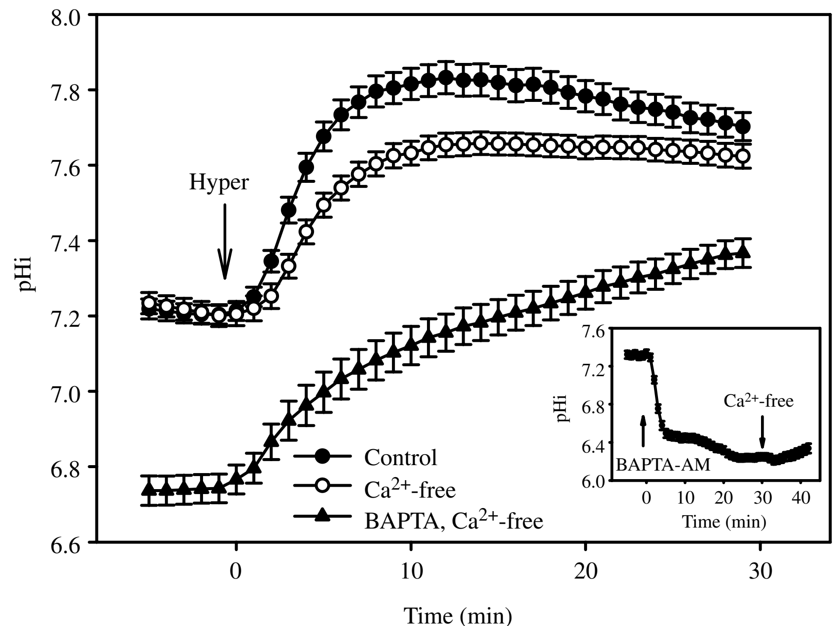


Fig. 4. Changes in pHi upon challenging cells with hypertonicity while using (a) $Ca^{2+}$-containing medium (control), (b) $Ca^{2+}$-free medium and (c) $Ca^{2+}$-free medium along with preincubation of cells with the intracellular $Ca^{2+}$-chelating agent BAPTA-AM. Values are means ± s.e.m. of 68–95 cells from 5–10 independent preparations. Inset: pHi changes upon exposing cells to 25 μmol $l^{-1}$ BAPTA-AM followed by $Ca^{2+}$-free medium. Values are means ± s.e.m. of 46 cells from three independent preparations.

Inhibiting PKA, using the inhibitor H-89, induced a slight and transient decrease of basal pHi, which then increased on exposing cells to hypertonicity (Fig. 6A). This increase, however, was significantly slower than in control hepatocytes, and the apparent rate of proton secretion amounted to 1.9±0.13 and 0.73±0.09 mmol $H^+$ $l^{-1}$ $min^{-1}$ in the absence and presence of H-89, respectively ($P<0.05$). Measurement of proton secretion indicated that H-89 had no effect on the basal rate of proton secretion, whereas upon challenging the cells with hypertonic medium the change in proton secretion rate was significantly ($P<0.05$) reduced by 41% compared to the change in rate seen in controls (Fig. 6B).

Inhibiting CaM with calmidazolium appeared to have only little effect on basal pHi (Fig. 7A), but reduced the hypertonicity-induced pHi increase by half of that seen in controls. Proton secretion determined in these measurements amounted to 1.9±0.13 and 0.9±0.07 mmol $H^+$ $l^{-1}$ $min^{-1}$ in the absence and presence of calmidazolium, respectively ($P<0.05$). Determination of proton secretion using the cytosensor indicated that calmidazolium had no effect on the basal rate, yet it reduced the hypertonicity-induced change in proton secretion rate by approximately 15% compared to controls (Fig. 7B). This decrease, however, was not significant ($P>0.05$).

In Fig. 8, a summary of the rates of proton secretion following hypertonic stimulation under the various conditions examined is given, allowing direct comparison of all treatments.

### *Effects of intracellular acidification*

#### *Effects of cariporide and SITS on acidification-induced pHi changes*

The effects of inhibitors of NHE-1 and the sodium-dependent $Cl^-/HCO_3^-$ exchanger on steady-state pHi and on pHi recovery following sodium-propionate-induced acidification are shown in Fig. 9A,B. In control saline, addition of sodium propionate caused a decrease in pHi from a basal value of 7.19±0.04 to a minimum pHi of 6.56±0.04 after 2 min. Subsequently, pHi increased again and after 15 min it had recovered to a value close to the initial one. In the presence of cariporide, given 5 min before sodium propionate, the initial acidification was similar to controls. The subsequent pHi recovery, however, was significantly retarded and proton secretion amounted to 0.9±0.03 and to 0.35±0.03 mmol $H^+$ $l^{-1}$ $min^{-1}$ in the absence and presence of cariporide, respectively ($P<0.05$).

