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OSMOTIC AND THERMAL EFFECTS ON *IN SITU* ATPase ACTIVITY IN PERMEABILIZED GILL EPITHELIAL CELLS OF THE FISH *GILLICHTHYS MIRABILIS*

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Summary

Long-jawed mudsuckers (*Gillichthys mirabilis*) were acclimated to sea water (SW) at 7 °C, SW at 26 °C or dilute sea water (DSW) at 26 °C for 5 months. Gill cells were isolated and the proportion of mitochondria-rich (MR) cells was determined. The number of cells harvested amounted to $4.7\times10^7\pm0.6\times10^7$ to $10.6\times10^7\pm1.1\times10^7$ and the yield was between $7.1\times10^8\pm0.6\times10^8$ and $10.7\times10^8\pm1.4\times10^8$ cells g^{-1} gill epithelial mass. Cell viability was 96.8±0.4 to 97.8±0.6 %. The number, size and volume of MR cells decreased significantly during DSW acclimation, but did not change during thermal acclimation. The protein content was not influenced by osmotic or thermal acclimation and ranged between 20.0±1.6 and 22.1±1.5 pg cell^{-1}. Using a new method, which is based on the formation of plasma membrane channels by alamethicin, we were able to permeabilize gill cells. For the first time, the Na^+/K^+-ATPase and H^+-ATPase activities of fish gills were determined in intact cells *in situ*. The activity of both ATPases was dependent on alamethicin concentration (optimum 100 μg mg^{-1} protein) and on preincubation time (optimum 10 min). The *in situ* activity of both ATPases was influenced by osmotic, but not thermal, acclimation. A positive linear correlation was found between *in situ* Na^+/K^+-ATPase activity and total MR cell volume. However, we show, for the first time, that a negative linear correlation exists between H^+-ATPase activity and total MR cell volume, suggesting a localization of H^+-ATPase in pavement cells. In permeabilized cells, the activity of both ATPases was 2.6–3.9 times higher than that of crude homogenates and 1.6–2.1 times higher than that of permeabilized homogenate vesicles. We hypothesize that in crude homogenates three-quarters of Na^+/K^+-ATPase and two-thirds of H^+-ATPase activity are not detectable both because of a mixture of inside-out and right-side-out vesicles and because of the disruption of membrane and enzyme integrity.

Key words: cell isolation, cell permeabilization, gill epithelial cells, H^+-ATPase, ion transport, mitochondria-rich cells, Na^+/K^+-ATPase, mudsucker, *Gillichthys mirabilis*.

Introduction

Gill epithelial cells of teleosts are responsible for active uptake and secretion of Na^+, Cl^- and other ions (Evans, 1993). Two cell types are present in gill epithelium, which are thought to be involved in active ion transport. These are mitochondria-rich (MR) cells and pavement cells (Goss *et al.* 1992). MR cells are the site for active NaCl secretion (Foskett and Scheffey, 1982), a process catalyzed by Na^+/K^+-ATPase located in the basolateral plasma membrane of these cells (Karnaky, 1986). Recently, an NEM (*N*-ethylmaleimide)-sensitive, V-type H^+-ATPase was identified in crude gill homogenates of *Oncorhynchus mykiss*; it showed higher activity in DSW-acclimated fish and was immunolocalized to the apical membrane of both MR cells and pavement cells (Lin and Randall, 1993; Lin *et al.* 1994). Current models of NaCl secretion and absorption across teleost gills involve different transport proteins, which are modified under hypo- or hyperosmotic conditions and include Na^+/K^+-ATPase and H^+-ATPase (Evans, 1993). These models describe steady-state mechanisms of ion transport across the gill epithelium of fully acclimated fish. Little is known about signal transduction mechanisms in response to osmotic changes and about factors that mediate modulation of membrane proteins, such as Na^+/K^+-ATPase and H^+-ATPase. The regulation of the enzymes responsible for ion transport may be affected by environmental factors other than medium osmolality. For example, a significant increase in Na^+/K^+-ATPase activity was found after cold-acclimation in crude gill homogenates of *Cyprinodon salinus* (Stuenkel and Hillyard, 1980), *Rutilus rutilus* and *Salvelinus alpinus* (Schwarzbaum *et al.* 1991). To reveal the nature of the mechanisms involved in modulating plasma-membrane-localized ion transport, new experimental approaches including the use of isolated cells will be needed. In the past, methods have been developed for the isolation and concentration of MR cells and pavement cells from different

species of teleosts (e.g. Kamiya, 1972; Hootman and Philpott, 1978; Naon and Mayer Gostan, 1983; Kültz and Jürss, 1993). Cell permeabilization is another important prerequisite for many approaches to the study of the physiological function of cells and tissues, including measurements of *in situ* enzyme activity and K_m (Aragon and Sols, 1991), the investigation of organelle biosynthesis and intracellular lipid transport (Voelker, 1991), fluorescent labeling and intraorganelle tracking of second messenger pools and other mediators (Hofer and Machen, 1993), and the study of the molecular mechanisms of membrane traffic and polarized sorting (Morre *et al.* 1993). All of these phenomena play a role during the adjustments that occur in the gill epithelium in response to osmotic changes. For example, the lipid metabolism of gill cells is influenced by osmotic acclimation of *Anguilla anguilla* (Hansen, 1987; Hansen *et al.* 1992), as is adenylate cyclase activity in *Oncorhynchus mykiss* gills (Guibbolini and Lahlou, 1987) and, of course, the polarity of NaCl transport is reversed after acclimation of euryhaline teleosts from hypo- to hyperosmotic water and *vice versa*. Important advantages of investigating physiological phenomena in permeabilized cells *in situ* are that the integrity of cellular organization is maintained and protein concentrations are maintained at realistic levels. We describe a method for chemically permeabilizing gill epithelial cells. Using this method, we investigated the effects of osmotic and thermal acclimation on the *in situ* activity of two important enzymes involved in energizing active NaCl transport, Na^+/K^+-ATPase and H^+-ATPase. We also analyzed the relationship between the activity of both ATPases and the proportion of MR cells in gill epithelium.

Materials and methods

Animals

Long-jawed mudsuckers (*Gillichthys mirabilis*, Gobiidae) were collected in bays and estuaries near San Diego, California, in summer 1993 and transported to Corvallis, Oregon, where the work was conducted. During the 6 h transportation, fish were maintained in sealed plastic bags half-filled with sea water and saturated with O_2. None of the fish died during transport. Fish ranged between 10.5 and 13.3 cm in total length and between 10.2 and 24.1 g in mass. They were divided into three groups. The first group was immediately transferred to sea water (SW, 34 ‰) and held at 26±1 °C, those of the second group were preacclimated to SW and held at 15±1 °C for 1 week and then acclimated to SW at 7±1 °C, and a third group of fish was kept in 50 % SW (17 ‰) at 26±1 °C for 1 week and then acclimated to dilute sea water (DSW, 1.5 ‰) at 26±1 °C. Fish were held under these conditions at an artificial photoperiod of 12 h:12 h L:D for 5 months. They received commercial fish pellets *ad libitum*. Aquaria were equipped with a filtering and de-ammonification system, and the water was aerated vigorously during the entire course of the experiment. After determination of body mass and total length, fish were killed by spinal transection. The pericardial cavity was opened, and the gills were perfused with Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (BSS, GIBCO) *via* the bulbus arteriosus. This procedure was continued until all blood had been removed from gills, as indicated by the disappearance of any red color from the gill arches. It was not necessary to add heparin or epinephrine to prevent clotting of blood. During perfusion, the whole fish was immersed in the appropriate water so that the epithelial cells continued to be exposed to acclimation conditions.

