The Journal of Experimental Biology 200, 1499–1508 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JEB0660

Na^+ AND Ca^{2+} HOMEOSTATIC MECHANISMS IN ISOLATED CHLORIDE CELLS OF THE TELEOST *OREOCHROMIS MOSSAMBICUS* ANALYSED BY CONFOCAL LASER SCANNING MICROSCOPY

JIE LI, JELLE EYGENSTEYN, ROBERT A. C. LOCK, SJOERD E. WENDELAAR BONGA AND GERT FLIK*
Department of Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Accepted 26 February 1997

Summary

Chloride cells (CCs; recognised by the vital mitochondrial stain DASPEI) and pavement cells (PCs) isolated from tilapia opercular epithelium were adhered to Cell-Tak-coated glass coverslips and loaded with fluorescent probes for the measurement of intracellular concentrations of Na^+ or Ca^{2+}. Basal levels of cytosolic Na^+ and Ca^{2+} ranged from 6.4 to 16.5 mmol l^{-1} and from 76 to 110 nmol l^{-1}, respectively, and did not differ between CCs and PCs. In CCs, inhibition of Na^+/K^+-ATPase by ouabain or Cu^{2+} increased intracellular $[Na^+]$. Replacing extracellular Na^+ with *N*-methyl-D-glucamine$^+$ led to a rise in cytosolic $[Ca^{2+}]$ that was dependent on the extracellular $[Ca^{2+}]$, indicating that a Na^+/Ca^{2+} exchanger was operating in reverse mode (importing Ca^{2+}). The forward mode of this exchanger could be demonstrated by inhibition with bepridil. The CC has various pathways for passive Na^+ influx: a tetrodotoxin-sensitive pathway, an amiloride-sensitive pathway and other as yet unidentified pathways.

Key words: tilapia, *Oreochromis mossambicus*, chloride cell, pavement cell, Na^+/Ca^{2+} exchange, Na^+/K^+-ATPase, homeostasis.

Introduction

Freshwater fish take up Na^+ and Ca^{2+} from the water *via* their gills (Lin and Randall, 1995; Flik *et al.* 1995). In the branchial epithelium, two cell types are implicated in ion transport: specialised ion-transporting cells, the so-called chloride cells (CCs; Foskett and Scheffey, 1982; Foskett *et al.* 1983; McCormick, 1995) and the pavement cells (PCs; see Lin and Randall, 1995). CCs are located mainly in the filamental epithelium. These cells are structurally characterized by a pit or crypt formed by the apical plasma membrane and by an abundance of mitochondria in association with an extensive tubular system of basolateral plasma membrane invaginations (Wendelaar Bonga *et al.* 1990; Li *et al.* 1995; Van der Heijden *et al.* 1997). A specific role in Na^+ uptake has been attributed to the PCs of the gills (Goss *et al.* 1992; Morgan *et al.* 1994): in these cells, an apically located H^+-ATPase may provide a proton-motive force for Na^+ influx *via* amiloride-sensitive channels (Lin and Randall, 1995). Cl^- is taken up specifically *via* the CCs (Marshall, 1995). This working model for Na^+ and Cl^- transport in fish gills is analogous to that proposed for the frog skin (Ehrenfeld *et al.* 1990; Potts, 1994), where the independent uptakes of Na^+ and Cl^- have been attributed, respectively, to skin cells and the mitochondria-rich cells. Although the branchial epithelium of fish may be anatomically reminiscent of frog skin, physiological or biochemical evidence for this model in fish gills is as yet fragmentary and circumstantial; the proton pump is also present in CCs, and the target for amiloride has not been specifically localised (Lin and Randall, 1995). Moreover, as yet, very few species (mainly salmonids) have been studied. Na^+ from the water that enters the cells of the branchial epithelium is extruded across the basolateral plasma membrane by the ubiquitous Na^+/K^+-ATPase. Consensus exists that the CC mediates the transport of Ca^{2+} (see McCormick, 1995). Ca^{2+} enters the CC down a steep electrochemical gradient, *via* stanniocalcin-sensitive Ca^{2+} channels, and is extruded from the cell to the blood *via* energized Ca^{2+} pumps (Flik *et al.* 1995), a Ca^{2+}-ATPase and/or a Na^+/Ca^{2+} exchanger (Verbost *et al.* 1994*b*).

The entry of Na^+ and Ca^{2+} from the water into the cell is considered to be the rate-limiting step of the transepithelial transport; the extrusion mechanisms must, considering their kinetic properties *in vitro*, operate *in vivo* far below their maximum capacity (Flik and Verbost, 1993). It thus seems that the idea of homeostasis of intracellular free Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) concentrations is warranted. The total concentrations of Na^+, Cl^- and Ca^{2+} in PCs and CCs have recently been reported, and these may vary in response to experimental manipulation of ion transport (Morgan and Potts, 1995). Data on free ion concentrations in gill cells have not previously been reported. This may relate to the difficulty of getting living gill cells to stick to glass coverslips, a prerequisite for the type of studies presented here.

It is generally assumed that the CC in the opercular

*Author for correspondence (e-mail: gertflik@sci.kun.nl).

membrane may serve as a model for the CC in the gills (Wendelaar Bonga *et al.* 1990; Marshall *et al.* 1992). As the opercular epithelium is less complex than the branchial epithelium, we isolated, plated and identified CCs and PCs of tilapia (*Oreochromis mossambicus*) opercular epithelium. We measured cytosolic $[Na^+]_i$ and $[Ca^{2+}]_i$ using specific fluorescent probes and confocal laser scanning microscopy. Some homeostatic mechanisms were studied in CCs by manipulating the entry and extrusion routes for Na^+ and Ca^{2+}, with an emphasis on the roles of the Na^+/K^+-ATPase and the Na^+/Ca^{2+} exchanger. Cu^{2+}, an inhibitor of Na^+/K^+-ATPase activity (Li *et al.* 1996) and of branchial Na^+ influx (Laurén and McDonald, 1987), was tested for its effects.

Materials and methods

Materials

Opercular epithelium was obtained from adult (75–100 g) Mozambique tilapia (*Oreochromis mossambicus* Peters), kept in Nijmegen tapwater at 26 °C. Reagent-grade chemicals, enzymes, ionophores, blockers, culture media and antibiotics were purchased from Sigma. Cell-Tak adhesive was from Bicton Dickinson Labware, Bedford, MA, USA. Fluorescent probes and the detergent pluronic F127 were from Molecular Probes, Eugene, OR, USA.

Isolation of opercular epithelial cells

Fish were quickly anaesthetised with phenoxy-ethanol (2 ml l^{-1}) and killed by transection of the spinal cord. Opercular epithelium was carefully separated from the underlying connective tissue and chopped with a razor blade into small segments (approximately 3 mm×3 mm). The chopped tissue was incubated for 30 min at room temperature (22 °C) in 10 ml of isolation medium, while being gently agitated. The isolation medium contained (in mmol l^{-1}): NaCl, 125; $NaHCO_3$, 10; K_2HPO_4, 3; $MgCl_2$, 1; $CaCl_2$, 1; aprotinin, 0.2 trypsin inhibitor units ml^{-1}; dithiothreitol, 0.1; Hepes, 10; adjusted to pH 7.4 with Tris, supplemented with glucose, 10 (Verbost *et al.* 1994*a*). After the incubation, cells were isolated from their tissue context by gently passing the tissue suspension through a wide-bore pipette. Increasing opacity of the suspension indicated the progress of the isolation procedure. The suspension was passed through cheesecloth (mesh size 100–200 μm) to remove larger tissue fragments; in this way, we also removed mucus that adsorbed to the filter cloth. The resulting cell suspension was centrifuged at low speed (125 ***g***, 5 min), and the medium was replaced with a physiological salt solution (PSS) containing (in mmol l^{-1}): NaCl, 125; $NaHCO_3$, 10; NaH_2PO_4, 1; KCl, 3; $MgSO_4$, 2; $CaCl_2$, 1.8; glucose, 10; and Hepes, 10; adjusted with Tris to pH 7.4). The loosely pelleted cells were resuspended in 2 ml of PSS.