Exposing hepatocytes to SITS induced a gradual decrease in pHi and a final steady-state was attained after 15 min (not shown). During this time basal pHi was reduced to 6.96±0.02 and upon addition of sodium propionate it dropped to 6.5±0.02 (Fig. 9B). The recovery of pHi then occurred at a rate of 0.60±0.02 mmol $H^+$ $l^{-1}$ $min^{-1}$, which was significantly lower than in controls ($P<0.05$). In the concurrent presence of both cariporide and SITS, basal pHi showed a continuous decrease during pre-incubation and upon addition of sodium propionate pHi decreased from 6.66±0.03 to 6.43±0.04 within 3 min. Recovery of pHi determined under these conditions was

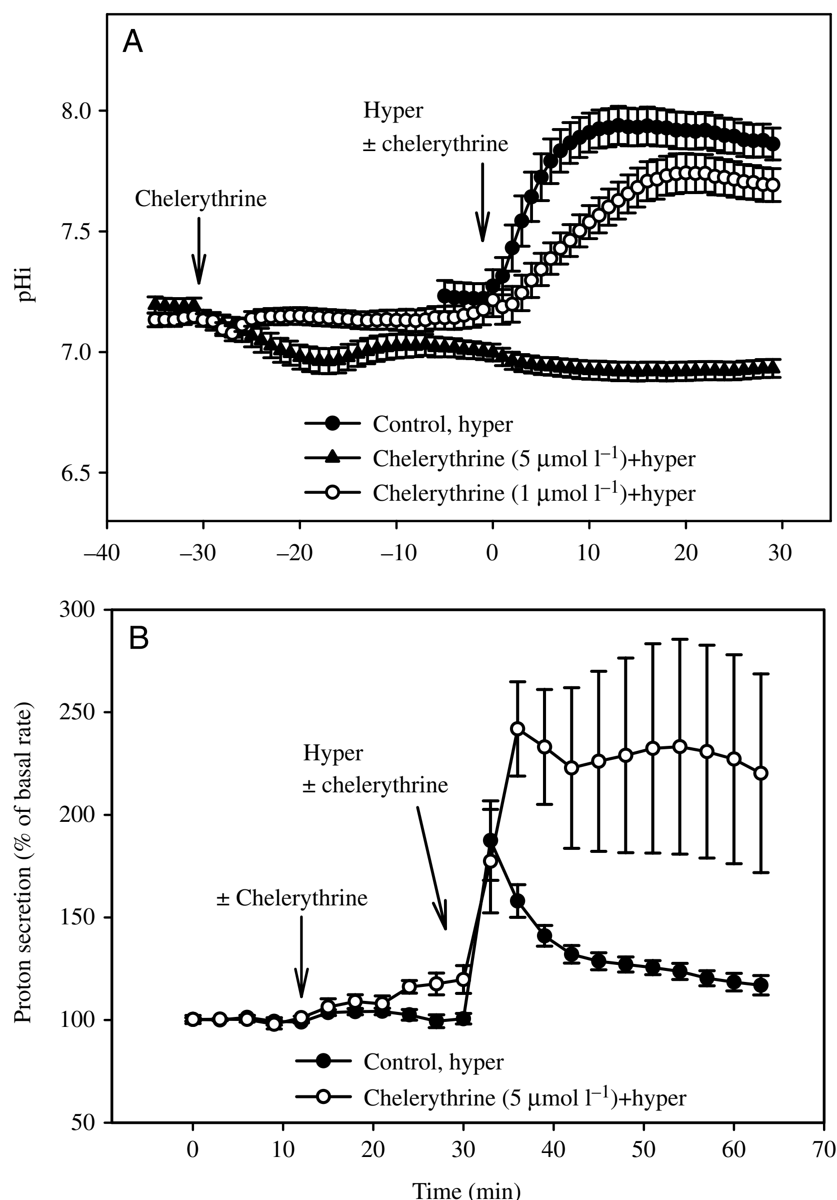


Fig. 5. Effect of 1 and 5 $\mu$mol $l^{-1}$ chelerythrine on basal pHi and on hypertonicity (hyper)-induced pHi changes (A) and the effect of 5 $\mu$mol $l^{-1}$ chelerythrine on basal and hypertonic proton secretion rate (B). Values are means ± s.e.m. of 23–51 cells from three or four independent preparations in A and from four or five independent preparations in B.

virtually abolished with proton secretion rate amounting to 0.07±0.04 mmol $H^+$ $l^{-1}$ $min^{-1}$.

*pH-dependence of pHi recovery following acidification*

To determine the effect of pHe, after adjustment of pHi, on the pHi recovery after acid loading, cells were incubated for 30 min in media with the pH adjusted to 6.8, 7.6 and 8.2 before sodium propionate exposure. Basal pHi values under these conditions were 6.78±0.03 (pH 6.8), 7.38±0.03 (pH 7.6) and 7.73±0.04 (pH 8.2). Upon exposing the cells to sodium propionate, pHi decreased within 2 min to 6.26±0.03 (pH 6.8), 6.84±0.03 (pH 7.6) and 7.1±0.05 (pH 8.2). The rate of proton secretion during pHi recovery for the three conditions amounted to 0.87±0.11 (6.8; $P$<0.05 *versus* controls), 0.88±0.04 (pH 7.6), and to 1.53±0.12 (8.2; $P$<0.05) mmol $H^+$ $l^{-1}$ $min^{-1}$.