Cell isolation

Gill arches were dissected and rinsed twice in Ca^{2+}/Mg^{2+}-free Hanks BSS. Epithelial tissue was scraped from the cartilage using a blunt-tipped scalpel and suspended in 20 ml of Ca^{2+}/Mg^{2+}-free Hanks BSS containing 5 mmol l^{-1} Na_2EDTA, 10 mmol l^{-1} glucose and 30 μmol l^{-1} Phenol Red. Accurate weighing of gill tissue prior to cell isolation resulted in the drying out of the cells and a strongly decreased cell viability, but was necessary to determine cell harvest and yield. Therefore, the relationship between gill wet mass and body mass or total length of fish was assessed using fish from all acclimation groups. The gills of these fish were not used for other purposes. Gill epithelial tissue used for cell isolation was not weighed. It was incubated for 30 min under moderate shaking at the appropriate acclimation temperature. During incubation, the atmosphere over the suspension was continuously renewed by a gentle flow of a 98 % O_2/2 % CO_2 gas mixture. The flow rate was adjusted so that the pH, indicated by Phenol Red, was maintained at the initial value (7.4). Following incubation, tissue pieces were dispersed by gentle resuspension by pipetting 20 times using a 1 ml automatic pipette (Oxford). Subsequent filtering through screens of 60 and 200 mesh (Sigma) resulted in complete dispersion of epithelial tissue into single cells. Cells were washed twice in Ca^{2+}/Mg^{2+}-free Hanks BSS by centrifugation at 100 ***g*** for 5 min (Sorvall RC-5B, SS-34 rotor, Du Pont) and counted using a hemocytometer. One-fifth of the final cell suspension was retained for labeling MR cells and for the determination of cell viability. Cell viability, determined by the Trypan Blue dye exclusion test (Sharpe, 1988) used at 30 min intervals after isolation, showed perfect stability of cell preparations in Ca^{2+}/Mg^{2+}-free Hanks BSS within the first 2 h. For this time, cells were kept at the acclimation temperature of the fish. All experimental procedures and measurements were performed within 2 h. The major part (four-fifths) of the cell suspension was used for cell permeabilization. Cellular protein content of each sample was determined prior to permeabilization using the bicinchoninic acid (BCA) method (Smith *et al.* 1985).

Mitochondria-rich cells

2 - (*p* - dimethylaminostyryl) - 1 - methyl - pyridiniumiodine (DASPMI, Aldrich, Milwaukee, WI, USA), a vital fluorescent dye, was added to the cell suspension at a final concentration of 10 μmol l^{-1}. Mitochondria-rich cells (MR cells) were labeled specifically during a 30 min incubation at 4 °C in the

dark. DASPMI binds directly to intact mitochondria (Bereiter-Hahn, 1976) and its specificity for the MR cells of teleost gills has been demonstrated previously (Karnaky *et al.* 1984). After labeling the MR cells, the dye was removed from the suspension by washing the cells twice in Ca^{2+}/Mg^{2+}-free Hanks BSS and centrifuging at 100 ***g*** for 5 min. A sample of the suspension was loaded into a hemocytometer and examined using an epifluorescence/phase contrast microscope (Axioscope, Zeiss). In epifluorescence mode, the exciter-barrier filter and reflector combination for an excitation wavelength of 450–490 nm (Axio-Line) was used. Micrographs were taken from an identical area of cell suspension in epifluorescence and phase contrast mode (Kodak TMax 400 ASA). The percentage of MR cells among all the gill epithelial cells and their size were determined on micrographs. Metric calibration of micrographs was achieved using an objective micrometer. Between 40 and 150 MR cells were counted, and the diameter of 20 MR cells was measured for each fish. The volume of a single MR cell was calculated according to the following equation:

$$V = (4/3)(D/2)^3\pi, \quad (1)$$

where V is the mean volume of a single MR cell (μm^3) and D is the mean diameter of the MR cells of one fish (μm). The volume of MR cells per gram gill epithelial mass was calculated as the product of the mean volume of a single MR cell and the number of MR cells per gram epithelial wet mass.

Cell permeabilization

Cells were chemically permeabilized using the channel-forming antibiotic alamethicin (Sigma). The channels formed by this antibiotic allow unlimited penetration of ATP and ADP through the plasma membrane. Alamethicin-treated cells thus are suitable for *in situ* assays of ATPase activity (Ritov *et al.* 1993). A stock solution of alamethicin was prepared as 20 mg ml^{-1} in 60 % ethanol. Duplicate determinations of the protein concentration of cell suspensions, from three different dilutions of the same cell suspension, were carried out immediately after cell isolation. Suspensions were then diluted to a protein concentration of 2 mg ml^{-1} in Ca^{2+}/Mg^{2+}-free Hanks BSS (pH 7.8). Preincubation of these cell suspensions with alamethicin prior to their addition to ATPase reagent was necessary to allow channel formation by the alamethicin. For the determination of the optimal alamethicin concentration, 1–64 μl of stock solution was added to a 1.6 ml cell suspension. Controls, in which the alamethicin stock solution was replaced by either a 60 % ethanol solution or water, were run in parallel to each assay. In order to compare ATPase activities in whole permeabilized cells, in homogenates and in permeabilized homogenate vesicles, cell suspensions obtained from fish acclimated to SW at 26 °C were divided into three samples. One sample was treated as described above, the second was homogenized using an Ultraturrax (Janke and Kunkel, Germany) at maximum speed for 60 s, and the third was also homogenized and then treated with alamethicin exactly as for the first sample. Before homogenization, protease inhibitors were added to give the following final concentrations: phenylmethanesulphonylfluoride 1 mmol l^{-1}; leupeptin 10 μg ml^{-1}; pepstatin 10 μg ml^{-1}; and aprotinine 1 μg ml^{-1}. Samples were kept on ice during and after homogenization. The method of sample preparation described above yielded the highest *in vitro* Na^+/K^+-ATPase and H^+-ATPase activities of all homogenization methods tested. These included different homogenization intensities with the Ultraturrax, different intensities of sonication (Branson Ultrasonic) and different intensities of homogenization with glass homogenizers (Kontes). Following preincubation, 25 μl of cell suspension or homogenate was added to 1.1 ml of ATPase reagent, and ATPase activities were determined after gentle mixing.

ATPase activity

The activities of Na^+/K^+-ATPase and H^+-ATPase were determined using a coupled assay modified from Penefsky and Bruist (1984), which is based on the equimolar coupling of ATPase activity to pyruvate kinase and lactate dehydrogenase. The assay mixture was optimized using *in vitro* assays for Na^+/K^+-ATPase and H^+-ATPase of *G. mirabilis* gill and contained 30 mmol l^{-1} imidazole, 125 mmol l^{-1} NaCl, 20 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Na_2EDTA, 0.4 mmol l^{-1} NaN_3, 1 mmol l^{-1} Na_2ATP, 0.2 mmol l^{-1} Na_2NADH, 2 mmol l^{-1} phosphoenol pyruvate, 3 i.u. ml^{-1} pyruvate kinase and 2 i.u. ml^{-1} lactate dehydrogenase (pH 7.4). Optimization of ATPase assays was performed for all of the above-listed salts and for the Na^+:K^+ ratio. The presence of sodium deoxycholate (0.1 %) during homogenization and assay did not result in increased *in vitro* activities of either ATPase (data not shown). Three assays were run for each sample: (1) 1 ml of ATPase reagent + 0.1 ml of water + 25 μl of sample; (2) 1 ml of ATPase reagent + 0.1 ml of 11.25 mmol l^{-1} ouabain + 25 μl of sample; and (3) 1 ml of ATPase reagent + 0.1 ml of 11.25 mmol l^{-1} *N*-ethylmaleimide (NEM) + 25 μl of sample. The difference in the ATPase activity between assays 1 and 2 represented Na^+/K^+-ATPase activity, and the difference between assays 1 and 3 represented H^+-ATPase activity. The inhibitory effects of ouabain and NEM on ATPase activity were additive (data not shown). In addition to all the substrates and coenzymes necessary for ATPase and coupling enzymes, the ATPase reagent also contained 0.4 mmol l^{-1} NaN_3 and 5 mmol l^{-1} Na_2EDTA and was Ca^{2+}-free. This ensured complete inhibition of F-type (mitochondrial) ATPase and Ca^{2+}-ATPase. All activities measured were linear throughout the time course of the assay. All assays were performed at 25 °C and activity was expressed as specific activity (international units mg^{-1} protein) or relative activity (%).

