The Journal of Experimental Biology 200, 2369–2376 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB1073

Na^+/H^+ EXCHANGE AND OSMOTIC SHRINKAGE IN ISOLATED TROUT HEPATOCYTES

B. FOSSAT[1,*], J. PORTHÉ-NIBELLE[1], S. PEDERSEN[1] AND B. LAHLOU[2]

[1]*Laboratoire de Physiologie et Toxicologie Environnementales* and [2]*Laboratoire de Physiologie Cellulaire et Moléculaire, UFR Faculté des Sciences, Université de Nice Sophia Antipolis, 06108 Nice Cedex 02, France*

Accepted 17 June 1997

Summary

The ability of rainbow trout liver cells to regulate their intracellular pH (pHi) was studied using two methods on hepatocytes isolated by collagenase digestion: (i) by monitoring pHi with the fluorescent dye BCECF-AM, and (ii) by measuring the amiloride-sensitive uptake of ^{22}Na, which represents Na^+/H^+ exchange.

In low-Na^+ medium ($\leqslant$16 mmol l^{-1}), Na^+ uptake was reduced by approximately 70 % in the presence of amiloride derivatives (DMA or MPA, 10^{-4} mol l^{-1}).

Changing separately either the extracellular pH (pHe) or the intracellular pH (pHi, clamped by treating the cells with nigericin in the presence of 140 mmol l^{-1} K^+) between 6 and 8 induced an increase in the rate of Na^+ uptake when pHe was raised or when pHi was reduced.

When transferred to hypertonic medium, hepatocytes shrank to nearly 72 % of their initial volume, and thereafter a slow and partial regulatory volume increase phase was observed, with an increase in the amiloride-sensitive rate of Na^+ uptake and an increase in intracellular pH. As DIDS-sensitive Cl^- uptake was concomitantly enhanced, it is suggested that hypertonic stress activates Na^+/H^+ and Cl^-/HCO_3^- exchange.

Key words: Na^+/H^+ exchange, hepatocytes, osmotic shrinkage, pHi, trout, *Oncorhynchus mykiss.*

Introduction

It has been shown in many cell types and appears to be ubiquitous in eukaryotic cells that the dominant housekeeping system involved in pHi regulation is the Na^+/H^+ exchanger, which extrudes protons produced by cellular metabolism.

Na^+/H^+ exchange has also been related to volume regulation in many cell models (Grinstein *et al.* 1984). The regulatory volume increase (RVI) induced by a hyperosmotic stress involves Na^+ and Cl^- uptake resulting from Na^+/H^+ and Cl^-/HCO_3^- exchange in parallel or from $Na^+/K^+/2Cl^-$ cotransport. The hyperosmotic treatment is achieved either by increasing the extracellular osmolarity or by submitting the cells to a hypo-osmotic challenge followed by transfer back to the isotonic medium (for reviews, see Al-Habori, 1994; Baumgarten and Feher, 1995; Hoffman and Dunham, 1995).

Fish hepatocytes have been little studied with respect to the regulation of their intracellular pH. In a series of studies on the rainbow trout *Oncorhynchus mykiss* (a freshwater species) and on the toadfish *Opsanus beta* (a marine species), Walsh and his coworkers described the regulation of pH by liver cells, especially in relation to the acid–base status of the animal (Walsh, 1986, 1987, 1989, 1990; Walsh and Mommsen, 1992).

Very little is known about the ionic mechanisms involved in volume regulation by fish hepatocytes. In previous studies on isolated trout hepatocytes, we tried to analyze how these cells adjust their volume following an osmotic challenge. Under hypo-osmotic conditions, the regulatory volume decrease (RVD) takes place as a consequence of loss of K^+, Cl^- and amino acids from the cells (Bianchini, 1989; Michel *et al.* 1994), as shown for other cell types (see Hoffman and Dunham, 1995). To our knowledge, there are no reports on fish hepatocytes subjected to hyperosmotic conditions.

In the present work, we attempted to analyze Na^+/H^+ exchange in relation to changes in extracellular and intracellular pH and as one of the mechanisms involved in RVI after osmotic shrinkage.