*Effect of chelerythrine, H-89 and calmidazolium on the acidification-induced pHi changes*

As seen in Fig. 10A, exposure to 5 $\mu$mol $l^{-1}$ chelerythrine induced a decrease in pHi from a basal value of 7.33±0.04 to a value of 6.86±0.04 within 15 min and upon addition of sodium propionate, pHi decreased to a value of 6.63±0.03 within 4 min, after which pHi remained constant with the proton secretion rate amounting to 0.15±0.04 mmol $H^+$ $l^{-1}$ $min^{-1}$. In the corresponding controls, which were also controls for H-89 and calmidazolium treatments, pHi decreased, upon acid loading, from a basal value of 7.19±0.02 to 6.5±0.06 within 2 min and thereafter, pHi started to increase with a rate of proton secretion amounting to 1.31±0.1 mmol $H^+$ $l^{-1}$ $min^{-1}$.

Following incubation with H-89, challenging the cells with sodium propionate acidified the hepatocytes from a basal pHi value of 7.17± 0.03 to 6.61±0.05 within 2 min (Fig. 10B). Proton secretion rate was significantly ($P$<0.05) decreased to 0.72±0.04 mmol $H^+$ $l^{-1}$ $min^{-1}$ compared with that of controls.

Finally, as shown in Fig. 10C, a slight transient decrease in basal pHi was noted during exposure to calmidazolium, and upon exposing the cells to sodium propionate, pHi decreased from 7.36±0.05 to 6.6±0.06 within 2 min. Thereafter pHi recovery took place at a reduced rate of 0.96±0.05 mmol $H^+$ $l^{-1}$ $min^{-1}$, which, however, was not significantly different from that of controls.

A summary of the rates of proton secretion following sodium-propionate-induced acidification under the various conditions examined is given in Fig. 11.

## Discussion

### *Hypertonicity-induced activation of NHE*

Our present findings demonstrate that hypertonicity induces an increase in pHi in trout hepatocytes, which is mainly due to enhanced $Na^+/H^+$ exchanger activity. This alkalinization was completely blocked upon selectively inhibiting NHE-1 using cariporide (Fig. 1A), confirming that, as observed in several other cell types (Grinstein et al., 1992; Kapus et al., 1994; Pedersen et al., 2002), this NHE-isoform is the main exchanger responsible for the hypertonicity-induced change in pHi. Furthermore, the acidification of pHi in the presence of

cariporide (Fig. 1A) suggested that the amiloride-sensitive proton secretion reported to be tonically active under steady-state conditions in trout hepatocytes (Furimsky et al., 1999; Krumschnabel et al., 2001b) also involves NHE-1. Looking at the acidification of the extracellular space, however, inhibition of the NHE-1 with cariporide reduced steady-state proton secretion only by about 3%, and NHE-1 activity accounted for only 80% of the hypertonicity-induced proton secretion rate (Fig. 1B). These findings indicate that there are other mechanisms working in parallel with NHE-1 under iso- and hyperosmotic conditions. In fact, it could be that other NHE isoforms are involved, since amiloride has been shown to reduce steady-state proton secretion by 20% (Krumschnabel et al., 2001b) and to completely block hypertonicity-induced proton secretion (Ebner et al., 2005). Surprisingly, although these isoforms appear to be activated by hypertonicity, there was no corresponding increase of pHi detectable, because cariporide completely eliminated the increase in pHi during hypertonic exposure. A speculative answer to this apparent paradox would be to postulate the presence of intracellular cariporide-sensitive mechanisms, normally operating to remove $H^+$ from the cytosol into intracellular compartments.

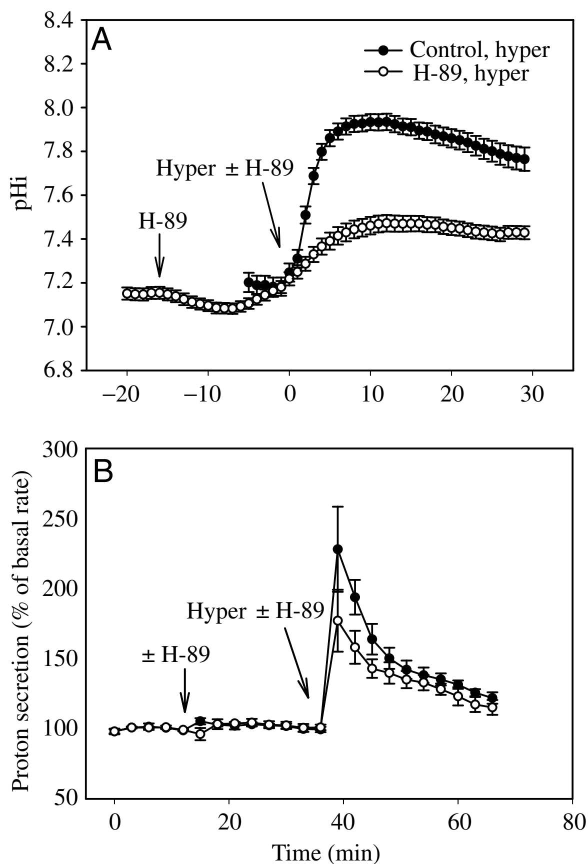


Fig. 6. Effect of 1 μmol $l^{-1}$ H-89 on basal pHi and on hypertonicity (hyper)-induced pHi changes (A) and on basal and hypertonic proton secretion rate (B). Values are means ± s.e.m. of 27–39 cells from three or four independent preparations in A and from three independent preparations in B.