Statistics

Results are expressed as means ± standard error of means (S.E.M.). The number of fish used from each group was five for the determination of the number, diameter and volume of MR cells, and six for all ATPase activities reported. Twenty-one fish were used for assessing the correlation between total length (TL) or body mass (BW) and the wet mass of the whole

gill basket (GBW) or the wet mass of gill epithelium (GEW), and 15 fish were used for assessing the correlation between MR cell volume and Na^+/K^+-ATPase or H^+-ATPase activity. Each pair of data used for linear regression analysis was from the same fish. Comparable data from different groups were analyzed using the *F*-test followed by an unpaired, two-sided *t*-test. The Mann–Whitney test was used instead of a *t*-test in cases of significant differences between variances of means. One-way analysis of variance (ANOVA) was used to test for significant differences between means within the series of experiments relating ATPase activity to alamethicin concentration and preincubation time, and a *t*-test for multiple comparisons, in which the *P* values were corrected by the Bonferroni method, was used to assess which means were significantly different within these series. All values stated as significant had $P<0.05$.

Results

Correlation between gill wet mass and body mass or total length of fish

Correlations between body mass (BW, in g) or total length (TL, in cm) of fish and wet mass of whole gill baskets (GBW, in mg) or wet mass of gill epithelium only (GEW, in mg) are shown in Fig. 1. It must be emphasized that the linear regression equations are only valid for fish in the length and mass ranges used for our experiments (see Discussion). Interestingly, only about one-third of GBW is due to GEW (Fig. 1). Even though the gill epithelium is the main site for major gill function, cartilaginous structures, which stabilize the organ and form a rigid support for the major blood vessels, contribute most to gill wet mass. We decided to use the correlation between TL and GEW for calculating cell harvest, because it was higher than the correlation between BW and GEW.

Cell harvest, cell viability and cellular protein content

The non-enzymatic cell isolation method presented here yields highly viable and highly dispersed cells (Fig. 2). Isolation efficiency, expressed as cell harvest and yield, cell viability and cellular protein content of fish acclimated to different osmotic and thermal conditions are shown in Table 1. Total cell harvest ranged between 4.7×10^7 and 10.6×10^7 cells. Since GEW ranged between 48 and 126 mg (Fig. 1), yield, expressed as the number of cells per gram GEW, is about an order of magnitude higher than cell harvest (Table 1). Interestingly, cell harvest and yield were significantly influenced by osmotic, but not thermal, acclimation. The viability of cells was not influenced by either acclimation treatment (Table 1). The protein content per gill epithelial cell was between 20.0±1.6 and 22.1±1.5 pg. It was slightly lower in cells of DSW-acclimated fish, but the difference from SW-acclimated fish was not significant. Thermal acclimation also had no significant effect on cellular protein content (Table 1).

Mitochondria-rich cells

MR cells were easily identified among other gill epithelial cells after DASPMI staining (Fig. 2). Under all conditions, they represented less than 3 % of total cell number. Although the absolute number (Table 1) and relative proportion (Fig. 3A) of MR cells are not significantly different between the two SW-acclimated groups, they are more than two times higher than values for the DSW-acclimated group. The diameter of MR cells is also significantly lower in DSW-acclimated fish, but did not change with thermal acclimation (Fig. 3B). MR cells, as well as other gill epithelial cells, readily round up after isolation (Fig. 2). Therefore, it is justifiable to use an equation to calculate a spherical volume from the radius of these cells. The mean volume of single MR cells ranges between 740±90 and 2790±290 μm^3 under different

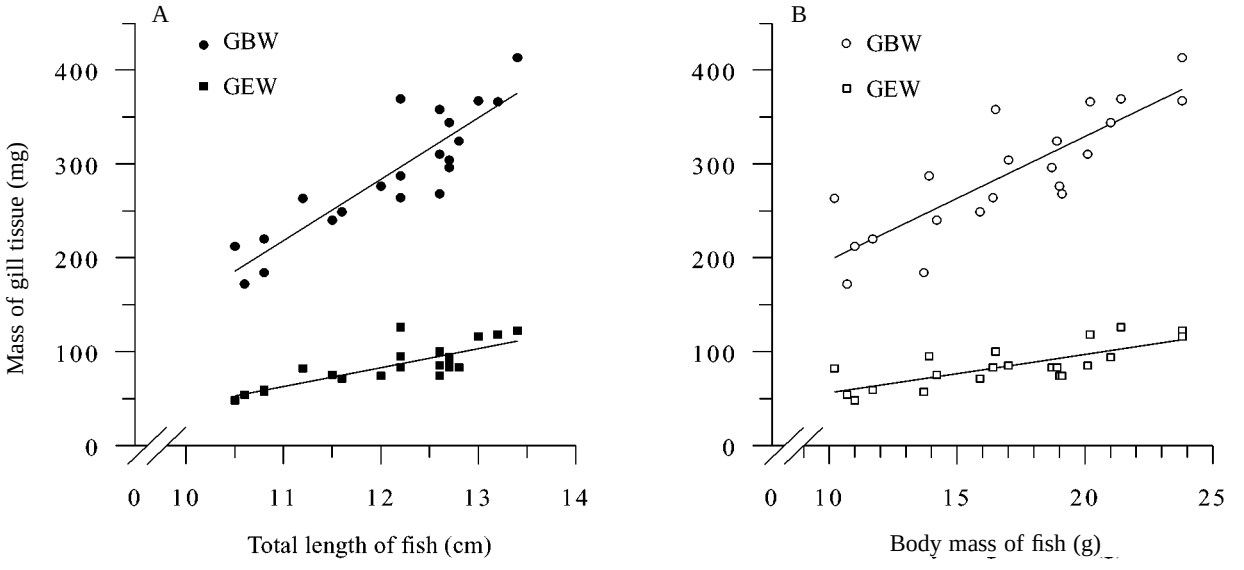

Fig. 1. Linear relationships between (A) total length of fish (TL) and gill basket mass (GBW, filled circles, GBW=65.43TL−501.23, *r*=0.89) or gill epithelial mass (GEW, filled squares, GEW=20.01TL−157.73, *r*=0.80), and (B) between body mass of fish (BW) and GBW (open circles, GBW=13.19BW+65.45, *r*=0.84) or GEW (open squares, GEW=4.10BW+15.18, *r*=0.77).