Plating of the cells

Isolated cells were allowed to adhere to round (diameter 22 mm) glass coverslips, coated with the polyphenolic protein adhesive Cell-Tak (Waite and Tanzer, 1981). Just before plating, 3 μl of Cell-Tak (1.3 mg ml^{-1} in 5 % acetic acid) was applied as a thin liquid film over the coverslip, and the acetic acid was allowed to evaporate. The coverslips were then rinsed with distilled water and dried at room temperature. Next, 500 μl of cell suspension (equivalent to 10×10^3 to 50×10^3 cells) was placed on each coverslip and left undisturbed for 15 min at room temperature under atmospheric conditions to allow the cells to adhere. Subsequently, non-adhering cells were removed by rinsing the coverslips with PSS. The coverslips with cells were then stored in L-15 Leibovitz culture medium supplemented with penicillin (100 i.u. ml^{-1}), streptomycin (100 μg ml^{-1}) and 10 % foetal calf serum (FCS). Over 90 % of the plated cells were viable, as routinely assessed 6 h after isolation by a Trypan Blue exclusion test.

Identification of CCs

Using phase-contrast optics, the CCs among the isolated cells were identified as large (diameter 10–20 μm), spherical to ovoid, finely granular cells with a centrally located nucleus. This identification was confirmed by staining the cells with the fluorescent vital stain for mitochondria, DASPEI (excitation at 488 nm and emission at 530 nm), using a Bio-Rad MRC-600 confocal laser scanning microscope (Li *et al.* 1995).

Determination of $[Na^+]_i$ and $[Ca^{2+}]_i$

Plated cells were loaded in the dark with the cell-permeant fluorescent probes Sodium Green (tetra-acetate salt), for the measurement of $[Na^+]_i$ (Amarino and Fox, 1995), and Fluo-3 or Fura Red, for the measurement of basal $[Ca^{2+}]_i$. Fura Red fluorescence decreases with increasing $[Ca^{2+}]_i$. Cells were loaded by exposure to the AM esters of these probes. Coverslips with the plated cells were placed in PSS, containing 1 μmol l^{-1} pluronic F127 and either Sodium Green (10 μmol l^{-1}) or one of the Ca^{2+} probes (5 μmol l^{-1} for Fluo-3 and 6 μmol l^{-1} for Fura Red). After loading with probe for 60 min at room temperature, the cells were washed three times with PSS to remove the extracellular probe and detached cells. Next, the coverslip was mounted at the bottom of a microscope chamber filled with 1 ml of PSS. The chamber was placed on the stage of an inverted microscope (Nikon, Diaphot, Tokyo, Japan) equipped with a 60× oil-immersion objective (numerical aperture 1.4). Excitation of probes at 488 nm was achieved by directing the light from a 15 mW argon ion laser through an excitation filter (488 DF 10). To reduce bleaching of fluorescent probes, the laser emission was attenuated to 10 % by introducing a neutral density filter. For Fluo-3 and Sodium Green, the 'BHS' block was used: fluorescent light was passed through a dichroic reflector (DR 510 LP) and a barrier filter (OG 515), and the emission at 530 nm was measured. For Fura Red, a combination of the BHS and the A2 block was used, with a dichroic reflector (DR 565 LP) and a long-pass filter (EF 600 LP), to collect the emission at 609 nm. Background fluorescence was continuously monitored and (automatically) corrected.

Optical sections of approximately 1 μm in the *z*-direction were obtained by opening the confocal pinholes to

approximately 50 % of their maximum setting. Fluorescence intensities were recorded with an AT-compatible 486-66 MH2 computer, equipped with time-course radiometric software (Bio-Rad Microscience), and all fluorescent recordings were stored on hard and optical discs for further analysis.

For calibration of basal $[Na^+]_i$, maximum fluorescence (F_{max}) was measured after 20 μmol l^{-1} digitonin had been added; the minimum fluorescence (F_{min}) could be equated to the background fluorescence (F_{bkg}). To calculate basal $[Ca^{2+}]_i$, a 'Mn^{2+} calibration' was performed (Kao *et al.* 1989). In short, F_{max} was obtained by addition of the Ca^{2+}-specific ionophore ionomycin (2.5 μmol l^{-1}), followed by addition of $MnSO_4$ (2 mmol l^{-1}) to obtain F_{Mn}; the predicted F_{max} would then be $[(F_{Mn}-F_{bkg})/0.2]+F_{bkg}$. Since the fluorescence intensity of Ca^{2+}-free Fluo-3 is only one-fortieth of that of the Ca^{2+} complex, F_{min} should be $[(F_{max}-F_{bkg})/40]+F_{bkg}$.

Intracellular concentrations of Na^+ or Ca^{2+} ($[X]_i$) were calculated using the equation:

$$[X]_i = K_d(F-F_{min})/(F_{max}-F),$$

where the values for the dissociation constants (K_d) for Na^+/Sodium Green and Ca^{2+}/Fluo-3 were taken as 21 mmol l^{-1} and 400 nmol l^{-1}, respectively.

The data on the changes in fluorescent signals upon manipulation of the cells were normalised to baseline values, which were designated 1.0; values for Fura Red fluorescence were inverted (as Fura Red fluorescence intensity decreases when $[Ca^{2+}]_i$ increases) before being normalised; during the experiments, no significant bleaching of the fluoroprobes was observed (Scheenen *et al.* 1996).

Homeostatic mechanisms in chloride cells

To study homeostatic control mechanisms for $[Na^+]_i$ and $[Ca^{2+}]_i$ of CCs, we measured the changes in the fluorescent signals of the Na^+ and Ca^{2+} probes after interfering with the entry and extrusion routes of Na^+ and Ca^{2+}. Intracellular $[Na^+]$ was manipulated by replacing Na^+ in the medium by equimolar amounts of *N*-methyl-D-glucamine ($NMDG^+$). Ouabain (1 mmol l^{-1}) was used to inhibit Na^+/K^+-ATPase activity. $Cu(NO_3)_2$ (4 μmol l^{-1}) was added to evaluate the effects of Cu^{2+}. Na^+ influx was manipulated by the addition of 2 μmol l^{-1} tetrodotoxin (TTX; Van Driessche and Zeiske, 1985) or 50 μmol l^{-1} amiloride (Zadunaiski *et al.* 1995).

Na^+/Ca^{2+} exchange activity requires the action of Na^+/K^+-ATPase for the maintenance of the Na^+ gradient across the membrane, which drives the extrusion of Ca^{2+} *via* this carrier. Changes in $[Ca^{2+}]_i$ were followed after replacement of extracellular Na^+ with $NMDG^+$, in the presence of high (1.8 mmol l^{-1}) and low (50 μmol l^{-1}) levels of Ca^{2+} in the medium. Under conditions of low Na^+ levels in the external medium, the Na^+/Ca^{2+} exchanger is predicted to operate in its reverse mode (Snowdowne and Borle, 1985). The Na^+/Ca^{2+} exchange blocker bepridil was tested at 50 μmol l^{-1} (Garcia *et al.* 1988). Since bepridil is a partial blocker with relatively low affinity (maximal inhibition 60 %, K_i=70 μmol l^{-1} for the exchanger in enterocyte plasma membranes; Flik *et al.* 1990) and has a lipophilic nature, we assessed membrane integrity when testing its effects. To this end, we used the monomeric cyanine nucleic acid stain TO-PRO-1 (Molecular Probes, T3602). This probe is cell-impermeant and fluoresces (excitation at 488 nm and emission at 515 nm) only upon binding to DNA or RNA. Measurements of dual emissions (for Fura Red and TO-PRO-1) were analysed using a ratiometric, time-course software package (TCSM, Bio-Rad). This allowed us to record the fluorescence signals of Fura Red and TO-PRO-1 in a single cell. TO-PRO-1 (2 μmol l^{-1}) was added to the medium at the start of the experiment.