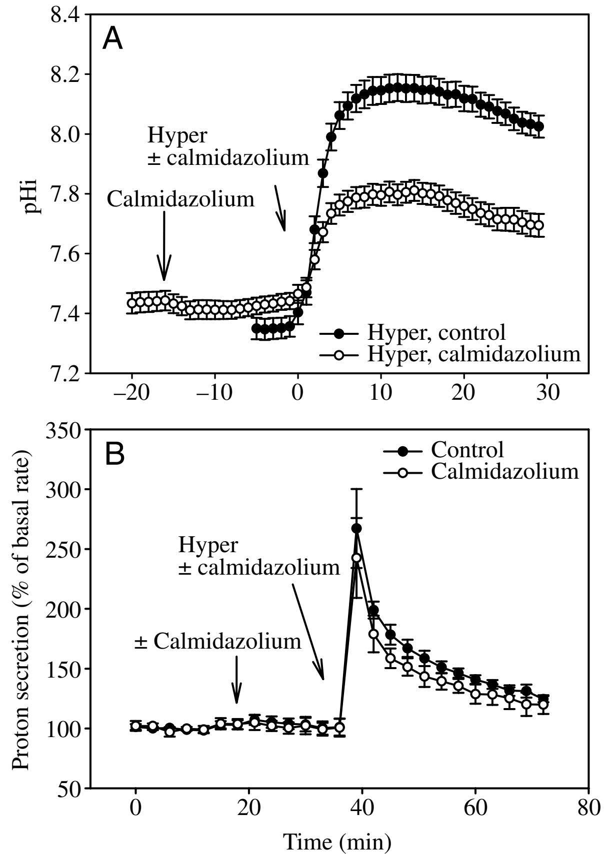


Fig. 7. Effect of 5 μmol $l^{-1}$ calmidazolium on basal pHi and on hypertonicity (hyper)-induced pHi changes (A) and on basal and hypertonic proton secretion rate (B). Values are means ± s.e.m. of 37–51 cells from three independent preparations in A and from four independent preparations in B.

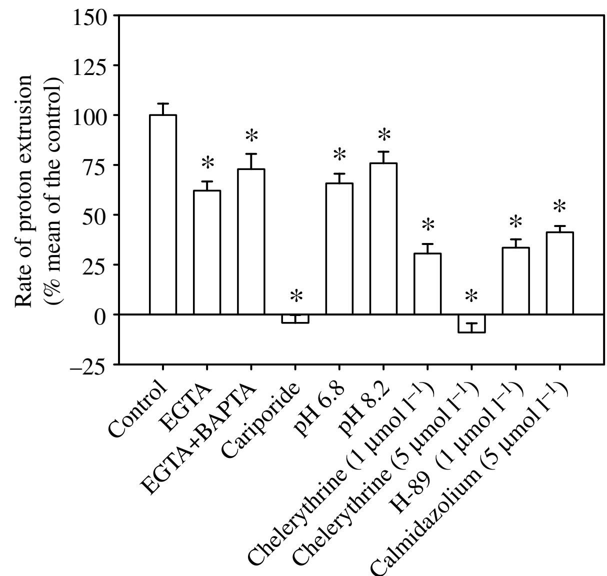


Fig. 8. Rates of proton secretion (calculated from the slope of the linear portion of the increase of pHi multiplied by β) following hypertonic exposure. Values are expressed as percentages of the mean of the corresponding controls of each treatment.

Thus, upon inhibition with cariporide these protons would remain in the cytoplasm and thus could counteract activities of the cariporide-insensitive NHE isoforms in the cell membrane. In support for this, the non-isoform-specific inhibitor amiloride also acidifies the cells but concurrently prevents enhanced acid secretion (Krumschnabel et al., 2003; Ebner et al., 2005). The presence of intracellular NHE isoforms acidifying Golgi- and post-Golgi compartments has been documented in human cells (Nakamura et al., 2005), and in cardiomyocytes cariporide was reported to inhibit the mitochondrial NHE, thereby affecting mitochondrial matrix pH (Ruiz-Meana et al., 2003).

### *The possible involvement of $[Ca^{2+}]i$*

A hypertonicity-induced increase in intracellular $Ca^{2+}$ has been observed in trout hepatocytes in a previous study (Krumschnabel et al., 2003), and our data indicated that this was due to $Ca^{2+}$ mobilization from intracellular stores accompanied by a sustained $Ca^{2+}$ influx from extracellular space (Fig. 3). Removal of intracellular $Ca^{2+}$ caused a significant reduction in pHi, emphasizing a connection between steady-state pHi and $Ca^{2+}$ movements. Similar observations have been reported for rat hepatocytes (Martin-Requero et al., 1997) and rat alveolar