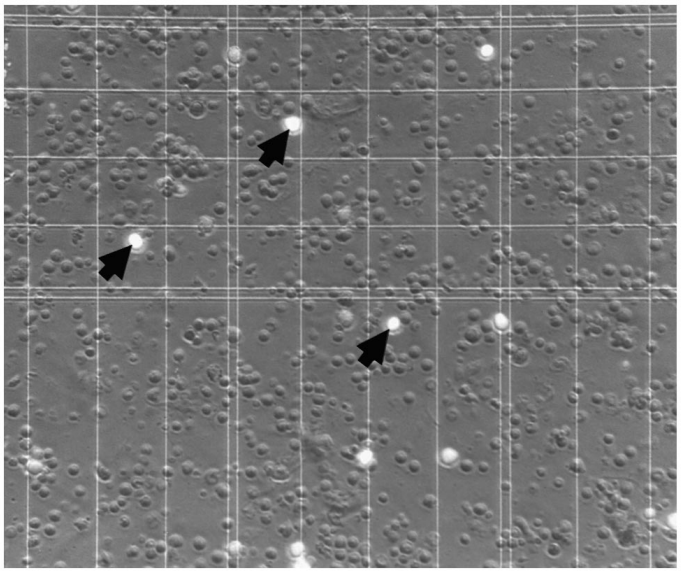

Fig. 2. Micrograph showing part of a gill epithelial cell suspension obtained from a fish acclimated to sea water at 26 °C. The micrograph was taken with the Axioscope set to epifluorescence and phase contrast mode, so that fluorescing and non-fluorescing cells can be viewed simultaneously. Cells are completely dispersed and mitochondria-rich cells (MR cells) are easily recognized by their bright fluorescence (arrows point to some MR cells). The size of a small square of the hemocytometer is 50 μm × 50 μm.

acclimation regimes. Both the mean volume of single MR cells and the mean total volume of MR cells per gram GEW are significantly influenced by osmotic, but not thermal, acclimation of fish (Table 1). Osmotic acclimation from SW to DSW affects MR cell number and size by a factor of about 2 (Fig. 3). Using equation 1 to obtain the mean volume of a single MR cell and multiplying this by the number of MR cells gives a total MR cell volume for SW-acclimated fish more than

Table 1. *Isolation efficiency, cell viability, cellular protein content and volume of mitochondria-rich cells determined after isolation of gill epithelial cells from fish acclimated to different osmotic and thermal conditions*

	SW, 7 °C	SW, 26 °C	DSW, 26 °C
10^{-7} × total cell harvest (cells)	9.8±2.1	10.6±1.1	4.7±0.6*
10^{-8} × yield (cells g^{-1} GEW)	10.7±1.4	10.1±1.2	7.1±0.6*
Viability† (%)	97.6±0.5	96.8±0.4	97.8±0.6
Cellular protein content (pg $cell^{-1}$)	22.0±0.7	22.1±1.5	20.0±1.6
10^{-7} × MR cell number (cells g^{-1} GEW)	2.64±0.27	2.43±0.49	0.84±0.13*
Volume of single MR cell (μm^3)	2790±290	2540±480	740±90*
Total volume of MR cells (mm^3 g^{-1} GEW)	73.1±9.0	59.7±14.7	5.8±0.6*

*Significantly different from other groups.
†Viability of cells 2 h after isolation.
Data are means ± S.E.M. (*N*=5).
GEW, mass of gill epithelium; SW, sea water; DSW, dilute sea water; MR cell, mitochondria-rich cell.

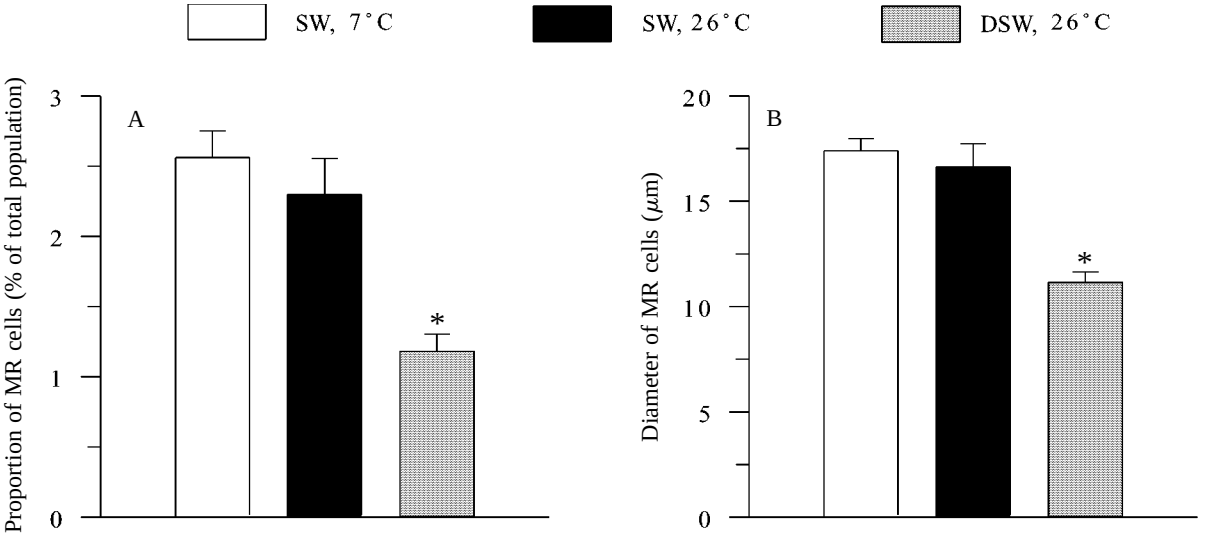

Fig. 3. (A) Relative proportion of mitochondria-rich (MR) cells in the total population of all gill epithelial cells and (B) the diameter of MR cells in relation to osmotic and thermal acclimation of fish. Data are means + S.E.M. (*N*=5); an asterisk indicates a significant difference from all other groups (P<0.005). SW, sea water; DSW, dilute sea water.

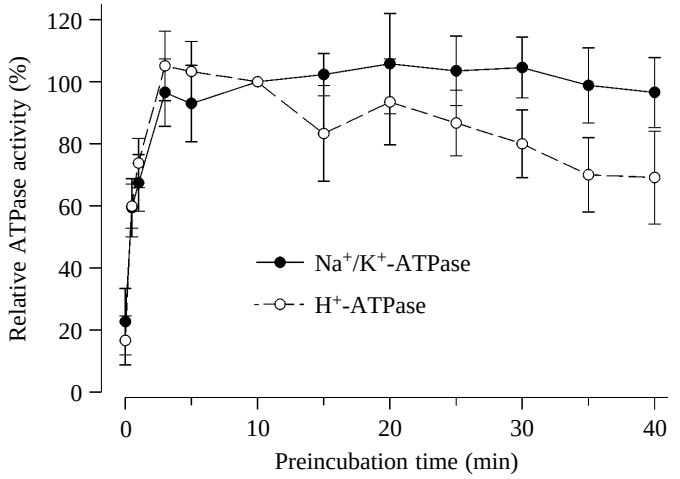

Fig. 4. Dependence of Na^+/K^+-ATPase activity (filled circles, solid line) and H^+-ATPase activity (open circles, dashed line) on the length of the preincubation period of cell suspensions in the presence of alamethicin. Preincubation time had a significant effect on Na^+/K^+-ATPase (P<0.001) and H^+-ATPase activity (P<0.001). However, for both ATPase activities, only the control values (preincubation time=0) were significantly different from the value at 10 min, which was designated 100 % to serve as a reference (P<0.01 in each case). Data were obtained from fish acclimated to sea water at 26 °C and represent means ± S.E.M. (N=6).