Statistics

Each experiment was carried out with at least six different batches of cells and, of each batch, at least 10 cells were measured per treatment. The values for changes in fluorescence signals are taken from representative experiments. Values for $[Na^+]_i$ and $[Ca^{2+}]_i$ are the mean ± S.E.M. of at least six experiments. Statistical significance of differences was assessed using Student's *t*-test; significance was accepted when $P<0.01$.

Results

Identification and viability of chloride cells and pavement cells

In the isolated cell population, two prominent cell types could be discerned at first sight: large (diameter 10–20 μm; Fig. 1A) spherical-to-ovoid cells with fine granules and prominent nuclei, and small round cells (diameter <10 μm). Since the larger cells accumulated more DASPEI (Fig. 1B) than any other cell in this suspension, we designated them as CCs (Li *et al.* 1995). The smaller cells in suspension were essentially DASPEI-negative and were therefore designated as pavement cells (PCs). As cell size and degree of granulation alone are not conclusive indicators of cell type, CCs were routinely identified by DASPEI-poststaining.

Basal $[Na^+]_i$ and $[Ca^{2+}]_i$ in chloride cells and pavement cells

The CCs and PCs selected for the measurements of basal $[Na^+]_i$ and $[Ca^{2+}]_i$ showed uniform fluorescence in the cytoplasmic compartment for each of the probes tested. The Na^+ ionophore gramicidin-D (5 μg ml^{-1}; Ahlemeyer *et al.* 1992) did not induce maximum fluorescence of Sodium Green, but addition of digitonin (20 μmol l^{-1}) increased the fluorescence to a maximum (data not shown). $[Na^+]_i$ in cells at rest is 12.6±3.9 mmol l^{-1} for PCs and 10.7±4.3 mmol l^{-1} for CCs and was not significantly different between these two cell types. Basal $[Ca^{2+}]_i$ did not differ ($P>0.15$) between PCs and CCs and was 89±13 and 95±15 nmol l^{-1}, respectively (Table 1). The values for resting levels of intracellular Na^+ and Ca^{2+} concentration in PCs and CCs are in line with observations on a variety of cells from vertebrate species (Negulescu *et al.* 1990; Ahlemeyer *et al.* 1992; Petersen *et al.* 1994) and confirm the viability of our cells in primary culture.

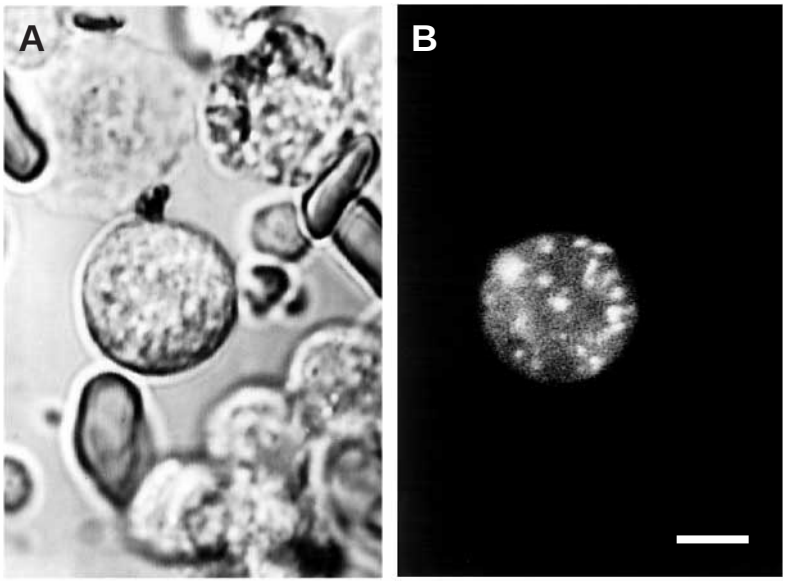

Fig. 1. Identification (using a confocal laser scanning microscope) of a mitochondria-rich cell (chloride cell) isolated from opercular epithelium, after vital staining of the plated suspension with DASPEI. (A) Opercular epithelial cells shown with light transmission microscopy. (B) Mitochondria-rich cell demonstrated by DASPEI fluorescence in the same cell preparation. Note the specific sequestering of the DASPEI in the mitochondria. Scale bar, 5 μm.

We then focused our studies on the mechanisms involved in maintaining intracellular levels of Na^+ and Ca^{2+}, specifically in the CCs.

Manipulating Na^+/K^+-ATPase activity

Effects of ouabain

Fig. 2 shows that a constant $[Na^+]_i$ in a CC depends on the activity of Na^+/K^+-ATPase: Na^+ efflux became inhibited approximately 10 min after the cells had been exposed to ouabain (1 mmol l^{-1}); this was indicated by an increase in the Sodium Green signal, reflecting an increasing $[Na^+]_i$. Intracellular $[Na^+]$ was further and maximally increased after the cell was rendered permeable by the addition of digitonin. Replacement of extracellular Na^+ by $NMDG^+$ in the presence of extracellular K^+ led to a larger decrease in the basal Sodium Green signal than that observed in the absence of extracellular K^+ (data not shown), providing further evidence that Na^+ efflux is mediated by a Na^+/K^+-ATPase.

Effects of Cu^{2+}

After exposure of the cells to $Cu(NO_3)_2$ for 5–15 min, the Sodium Green signal increased, indicating an increase in $[Na^+]_i$. Addition of *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a membrane-permeant chelator with great affinity for Cu^{2+} (Arslan *et al.* 1985), reversed the rise in $[Na^+]_i$ caused by Cu^{2+} within approximately 15 min, albeit only partly (Fig. 3).

Table 1. *Basal concentrations of intracellular Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) in isolated chloride cells and pavement cells from opercular epithelium of tilapia*

	$[Na^+]_i$ (mmol l^{-1})	$[Ca^{2+}]_i$ (nmol l^{-1})
Chloride cells	10.7±4.3 (20)	95±15 (20)
Pavement cells	12.6±3.9 (25)	89±13 (25)

Values are means ± S.E.M.; the number of cell suspensions tested is given in parentheses.

Manipulation of Na^+/Ca^{2+} exchange activity

Evidence for Na^+/Ca^{2+} exchange activity in CCs is shown in Figs 4 and 5. Replacement of extracellular Na^+ by $NMDG^+$ increased the values for normalised fluorescence (an absolute decrease in Fura Red fluorescence occurs under these conditions), indicating an increase in $[Ca^{2+}]_i$. This rise in $[Ca^{2+}]_i$ was greater in the presence of 1.8 mmol l^{-1} extracellular Ca^{2+} (Fig. 4A), than in the presence of 50 μmol l^{-1} extracellular Ca^{2+} (Fig. 4B). Re-introduction of Na^+ into the medium (replacing the medium with PSS) restored the basal Fura Red signal. Ionomycin, a specific Ca^{2+} ionophore, induced a strong Ca^{2+} influx, which was reversed by the addition of 2 mmol l^{-1} EGTA (Fig. 4A,B).

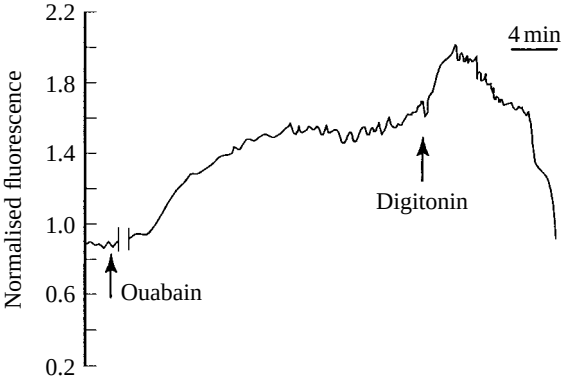

Fig. 2. Effect of ouabain on intracellular Na^+ concentration ($[Na^+]_i$) in an isolated chloride cell. The fluorescence intensity of Sodium Green started to increase approximately 10 min (represented by the break in the trace) after the addition of 1 mmol l^{-1} ouabain. Addition of 20 μmol l^{-1} digitonin further and maximally increased the fluorescence intensity. Values for fluorescence intensity were normalised and are presented in arbitrary units; baseline fluorescence was designated as 1.0.