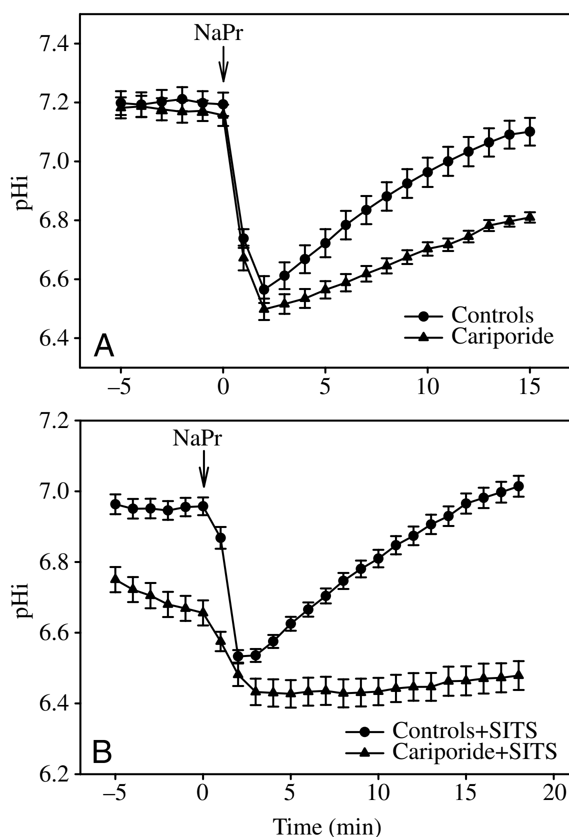


Fig. 9. pHi recovery following 30 mmol $l^{-1}$ sodium-propionate-induced acidification in the absence and presence of 10 μmol $l^{-1}$ cariporide (A) and in the presence of 0.5 mmol $l^{-1}$ SITS or SITS + cariporide (B). Values are means ± s.e.m. of 40–79 cells from 3–5 independent preparations in A and of 44–69 cells from three independent preparations in B.

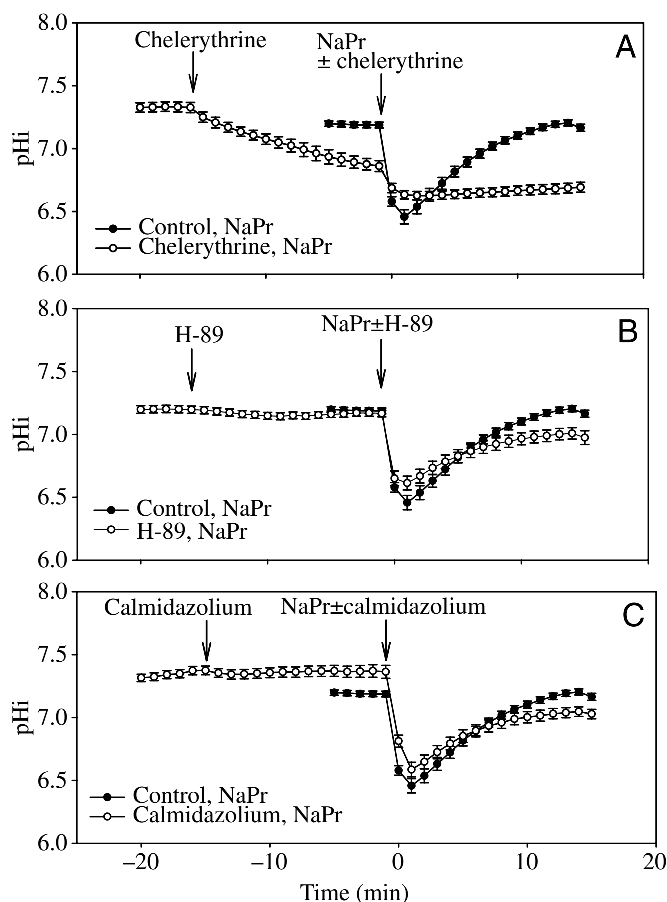


Fig. 10. Effects of 5 μmol $l^{-1}$ chelerythrine (A), 1 μmol $l^{-1}$ H-89 (B) and 5 μmol $l^{-1}$ calmidazolium (C) on basal pHi and on pHi recovery after sodium-propionate-induced acidification. Values are means ± s.e.m. of ⩾40 cells from ⩾4 independent preparations.

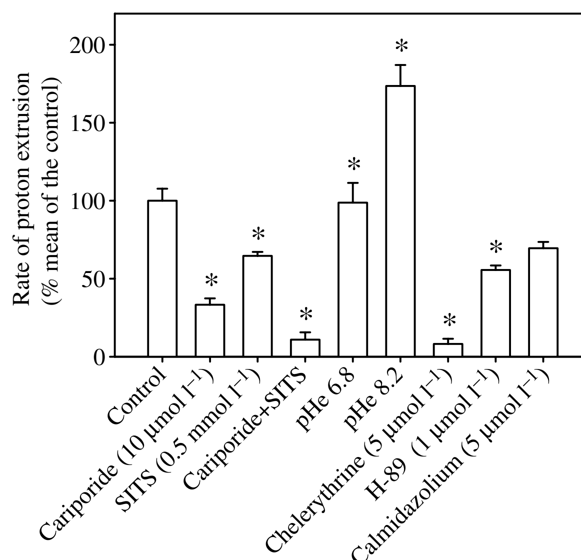


Fig. 11. Rates of proton secretion (calculated from the slope of the linear portion of the increase of pHi multiplied by β) following acid loading. Values are expressed as percentages of the mean of the corresponding controls for each treatment.