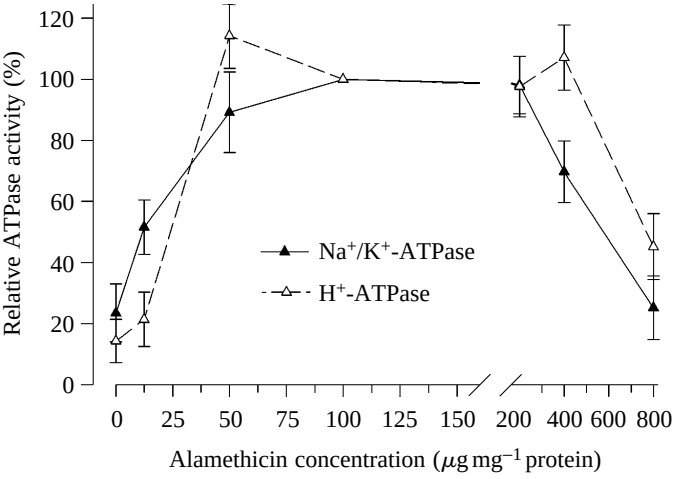

Fig. 5. Dependence of Na^+/K^+-ATPase activity (filled triangles, solid line) and H^+-ATPase activity (open triangles, dashed line) on alamethicin concentration. Alamethicin concentration had a significant effect on Na^+/K^+-ATPase (P<0.001) and H^+-ATPase activity (P<0.001). At the following alamethicin concentrations, Na^+/K^+-ATPase activity was significantly different from the value at a concentration of 100 μg alamethicin mg^{-1} protein, which was designated 100 % to serve as a reference: 0 μg mg^{-1} protein (P<0.01), 12.5 μg mg^{-1} protein (P<0.05) and 800 μg mg^{-1} protein (P<0.01). At the same concentrations, H^+-ATPase activity was significantly different from the reference value: 0 μg mg^{-1} protein (P<0.001), 12.5 μg mg^{-1} protein (P<0.001) and 800 μg mg^{-1} protein (P<0.01). Data were obtained from fish acclimated to sea water at 26 °C and represent means ± S.E.M. (N=6).

an order of magnitude higher than the total MR cell volume for DSW-acclimated fish (Table 1).

Cell permeabilization and in situ *ATPase activity*

In order to optimize permeabilization of gill epithelial cells, the dependence of Na^+/K^+-ATPase and H^+-ATPase activity on alamethicin concentration and time of preincubation was determined. Since alamethicin effects depend on the density of the cell suspension (data not shown), it was important to ensure that the ratio of alamethicin concentration to cell density was constant in all assays. Therefore, all preincubations were carried out in cell suspensions with a constant protein content of 2 mg ml^{-1} in Ca^{2+}/Mg^{2+}-free Hanks BSS (pH 7.8). Alamethicin channels formed very rapidly during preincubation, with Na^+/K^+-ATPase and H^+-ATPase activity saturating after a 3 min preincubation (Fig. 4). After 15 min, a slight, but insignificant, decrease in H^+-ATPase activity was detectable. This decrease was not seen for Na^+/K^+-ATPase activity. A preincubation time of 10 min was optimal for both ATPases and was chosen as standard for all subsequent assays. The dependence of Na^+/K^+-ATPase activity and H^+-ATPase activity on alamethicin concentration is shown in Fig. 5. Saturation of activity of both ATPases was achieved at alamethicin concentrations of 50 μg mg^{-1} protein and was maintained up to a concentration of 200 μg mg^{-1} protein for Na^+/K^+-ATPase and 400 μg mg^{-1} protein for H^+-ATPase (Fig. 5). An alamethicin concentration of 100 μg mg^{-1} protein resulted in optimal activity of both ATPases and was therefore chosen as standard for all subsequent assays.

Ethanol had no effect on the activity of either ATPase at the concentrations used (Fig. 6). In water and ethanol controls, about 20 % of the Na^+/K^+-ATPase and H^+-ATPase activity obtained in the presence of alamethicin was measured (Fig. 6). The specific activities of Na^+/K^+-ATPase and H^+-ATPase were only between one-third and one-quarter in homogenates compared with permeabilized cells (Fig. 7). The difference was highly significant. Homogenate vesicles, permeabilized with alamethicin in the same manner as cell suspensions, still showed significantly lower activity of both ATPases. Nevertheless, compared with non-permeabilized homogenate vesicles, their activity was approximately twofold higher (Fig. 7). Notably, Na^+/K^+-ATPase and H^+-ATPase activity showed the same pattern when their activities were compared in permeabilized cells, homogenate vesicles and permeabilized homogenate vesicles (Fig. 7).

Na^+/K^+-ATPase and H^+-ATPase activities were not significantly influenced by thermal acclimation of fish (Fig. 8). However, acclimation of fish from SW to DSW significantly affected the *in situ* activity of both ATPases. While Na^+/K^+-ATPase activity is only about half as high in gill epithelial cells isolated from DSW-acclimated fish compared with SW-acclimated fish, H^+-ATPase activity increased by a factor of almost 2 (Fig. 8). Despite this contrary osmotic influence, Na^+/K^+-ATPase activity is higher under all conditions than H^+-ATPase activity. It exceeds the latter by factors of 3.9 (SW, 7 °C), 4.2 (SW, 26 °C) or 1.4 (DSW, 26 °C). Not only are the

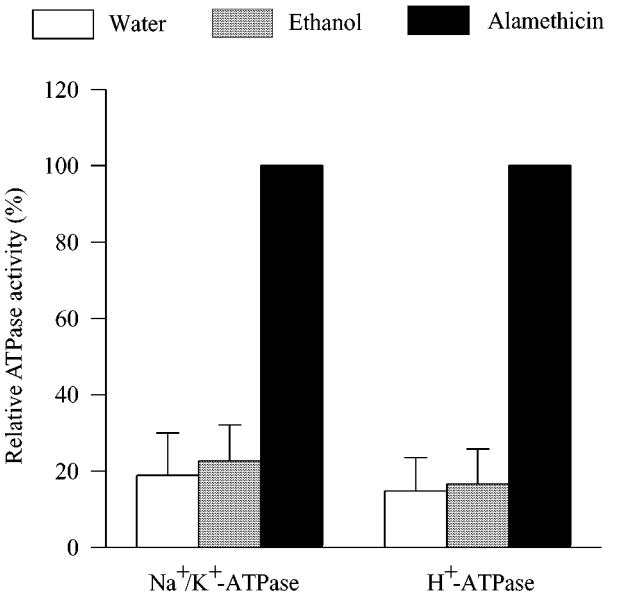

Fig. 6. Effects of ethanol on Na^+/K^+-ATPase and H^+-ATPase activity. Ethanol concentration in the control was the same as in cell suspensions treated with 100 μg alamethicin mg^{-1} protein (0.3 %). Preincubation time was 10 min. The activity of both ATPases was significantly different in both water and ethanol controls from that in alamethicin-treated cells (P<0.01), but not between the two controls. Data were obtained from fish acclimated to sea water at 26 °C and represent means + S.E.M. (N=6).

