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TRANSPORT PROPERTIES OF CULTURED BRANCHIAL EPITHELIA FROM FRESHWATER RAINBOW TROUT: A NOVEL PREPARATION WITH MITOCHONDRIA-RICH CELLS

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Summary

A new double-seeded insert (DSI) technique is described for culture of branchial epithelial preparations from freshwater rainbow trout on filter supports. DSI epithelia contain both pavement cells and mitochondria-rich (MR) cells (15.7±2.5 % of total cell numbers). MR cells occur singly or in clusters, are voluminous, open apically to the 'external environment' and exhibit ultrastructural characteristics similar to those found in the 'chloride cells' of freshwater fish gills. After 6–9 days in culture with Leibovitz's L-15 medium on both surfaces (symmetrical conditions), transepithelial resistance (TER) stabilized at values as high as 34 kΩ cm^2, indicative of electrically 'tight' epithelia. The density of MR cells, the surface area of their clusters and transepithelial potential (TEP; up to +8 mV basolateral positive, mean +1.9±0.2 mV) were all positively correlated with TER. In contrast, preparations cultured using an earlier single-seeded insert (SSI) technique contained only pavement cells and exhibited a negligible TEP under symmetrical conditions. Na^+/K^+-ATPase activities of DSI preparations were comparable with those in gill filaments, but did not differ from those of SSI epithelia. Replacement of the apical medium with fresh water to mimic the *in vivo* situation (asymmetrical conditions) induced a negative TEP (–6 to –15 mV) and increased permeability to the paracellular marker PEG-4000. Under symmetrical conditions, unidirectional Na^+ and Cl^- fluxes were in balance, and there was no active transport by the Ussing flux ratio criterion. Under asymmetrical conditions, there were large effluxes, small influxes and evidence for active Cl^- uptake and Na^+ extrusion. Unidirectional Ca^{2+} fluxes were only 0.5–1.0 % of Na^+ and Cl^- fluxes; active net Ca^{2+} uptake occurred under symmetrical conditions and active net extrusion under asymmetrical conditions. Thus, DSI epithelia exhibit some of the features of the intact gill, but improvements in culture conditions are needed before the MR cells will function as true freshwater 'chloride cells'.

Key words: gill, cell culture, filter insert, chloride cell, mitochondria-rich cell, pavement cell, transepithelial potential, transepithelial resistance, Na^+/K^+-ATPase activity, Ca^{2+} transport.

Introduction

The branchial epithelium of freshwater fish is responsible for the active uptake of Na^+, Cl^- and Ca^{2+}, but the actual sites and mechanisms of transport remain controversial (Goss et al., 1995; Kirschner, 1997; Perry, 1997; Claiborne, 1997). There is a pressing need for a simplified flat *in vitro* model of the freshwater gill comparable with the opercular epithelium of marine teleosts, which has proved invaluable in understanding the transport mechanisms of the seawater gill (Zadunaisky, 1984; Marshall, 1995). Various isolated epithelia from freshwater teleosts have been evaluated, with mixed results (Foskett et al., 1981; Marshall, 1985; McCormick et al., 1992; Marshall et al., 1992, 1995, 1997; Wood and Marshall, 1994; Burgess et al., 1998). Only the the opercular epithelium of the freshwater tilapia *Oreochromis niloticus* actively transported all three ions in the uptake direction, but the actual flux rates of Na^+ and Cl^- were extremely low (Burgess et al., 1998).

An entirely different approach to the problem is to culture a 'reconstructed' branchial epithelium *in vitro*. Wood and Pärt (1997) developed a method for growing branchial epithelia from freshwater rainbow trout gills on permeable filter supports ('inserts'). These epithelia can be exposed to different media on their apical and basolateral surfaces and, indeed, survive apical freshwater exposure for 24–48 h. This method, hereafter designated as the single-seeded insert (SSI) approach, involves tryptic digestion of gill filaments to obtain epithelial cells, initial culture of the cells in flasks for 6 days, retrypsination and then seeding of these cells onto filter inserts for a further 6–9 days of culture. By morphological and

physiological criteria (Wood and Pärt, 1997; Wood et al., 1998; Gilmour et al., 1998b), the resulting SSI preparations consist entirely of pavement ('respiratory') cells, duplicate the electrical and passive permeability properties of the intact gill quite well, but show almost negligible ion transport function except for a very small active uptake of Cl^-. This latter deficit is probably due in part to the absence of mitochondria-rich (MR) cells ('chloride cells'), which are thought to play key but controversial roles in ion uptake *in vivo* (Perry, 1997; Claiborne, 1997). These cells are absent because they fail to attach and die out after a few days in the initial flask culture (Pärt et al., 1993; Wood and Pärt, 1997).