Materials and methods

Cell separation

Isolated hepatocytes were prepared from the rainbow trout *Oncorhynchus mykiss* (Walbaum) following the collagenase procedure described previously (Porthé-Nibelle and Lahlou, 1981; Bianchini *et al.* 1988). The cells were finally suspended in Eagle's Minimum Essential Medium (H-MEM)

*e-mail: fossatb@unice.fr.

supplemented with Hanks' salts (Boehringer). Cell concentration was brought to approximately 17.5×10^6 cells ml^{-1}, corresponding to 3.3 mg protein ml^{-1}. The hepatocytes were preincubated with gentle shaking for 40 min to ensure recovery of the steady-state ionic contents. All experiments were conducted at room temperature (20 °C) with no gassing of the vials. Cell viability assessed by Trypan Blue exclusion and lactate dehydrogenase (LDH) assay (Sigma) was approximately 85 %.

Incubation media

The iso-osmotic control medium, H-MEM, contained (in mmol l^{-1}): NaCl 136.8; KCl 5.4; $CaCl_2$ 1.3; KH_2PO_4 0.44; $MgSO_4$ 0.81; Na_2HPO_4 0.34; Hepes 10; and (in mg l^{-1}): vitamins 8.1; glucose 1000; Phenol Red 10; amino acids 566; glutamine 292; pH 7.4. The Hepes concentration used in the present experiments (10 mmol l^{-1}) is in agreement with the recommendations of Walsh (1990) concerning the appropriate concentration of this buffer in incubation medium.

Low-Na^+ media were obtained by replacing Na^+ with *N*-methyl-D-glucamine (NMDG), high-K^+ media by replacing Na^+ with K^+ without changing the osmolarity.

Radioisotopes

^{22}Na (33 GBq mg^{-1}) and ^{36}Cl (0.43 MBq mg^{-1}) were obtained from the Radiochemical Centre, Amersham, England, ^3H_2O (5.5 MBq ml^{-1}) from the Commissariat à l'Energie Atomique, Saclay, France, and [^{14}C]dextran (0.1 MBq mg^{-1}) from the New England Nuclear, Boston, USA.

Unidirectional ion fluxes

Measurement of amiloride-sensitive Na^+ flux

Na^+/H^+ exchange was assessed by measuring the cellular uptake of ^{22}Na in the presence and in the absence of amiloride or one of its derivatives: DMA (dimethylamiloride) and MPA (methylpropyl-amiloride). These drugs (from Merck, Sharpe and Dohme), known to be relatively specific inhibitors of the Na^+/H^+ antiporter in many cells, were used at appropriate concentrations.

After ^{22}Na had been added to the medium, samples of the cell suspension were taken at defined intervals to determine the radioactivity accumulated into the hepatocytes (Bianchini *et al.* 1988). The initial flux could be estimated from the initial rate of ^{22}Na accumulation, in practice during the first 5 min of incubation. Na^+ influx was expressed per milligram of protein to allow for comparisons between samples.

Radioactive pulse experiments

Unidirectional influx experiments were performed according to the previously reported 'pulse' method (Bianchini *et al.* 1988). At timed intervals (between 0 and 120 min), cells were added to a solution containing ^{22}Na or ^{36}Cl to measure uptake during an exposure period of 1 min. In low-Na^+ medium, cells were washed twice with the substitution medium just before the start of the experiment.

Cell water measurements

Intracellular water content (as an estimate of cell volume) was determined by using a double radioisotopic procedure with ^3H_2O (0.55 MBq ml^{-1}) to label the total water volume and [^{14}C]dextran (0.05 MBq ml^{-1}) to determine the extracellular space, as previously described (Bianchini *et al.* 1988).

'True' hypertonic challenge was made by adding sucrose to H-MEM to raise the osmolarity, *p*, to 410 mosmol l^{-1} (i.e. Δp=+80 mosmol l^{-1}). To produce 'pseudo-hypertonic' conditions (the term used by Lewis and Donaldson, 1990), hepatocytes were exposed to a hypotonic medium (one-third dilution: 2 parts H-MEM to 1 part H_2O, see Bianchini *et al.* 1988) for a period of 30 min and then transferred to isotonic H-MEM after centrifugation to collect the cells.

Measurement of intracellular pH

We made use of the fluorescent dye 2,7-bis(carboxyethyl)-carboxyfluorescein, BCECF-AM (Sigma), which is highly sensitive to changes in pHi and provides continuous monitoring of intracellular pH.

The general procedure applied was adapted from the method of Restrepo *et al.* (1988). The isolated cells were loaded by exposure to the dye (2 μmol l^{-1}) for 60 min, then washed and resuspended in fresh unlabelled medium. Fluorescence was measured on samples (3 ml) at 530 nm, with excitation at 500 nm and 450 nm alternatively. Salt or chemical reagents were added as small volumes (<20 μl) of stock solutions in order not to interfere with the light signal by dilution.