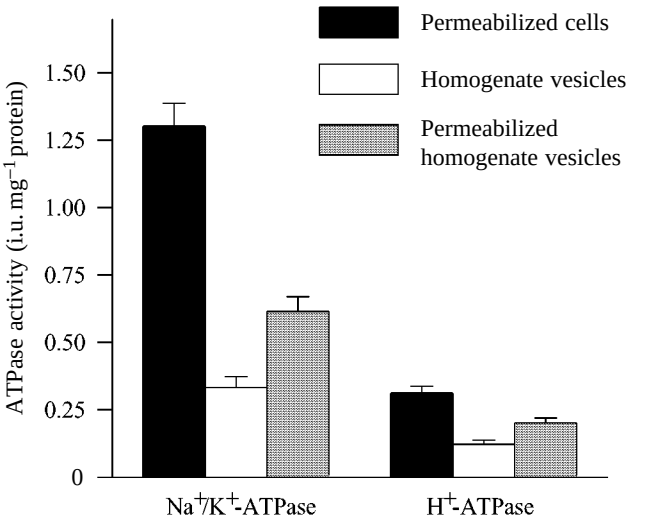

Fig. 7. Comparison of Na^+/K^+-ATPase and H^+-ATPase activities in permeabilized gill epithelial cells, homogenate vesicles and permeabilized homogenate vesicles. Cells and homogenate vesicles were permeabilized by a 10 min preincubation in the presence of 100 μg alamethicin mg^{-1} protein. For both ATPases, values for all three groups are significantly different from each other (P<0.01). Data were obtained from fish acclimated to sea water at 26 °C and represent means + S.E.M. (N=6).

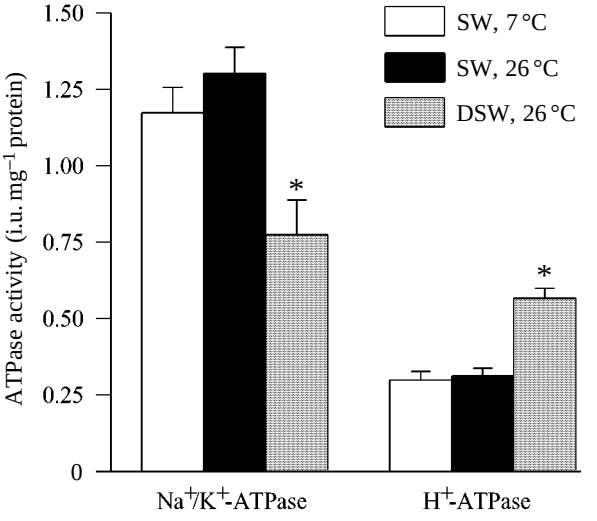

Fig. 8. The influence of osmotic and thermal acclimation on Na^+/K^+-ATPase and H^+-ATPase activity in permeabilized gill epithelial cells *in situ*. Thermal acclimation had no significant effect on the activity of either ATPase. Osmotic acclimation to diluted sea water (DSW) resulted in significantly lower Na^+/K^+-ATPase (P<0.05) and significantly higher H^+-ATPase (P<0.01) activity. An asterisk indicates significant differences from both other groups. Data are means + S.E.M. (N=6). SW, sea water.

osmotic effects on H^+-ATPase different from those on Na^+/K^+-ATPase, but so are the osmotic influences on the number, size and volume of MR cells. This is directly reflected in a negative slope of the regression line when plotting *in situ* H^+-ATPase activity of gill epithelial cells against total MR cell volume in gills from the same fish (Fig. 9B). The opposite is true for *in situ* Na^+/K^+-ATPase activity, which shows a positive linear correlation with total MR cell volume (Fig. 9A). The positive correlation between total MR cell volume and Na^+/K^+-ATPase activity is low (r=0.60), which might be explained by the low abundance of MR cells in *G. mirabilis* gills (<3 %, Fig. 3). The better negative correlation between total MR cell volume and H^+-ATPase activity (r=0.77) may result from two opposite effects induced by DSW acclimation, these being a decrease in total MR cell volume and a simultaneous increase in H^+-ATPase activity in pavement cells, which are much more abundant in *G. mirabilis* gill epithelium than are MR cells. Thus, osmotic acclimation of fish resulted in strikingly different responses of *in situ* activity of Na^+/K^+-ATPase and H^+-ATPase in gill epithelial cells.

Discussion

We used a non-enzymatic cell isolation procedure for dispersion of gill epithelium into single cells. An important advantage of this approach is that proteases, frequently used in many other cell isolation protocols, were absent during cell isolation, and integral membrane proteins, such as Na^+/K^+-ATPase and H^+-ATPase, were not subject to protease digestion. In addition, no serum or hormone supplements were used, preventing any possible effects of these exogenous factors on ATPases. Nevertheless, cell isolation was very

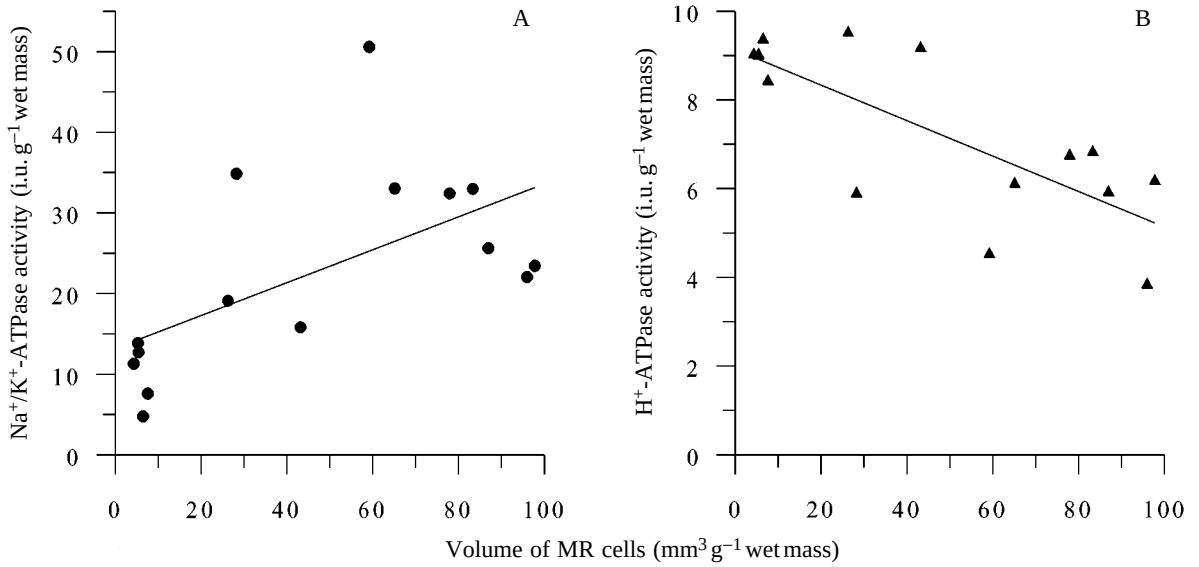

Fig. 9. Linear relationships between the *in situ* ATPase activity and the total volume of mitochondria-rich (MR) cells per gram gill epithelial wet mass. (A) Na^+/K^+-ATPase activity (circles, $A=0.20V+13.22$, $r=0.60$, where A is enzyme activity and V is total MR cell volume); (B) H^+-ATPase activity (triangles, $A=-0.04V+9.13$, $r=0.77$). Each pair of data was from the same fish.

efficient and yielded highly viable and completely dispersed cells. High viability was achieved in part by avoiding desiccation of cells during weighing. To estimate the wet mass of gill epithelium, we used an equation derived from linear regression analysis of TL and GEW. The total length of fish is much less dependent on nutritional status than is body mass, and the lower coefficient of correlation between BW and GEW was probably due to the unequal feeding rates of cold- and warm-acclimated fish. It must be stressed that the equation used for calculating GEW is only valid in the size range of fish used in our experiments. It is not known whether this linear relationship is valid for the full size range of *G. mirabilis*. Considering all developmental stages, in several other fishes, the relationship between gill area and body mass is logarithmic (Hughes, 1972).