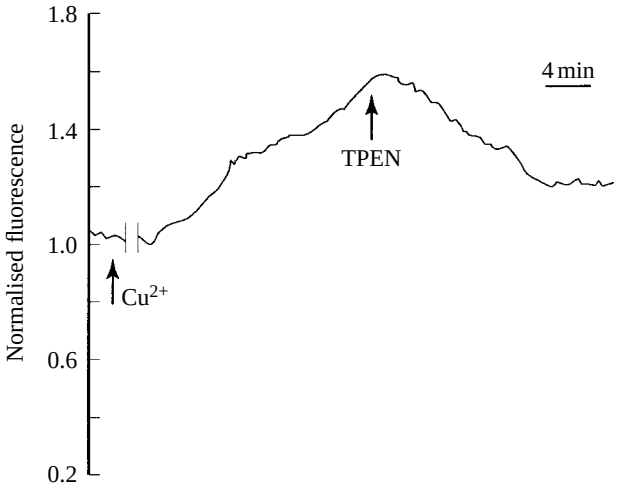

Fig. 3. Effects of copper (Cu^{2+}) on Sodium Green fluorescence of an isolated chloride cell and the effect of the addition of the cell-permeant Cu^{2+} chelator TPEN. Following addition of $Cu(NO_3)_2$ (4 μmol l^{-1}), the Sodium Green fluorescence started to increase after approximately 10–15 min (indicated by the break in the trace). Subsequent addition of 100 μmol l^{-1} TPEN restored the signal to near basal level. Values for fluorescence intensity were normalised and are presented in arbitrary units; baseline fluorescence was designated as 1.0.

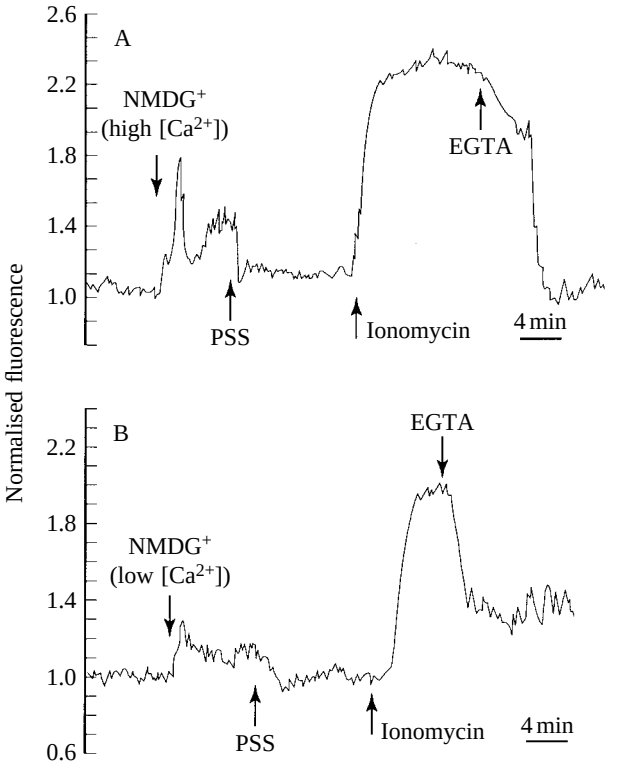

Fig. 4. Change in Fura Red fluorescence in an isolated chloride cell subjected to different experimental regimes. (A) Replacement of extracellular Na^+ by $NMDG^+$ (in the presence of 1.8 mmol l^{-1} $[Ca^{2+}]_o$) induced a large increase in $[Ca^{2+}]_i$. Re-addition of extracellular Na^+ (in PSS) rapidly brought $[Ca^{2+}]_i$ back to the basal level. A subsequent addition of 2 μmol l^{-1} ionomycin induced a further increase in $[Ca^{2+}]_i$, which was reversed by 2 mmol l^{-1} EGTA. (B) In the presence of 50 μmol l^{-1} $[Ca^{2+}]_o$, removal of extracellular Na^+ resulted in a smaller rise in $[Ca^{2+}]_i$. Values for Fura Red fluorescence were inverted and fluorescence intensity was subsequently normalised and presented in arbitrary units; baseline fluorescence was designated as 1.0.

Addition of 50 μmol l^{-1} bepridil increased the normalised fluorescence of Fura Red, indicating an increase in $[Ca^{2+}]_i$ (Fig. 5), and this may be explained by an inhibition of the exchanger in its forward mode. Exposure of the cells to bepridil typically resulted in a biphasic response: a slow rise in intracellular $[Ca^{2+}]$ and constant TO-PRO-1 fluorescence (phase a) was followed by a sudden decrease in Fura Red fluorescence and an increase in TO-PRO-1 fluorescence, indicating a marked rise in $[Ca^{2+}]_i$, which could result from an increase in membrane permeability to Ca^{2+} (phase b).

Manipulating Na^+ influx

To characterise Na^+ influx routes into the CCs, they were challenged with a Na^+-free medium (replacing Na^+ with $NMDG^+$) to lower the levels of cytosolic Na^+. Next, Na^+ influx was stimulated by replacement of the Na^+-free medium with PSS. In the controls, $[Na^+]_i$ returned to near basal levels (Fig. 6A). The addition, prior to Na^+ replacement, of the Na^+ channel blocker TTX, of the Na^+/H^+ exchange inhibitor amiloride or of a combination of these inhibitors two prevented the recovery to basal levels of $[Na^+]_i$ (Fig. 6B–D). The combination of TTX and amiloride never fully blocked Na^+ influx.

Discussion

We report here, for the first time, on free intracellular Na^+ and Ca^{2+} concentrations in chloride cells (CCs) and pavement cells (PCs) isolated from the opercular epithelium of a teleost fish. The operation of Na^+/K^+-ATPase and Na^+/Ca^{2+} exchanger activities in the CCs and the involvement of these carriers in intracellular Na^+ and Ca^{2+} homeostasis are demonstrated. The CC has tetrodotoxin-sensitive, amiloride-sensitive and other, as yet unidentified, influx routes for Na^+.

Isolation of branchial cells

To identify chloride cells in our suspensions of isolated opercular cells, we used the vital stain DASPEI. In numerous other studies on the morphology and biochemistry of PCs and CCs from fish gills (Hootman and Philpott, 1979; Perry and Walsh, 1989; Verbost *et al.* 1994*a*), CCs were discriminated from PCs by probes for either mitochondria (e.g. the vital stain DASPMI; Li *et al.* 1995; Van der Heijden *et al.* 1997) or for Na^+/K^+-ATPase (e.g. the fluorescent anthroyl-ouabain; McCormick *et al.* 1992); mitochondria and Na^+/K^+-ATPase are more or less confined to the CCs of the epithelium. We cannot, at this moment, discriminate subtypes of chloride cells (e.g. accessory cells, immature and mature cells; Wendelaar Bonga *et al.* 1990; α- or β-type CCs; Pisam *et al.* 1995) nor have we applied probes for proliferation or apoptosis to

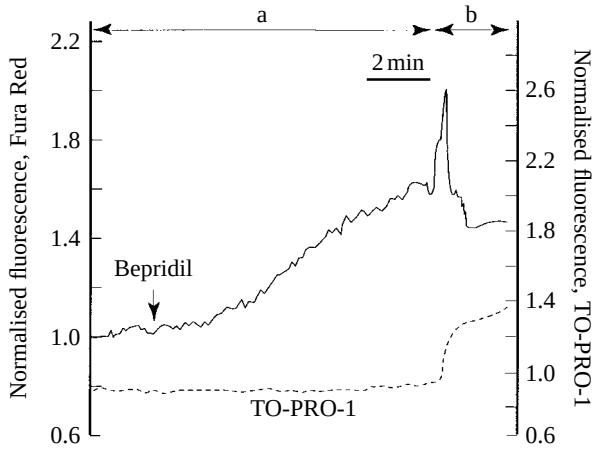

Fig. 5. Effects of bepridil on Fura Red and TO-PRO-1 fluorescence. Phase a: after addition of 50 μmol l^{-1} bepridil (and 0.2 μmol l^{-1} TO-PRO-1), $[Ca^{2+}]_i$ increased probably as a result of inhibition of Na^+/Ca^{2+} exchange activity. Phase b: a sudden rise in $[Ca^{2+}]_i$ coincided with an increase in the TO-PRO-1 signal, indicating loss of membrane integrity. Values for Fura Red fluorescence were inverted and fluorescence intensity was subsequently normalised and presented in arbitrary units; values for TO-PRO-1 fluorescence were also normalised and are presented in arbitrary units; baseline fluorescence was designated as 1.0.