At the end of each run, a calibration curve was established. For this purpose, digitonin (Sigma, 50 μg ml^{-1} cell suspension) was added to release BCECF from the incubated cells. By stepwise addition of HCl or NaOH (1 mol l^{-1}) to modify the pH of the suspension, a curve was obtained relating pH to fluorescence. A similar calibration curve was prepared using the K^+/nigericin technique (Thomas *et al.* 1979). The difference between the intracellular (nigericin) and the extracellular (digitonin) calibration curves showed that, in the latter case, pH values were underestimated by 0.025±0.05 unit (mean ± S.E.M., *N*=5). Since the size of the correction was very small and since the main purpose of using BCECF was to detect changes in pHi, the values reported here were not corrected.

Protein

Total protein was assayed using the method of Lowry *et al.* (1951), adapted for a Technicon autoanalyzer, after radioactivity counting and ultrasonic homogenization of the cells.

Results

Amiloride-sensitive Na^+/H^+exchange

Effects of amiloride derivatives

In Hanks' medium, derivatives of amiloride, DMA or MPA (10^{-4} mol l^{-1}), reduced the initial rate of uptake of Na^+ by approximately 30 % (results not shown).

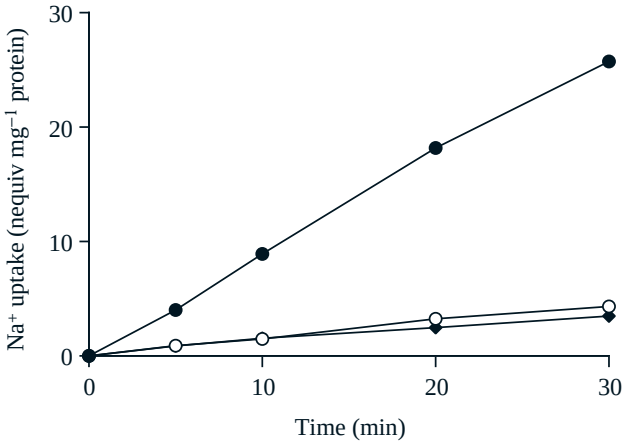

Fig. 1. Uptake of Na^+ by hepatocytes in low-Na^+ medium (16 mmol l^{-1} Na^+) in the absence (control, upper curve) and presence (lower curves) of an amiloride analogue (10^{-4} mol l^{-1}): DMA (○) or MPA (◆). The experiments were repeated three times, the most representative being presented here.

In the presence of lower Na^+ concentrations (≤16 mmol l^{-1}), DMA or MPA, at 10^{-4} mol l^{-1}, reduced the rate of Na^+ influx by 60–77 % (Fig. 1).

Relationship between Na^+ influx and pH

In normal medium (H-MEM, pHe 7.4), the resting pHi was 7.24±0.04 (mean ± S.E.M., N=11), significantly lower than pHe ($0.001<P<0.01$, Student's t-test).

Na^+/H^+ exchange depends on the ability of the cell to extrude protons into the extracellular medium and therefore on the pH gradient prevailing across the membrane. For this experiment, cells equilibrated at pH 7.4 were exposed to an extracellular pH (pHe) varying between 6.5 and 8 (Fig. 2). Although pHi was not measured, the pH gradient (or proton gradient) was obviously retained and ensured a corresponding, sustained Na^+ uptake. The rate of uptake revealed differences between the four curves of ^{22}Na accumulation even after 30 min of incubation. As expected, accumulation of Na^+ was greater at alkaline than at acidic pHe.

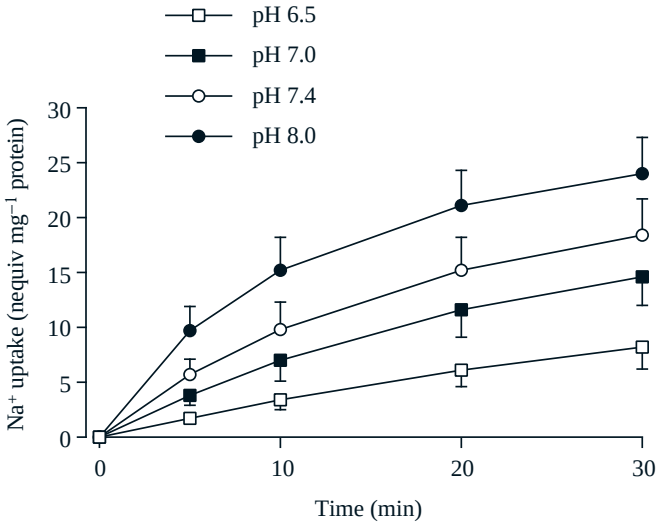

Fig. 2. Uptake of Na^+ by hepatocytes at various values of extracellular pH (pHe from 6.5 to 8). Values are means ± S.E.M. for five experiments.