cells (Murao et al., 2005). Although neither removal of extracellular $Ca^{2+}$ nor a chelation of intra- and extracellular $Ca^{2+}$ could abolish the hypertonicity-induced alkalinization, the kinetics and magnitude of this alkalinization were significantly affected by the absence of $Ca^{2+}$ (Fig. 4). This contradicts previous findings in other cell types, where shrinkage-induced activation of NHE was found to be independent of changes in $[Ca^{2+}]$i (Grinstein et al., 1985b; Mitsuhashi and Ives, 1988; Dascalu et al., 1992; Shrode et al., 1995; Pedersen et al., 1996). On the other hand, $Ca^{2+}$-dependent activation was reported for other conditions, including mitogenic stimulation (Mitsuhashi and Ives, 1988; Garnovskaya et al., 2003a) and elevation of $[Ca^{2+}]$i by a calcium ionophore or inhibition of the endoplasmic reticulum $Ca^{2+}$-ATPase (Maly et al., 2002). In human alveolar type II cells a decrease of $[Ca^{2+}]$i has been shown to elicit cell shrinkage (Murao et al., 2005).

The conclusion that $Ca^{2+}$ may be involved in the activation of NHE is also supported by the observation that calmidazolium reduces the hypertonicity-induced alkalinization. $Ca^{2+}$-calmodulin (CaM) has been shown to be involved in the activation process of NHE-1 in response to a variety of stimuli including mitogenic factors, ionomycin and serotonin (Wakabayashi et al., 1994; Bertrand et al., 1994; Garnovskaya et al., 2003a), and, importantly, osmotic shrinkage (Dascalu et al., 1992; Shrode et al., 1995; Shrode et al., 1997; Garnovskaya et al., 2003b). Consistent with the latter studies, our data confirmed an important role of a CaM-dependent pathway in the hypertonicity-induced NHE-1 activation in trout hepatocytes, as the inhibition of CaM by calmidazolium reduced the hypertonicity-induced pHi increase by 54% (Fig. 7A). A similar degree of inhibition of hypertonicity-induced pHi increase, ranging from 40–50% has been found in other cells with different CaM inhibitors (Dascalu et al., 1992; Pedersen et al., 1996). Deletion of the CaM binding subdomain in the C-terminal of the human NHE-1 reduced its activation by shrinkage by about 80% (Bertrand et al., 1994). In parallel with its reduction in the intracellular alkalinization, calmidazolium reduced the hypertonicity-induced proton secretion rate by 15% in the trout cells (Fig. 7B). This is considerably less than the 55 to 94% inhibition of hypertonicity-induced proton secretion seen in CHO-K1 cells exposed to various CaM inhibitors (Garnovskaya et al., 2003b). Compared with the severe reduction in alkalinization, indicating that a significant amount of protons remained in the cytoplasm during hypertonic stress in the presence of calmidazolium, the reduction in the rate of proton secretion was smaller than might be expected. This could indicate that again a proton movement between intracellular compartments and the cytoplasm is involved under these conditions (see above). A point that needs to be clarified regarding the action of CaM in trout hepatocytes is, whether it directly acts on NHE-1 *via* CaM binding sites at the cytoplasmic tail of the protein (Wakabayashi et al., 1997a; Garnovskaya et al., 2003b; Orlowski and Grinstein, 2004), or if it affects NHE-1 *via* stimulation of CaM-dependent kinases, as shown in an astrocyte cell model (Shrode et al., 1997). Interestingly, the CaM-dependent myosin light chain kinase involved in NHE-1 stimulation in these cells provides a link between antiporter activity and the cytoskeleton. A similar interaction might thus underlie the pronounced inhibition of NHE-1 we have recently observed in trout hepatocytes upon disruption of the cytoskeleton (Ebner et al., 2005).

### *pH-dependence of the NHE under hyperosmotic conditions*

Intracellularly two proton binding sites have been identified, one involved in the transfer of protons, whereas the other binding site appears to be an allosteric binding site modifying the transport characteristics of NHE-1 (Wakabayashi et al., 1992). Accordingly, NHE-1 has been shown to be sensitive to changes in pHi, and a reduced pHi activates the protein (Grinstein et al., 1985a; Takaichi et al., 1993; Bianchini et al., 1995). The pH dependence of NHE in trout hepatocytes has only been investigated in isotonic conditions, showing, at acutely altered pHi or pHe, a decrease in NHE activity when pHi was increased, and an increase when pHe was elevated (Fossat et al., 1997). This reflects that NHE activity of trout cells, similar to that of other species (Grinstein et al., 1985b), is independently affected by extra- and intracellular protons. In our experiments, hepatocytes were allowed to adjust their pHi to altered pHe conditions before hyperosmotic challenge, which, as expected, resulted in an increase and decrease of basal pHi at elevated and diminished pHe, respectively. Under these conditions, hypertonic NHE activity was reduced both at higher and lower pHe when compared to that seen in our standard pH 7.6 saline. Stimulation and inhibition by intracellular and extracellular protons, respectively, thus results in a rather complex activation pattern of NHE by hypertonicity, which we believe would be best explained by assuming a pH optimum for the antiporter under these conditions. In rat astrocytes (Shrode et al., 1997) and rat mesangial cells (Bevensee et al., 1999) a shift of the pHi dependence of NHE to more alkaline values under hyperosmotic conditions was observed, and according to these authors this shift may explain why cell shrinkage causes steady-state pHi to increase. Nevertheless, at alkaline pH the activity of the NHE typically was reduced, and a saturation of NHE activity in both iso- and hyperosmotic saline at pHe above 7.8 has previously been documented for rat lymphocytes (Grinstein et al., 1985b).