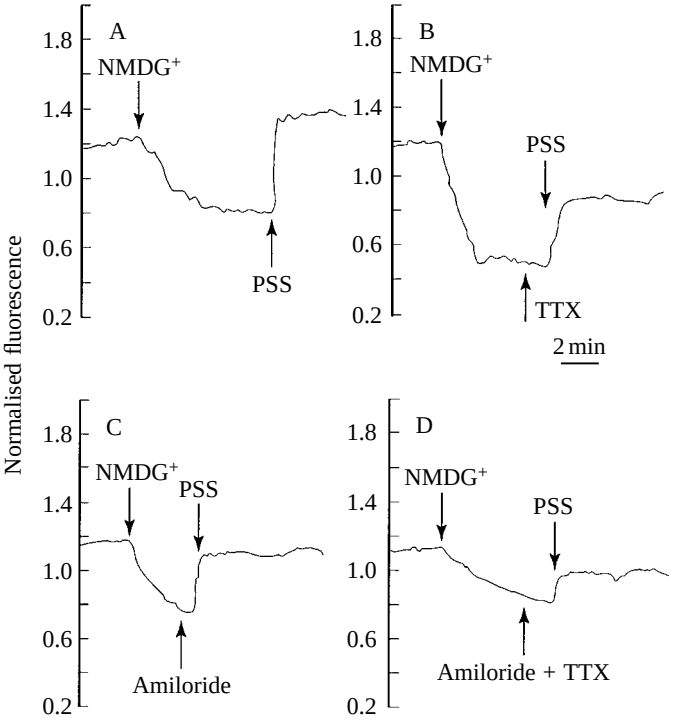

Fig. 6. Effects of amiloride and tetrodotoxin (TTX) on Na^+ influx in isolated chloride cells. (A) Substitution of extracellular Na^+ with $NMDG^+$ resulted in a fall in $[Na^+]_i$. Re-addition of extracellular Na^+ (in PSS) rapidly returned $[Na^+]_i$ to slightly above the basal level. (B) Addition of 2 μmol l^{-1} TTX, prior to re-addition of extracellular Na^+ (in PSS), prevented complete restoration of $[Na^+]_i$ to the basal level. (C) Addition of 50 μmol l^{-1} amiloride, prior to re-addition of extracellular Na^+ (in PSS), had a similar, but less pronounced, effect to that of TTX. (D) Addition of both TTX (2 μmol l^{-1}) and amiloride (50 μmol l^{-1}), prior to re-addition of extracellular Na^+ (in PSS), only partly prevented restoration of $[Na^+]_i$ to the basal level. Values for fluorescence intensity were normalised and are presented in arbitrary units; baseline fluorescence was designated as 1.0.

discriminate different phases in the cell cycle. The relatively broad range of basal levels of cytosolic Na^+ and Ca^{2+} may be related to this kind of inhomogeneity of the cells under investigation.

Cell-Tak, but not poly-L-lysine or gelatin, proved to be suitable to immobilize CCs on glass coverslips. Cell-Tak contains a polyphenolic protein from the mussel *Mytilus edulis* (Waite and Tanzer, 1981); the mussel secretes this protein to glue its byssus to a substratum. To avoid possible damage during the isolation of CCs, we omitted enzymatic treatments with trypsin or hyaluronidase. Such treatments are commonly used in cell biology, but bear the potential risk of damaging extracellular components of intrinsic proteins. The fact that we were able to demonstrate the operation of Na^+/K^+-ATPase and Na^+/Ca^{2+} exchanger activities in CCs lends support to our approach.

Intracellular Na^+ and Ca^{2+} concentrations of chloride cells and pavement cells

Several studies (Foskett and Scheffey, 1982; Marshall *et al.* 1993; Marshall, 1995) have characterized ion tranport mediated by CCs, using opercular epithelium. However, reports on investigations into intracellular Ca^{2+} and Na^+ levels are, to the best of our knowledge, lacking. This gap, at least in our opinion, is related primarily to technical problems. One complication, in our experience, is that freshly isolated CCs do not readily adhere to glass coverslips, a requirement for microscopic analysis with fluorescent probes. Of many (combinations of) glass coatings, so far only Cell-Tak has given satisfactory results. Another complication is that we had to use freshly isolated cells, as isolated CCs do not survive prolonged periods of culture (Pärt and Bergström, 1995). The removal of cells from their epithelial context and the subsequent process of adherence to a support may be anticipated to affect intracellular signal pathways and thus basal levels of $[Na^+]_i$ and $[Ca^{2+}]_i$.

The $[Na^+]_i$ measured in isolated CCs and PCs ranged from 6.4 to 16.5 mmol l^{-1}. Under our experimental conditions, no significant difference between the mean values for $[Na^+]_i$ of CCs and PCs was observed. These resting levels are approximately 20 % of the total $[Na^+]$ in the cell, which amounts to 50–80 mmol l^{-1} (Wood and LeMoigne, 1991; Eddy and Chang, 1993; Morgan *et al.* 1994). We cannot exclude the possibility that $[Na^+]_i$ was affected by the isolation procedure; also, the subsequent incubation of the cells in PSS, which differs from the *in situ* situation in that the apical membrane no longer faces fresh water, may have had unknown effects on $[Na^+]_i$. A cytosolic Na^+ level of approximately 10 mmol l^{-1} is in agreement with the $K_{0.5}$ of Na^+/K^+-ATPase for Na^+ of 13 mmol l^{-1} in trout gills (Flik *et al.* 1996) and 9.9 mmol l^{-1} in

tilapia gills (Flik, 1997); cytosolic Na^+ is a pivotal regulator of Na^+/K^+-ATPase activity (Ewart and Klip, 1995). Moreover, the $[Na^+]_i$ of the CCs and PCs in our study falls within the range 4–26 mmol l^{-1} reported for a variety of other vertebrate cell types (Harootunian *et al.* 1989; Negulescu *et al.* 1990; Ahlemeyer *et al.* 1992; Törnquist and Ekokoski, 1993).

Our data on $[Ca^{2+}]_i$ in the CCs and PCs are in excellent agreement with the concentrations reported for other vertebrate cells (Petersen *et al.* 1994). It seems to be a general rule that $[Ca^{2+}]_i$ in cells of vertebrates at rest is approximately 100 nmol l^{-1}. This value for the CC is consistent with the $K_{0.5}$ for Ca^{2+} of the Ca^{2+}-extruding ATPases and thus with a regulatory role for cytosolic $[Ca^{2+}]$ in the activity of this pump (Flik and Verbost, 1993; Verbost *et al.* 1994*a*; Flik *et al.* 1995). Cytosolic free $[Ca^{2+}]$ is an important regulator of many cellular physiological processes. As high levels of Ca^{2+} are toxic, cytosolic $[Ca^{2+}]$ is maintained at a submicromolar level; in contrast, extracellular $[Ca^{2+}]$ is maintained at a millimolar level. This asymmetrical distribution of Ca^{2+} is crucial to the brief rises in $[Ca^{2+}]_i$ for signal transduction and for signals that initiate cytotoxic processes (Orrenius *et al.* 1989; Petersen *et al.* 1994). The $[Ca^{2+}]_i$ of branchial epithelial cells from the tilapia is in agreement with the value for enterocytes of the same species (Schoenmakers *et al.* 1992). We conclude that these freshly isolated cells are able to control their basal $[Na^+]_i$ and $[Ca^{2+}]_i$ at a physiological level.