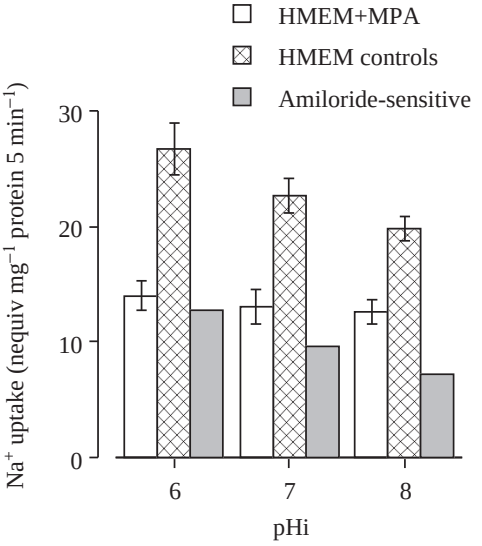

Fig. 3. Uptake of Na^+ by hepatocytes clamped at three different values (6, 7, 8) of intracellular pH (pHi). For each pH value, amiloride-sensitive Na^+ uptake was calculated by difference from control HMEM and HMEM+MPA (10^{-4} mol l^{-1}) values. Values are means ± S.E.M. for four experiments.

Similarly, Na^+ uptake was expected to vary with pHi. To achieve a preset intracellular pH, pHi was initially clamped at three different values (6, 7 and 8) by exposing hepatocytes for 10 min to nigericin (10 μmol l^{-1}) in the presence of 140 mmol l^{-1} K^+. The cells were then removed and transferred to medium at pHe 7.4. Na^+ influx was measured in the presence or absence of MPA (10^{-4} mol l^{-1}) during a period of 5 min. Na^+/H^+ exchange diminished when pHi was raised (Fig. 3).

Na^+/H^+ exchange and osmotic shrinkage

Cell content

In isotonic conditions, isolated trout hepatocytes maintain an intracellular water content of 3.10±0.03 μl mg^{-1} protein (mean ± S.E.M., N=15; Bianchini *et al.* 1988).

True hypertonic stress was achieved by direct transfer of hepatocytes to a hypertonic medium (Δp=+80 mosmol l^{-1}). In this medium, the cells shrank immediately (Fig. 4); the maximum volume decrease occurred within 10 min, and cells reached 72 % of their control volume (significantly different from control, $0.01<P<0.02$, Student's t-test, N=4). After 20 min in hypertonic medium, a regulatory volume increase (RVI) began, at a slow rate, and the cells returned to 90 % of their initial volume after approximately 2 h.

In the 'pseudo-hypertonic' conditions, trout hepatocytes were first exposed to a hypotonic medium (220 mosmol l^{-1}). We observed an initial rapid increase in cell water content (Fig. 5), followed by a shrinkage phase of RVD as described by

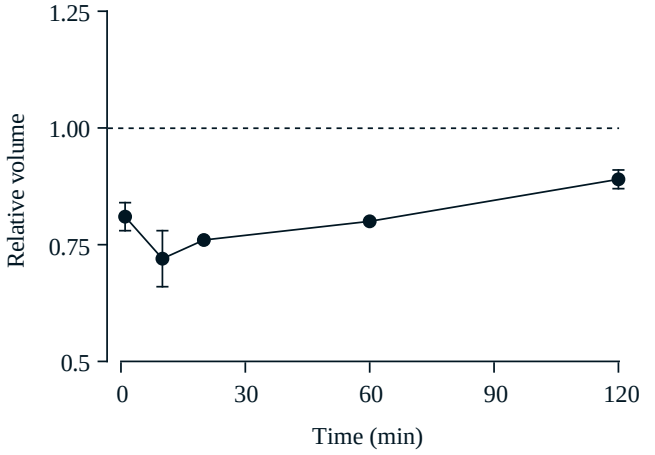

Fig. 4. Relative changes in cell water content (relative volume) of hepatocytes in hypertonic conditions, Δp=+80 mosmol l^{-1}. The water content of cells continuously incubated in isotonic medium is taken as unity. Values are means ± S.E.M. for four experiments. Where not illustrated, error bars were equal to or smaller than symbols.