We reasoned that *in vivo* chloride cells are nested amongst pavement cells, and *in vitro* they may fail to attach because they do not receive the correct attachment signals and surfaces. In support of this idea, Witters et al. (1996) reported that some MR cells persisted in explant cultures of trout gills, a preparation in which they could presumably retain their position amongst pavement cells. However, three other explant studies reported that MR cells did not survive in culture (Fernandes et al., 1995; Avella et al., 1994; Leguen et al., 1998). A more reliable way to create the correct environment for MR cells might be to establish a 'lawn' of attached pavement cells prior to seeding of chloride cells. In the present study, we have developed a novel double-seeded insert (DSI) preparation that employs this principle to incorporate MR cells successfully into the cultured branchial epithelium of freshwater trout. The initial period of flask culture is omitted (a considerable saving in time), and repetitive seeding is performed directly on the filter inserts. Having two different cultured preparations, one (SSI) with pavement cells only and one (DSI) with both pavement cells and MR cells, may prove extremely valuable for future mechanistic analyses.

The objectives of the present report, in addition to describing this new technique, were to describe the basic physiological properties of these new DSI epithelia and to compare them with SSI epithelia in terms of structure, electrical and permeability properties, Na^+/K^+-ATPase activity and Na^+, Cl^- and Ca^{2+} transport activity.

Materials and methods

Experimental animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from local aquaculture for experiments in both Hamilton, Canada, and Uppsala, Sweden. In Hamilton, the fish (50–150 g) were held in dechlorinated running tapwater (in mmol l^{-1}: $[Na^+]$, 0.55; $[Cl^-]$, 0.70; $[Ca^{2+}]$, 1.00; $[Mg^{2+}]$, 0.15; $[K^+]$, 0.05; pH 7.8–8.0). Photoperiod and temperatures (4–17 °C) varied seasonally. In Uppsala, fish (100–200 g) were held in running temperature-controlled fresh water (11–13 °C) (in mmol l^{-1}: $[Na^+]$, 0.06; $[Cl^-]$, 0.05; $[Ca^{2+}]$, 0.20; $[Mg^{2+}]$, 0.04; pH 7.0), which is a 10-fold dilution of regular Uppsala tapwater with deionized water. Thus, Uppsala fish were acclimated to a more dilute medium than Hamilton fish. In Uppsala, fish were exposed to a photoperiod that matched that of Hamburg, Germany, to allow for longer daylight in the winter months.

Cell isolation

Sterile techniques were used throughout. Cells for both single-seeded insert (SSI) and double-seeded insert (DSI) preparations were initially isolated from excised gill filaments by two consecutive cycles of trypsination. The isolation procedure is based on methods originally developed by Pärt et al. (1993), with slight modifications described by Wood and Pärt (1997).

Single-seeded insert (SSI) preparations

SSI preparations were grown in both Uppsala and Hamilton according to the two-stage methods described by Wood and Pärt (1997): 6–8 days of flask culture, followed by retrypsination and 6 days of culture on permeable Falcon filter inserts (cyclopore polyethylene terephthalate 'filters'; Becton Dickinson, Franklin Lakes, New Jersey, USA; pore density 1.6×10^6 pores cm^{-2}, pore diameter 0.45 μm, growth surface area 0.83 cm^2). Leibovitz's L-15 medium plus 2 mmol l^{-1} glutamine and 5 % foetal bovine serum (FBS) was used throughout and was supplemented with antibiotics [2 % PEST solution (5000 i.u. ml^{-1} penicillin and 5000 i.u. ml^{-1} streptomycin) (Gibco) and 2 % gentamicin solution (10 mg ml^{-1} gentamicin sulphate in distilled water (Gibco)] for the first 3 days of flask culture only.

Double-seeded insert (DSI) preparations

DSI preparations were grown only in Hamilton. The production of an initial primary culture in flasks was omitted. Instead, gill epithelial cells freshly isolated from filaments by trypsination were seeded directly onto Falcon culture inserts (as for SSI), but at a very high density of 2×10^6 to 2.5×10^6 viable cells cm^{-2}. At this stage, the culture medium was supplemented with antibiotics as above for SSI preparations, with volumes of 0.8 ml on the apical side and 1.0 ml on the basolateral side. One day after seeding, each insert was rinsed three times, or until visual inspection ensured that the mucous layer was removed, with 0.4 ml of antibiotic-supplemented culture medium. At this time, new cells freshly isolated from a second fish were seeded at the same very high density onto the layer of cells already established in the inserts. On the day following the second seeding, inserts were rinsed until the mucous layer was once again removed. Samples of 1.5 ml (apical) and 2.0 ml (basolateral) of antibiotic-free medium were added to inserts and wells, and the preparations were returned to the incubator. Bathing medium remained antibiotic-free and was changed every 48 h until the epithelia were used. Experimental and culture temperature was 17–19 °C throughout for both DSI and SSI preparations.