The importance of Na^+/K^+-ATPase for the chloride cell

In an isolated CC, $[Na^+]_i$ increased after addition of the specific Na^+/K^+-ATPase inhibitor ouabain, confirming that this Na^+ pump plays a role in intracellular Na^+ homeostasis. As the addition of digitonin after application of ouabain further increased the fluorescence, the rise in $[Na^+]_i$ in the presence of ouabain did not result from a non-specific influx. At least three phenomena may underlie the establishment of the new plateau level of $[Na^+]_i$ in the presence of a ouabain block. First, inhibition of the Na^+/K^+-ATPase activity stops Na^+ export from the cell and thus depolarizes the membrane, which in turn will slow down Na^+ influx. Second, Na^+ channels (see below) in the CC could be closed down by the rise in $[Na^+]_i$ resulting from the inhibition of the Na^+ pump and the consequent change in the membrane potential difference. Third, when $[Na^+]_i$ increases above approximately 35 mmol l^{-1}, the Na^+/Ca^{2+} exchanger reverses its mode (Snowdowne and Borle, 1985), exporting Na^+ (see below); the import of Ca^{2+} could be counteracted by Ca^{2+}-ATPase activity.

Effects of Cu^{2+}

Cu^{2+} reversibly disturbed intracellular Na^+ homeostasis, but never significantly increased $[Ca^{2+}]_i$ (data not shown). We relate these effects to an inhibition of Na^+/K^+-ATPase activity in the CC, resulting in an increase in $[Na^+]_i$. Cu^{2+} specifically inhibits membrane Na^+/K^+-ATPase activity in many cell types (Laurén and McDonald, 1987; Benders *et al.* 1994; Li *et al.* 1996) and was therefore used here as a tool to manipulate $[Na^+]_i$ in a predictable manner. In cultured human muscle cells, exposure to Cu^{2+} leads to a direct increase in $[Na^+]_i$ and a subsequent rise in $[Ca^{2+}]_i$ (Benders *et al.* 1994) through the activation of the reverse mode of a Na^+/Ca^{2+} exchanger. A similar rise in $[Ca^{2+}]_i$ did not occur in CCs exposed to Cu^{2+} (data not shown). We explain the effects of Cu^{2+} on the CC as follows. First, Cu^{2+} increases $[Na^+]_i$ in a manner comparable to that of ouabain, indicating Na^+/K^+-ATPase as a target. Second, TPEN, when used as a specific cell-permeant chelator of Cu^{2+}, reduced $[Na^+]_i$ back almost to the basal level, which indicates a reactivation of the pump. The effect of TPEN on the Cu^{2+}-intoxicated cell is in perfect agreement with biochemical data on Na^+/K^+-ATPase: Cu^{2+} competes with Mg^{2+} when it inhibits the Na^+/K^+-ATPase, and this inhibition is overcome by chelating Cu^{2+} to dithiothreitol (Li *et al.* 1996). These results would further suggest that, in our Cu^{2+}-exposed cells, Cu^{2+} binds to a cytosolic site of the ATPase. We conclude from the observation that TPEN never fully reversed the effects of Cu^{2+} that some Cu^{2+} is covalently bound to cell components. Third, changes in $[Ca^{2+}]_i$ seen in muscle cells exposed to Cu^{2+} resulted from the operation of the Na^+/Ca^{2+} exchanger in its reverse mode, but in the CC this action of the exchanger may be counteracted by the presumed Ca^{2+}-exporting activity of Ca^{2+}-ATPase activity (Flik *et al.* 1996). Our results do not favour the hypothesis of a general increase in membrane permeability induced by Cu^{2+}. If such a general increase were to occur, an abrupt rise in $[Ca^{2+}]_i$ would be predicted, because of the large Ca^{2+} gradient across the plasma membrane. Moreover, a general change of membrane permeability caused by Cu^{2+} would have led to an increased loss of the fluorescent probes from the cell (Rose *et al.* 1993; Benders *et al.* 1994), which we did not observe. Fourth, the half-maximum inhibitory concentration ($K_{0.5}$) of Cu^{2+} for Na^+/K^+-ATPase is 100 nmol l^{-1} (Li *et al.* 1996), making this enzyme a sensitive target for the heavy metal. We stress that, in our experiments, the free $[Cu^{2+}]$, which is the most toxic species, was unknown.

The importance of Na^+/Ca^{2+} exchanger activity for the chloride cell

Na^+/Ca^{2+} exchangers have been demonstrated in the plasma membrane of most cell types (Chern *et al.* 1992; Ye and Zadunaisky, 1992; Benders *et al.* 1994), including the chloride cells of the gills of the tilapia used in this study (Verbost *et al.* 1994*b*). The Na^+/Ca^{2+} exchanger in the CC is presumed to extrude Ca^{2+} from the cells (i.e. to operate in its forward mode), using the energy stored in the Na^+ gradient.

Analyses of plasma membrane preparations from intestinal and renal epithelia of tilapia indicate that either the Na^+/Ca^{2+} exchanger (intestine) or a Ca^{2+}-ATPase (kidney) drives transcellular Ca^{2+} uptake in these epithelia (Flik *et al.* 1996). In the branchial epithelium, however, both carriers are present and equally active (Verbost *et al.* 1994*b*), and so far no specific role for either carrier in homeostatic or vectorial transport is indicated.

The activity of the Na^+/Ca^{2+} exchangers can now be demonstrated at the level of the isolated CC. We provide two

lines of evidence. First, in its reverse mode – realised by removal of external Na^+ (Snowdowne and Borle, 1985) – the Na^+/Ca^{2+} exchanger should become dependent upon $[Ca^{2+}]_o$. Indeed, the Ca^{2+} influx was lower when the concentration of extracellular Ca^{2+} was reduced. The rapid Ca^{2+} influx caused by removal of extracellular Na^+ and the dependence on extracellular $[Ca^{2+}]$ strongly support the idea that the Na^+/Ca^{2+} exchanger operated in a reverse mode (Snowdowne and Borle, 1985; Herchulz and Lebrun, 1993; Benders *et al.* 1994). Second, bepridil, a blocker of the exchanger *via* one of its three Na^+ sites (Slaughter *et al.* 1988), increased $[Ca^{2+}]_i$ (and decreased $[Na^+]_i$; data not shown) in accordance with an inhibition of the forward mode of the carrier. Such inhibition should prevent Ca^{2+} efflux, causing the cell to gain Ca^{2+}. However, bepridil is not a specific inhibitor in an assay of whole cells. Following an initial rise in $[Ca^{2+}]_i$, a second increase in $[Ca^{2+}]_i$ and a change in membrane permeability were observed (as indicated by the concurrent fluorescent changes of Fura Red and of TO-PRO-1). We attribute this second increase in $[Ca^{2+}]_i$ to membrane damage caused directly by bepridil or indirectly by the cytotoxicity of a raised $[Ca^{2+}]_i$, or by a combination of these effects. Nor can we exclude the possibility that bepridil affected Ca^{2+}-ATPase activity (Younes *et al.* 1981) in the CC or blocked Ca^{2+} channels (Capparelli, 1992); clearly, such interactions with the cell cannot easily be discriminated, but all would lead to a rise in intracellular $[Ca^{2+}]$.

Our data support a role for Na^+/Ca^{2+} exchange in intracellular Ca^{2+} homeostasis of the CCs in tilapia. The electrochemical conditions *in vivo* predict a forward mode of the exchanger. We predict that its regulation is governed more by the electrical than by the chemical conditions of the cell. The affinity of the carrier for Ca^{2+} (2.04 μmol l^{-1}; Verbost *et al.* 1994*b*) is too low to allow regulation by the much lower cytoplasmic levels of Ca^{2+}; the affinity of the carrier for Na^+ (48 mmol l^{-1} in trout gills; Flik *et al.* 1997) would also predict continuous saturation by plasma Na^+ levels.