In suspensions of isolated gill epithelial cells, less than 3 % of total cells were MR cells. The proportion of MR cells in SW-acclimated fish (2.29–2.56 %) was more than twice as high as in DSW-acclimated fish (1.14 %). This trend is also reflected in the absolute numbers of these cells per gram GEW. A lower number of MR cells in DSW-acclimated fish compared with SW-acclimated fish has been found in other teleosts (Pisam and Rambourg, 1991). Unlike osmotic acclimation, thermal acclimation had no effect on the numbers of MR cells in fish held in SW. In general, the proportion of MR cells in gills of *G. mirabilis* was low compared with that in other hypo-osmoregulating or euryhaline teleosts. For example, in SW-acclimated *Opsanus beta*, MR cells make up 13 % of total gill epithelial cells (Perry and Walsh, 1989), and in the gill of *Oreochromis mossambicus* the proportion of MR cells is 6 % at 0.4 ‰ and 16 % at 60 ‰ salinity (Kültz and Jürss, 1993). A possible explanation for the low proportion of MR cells in *G. mirabilis* gills could be the high density of these cells in the elaborate jaw skin (Yoshikawa *et al.* 1993). MR cells in the gills of *G. mirabilis* were significantly larger in SW-acclimated fish than in fish held in DSW. No effects of thermal acclimation on the size of MR cells could be detected in fish held in SW. To our knowledge, no comparable data are available concerning the effects of thermal acclimation on MR cell number and size for other fishes. However, an increase in size of these cells with increasing osmotic concentration of water is very common among euryhaline teleosts (Pisam and Rambourg, 1991). In contrast to other teleosts (Pisam and Rambourg, 1991), MR cells in the gills of SW-acclimated *G. mirabilis* were not ovoid in shape when dispersed, but were round. This difference could be due to the different isolation procedures or to differences in membrane and cytoskeleton structure that affect the ability of MR cells to round up after isolation. Contrary to our results on gill MR cells, Yoshikawa *et al.* (1993) found no effect of osmotic acclimation on MR cell number and size in the jaw skin of *Gillichthys mirabilis*. This difference may be due to the length of the acclimation period, which was 5 months in our study and only 3 weeks in the study of Yoshikawa *et al.* (1993), suggesting that 3 weeks might not be sufficient for full acclimation. This seems unlikely, however, since adjustments in MR cell number and size after acclimation of *Oreochromis mossambicus* from DSW to SW were completed within about 1 week (Foskett *et al.* 1981). It is more likely that qualitative tissue-specific differences with respect to osmoregulatory function exist between the gill and the jaw skin of *G. mirabilis*, because such differences have been shown to exist between the gill and the opercular epithelium of *Oreochromis mossambicus* (Kültz *et al.* 1995).

In the present study, for the first time, isolated gill cells of fishes were permeabilized and used for determination of *in situ* ATPase activity. Isolated gill epithelial cells were maintained and permeabilized in Ca^{2+}/Mg^{2+}-free Hanks BSS so that Ca^{2+}

was not introduced to the ATPase assay during sample addition, thus ensuring that Ca^{2+}-ATPase was inactive. Xie *et al.* (1989) used 10 mmol l^{-1} Tris/EGTA as a medium for permeabilizing freshly isolated rat cardiac myocytes, pheochromocytoma cells (clone PC 12) and neuroblastoma×glioma hybrid cells (clone NG 108-15) with alamethicin, but we found that the viability of fish gill epithelial cells decreased substantially when these cells were maintained in 10 mmol l^{-1} Tris/EGTA for 2 h (data not shown). Alamethicin is one of several substances that form channels in biological membranes. It was the chemical of choice for our studies, since alamethicin channels allow unlimited penetration of ATP and ADP (Ritov *et al.* 1993). In gill epithelial cells of *G. mirabilis*, alamethicin channels formed best at alamethicin concentration of 100 μg mg^{-1} cellular protein and with a 10 min preincubation time. The time of preincubation was not as critical for good channel formation as was alamethicin concentration. Both ATPase activities showed linear increases with increasing protein concentration in the assay if the alamethicin:protein ratio was kept constant. The optimal concentration of alamethicin for permeabilizing purified skeletal muscle triads from New Zealand white rabbits was 600 μg mg^{-1} protein (Mitchell *et al.* 1983). For efficient permeabilization of sarcoplasmic reticulum vesicles from white skeletal muscles of rabbit hind limbs, an alamethicin concentration of 0.5–5 mg mg^{-1} protein was necessary (Ritov *et al.* 1993). At these high concentrations, alamethicin had inhibitory effects on Na^+/K^+-ATPase and H^+-ATPase in gill epithelial cells of fish (Fig. 5). Controls showed that this inhibition was not due to increased ethanol concentration, but was truly due to high alamethicin concentration. The effects of alamethicin concentration on Na^+/K^+-ATPase activity in PC 12 cells, NG 108-15 cells and rat cardiac myocytes were comparable with those in *G. mirabilis* gill epithelial cells. The optimal alamethicin concentration for permeabilizing these cells was between 50 and 250 μg mg^{-1} protein (Xie *et al.* 1989). It was also shown that, at alamethicin concentrations higher than 1 mg mg^{-1} protein, purified Na^+/K^+-ATPase of rat kidney was inhibited (Xie *et al.* 1989). The chemical nature of this inhibitory effect is unknown. As mentioned above, ethanol had no effect on Na^+/K^+-ATPase and H^+-ATPase activities at the concentrations used. About 20 % of Na^+/K^+-ATPase and H^+-ATPase activity was detectable, even if cells were not permeabilized (i.e. in controls). In theory, this value should not have exceeded 4 %, since cell viability determined using the Trypan Blue dye exclusion test was greater than 96 %. It is therefore possible that about one-sixth of the cells had minor membrane lesions causing ATP and ADP leakage, but not permitting penetration of Trypan Blue. In this sense, the quotient of ATPase activity in the presence and absence of alamethicin is a more sensitive cell viability assay than the Trypan Blue dye exclusion test. In addition to the determination of *in situ* ATPase activity and cell viability, gill epithelial cells of fishes, permeabilized by the method described in this study, might also be suitable for intraorganelle tracking of second messenger pools (Hofer and Machen, 1993), for cell polarization studies (Morre *et al.* 1993) and for the determination of *in vivo* K_m values of several enzymes (Aragon and Sols, 1991).

ATPase activities in permeabilized cells, homogenate vesicles and permeabilized homogenate vesicles have been measured under exactly the same conditions (the composition of the assay media and the measurement procedures were identical in all cases). The conditions were optimized for *in vitro* ATPase activity. If *in situ* ATPase activities, as measured under the conditions in our assay, do not represent maximal activities, the difference from *in vitro* ATPase activities would be even greater than demonstrated. Our results show that at least three-quarters of Na^+/K^+-ATPase and two-thirds of H^+-ATPase activity are not detectable in crude homogenates of gill epithelium. Because a broad spectrum of protease inhibitors was added at concentrations that have no effect on non-proteolytic enzymes (Eisenthal and Danson, 1992), the loss of enzyme activity compared with permeabilized cell preparations was not caused by a partial digestion of ATPase pumps by endogenous proteases released during homogenization. In fact, latent activity of Na^+/K^+-ATPase and H^+-ATPase was recovered after permeabilization of homogenate vesicles. Because permeabilization of homogenate vesicles resulted in an almost 100 % increase of activity of both ATPases, it is likely that about 50 % of the membrane vesicles had an inside-out orientation. During homogenization, vesicles probably form randomly with a correct or an inside-out orientation of the plasma membrane. In crude homogenates, ATPase activity is only detectable in those vesicles with an inside-out orientation since the ATP binding domains are exposed to the outside of these vesicles, but cannot be accessed by external ATP in vesicles with the correct orientation. Nevertheless, even when considering latent ATPase activity in homogenate vesicles (i.e. after permeabilization of vesicles), a significant difference in activity of Na^+/K^+-ATPase and H^+-ATPase between homogenates and whole cells was found. Two possible explanations for this difference are the enzyme concentration and the structural integrity of the ATPases and their lipid environment. Enzyme concentration is much higher *in situ* in intact cells than after homogenization, and significant effects of enzyme concentration on activity of some enzymes have been reported (Aragon and Sols, 1991). It is also likely that some structural damage occurs during homogenization to some of the Na^+/K^+-ATPase and H^+-ATPase molecules or to their immediate lipid environment, resulting in a loss of activity. Even though permeabilization of cells may also lead to some changes in plasma membrane lipid arrangement and ATPase concentration because of the insertion of alamethicin channels, these changes are likely to be minor in comparison with the drastic effect of homogenization. Na^+/K^+-ATPase activity of teleost gills was shown to be significantly influenced by the lipid composition of the plasma membrane (Gibbs and Somero, 1990). Raynard and Cossins (1991) reported that membrane order may influence Na^+/K^+-ATPase activity, without changing the total number of pump sites, by altering its substrate turnover