Electrophysiological measurements

Transepithelial resistance (TER) was monitored using STX-2 chopstick electrodes connected to a EVOM epithelial volt/ohmeter (World Precision Instruments, Sarasota, Florida,

USA). TER measurements made in Uppsala could be determined only for membranes with TERs of $\leqslant 20\,k\Omega\,cm^2$. In Hamilton, a custom-modified EVOM voltohmeter was employed measuring TER as high as $100\,k\Omega\,cm^2$. Transepithelial potential (TEP) was monitored using agar/salt bridges ($3\,mol\,l^{-1}$ KCl in 4% agar) connected through Ag/AgCl electrodes (WPI) to a pH meter used as a high-impedance electrometer (Radiometer pHM 84). All TEP values were expressed relative to the apical side as 0 mV. Appropriate blank corrections for the influence of the apical and basolateral solutions (i.e. culture medium or fresh water) on measurements of both TER and TEP were performed as described by Wood et al. (1998). Daily measurements of TER and TEP across filter inserts with culture medium on both surfaces were made starting 48 h after the initial seeding of SSI membranes and 72 h after the initial seeding of DSI membranes. At selected times, TER and TEP values were also recorded for inserts with fresh water substituted on the apical (i.e. insert) side of the preparation, with culture medium remaining on the basolateral (well) side. Fresh water was either dechlorinated Hamilton tapwater sterilized by autoclaving or full-strength Uppsala tapwater diluted 50:50 with double-distilled water and sterilized by filtration through a filter with a pore diameter of 0.22 μm. The latter was chosen so as to match Ca^{2+} levels ($1.0\,mmol\,l^{-1}$) at the two locations.

Microscopy

Phase-contrast microscopy was used routinely to monitor cell growth in flasks and on inserts and to determine the extent of detachment during trypsinations. To determine whether mitochondria-rich (MR) cells were present in the cultures, 5 μl of Rhodamine 123 stock (methyl-*o*-6-amine-3′-imino-3H-xanthen-9-yl benzoate monohydrochloride, $1\,mg\,ml^{-1}$ in sterile phosphate-buffered saline; Molecular Probes, R302, Eugene OR, USA) was added to the apical and/or basolateral media for 30 min, at which point the stain was washed away with three consecutive 2 min rinses of culture medium prior to viewing. The fluorescence of MR cells was detected using a Zeiss inverted microscope with epifluorescence and automatic exposure control for photography. The dimensions of fluorescing clusters were measured using an ocular micrometer, and the calculated area of fluorescing clusters in each insert was expressed as a percentage of the total area of the insert ($0.83\,cm^2$). The total percentage of fluorescing cells was determined by trypsinating the cells from the confluent DSI membranes. The isolated cells were collected in a centrifuge tube, resuspended in 3 ml of culture medium and stained with 10 μl of Rhodamine 123 for 30 min. After three rinses with culture medium, the number of fluorescent cells was counted on a haemocytometer and expressed as a percentage of the total number of cells.

Selected filter inserts, cultured until fixation under symmetrical conditions, were fixed for transmission electron microscopy. Apical and basolateral media were replaced with 5% glutaraldehyde in $0.1\,mol\,l^{-1}$ sodium cacodylate buffer (pH 7.4), and the inserts were fixed for 2 h at 0–4 °C. Inserts were post-fixed with 1% osmium tetroxide in $0.1\,mol\,l^{-1}$ sodium cacodylate buffer (pH 7.4) at room temperature for 1 h and then dehydrated in a graded ethanol series (50–100%). After dehydration, the preparations were embedded in Spurr's resin (Marivac Ltd., Halifax, Nova Scotia, Canada). Blocks were sectioned on an ultramicrotome (MT-7, TMC) using a diamond knife (Micro Star, Huntsville Texas, USA) and stained with lead citrate/uranyl acetate (Lewis and Knight, 1977). The sections were examined using a Jeol transmission electron microscope (JEM 1200 EX2)

[^{3}H]PEG-4000 permeability

The permeability of cultured DSI epithelia to [^{3}H]polyethylene glycol-4000 (NEN-Dupont; 1 μCi (1 Ci= 3×10^{10} Bq) added to basolateral media of each insert) was measured on days 6–9 using methods and equations identical to those described earlier for SSI epithelia (Wood et al., 1998; Gilmour et al., 1998b).