Na^+ influx

The partial blockage of the Na^+ influx by TTX, amiloride or a combination of these chemicals suggested that Na^+ can move into the CC along at least three pathways other than through the Na^+/Ca^{2+} exchanger (in its reverse mode): through a TTX-sensitive Na^+ channel, through an amiloride-sensitive Na^+/H^+ exchanger, and through as yet undefined Na^+ pathway(s). A similar set of Na^+ entry routes has been described for a variety of other non-excitable cell types (Watsky *et al.* 1991; Wen *et al.* 1994; Negulescu *et al.* 1990; Lin and Randall, 1995). We have no data that would allow us to discriminate between the apical or basolateral subdomain of the plasma membrane as possible sites of the effects of these inhibitors. Interestingly, Zadunaiski *et al.* (1995) have presented evidence for a Na^+/H^+ exchanger in the apical membrane of the CC in *Fundulus heteroclitus* opercular epithelium. We have the impression that the CC tends to adhere to the coverslip with its basolateral side and, given the possible resolution of confocal laser scanning microscopy analysis, Na^+ flux through channels may eventually be specifically localised in the basolateral or the apical plasma membrane domain by demonstrating subcellular changes in ion levels. At present, we are investigating the possibility of applying concanavalin-A–fluorescein conjugates to stain the apical membrane of the isolated CC (Li *et al.* 1995; Van der Heijden *et al.* 1997). The incomplete blockage of Na^+ influx by TTX plus amiloride suggests that other, as yet unknown, pathways for Na^+ influx exist in CCs.

The authors thank Dr Julie Fenwick-Ghikas for reading the manuscript critically. Dr Werner Koopman is gratefully acknowledged for his advice on data presentation. J.L. was supported by a grant from the Graduate School of Environmental Chemistry and Toxicology (M&T).

References

AHLEMEYER, B., WEITRAUT, H. AND SCHONER, W. (1992). Cultured chick-embryo heart cells respond differently to ouabain as measured by increase in their intracellular Na^+ concentration. *Biochim. biophys. Acta* **1137**, 135–142.

AMARINO, G. P. AND FOX, M. H. (1995). Intracellular Na^+ measurements using sodium green tetraacetate with flow cytometry. *Cytometry* **21**, 248–256.

ARSLAN, P., VIRGILIO, D. F., BELTRAME, M., TSIEN, R. Y. AND POZZAN, T. (1985). Cytosolic Ca^{2+} homeostasis in Ehrlich and Yoshida carcinomas: a new, membrane-permeant chelator of heavy metals reveals that these ascites tumour cell lines have normal cytosolic free Ca^{2+}. *J. biol. Chem.* **260**, 2719–2727.

BENDERS, A. A. G. M., LI, J., LOCK, R. A. C., BINDELS, R. J. M., WENDELAAR BONGA, S. E. AND VEERKAMP, J. H. (1994). Copper toxicity in cultured human skeletal muscle cells: the involvement of Na^+/K^+-ATPase and the Na^+/Ca^{2+}-exchanger. *Pflügers Arch.* **428**, 461–467.

CAPPARELLI, E. V. (1992). New calcium channel blockers: improved therapy? *Clin. Pharmac.* **11**, 549–552.

CHERN, Y. J., CHUEH, S. H., LIN, Y. J., HO, C. M. AND KAO, L. S. (1992). Presence of Na^+/Ca^{2+} exchange activity and its regulation of intracellular calcium concentration in bovine adrenal chromaffin cells. *Cell Calcium* **13**, 99–106.

EDDY, F. B. AND CHANG, Y. G. (1993). Effects of salinity in relation to migration and development in fish. In *The Vertebrate Gas Transport Cascade; Adaptation to Enviroment and Mode of Life* (ed. J. Eduardo and P. W. Bicudo), pp. 35–42. Boca Raton, FL: CRC Press.

EHRENFELD, J., LACOSTE, I., GARCIA-ROMEU, F. AND HARVEY, B. (1990). Interdependence of Na^+ and H^+ transport in frog skin. In *Animal Nutrition and Transport Processes*, vol. 2, *Transport, Respiration and Excretion: Comparative and Environmental Aspects* (ed. J. P. Truchot and B. Lahlou), pp. 152–170. Basel: Karger.

EWART, H. S. AND KLIP, A. (1995). Hormonal regulation of the Na^+/K^+-ATPase: mechanisms underlying rapid and sustained changes in pump activity. *Am. J. Physiol.* **269**, C295–C311.

FLIK, G. (1997). Transport and housekeeping of calcium in fish gills. In *Society for Experimental Biology Symposium*/Bill Potts Springer (in press).

FLIK, G., KANEKO, T., GRECO, A. M., LI, J. AND FENWICK, J. C. (1997).

Sodium dependent ion transporters in trout gills. *Fish Physiol. Biochem*. (in press).

FLIK, G., KLAREN, P. H. M., SCHOENMAKERS, T. J. M., BIJVELDS, M. J. C., VERBOST, P. M. AND WENDELAAR BONGA, S. E. (1996). Cellular calcium transport in fish: unique and universal mechanisms. *Physiol. Zool.* **69**, 403–417.

FLIK, G., SCHOENMAKERS, T. J. M., GROOT, J. A., VAN OS, C. H. AND WENDELAAR BONGA, S. E. (1990). Calcium absorption by fish intestine: the involvement of ATP- and sodium-dependent calcium extrusion mechanisms. *J. Membr. Biol*. **113**, 13–22.

FLIK, G. AND VERBOST, P. M. (1993). Calcium transport in fish gills and intestine. *J. exp. Biol*. **184**, 17–29.

FLIK, G., VERBOST, P. M. AND WENDELAAR BONGA, S. E. (1995). Calcium transport processes in fishes. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 317–343. New York: Academic Press.

FOSKETT, J. K., BERN, H. A., MACHEN, T. E. AND CONNER, M. (1983). Chloride cells and the hormonal control of teleost fish osmoregulation. *J. exp. Biol*. **106**, 255–281.

FOSKETT, J. K. AND SCHEFFEY, C. (1982). The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* **215**, 164–166.

GARCIA, M. L., SLAUGHTER, R. S., KING, V. F. AND KACZOROWSKI, G. J. (1988). Inhibition of sodium–calcium exchanger in cardiac sarcolemmal membrane vesicles. II. Mechanism of inhibition by bepridil. *Biochemistry* **27**, 2410–2415.

GOSS, G. G., PERRY, S. F., WOOD, C. M. AND LAURENT, P. (1992). Mechanisms of ion and acid–base regulation at the gill of freshwater fish. *J. exp. Zool*. **263**, 143–159.

HAROOTUNIAN, A. T., KAO, J. P., ECKERT, B. K. AND TSIEN, R. Y. (1989). Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. biol. Chem.* **264**, 19458–19467.

HERCHULZ, A. AND LEBRUN, P. (1993). A role for Na/Ca exchange in the pancreatic B cell. *Biochem. Pharmac*. **45**, 7–11.

HOOTMAN, S. R. AND PHILPOTT, C. W. (1979). Ultracytochemical localization of Na^+/K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. *Anat. Rec.* **193**, 99–130.

KAO, J. P. Y., HARROOTUNIAN, A. T. AND TEIEN, R. Y. (1989). Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. *J. biol. Chem.* **264**, 8179–8184.

LAURÉN, D. J. AND MCDONALD, D. G. (1987). Acclimation to copper by rainbow trout, *Salmo gairdneri. Can. J. Fish aquat. Sci.* **44**, 105–111.

LI, J., EYGENSTEYN, J., LOCK, R. A. C., VERBOST, P. M., VAN DER HEIJDEN, A. J. H., WENDELAAR BONGA, S. E. AND FLIK, G. (1995). Branchial chloride cells in larvae and juveniles of freshwater tilapia *Oreochromis mossambicus*. *J. exp. Biol*. **198**, 2177–2184.

LI, J., LOCK, R. A. C., KLAREN, P. H. M., SWARTS, H. G. P., SCHUURMANS STEKHOVEN, F. M. A. H., WENDELAAR BONGA, S. E. AND FLIK, G. (1996). Kinetics of Cu^{2+} inhibition of Na^+/K^+-ATPase. *Toxicol. Lett.* **87**, 31–38.