### *PKC- and PKA-dependent pathways are involved in the hypertonicity-induced activation of NHE*

Evidence about the involvement of PKC in hypertonicity-induced activation of NHE is contradictory in different cell types. In a study on Ehrlich ascites tumour cells, the PKC inhibitor chelerythrine inhibited hypertonicity-induced amiloride-sensitive pHi increase (Pedersen et al., 1996; Pedersen et al., 2002), whereas in rat bone cells (Dascalu et al., 1992), rat astrocytes (Shrode et al., 1995), rat hepatocytes (Heinzinger et al., 2001) and CHO-K1 cells (Garnovskaya et al., 2003b) no PKC-dependent hypertonicity-induced activation of NHE was noted. In our study, the concentration-dependent decrease in basal pHi upon addition of 1 or 5 $\mu mol\,l^{-1}$ (and 10 or

30 μmol $l^{-1}$, not shown) chelerythrine (Fig. 5A), indicates a potential role for PKC in the steady-state pHi regulation in trout hepatocytes. Importantly, the decrease of pHi was accompanied by an increase in proton secretion, indicating that not only NHE activity but also other pHi regulatory mechanisms are influenced by the activity of PKC, some of which appear to be stimulated upon PKC inhibition. This conclusion was supported by the observation that the decrease in pHi after addition of chelerythrine exceeded the intracellular acidification observed after complete inhibition of NHE by EIPA, and by the fact that even after blockade of NHE chelerythrine was still able to reduce pHi (Gende, 1996). In addition, we observed that following the addition of chelerythrine, oxygen consumption of trout hepatocytes is increased by up to a factor of two within 15 min and this stimulation persists for at least 30 min (unpublished). Although the underlying mechanism is not clear at present, this enhanced metabolic activity could have contributed to elevated proton production. Under hypertonic conditions chelerythrine completely inhibited the intracellular alkalinization in a dose-dependent manner, but at the same time it again increased the rate of proton secretion. As stated before, this can be explained by assuming a stimulation of proton production within the cell due to the inhibition of PKC. Secretion of these protons would result in a constant intracellular pH and a lowering of extracellular pH. This again suggests that besides possible membrane effects inhibition of PKC affected intracellular mechanisms involved in the acid–base homeostasis. Pedersen et al. (Pedersen et al., 1996; Pedersen et al., 2002), however, interpreted the reduction in the hypertonicity-induced increase in pHi by chelerythrine as an attenuation of NHE activity in Ehrlich ascites tumour cells. At higher concentrations (30 μmol $l^{-1}$) chelerythrine completely blocked the hypertonicity-induced proton secretion rate, but intracellular pH measurements indicated a reduced viability of the cells, and therefore these data were not included in this study. Negative effects of higher concentrations of chelerythrine on the viability of cells have also been reported (Zhang et al., 2005). However, the specificity of chelerythrine has been questioned (Davies et al., 2000). The dose-dependent effect observed in the lower concentration range in our study suggests, however, that unspecific side effects did not affect our results.

Upon pharmacological stimulation, the activation of PKA by increased cAMP levels has been shown to decrease activity of NHE-3 (Kandasamy et al., 1995; Moe et al., 1995) and NHE-5 (Attaphitaya et al., 2001), while the activity of NHE-1 and NHE-2 was enhanced (Kandasamy et al., 1995). In line with the first observation inhibition of PKA by decreasing cAMP production has been shown to increase NHE activity (Saccomani et al., 1990). In contrast, in hyperosmotic conditions PKA is apparently not involved in the activation of NHE, as cell shrinkage often, although not universally (Orlic et al., 2002), fails to raise cAMP concentrations (Kregenow et al., 1976; Shrode et al., 1995) and even pharmacological elevation of cAMP was found to be without effect in several mammalian cell types (Dascalu et al., 1992; Shrode et al., 1995). However, our present data indicate that, in trout hepatocytes, PKA appears to contribute to the activation of NHE-1, both under steady-state conditions as well as in response to hyperosmotic stress. Thus, the addition of the PKA inhibitor H-89 decreased steady-state pHi, reduced the hypertonicity-induced pHi increase by 58% (Fig. 6A), and attenuated the hypertonicity-induced proton secretion rate by 41% (Fig. 6B). This different responsiveness of trout and mammalian NHE-1 is consistent with molecular features of the antiporter. The cytoplasmic domain of the mammalian NHE-1 homologues, which is the main site of NHE-1 regulation, lacks PKA consensus sites, whereas in teleosts these domains appear to be consistently present (Pedersen and Cala, 2004). Whether the osmotic activation in trout hepatocytes involves a direct phosphorylation of NHE-1 remains to be determined. In winter flounder, cAMP-mediated stimulation of NHE-1 caused PKA-sensitive phosphorylation of the antiporter (Pedersen and Cala, 2004), whereas direct phosphorylation appeared to play no role in hyperosmotic activation (Pedersen et al., 2003).