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activities have not previously been reported in cultured gill cells and so were measured at various stages in the preparation of both SSI and DSI epithelia. In Uppsala, measurements were made (i) on the original gill filaments from which the preparations were derived, (ii) on cells at days 3 and 6 from primary culture flasks and (iii) on SSI preparations at day 6 after re-seeding on filter inserts, with all cells coming from the same fish. Cell samples from inserts and flasks were collected by trypsinization and pooled to approximately 2×10^6 cells for each sample. In a parallel study in Hamilton, activities were measured (i) on original gill filaments, (ii) on freshly isolated cells at day 0, (iii) on cells at days 3 and 6 from primary culture flasks and (iv) on SSI preparations at day 6–7 after re-seeding on filter inserts. In addition, as a direct comparison between DSI and SSI preparations, cells were harvested from both types of epithelia at the point of peak TER. Cell and filament samples were frozen at −70 °C in homogenizing medium ($0.25\,mol\,l^{-1}$ sucrose, $6\,mmol\,l^{-1}$ disodium EDTA; pH 4.8) for later analysis.

Na^+/K^+-ATPase activity was determined as the difference in the rate of phosphate liberation from adenosine triphosphate (ATP) in the presence of K^+ and in the absence of K^+ with ouabain present. The assay, developed by Holliday (1985) for fiddler crab gills, was adjusted to accommodate fish gill samples by lowering the [NaCl] in both assay solutions to $100\,mmol\,l^{-1}$ in the '$+K^+$' solution and $150\,mmol\,l^{-1}$ in the '$-K^+$' solution. In addition, more ouabain was added to the '$-K^+$' solution ($1.67\,mmol\,l^{-1}$ instead of $1\,mmol\,l^{-1}$); otherwise, the assay remained unchanged. Protein content was determined using the Bradford (1976) reagent according to the directions for the micro-method of Sigma kit B-6916.

Unidirectional ion fluxes

Unidirectional Na^+ and Cl^- flux measurements were performed across cultured DSI epithelia on days 6–9 with simultaneous measurements of TER and TEP. Unidirectional

Ca^{2+} fluxes were measured across both DSI and SSI preparations, since they had never previously been measured in any cultured gill epithelium. Equations for the calculation of influxes (J_{in}; positive sign) and effluxes (J_{out}; negative sign) were identical to those used previously for SSI epithelia (Wood and Pärt, 1997; Wood et al., 1998). In brief, with culture media in both apical and basolateral compartments, unidirectional flux measurements were conducted by adding 1 μCi of the appropriate isotope (^{22}Na, ^{36}Cl or ^{45}Ca, all NEN-Dupont) to one side of the membrane and monitoring the appearance of radioactivity on the other side over flux periods ranging from 5 to 9 h. The cultured epithelia were chosen on the basis of the stability of the TER throughout the flux procedures. Only those membranes for which TER was initially above 10 kΩ cm^2 and declined by less than 50 % by 24 h following a flux experiment were included (Wood et al., 1998). This protocol excludes preparations that may have suffered from mechanical damage or microorganism contamination during the flux procedures.

For flux measurements in the presence of fresh water, apical culture medium was replaced with sterile fresh water for a 4 h period of acclimation. To ensure that the fresh water was not contaminated with remnants of medium, the membrane was rinsed four times with 1 ml of fresh water before the final 1.5 ml was added to the apical compartment. At the 4 h mark, fresh water was replaced again using the same procedure, and isotope was added to the 'hot side' for either influx or efflux; TEP and TER were measured and solution samples were taken at this time and at the end of the flux period.

Each insert preparation could be used for influx or efflux experiments, but not both. Therefore, to calculate net flux (J_{net}), and to apply the Ussing criterion, it was necessary to pair cultured membranes that underwent opposite procedures. Pairs were matched according to the most similar average TER and average TEP (the two criteria yielded the same pairings), and each membrane was used only once in the pairing procedure. The criterion used to detect the presence of active transport was inequality between the measured flux ratio (J_{in}/J_{out}) and that predicted for entirely passive fluxes by the Ussing flux ratio equation (Kirschner, 1970). The predicted passive flux ratio was calculated according to the following equation:

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap}e^{(-zFV/\boldsymbol{R}T)}}{A_{Bl}} , \qquad (1)$$

where A_{Ap} and A_{Bl} are the activities of the ions (Na^+, Cl^- or Ca^{2+}) on the apical and basolateral sides, respectively, z is the ionic valence, V is the measured TEP (in V; average for the membrane pairs), and F, $\boldsymbol{R}$ and T have their usual thermodynamic values. A_{Na} and A_{Cl} in L-15 medium supplemented with 5 % FBS were 75 % of the total concentrations of 155 mmol l^{-1} and 143 mmol l^{-1} respectively (see Wood et al., 1998). Similar microelectrode methods determined that A_{Ca} in the media was 1.50±0.05 mmol l^{-1} (mean ± S.E.M., N=5). For fresh water, the activities of all three ions were taken as equal to their measured concentrations.