Bianchini *et al.* (1988), and the volume returned to its original value after 30 min. Restoring isotonicity at this stage resulted in a second phase of volume modification. There was a rapid shrinkage to 80 % of the isotonic control volume, followed by a steady volume increase towards the control value. The rate of volume recovery was slow and similar to that observed in true hypertonic conditions.

Ionic fluxes

Because we found that RVI was identical in both conditions described above, the associated ionic events were studied during the true hypertonic challenge (Δp=+80 mosmol l^{-1}).

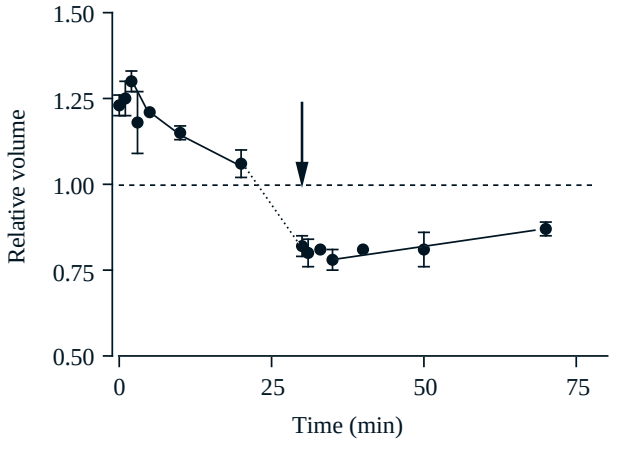

Fig. 5. Relative changes in cell water content (relative volume) of hepatocytes in 'pseudo-hypertonic' conditions. Cells were exposed to hypotonic medium (isotonicity × 0.70) for 25 min, then centrifuged and transferred to isotonic medium (at the arrow). The water content of cells maintained in isotonic medium is taken as unity. Values are means ± S.E.M. for four experiments. Where not illustrated, errors bars were equal to or smaller than symbols.

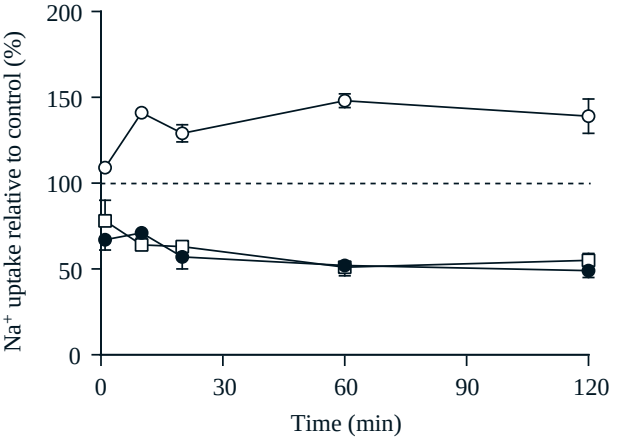

Fig. 6. Time course of Na^+ uptake in hypertonic medium (○) and in the presence of amiloride (10^{-3} mol l^{-1}) in isotonic H-MEM (□) or hypertonic conditions (●). The uptake of isotope during a labelling period of 1 min is expressed as percentage of the mean uptake by hepatocytes from the same preparation kept in isotonic conditions. Values are means ± S.E.M. for four experiments. Where not illustrated, errors bars were equal to or smaller than symbols.

Na^+ uptake. When hepatocytes were transferred to hypertonic medium, Na^+ uptake (measured in 'pulse' experiments) increased during the first hour, up to 148±4 % (N=4) of the control influx in isotonic conditions (taken as 100 %) (Fig. 6). In the presence of amiloride (10^{-3} mol l^{-1}), the Na^+ uptake was significantly lower than under control conditions; no increase was observed in hypertonic medium.

Intracellular $[H^+]$. The pHi measured with BCECF increased gradually after addition of sucrose to the incubation medium, as a result of export of H^+ from cells in hypertonic conditions. This effect was abolished in the presence of amiloride 10^{-3} mol l^{-1} (Fig. 7).

Cl^- uptake. Cells subjected to a hypertonic medium demonstrated a higher Cl^- uptake than in isotonic conditions.