LIN, H. AND RANDALL, D. (1995). Proton pumps in fish gills. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 229–256. New York: Academic Press.

MARSHALL, W. S. (1995). Transport processes in isolated teleost epithelia: opercular epithelium and urinary bladder. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 1–25. New York: Academic Press.

MARSHALL, W. S., BRYSON, S. E. AND GARG, D. (1993). α_2-Adrenergic inhibition of Cl^- transport by opercular epithelium is mediated by intracellular Ca^{2+}. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5504–5508.

MARSHALL, W. S., BRYSON, S. E. AND WOOD, C. M. (1992). Calcium transport by isolated skin of rainbow trout. *J. exp. Biol*. **166**, 297–316.

MCCORMICK, S. D. (1995). Hormonal control of chloride cell function. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 285–315. New York: Academic Press.

MCCORMICK, S. D., HASEGAWA, S. AND HIRANO, T. (1992). Calcium uptake in the skin of a freshwater teleost. *Proc. natn. Acad. Sci. U.S.A.* **89**, 3635–3638.

MORGAN, I. J. AND POTTS, W. T. W. (1995). The effects of thiocyanate on the intracellular ion concentrations of branchial epithelial cells of brown trout. *J. exp. Biol*. **198**, 1229–1232.

MORGAN, I. J., POTTS, W. T. W. AND OATES, K. (1994). Intracellular ion concentrations in branchial epithelia cells of brown trout (*Salmo trutta*) determined by X-ray microanalysis. *J. exp. Biol.* **194**, 139–151.

NEGULESCU, P. A., HAROOTUNIAN, A., TSIEN, R. Y. AND MACHEN, T. E. (1990). Fluorescence measurements of cytosolic free Na concentration, influx and efflux in gastric cells. *Cell Reg.* **1**, 259–268.

ORRENIUS, S., MCCONKEY, D. J., BELLOMO, G. AND NICOTERA, P. (1989). Role of Ca^{2+} in toxic cell killing. *Trends pharmac. Sci.* **10**, 281–285.

PÄRT, P. AND BERGSTRÖM, E. (1995). Cellular and molecular approaches to fish ionic regulation. In *Fish Physiology*, vol. 14, *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. C. M. Wood and T. J. Shuttleworth), pp. 207–228. New York: Academic Press.

PERRY, S. F. AND WALSH, P. J. (1989). Metabolism of isolated fish gill cells: contribution of epithelial chloride cells. *J. exp. Biol*. **144**, 507–520.

PETERSEN, O. H., PETERSEN, C. C. H. AND KASAI, H. (1994). Calcium and hormone action. *A. Rev. Physiol*. **56**, 297–319.

PISAM, M., LE MOAL, C., AUPERIN, B., PRUNET, P. AND RAMBOURG, A. (1995). Apical structures of "mitochondria-rich" alpha and beta cell in euryhaline fish gill: their behaviour in various living conditions. *Anat. Rec*. **241**, 13–24.

POTTS, W. T. W. (1994). Kinetics of sodium uptake in freshwater animals: a comparison of ion-exchange and proton pump hypotheses. *Am. J. Physiol*. **266**, R315–R320.

ROSE, U. M., BINDELS, R. J. M., VIS, A., JANSEN, J. W. C. M. AND VAN OS, C. H. (1993). The effects L-type Ca^{2+} channel blockers on anoxia-induced increase in intracellular Ca^{2+} concentration in rabbit proximal tubule cells in primary culture. *Pflügers Arch*. **423**, 378–386.

SCHEENEN, W. J. J. M., MAKINGS, L. R., GROSS, L. R., POZZAN, T. AND TSIEN, R. Y. (1996). Photodegradation of indo-1 and its effects on apparent Ca^{2+} concentrations. *Chem. Biol.* **3**, 765–774.

SCHOENMAKERS, T. J. M., KLAREN, P. H. M., FLIK, G., LOCK, R. A. C., PANG, P. K. T. AND WENDELAAR BONGA, S. E. (1992). Actions of cadmium on basolateral plasma membrane proteins involved in calcium uptake by fish intestine. *J. Membr. Biol.* **127**, 161–172.

SLAUGHTER, R. S., GARCIA, M. L., CRAGOE, E. J., JR, REEVES, J. P. AND KACZOROVSKI, G. J. (1988). Inhibition of sodium–calcium exchange in cardiac sarcolemmal membrane vesicles. I. Mechanism of inhibition by amiloride analogues. *Biochemistry* **27**, 2403–2409.

SNOWDOWNE, K. W. AND BORLE, A. B. (1985). Effects of low extracellular sodium on cytosolic ionized calcium. *J. biol. Chem.* **260**, 14998–15007.

TÖRNQUIST, K. AND EKOKOSKI, E. (1993). Intracellular free sodium concentration in GH_4C_1 cells. *J. cell. Physiol.* **154**, 608–614.

VAN DER HEIJDEN, A. J. H., VERBOST, P. M., EYGENSTEYN, J., LI, J., WENDELAAR BONGA, S. E. AND FLIK, G. (1997). Chloride cells in gills of tilapia *Oreochromis mossambicus* adapted to freshwater or seawater, quantification by confocal laser scanning microscopy. *J. exp. Biol.* **200**, 55–64.

VAN DRIESSCHE, W. AND ZEISKE, W. (1985). Ionic channels in epithelial membranes. *Physiol. Rev.* **65**, 833–903.

VERBOST, P. M., FLIK, G. AND COOK, H. (1994*a*). Isolation of gill cells. In *Biochemistry and Molecular Biology of Fishes*, vol. 3 (ed. P. W. Hochachka and T. P. Mommsen), pp. 239–247. Amsterdam: Elsevier Science Ltd.

VERBOST, P. M., SCHOENMAKERS, TH. J. M., FLIK, G. AND WENDELAAR BONGA, S. E. (1994*b*). Kinetics of ATP- and Na^+-gradient-driven Ca^{2+} transport in basolateral membranes from gills of freshwater- and seawater-adapted tilapia. *J. exp. Biol.* **186**, 95–108.

WAITE, J. H. AND TANZER, M. L. (1981). Polyphenolic substance of *Mytilus edulis*: novel adhesive containing L-dopa and hydroxyproline. *Science* **212**, 1038–1040.

WATSKY, M. A., COOPER, K. AND RAE, J. L. (1991). Sodium channels in ocular epithelia. *Plügers Arch.* **419**, 454–459.

WEN, R., LUI, G. M. AND STEINBERG, R. H. (1994). Expression of a tetrodotoxin-sensitive Na^+ current in cultures human retinal pigment epithelial cells. *J. Physiol., Lond.* **15**, 187–196.

WENDELAAR BONGA, S. E., FLIK, G., BALM, P. H. M. AND VAN DER MEIJ, J. C. A. (1990). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell Tissue Res.* **259**, 575–585.

WOOD, C. M. AND LEMOIGNE, J. (1991). Intracellular acid–base responses to environmental hyperoxia and normoxic recovery in rainbow trout. *J. exp. Biol.* **126**, 499–512.

YE, J. J. AND ZADUNAISKY, J. A. (1992). Study of the Ca^{2+}/Na^+ exchange mechanism in vesicles isolated from apical membranes of lens epithelium of spiny dogfish (*Squalus acanthias*) and bovine eye. *Exp. Eye Res.* **55**, 243–250.

YOUNES, A., FONTANARAVA, C. AND SCHNEIDER, J. M. (1981). Effects of bepridil on the Ca^{2+} dependent ATPase activity of sarcoplasmic reticulum. *Biochem. Pharmac.* **30**, 2979–2981.

ZADUNAISKI, J. A., CARDONA, S., AU, L., ROBERTS, D. M., FISHER, E., LOWENSTEIN, B., CRAGOE, E. J., JR AND SPRING, K. R. (1995). Chloride transport activation by plasma osmolarity during rapid adaptation to high salinity of *Fundulus heteroclitus. J. Membr. Biol.* **143**, 207–217.