Analytical methods

Na^+ and Ca^{2+} concentrations were measured in a Varian AA1275 atomic absorption unit in Hamilton. In Uppsala, the Ca^{2+} concentration of samples was measured using a colorimetric assay (Sigma KC308). All Cl^- concentrations were determined by a colorimetric assay (Zall et al., 1956). Radioactivity was determined by scintillation counting using either a Packard Tricarb 1900 CA (Uppsala) or LKB 1217 Rackbeta instrument (Hamilton) with quench correction by internal standardization.

Statistical analyses

Data have been expressed as means ±1 S.E.M. (N), with N being the number of cultured epithelia or cell samples used in the calculation. Regression lines were fitted by the method of least squares, and the significance of Pearson's correlation coefficient (r) was determined. The statistical significance of differences between means was calculated using Student's two-tailed t-tests, paired, unpaired or one-sample tests as needed. The limit of significance in all statistical analyses was 5 %. The study is based on more than 200 DSI preparations from 46 fish and more than 300 SSI membranes from 50 fish.

Results

DSI epithelia were grown only in Hamilton. SSI epithelia were grown in both Uppsala and Hamilton; unless noted otherwise, results were similar for SSI preparations from the two locations.

Transepithelial resistance (TER)

TER, with culture media on both surfaces, increased in a sigmoidal fashion over time in DSI preparations (Fig. 1A), similar to the pattern in SSI preparations (Fig. 1B), and reached a stable plateau at approximately days 6–9. The growth curves illustrated in Fig. 1 end on days 6 and 7 because most of the preparations were harvested for experiments starting at this time. For both epithelia, there was considerable variability amongst different batches, with generally higher values occurring in the winter months. Comparing epithelia made at the same time of year, DSI preparations exhibited generally higher and later plateau values: absolute mean DSI values ranged from 1.3 to 34 kΩ cm^2 compared with 1.2–21 kΩ cm^2 in SSI preparations.

The TER response to apical freshwater exposure of SSI preparations was identical to that reported earlier (see Fig. 5 of Wood and Pärt, 1997): a substantial rise that remained stable over 3 h, then a dramatic fall when culture medium was returned to the apical side, and finally recovery within a short period to near initial values. However, in DSI preparations, the response of TER to apical fresh water was rather variable. When the starting TER was relatively low, the response was comparable with that seen in SSI preparations. However, in the more usual circumstance where starting TER was high (>10 kΩ cm^2), the TER exhibited little or no increase and often declined towards the end of the 3 h freshwater exposure. In