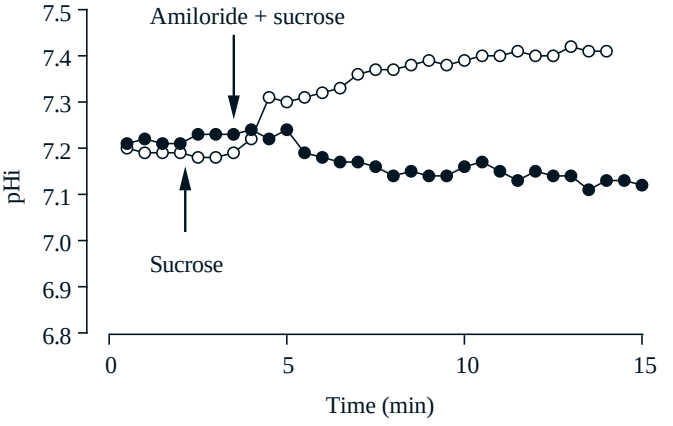

Fig. 7. Effect of hypertonic challenge (sucrose) on the intracellular pH of hepatocytes in control conditions (○) or in the presence of 10^{-3} mol l^{-1} amiloride (●). The experiments were repeated three times, the most representative being presented here.

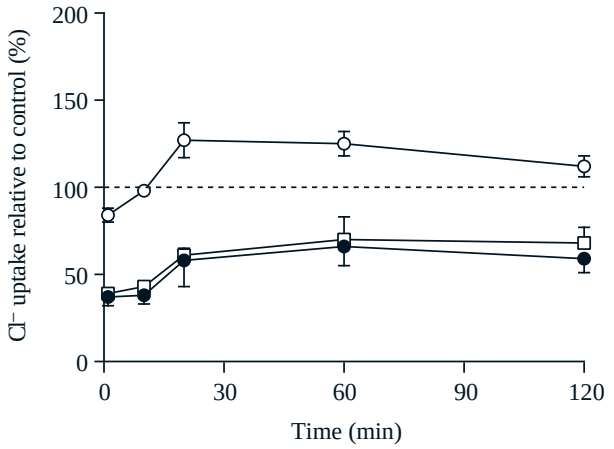

Fig. 8. Time course of relative uptake of Cl^- in hypertonic medium (○, N=7) and in the presence of DIDS (10^{-4} mol l^{-1}, N=4) in isotonic H-MEM (□) or hypertonic conditions (●). The uptake of isotope during a labelling period of 1 min is expressed as a percentage of the mean uptake by hepatocytes from the same preparation kept in isotonic conditions. Values are means ± S.E.M. Where not illustrated, errors bars were equal to or smaller than symbols.

The flux rate increased to 127±10 % (N=7) after 20 min ($0.02<P<0.05$, Student's t-test, control value taken as 100 %). DIDS caused an inhibition of Cl^- fluxes in both isotonic and hypertonic media. Blockade of the hypertonicity-induced ^{36}Cl flux was complete (Fig. 8).

In the same conditions, the $Na^+/K^+/2Cl^-$ cotransport inhibitor bumetanide (10^{-4} mol l^{-1}) had no effect on the increased Cl^- flux (Fig. 9). However at 20 and 60 min, in the presence of bumetanide, the differences in Cl^- uptake between hypertonic and control medium were highly significant ($0.001<P<0.01$).

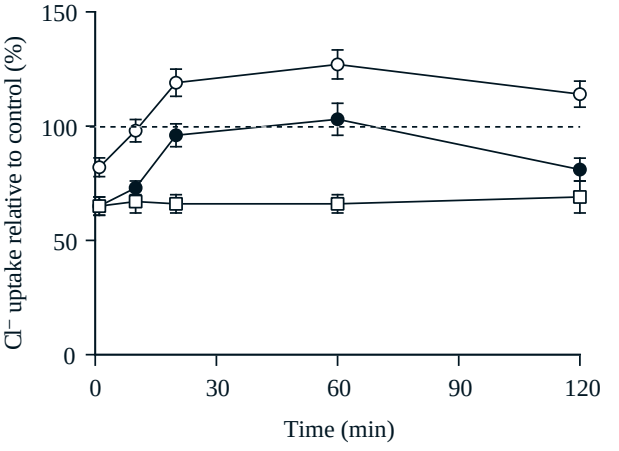

Fig. 9. Time course of Cl^- uptake in hypertonic medium (○) and in the presence of bumetanide (10^{-4} mol l^{-1}) in isotonic H-MEM (□) or hypertonic conditions (●). The uptake of isotope during a labelling period of 1 min is expressed as a percentage of the mean uptake by hepatocytes from the same preparation kept in isotonic conditions. Values are means ± S.E.M. for four experiments.