### *Involvement of PKC-, PKA- and CaM-dependent pathways in the acid loading-induced pH regulation mechanism*

Although the dependence of NHE activity on pHi or pHe during cell recovery from acidification has been studied in great detail (Grinstein et al., 1985a; Takaichi et al., 1993; Bianchini et al., 1995; Bevensee et al., 1999), comparatively little is known regarding the role of other factors possibly contributing to NHE regulation under this condition. In general, the stimulation of NHE upon acidification is believed to reflect its activation by cytoplasmic $H^+$, which interact with a $H^+$ sensor site at the N-terminal transmembranous region of the antiporter (Wakabayashi et al., 1992). The concentration of intracellular $H^+$ thus appears to be the primary determinant of NHE activity. In addition, however, some studies have documented alterations of amiloride-dependent pHi recovery in the presence of a CaM antagonist, of PKC stimulatory agents, or of elevated cAMP, all of which appear to act by altering the pH sensitivity of NHE (Moule and McGivan, 1990; Takaichi et al., 1993; Kandasamy et al., 1995).

Our present data showed that, in line with previous data (Walsh, 1986; Furimsky et al., 1999; Krumschnabel et al., 2001b; Tuominen et al., 2003), in trout hepatocytes pHi recovery following sodium propionate addition is governed by NHE and a SITS-sensitive mechanism, presumably a $Na^+$-dependent $Cl^-/HCO_3^-$ exchanger. Specifically, we observed that using cariporide and SITS alone reduced pHi recovery by 61% and 33%, respectively, and upon using both inhibitors simultaneously, the reduction in proton secretion rate (93%) was almost equal to the sum of their individual inhibition (Fig. 9A,B). Thus, the main share of alkalinization was due to NHE-1 activity, and the impact on pHi recovery of the various agents applied will therefore largely reflect their effect on NHE-1 activity. In principle, all measurements could have been conducted with SITS present, but since SITS significantly reduced the resting pHi of the cells and could thereby have affected the action of other inhibitors tested it was omitted from the subsequent experiments.

In our experiments examining the interactive effect of, on the one hand, pHe and spontaneously adjusted pHi levels and, on the other hand, pHi recovery after an acidic load, we obtained results indicating a positive correlation between these parameters (Fig. 11), similar to findings on rat lymphocytes (Grinstein et al., 1985b). As outlined above, regarding the impact of pHi, this differs from previous findings, where, at constant pHe, NHE activity decreased with an increase of pHi (Fossat et al., 1997). By contrast, the increase of NHE activity with increasing pHe would be in line with the study of Fossat et al. (Fossat et al., 1997). Together, this seems to suggest that when pHi is allowed to adjust to the prevailing pHe, the latter is a quite strong determinant of acidification-induced NHE activity. Although at first glance this may seem to contradict the well known pivotal importance of intracellular protons as a major determinant of NHE activity, it may also reflect an additive effect of stimulation by intracellular protons and the favourable proton gradient prevailing at high pHe.

Besides the impact of pHe, we observed that inhibition of PKA, and CaM to a some extent (though not significant), affected NHE activity by reducing pHi recovery (Fig. 11). The same may be true for inhibition of PKC by chelerythrine, with the possibility that PKC inhibition might have activated other hydrogen-producing mechanisms acidifying the cell, as discussed in the previous section. These findings were surprising in the light of previous reports showing that addition of agents stimulating these pathways produced an acceleration of NHE activity, which was additive to, and thus presumably independent from, the stimulation of NHE activity by acidification (Moule and McGivan, 1990; Kandasamy et al., 1995). By contrast, our data suggest that these pathways contribute to pHi recovery without prior stimulation of PKA, CaM or PKC. This would imply that these pathways are, at least to some extent, already stimulated during acidification. To our knowledge, this has not yet been tested explicitly, but previous studies found enhanced PKC stimulation of NHE-1 in acidified outer hair cells (Ikeda et al., 1996), but also reduced cAMP responses to isoproterenol and ionomycin in rat pinealocytes at low pHi (Ho et al., 1992).

In summary our study provides evidence that, in trout hepatocytes, the NHE-1 isoform of the $Na^+/H^+$ exchanger is the main mechanism mediating pH changes under hypertonic stress and following acidification. Similar to other cells, NHE-1 activity is sensitive to alterations of pHe and pHi, with somewhat different activation profiles detectable under both conditions. Furthermore, hypertonic NHE-1 activation is partly dependent on $Ca^{2+}$, which differs from most mammalian cells, where $Ca^{2+}$ is mainly important in response to mitotic stimuli. Other signalling pathways contributing to activation of trout hepatocyte NHE-1 involve PKA, PKC and, at least in hypertonic conditions, CaM. The role of these pathways in hypertonic NHE-1 activation agrees with findings on many, although not all, mammalian cells. In contrast, their contribution to NHE-1-mediated acid recovery has, to our knowledge, not yet been documented in mammalian cells.

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