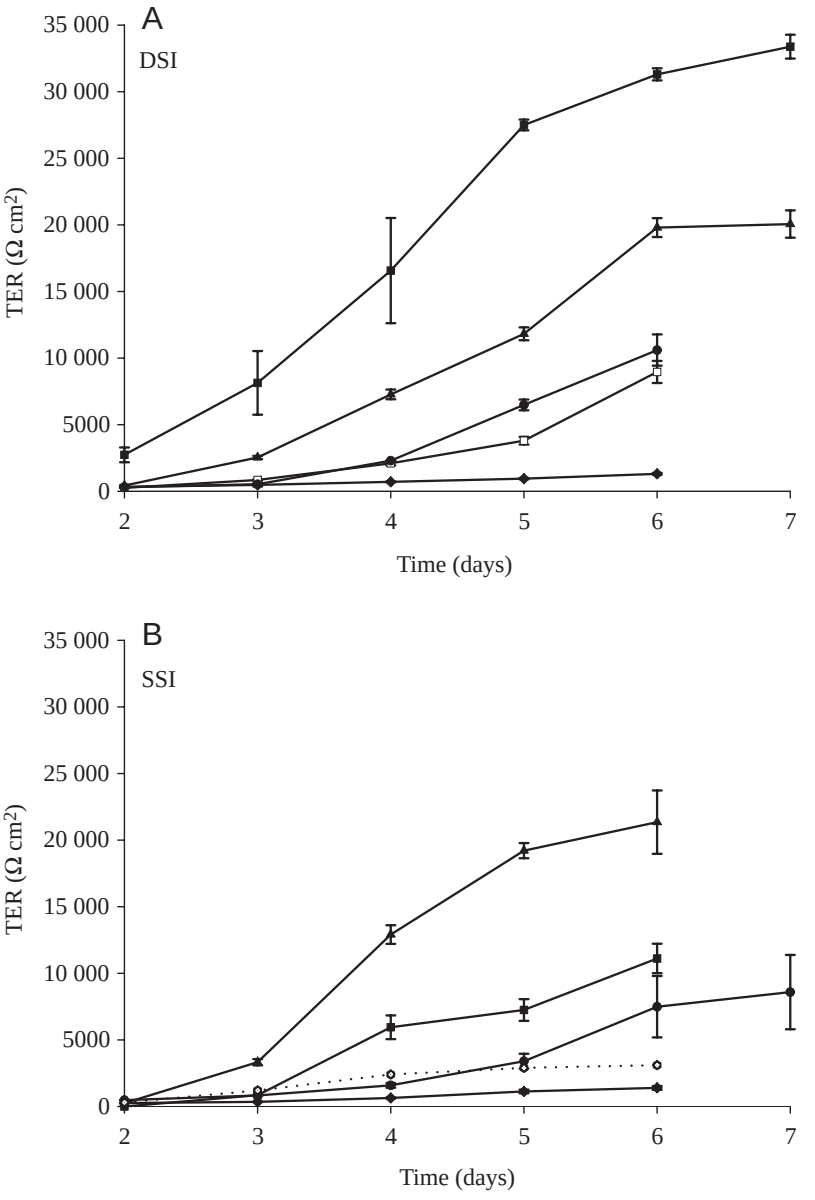

Fig. 1. Changes in transepithelial resistance (TER) with time in culture for representative batches of (A) double-seeded insert (DSI) epithelia and (B) single-seeded insert (SSI) epithelia. Measurements were taken with culture medium on both the apical and basolateral sides (symmetrical conditions). Each curve represents a different group of membranes, each of which was prepared within a 3 week period. TER was first measured 2 days after seeding onto inserts. Measurements end at days 6–7 because the epithelia were harvested for experiments at this time. Values are expressed as means ±1 S.E.M. (A) *N*=20 (filled squares); *N*=28 (diamonds); *N*=22 (circles); *N*=22 (open squares); *N*=30 (diamonds). (B) *N*=124 (triangles); *N*=62 (squares); *N*=8 (circles); *N*=14 (open circles, dotted line); *N*=5 (diamonds).

these cases, only partial recovery was seen even 24 h after restoration of culture medium to the apical side.

Morphology

A fundamental difference between SSI and DSI preparations was the occurrence of mitochondria-rich (MR) cells only in the latter. No brightly fluorescing cells were evident in any of the SSI membranes (not shown) when stained with the mitochondrial fluorophore Rhodamine 123 on the apical and basolateral surfaces. However, brightly fluorescing cells were seen in all membranes cultured using the new DSI technique. Fig. 2 is an example of a DSI epithelium with a TER of 10.1 kΩ cm^2. Typically, there were numerous single fluorescent cells (Fig. 2b) and prominent clusters of fluorescent cells (Fig. 2d). Under phase contrast (Fig. 2a), these single cells could not easily be distinguished from the pavement cell majority. However, under fluorescent illumination (Fig. 2b), a heterogeneous staining pattern was observed, with MR cells fluorescing brightly in contrast to the poorly stained surrounding pavement cells. Regardless of whether DSI epithelia were stained with Rhodamine (e.g. Fig. 2c) or not, prominent cell clusters could be detected on DSI epithelia using phase contrast alone (Fig. 2c). Under fluorescent illumination, an intense signal was seen in part or all of the cell cluster in Rhodamine-stained preparations (Fig. 2d), indicating the presence of specific regions in the epithelium where groups of MR cells had accumulated. The multiple layers of fluorescent cells in these clusters prevent a clear view of the unstained central nuclei.

Transmission electron microscopy of the MR cells revealed a cell type that is voluminous compared with the surrounding squamous pavement cells and open apically to the ‘external’ environment (Fig. 3A,B). These MR cells contain numerous large mitochondria (with mitochondrial granules), a branching