Discussion

In fish, changes in physiological state, levels of exercise and environment disturb the acid–base balance of the animal, affecting both extracellular and intracellular pH. Salmonids in particular (such as the rainbow trout) are very sensitive to these variations. A number of authors have convincingly argued that ionic transport taking place across the gills to ensure osmoregulation also provides the basic mechanism by which the extracellular pH is adjusted in the blood (see Avella and Bornancin, 1990). At the cellular level, Na^+/H^+ exchange has been shown to be the most efficient mechanism for extruding protons from the cytoplasm of many eukaryotic cells and thus plays a major role in the regulation of pHi (Moolenar, 1986). Since the exchange is between the cell and its immediate extracellular environment, the two measures of pH regulation, cellular and organismic, may be expected to be linked. However, in red blood cells of the trout, Motais *et al.* (1992) have shown that the exchanger cannot regulate pHi, for Na^+/H^+ exchange remains inactivated even when pHi drops to as low as 6.3. Instead, these authors have proposed that the trout erythrocyte combines this ion exchange with molecular shifts in haemoglobin to regulate the extracellular pH of the whole fish.

Intracellular pH determination by the BCECF method shows that pHi is only slightly different from pHe in these fish. In physiological conditions (pHe 7.4–7.8), pHi is lower than pHe by approximately 0.2 unit. Using the DMO method, in parallel with BCECF, Walsh (1989) measured a similar value in toadfish and also in the eel *Anguilla rostrata* (Walsh and Moon, 1983).

It is usually held that protons are at a lower electrochemical potential inside the cells than outside, in such a way that they should be extruded by an active process, whether direct or indirect (Roos and Boron, 1981). For an external pH of 7.4, if protons were in electrochemical equilibrium, pHi would be 6.4 in most cells (membrane potential −60 mV; Frelin *et al.* 1988) or 6.8 in fish hepatocytes (membrane potential −39 mV in *Raja erinacea*, Wondergem *et al.* 1985; membrane potential −34 mV in *Opsanus beta*, Walsh, 1989). However, these calculated values are lower than those actually determined. In isolated trout hepatocytes, we have measured the membrane potential using several methods (radioactive probes, patch-clamp) and found no values larger than −10 mV (J. Porthé-Nibelle and B. Fossat, unpublished observations). Using this figure and within the limits of experimental error, the calculated difference between pHi and pHe would be only 0.2 unit, which is what we observed using the nigericin technique (results not shown). Therefore, we consider that trout hepatocytes are normally in equilibrium under physiological conditions and are not driven to extrude H^+.

However, as in other cell models, the system is not in equilibrium when pHi is disturbed. This is revealed by modifying pHe (Fig. 2) or pHi (Fig. 3). When pHi was not clamped, recovery was relatively fast (in 10–15 min) provided that Na^+ was present at a concentration of 20 mmol l^{-1} or greater (see Results).

The use of amiloride derivatives specific for the inhibition of Na^+/H^+ exchange confirmed the presence of this antiporter in trout hepatocytes. This antiporter has also been detected in toadfish cells (Walsh, 1989).

The major problem is to determine what cellular or systemic functions the Na^+/H^+ exchange serves in fish. In addition to the regulation of pHi, shown by many cell types except trout red blood cells, Na^+/H^+ exchange is presumably useful in a more specific way for fish such as the rainbow trout that live in a freshwater environment. Unlike sea water, this fresh water is not buffered and therefore may impose direct constraints (e.g. by chemicals or acid rain) on the extracellular pH of fish by disturbing gill ion transport.

Also, in any environment, fish behaviour and metabolism may produce alterations in acid–base balance. For example, during exercise stress, *in vivo* studies have shown that the extracellular pH, but not pHi, was greatly affected in the liver of rainbow trout (Milligan and Wood, 1986, 1987). Isolated hepatocytes of toadfish (*O. beta*) exposed to high concentrations of lactate in the medium displayed a strict regulation of pHi: this regulation was impaired in the presence of $0.5\,mmol\,l^{-1}$ amiloride (Walsh, 1989). In the complex relationships detected between pHe, pHi and metabolism, 'hepatic' tissues represent a site of diversity in vertebrates and invertebrates (Walsh and Mommsen, 1992).

Na^+/H^+ exchange is also currently thought to play a role in cell volume regulation, although the causal relationships between the two phenomena are not clear. In isotonic medium, trout erythrocytes swell when they are presented with catecholamines and remain swollen as long as the hormone remains in the medium. This effect is mediated by β-receptors and results from Na^+/H^+ and Cl^-/HCO_3^- exchanges acting in parallel (Baroin *et al.* 1984) and leading to the intracellular accumulation of NaCl followed passively by water. In these conditions, Na^+/H^+ exchange occurs *via* a β-NHE transporter (Motais *et al.* 1990).