rate. In erythrocytes from *Oncorhynchus mykiss*, an increase in membrane order was paralleled by a decrease in Na^+/K^+-ATPase turnover (Raynard and Cossins, 1991). The Na^+/K^+-ATPase activity of rat hepatocytes was also shown to depend dramatically on membrane order, being distributed in both the apical and basolateral domains, but being active only in the basolateral membrane because of its lower fluidity (higher membrane order) (Sutherland *et al.* 1988). Another explanation for the differences between *in vitro* and *in situ* ATPase activities could be differences in ion concentrations (in particular Na^+ and K^+) at the inner side of the plasma membrane of permeabilized cells or the inside of homogenate vesicles. These concentrations are presumably different in permeabilized cells and homogenate vesicles. Because alamethicin channels do not allow free diffusion of ions from one side of the membrane to the other side (Vodyanoy *et al.* 1993; Bezrukov and Kasianowicz, 1993), it cannot be assumed *a priori* that ion concentrations are equal on both sides of the membrane in cells or homogenate vesicles permeabilized with alamethicin. Thus, the exact concentrations of ions inside permeabilized cells and homogenate vesicles are not known.

The independence of both Na^+/K^+-ATPase and H^+-ATPase activity on thermal acclimation in gill epithelial cells of *G. mirabilis* is surprising, because in crude gill homogenates of other teleosts Na^+/K^+-ATPase activity at least was significantly increased after cold-acclimation (Stuenkel and Hillyard, 1980; Schwarzbaum *et al.* 1991). Thermal compensation of Na^+/K^+-ATPase activity in parallel to homeoviscous adaptation was also found in crude homogenates of teleost gills (Gibbs and Somero, 1990) and erythrocytes (Raynard and Cossins, 1991). We are unable to state, at this point, whether the different response of Na^+/K^+-ATPase to cold-acclimation in gill epithelial cells of *G. mirabilis* is species-specific or caused by methodology (i.e. *in vitro* compared with *in situ* enzyme assays). In some studies, no increase in *in vitro* Na^+/K^+-ATPase activity of teleost gills after cold-acclimation was observed (Olsen *et al.* 1993; Staurnes *et al.* 1994). Changes in Na^+/K^+-ATPase and H^+-ATPase activities may be necessary to compensate for possible thermal effects on the energetic cost of osmoregulation. However, Na^+/K^+-ATPase activity has been shown to be subject to regulation by mechanisms other than a change in the number of active pumps. These activity changes are not detectable by *in situ* or *in vitro* enzyme assays, because in such assays the maximal activity determined by the number of pumps is measured. For instance, in *G. mirabilis* gill epithelial cells, the kinetic efficiency of Na^+/K^+-ATPase can be modulated by a phosphocreatine circuit (Kültz and Somero, 1995) and in *Oncorhynchus mykiss* erythrocytes a modulation of pumping and turnover rates of Na^+/K^+-ATPase results from changes in the immediate lipid environment of this enzyme (Raynard and Cossins, 1991). It is possible that an increase in the maximal Na^+/K^+-ATPase activity (number of active pumps) measured after cold-acclimation of fish *in vitro* represents an artifact. The degree of destruction of plasma membrane and Na^+/K^+-ATPase during homogenization may depend on the lipid composition of the plasma membrane, which is known to be modulated by the process of homeoviscous adaptation during thermal acclimation (Prosser and Heath, 1991). Thus, increased Na^+/K^+-ATPase activity measured *in vitro* might simply be due to the higher stability of this enzyme in cold-acclimated plasma membranes during homogenization. It is also known that enzyme concentration, which is much higher *in situ* than *in vitro*, has significant effects on enzyme activity (Aragon and Sols, 1991). Moreover, it is possible that the use of detergents (e.g. sodium deoxycholate) during sample preparation and *in vitro* ATPase assay leads to different results by unmasking latent ATPase activity in the vesicles of the endoplasmic reticulum or Golgi apparatus. However, this intracellular fraction of ATPase activity not bound to plasma membranes represents pumps that are immature under *in vivo* conditions and are not involved in active ion transport across the plasma membrane (Mircheff *et al.* 1993). Comparable data on the effects of thermal acclimation on H^+-ATPase in fish gills are not yet available.

In contrast to thermal acclimation, osmotic acclimation resulted in significant changes in both the Na^+/K^+-ATPase and the H^+-ATPase activity of gill epithelial cells. The activity of Na^+/K^+-ATPase was significantly increased in SW-acclimated compared with DSW-acclimated fish. This is a common response in many other teleosts (De Renzis and Bornancin, 1984). In contrast, Yoshikawa *et al.* (1993) reported that osmotic acclimation had no effect on Na^+/K^+-ATPase activity in crude homogenates of *G. mirabilis* gill and jaw skin. Whether these different results are due to a difference in methodology, to different acclimation times or to different populations of fish is not clear. In gill epithelial cells of *G. mirabilis*, H^+-ATPase activity responded in a different manner from Na^+/K^+-ATPase to osmotic acclimation, being significantly higher in DSW-acclimated than in SW-acclimated fish. The same effect of osmotic acclimation on the activity of H^+-ATPase was also found in crude homogenates of *Oncorhynchus mykiss* gills (Lin and Randall, 1993). An increasing amount of evidence supports the hypothesis that H^+-ATPase creates an electrical gradient for Na^+ absorption through an apical Na^+ channel in gill epithelial cells (Avella and Bornancin, 1989; Lin and Randall, 1991; Goss *et al.* 1992). It is not known whether this process is located in MR cells or pavement cells. A recent study has shown that H^+-ATPase is localized in the apical membranes of both the MR cells and the pavement cells of *Oncorhynchus mykiss* gills (Lin *et al.* 1994). This finding is in agreement with the idea that MR cells and pavement cells are electrically coupled (Goss *et al.* 1992). Since the correlation between H^+-ATPase and MR cell number is negative and opposite to the correlation between Na^+/K^+-ATPase and MR cell number, it is likely that most of the H^+-ATPase of *G. mirabilis* gills is localized in pavement cells. An alternative explanation, which we view as less likely, would be a marked increase in H^+-ATPase pump numbers in the smaller MR cells of DSW-acclimated fish. Further investigations, which could involve the use of permeabilized gill epithelial cells, are necessary to elucidate the molecular mechanisms and cellular organization of active, independent absorption of Na^+ and Cl^- across teleost gill epithelium.

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