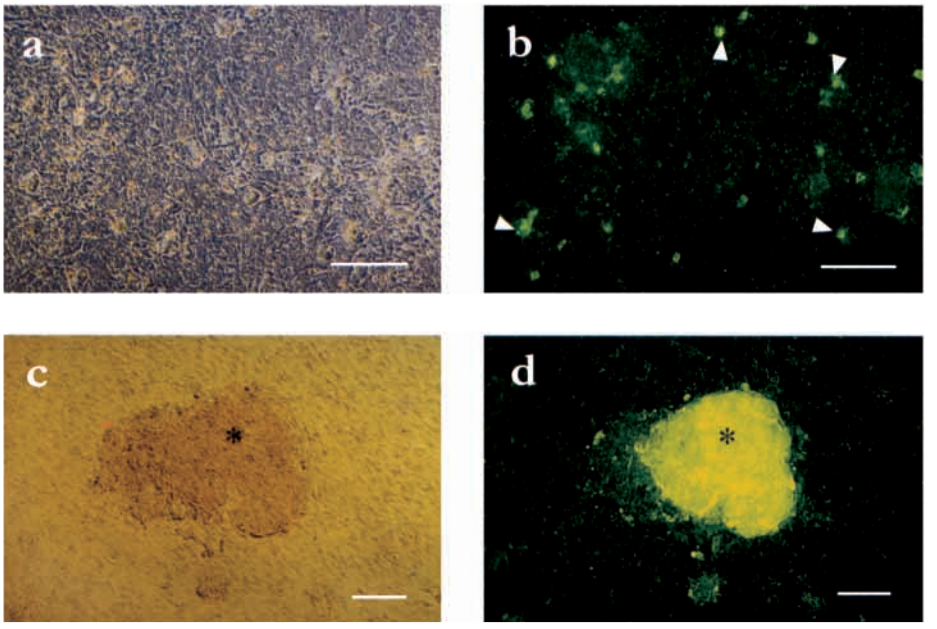

Fig. 2. Photomicrographs showing double-seeded insert (DSI) epithelia stained with Rhodamine 123 and viewed either with (b,d) or without (a,c; i.e. phase-contrast only) fluorescent illumination. In a and b, an area with only single mitochondria-rich (MR) cells (indicated by arrowheads in b) is shown; in c and d, a representative mitochondria-rich cell cluster is indicated by an asterisk. Scale bars, 200 μm.

tubular system (Fig. 3B) and well-defined tight junctions (Fig. 3C) in the apical region between the MR cells and adjacent pavement cell, comparable with those between pavement cells. These tight junctions were similar to those previously described between pavement cells in SSI epithelia (Gilmour et al., 1998b).

In a set of DSI epithelia for which the mean TER was 6.73±1.14 kΩ cm^2 (N=24) (with culture medium on both surfaces), the surface area occupied by discrete clusters of fluorescing cells was 0.17±0.03 % (N=24), ranging from only 0.03 to 0.44 % of the total surface area of the epithelia. There was a significant positive correlation (r=0.76, P<0.0001, N=24) between the fractional surface area of these fluorescent clusters and TER (Fig. 4A). While a straight line has been fitted to these data, an alternative interpretation, based on the distribution of data points, is that a critical number of MR cell clusters may evoke a change to a higher TER.

These fractional areas do not include the area covered by single fluorescing cells, which was clearly substantial but impossible to quantify using visual microscopy. Therefore, in a separate set of confluent DSI preparations with a mean TER of 20.37±4.40 kΩ cm^2 (N=12) (with culture medium on both surfaces), the number of MR cells was quantified by isolating all the cells by mild trypsination, staining them with Rhodamine 123, and expressing the number of fluorescent cells as a percentage of the total cell count. This method yielded values ranging from 0.1 to 29 % of the total cells, with a mean MR cell fraction of 15.7±2.5 % (N=12). As with the fractional surface area of the clusters, the fractional numbers of fluorescing cells (as a percentage of the total) was positively correlated with TER (r=0.58, P<0.01, N=12; Fig. 4B). Thus, part of the quantitative difference between the mean fractional surface area and the mean fractional number of MR cells was probably due to the higher TER range (mean 20.37 kΩ cm^2 compared with 6.73 kΩ cm^2) in the latter series. However, the greater portion of the difference was undoubtedly due to the fact that most of the fluorescent cells were individual cells outside clusters, and that the individual surface areas of these cells *in situ* were much smaller than those of pavement cells.

Na^+/K^+-ATPase activity

Direct comparison of Na^+/K^+-ATPase activities between DSI and SSI epithelia in Hamilton, relative to the original activities in the filaments from which they were made (Fig. 5A), revealed no significant differences, despite the presence of MR cells in the DSI epithelia. Interestingly, freshly harvested gill filaments from Uppsala trout exhibited two- to threefold higher activities than Hamilton trout (Fig. 5B,C), presumably because of the lower ion content of Uppsala water, but the activities of the final SSI epithelia grown from these filaments were identical. In initial flask cultures in Uppsala, Na^+/K^+-ATPase activity had declined by 65 %, relative to filamental values, by day 3, and this same lower level was maintained at day 6 (just prior to trypsinization for SSI filter culture). When the resulting SSI epithelia were harvested at peak TER, this same low level of activity was still seen (Fig. 5B). In Hamilton, this low Na^+/K^+-ATPase level was seen starting with filaments and continuing at day 0 (freshly dispersed cells), at day 3 and day 6 of flask culture, and in SSI epithelia (Fig. 5C). Rhodamine 123 staining demonstrated that MR cells declined from 12.8±4.2 % (N=4) of the total at day 0 to 0±0 % (N=4) at days 3 and 6, and in SSI preparations. At the same time, red blood cells declined from 2.1±0.3 % (N=4) at day 0 to 0.5±0.2 % (N=4) at day 3 and 0±0 % (N=4) at day 6 and in SSI preparations.