When transferred to hypertonic medium, cells behave like perfect osmometers and, after a shrinking phase, restoration of cell volume (RVI) occurs by activation of ionic transport. However, RVI does not always occur in these conditions, and in most cell types only a 'pseudo-hypertonic' RVI is observed. This phenomenon has been described, for example, for human lymphocytes (Grinstein *et al.* 1984), Ehrlich ascites tumour cells (Hoffmann and Simonsen, 1989; Pedersen *et al.* 1996), rat hepatocytes (Corasanti *et al.* 1990), frog red blood cells (Jorgensen, 1995) and eel erythrocytes (Gallardo *et al.* 1996). In the present study, we observed that isolated trout hepatocytes showed a slow and partial RVI following either hypertonic or 'pseudo-hypertonic' treatment.

Two mechanisms increasing intracellular osmolarity by way of NaCl accumulation during RVI have been described: $Na^+/K^+/2Cl^-$ cotransport or Na^+/H^+ and Cl^-/HCO_3^- exchange (for reviews, see Al-Habori, 1994; Baumgarten and Feher, 1995; Hoffman and Dunham, 1995). In trout hepatocytes, amiloride-sensitive Na^+ uptake, DIDS-sensitive, but not bumetanide-sensitive, Cl^- uptake and alkalisation of intracellular pH suggest activation of a coupled Na^+/H^+ and Cl^-/HCO_3^- cotransport. Such a mechanism has been reported in urodele (*Amphiuma*) red blood cells (Cala, 1985), dog red blood cells (Parker, 1983), human lymphocytes (Grinstein *et al.* 1984), rat hepatocytes (Graf *et al.* 1988; Corasanti *et al.* 1990) and Ehrlich ascites tumour cells (Pedersen *et al.* 1996).

The mechanism whereby cell shrinkage induces Na^+/H^+ activity is not clear. Dascalu *et al.* (1992) described the involvement of actin and calmodulin-like elements of the cytoskeleton in hyperosmotic activation of Na^+/H^+ exchange in an osteoblastic cell line, but no role was found for cyclic AMP, cyclic GMP, cytosolic Ca^{2+} and protein kinase C (PKC). Davis *et al.* (1992) suggested that, in barnacle muscle fibres, the stimulation of Na^+/H^+ exchange by shrinkage involves the activation of a G-protein that acts *via* neither cyclic AMP nor PKC.

A model for Na^+/H^+ exchange has been proposed by Grinstein *et al.* (1992*a*) involving protein kinases and phosphorylation in RVI and protein phosphatases and dephosphorylation in RVD. Bianchini *et al.* (1991) have reported activation of the Na^+/H^+ antiporter in rat thymic lymphocytes and human carcinoma cells in the presence of okadaic acid, a potent inhibitor of phosphatases 1 and 2A. However, Grinstein *et al.* (1992*b*) concluded that an indirect activation of the antiporter occurred, not requiring direct phosphorylation. In hyperosmotic conditions, changes in protein phosphorylation of a nuclear protein similar to histones have been reported in endothelial cells (Santell *et al.* 1993). In a recent review, Grinstein *et al.* (1994) discussed the possibility of an interaction between auxiliary proteins (cytoskeletal or other proteins) and the antiporter NHE-1, the isoform seemingly involved in RVI. In Ehrlich cells, Pedersen *et al.* (1996) reported that shrinkage-induced activation of the Na^+/H^+ antiporter is dependent on the presence of ATP and involves phosphorylation by PKC or a similar protein kinase.

The role of macromolecular crowding and confinement in volume regulation of cells exposed to hypertonicity has been reported in red blood cells (Garner and Burg, 1994), but in more complex cells such as hepatocytes this role is still doubtful.

Is Ca^{2+} a transduction signal involved in RVI? In a review, McCarty and O'Neil (1992) reported a variable role for this ion in the control of hypotonic cell volume regulation and RVD, but in hypertonic conditions Ca^{2+} did not seem to be involved. Similarly, Pedersen *et al.* (1996) concluded that intracellular Ca^{2+} plays no part in Na^+/H^+ activation during a hypertonic challenge.

In conclusion, in trout hepatocytes, Na^+/H^+ exchange is activated when cells are hyperosmotically stressed and may contribute to cell volume regulation. The mechanism involved is to be investigated further.

This work was supported by the Centre National de la Recherche Scientifique (ERS 78) and aided in part by the Commissariat à l'Energie Atomique. The authors wish to thank Professor J. C. Ellory for helpful discussion. During this work,

S.P. was an Erasmus exchange student from the Laboratory for Biological Chemistry, August Krogh Institute, University of Copenhagen, Denmark.

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