Transepithelial potential (TEP)

The overall mean TEP of DSI membranes under symmetrical conditions (culture medium on both surfaces, with reference to the apical surface as zero) was +1.9±0.2 mV

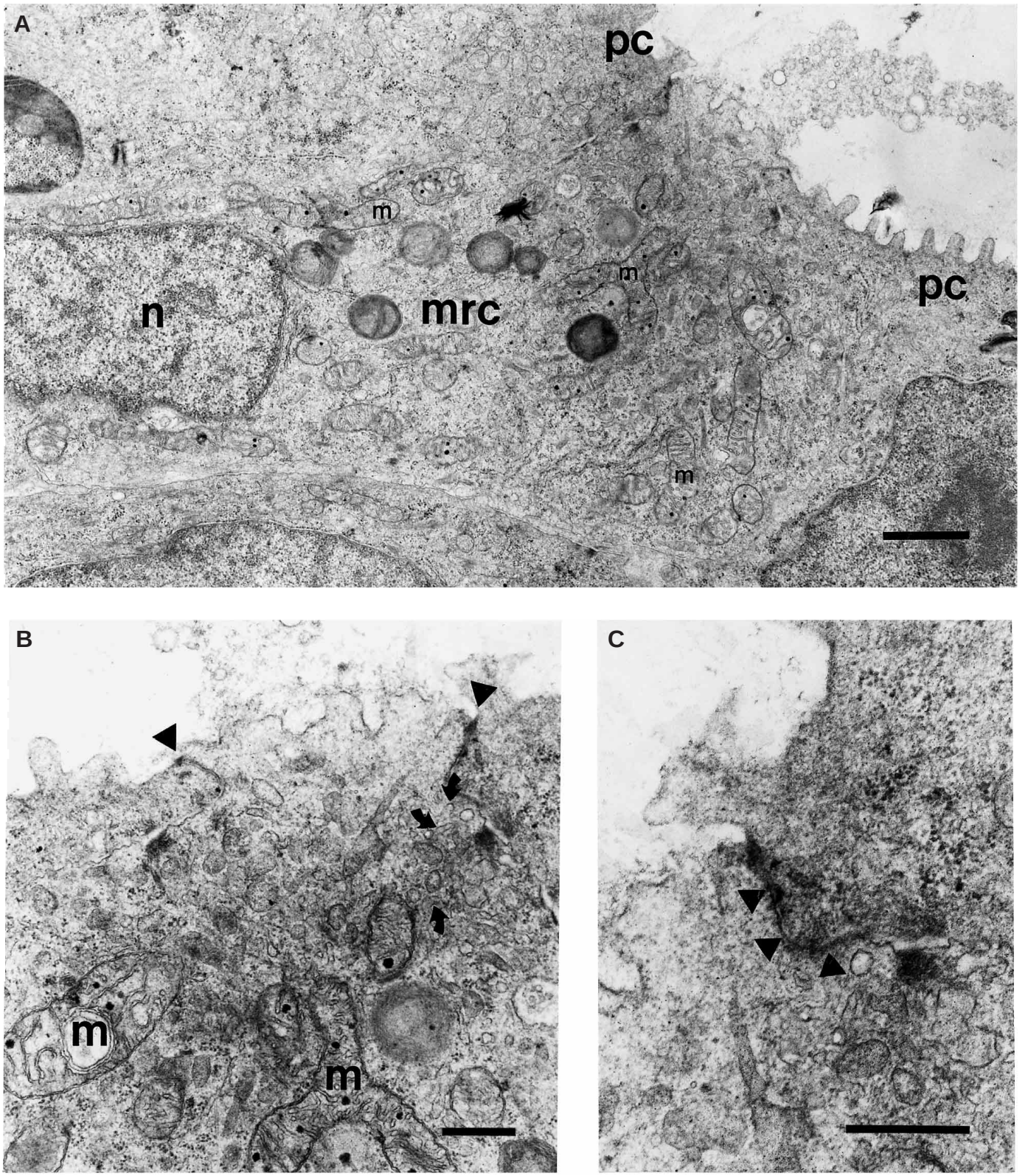

Fig. 3. Transmission electron micrographs of (A) a mitochondria-rich cell in a cultured double-seeded insert (DSI) epithelium; (B) a magnified portion of the apical area of the mitochondria-rich cell (apical exposure delineated by arrowheads, and branching tubular system indicated by curved arrows); and (C) a tight junction (indicated by arrowheads) between the mitochondria-rich cell and an adjacent pavement cell (m, mitochondrion; mrc, mitochondria-rich cell; n, nucleus; pc, pavement cell). Scale bars: (A) 1 μm; (B,C) 400 nm.

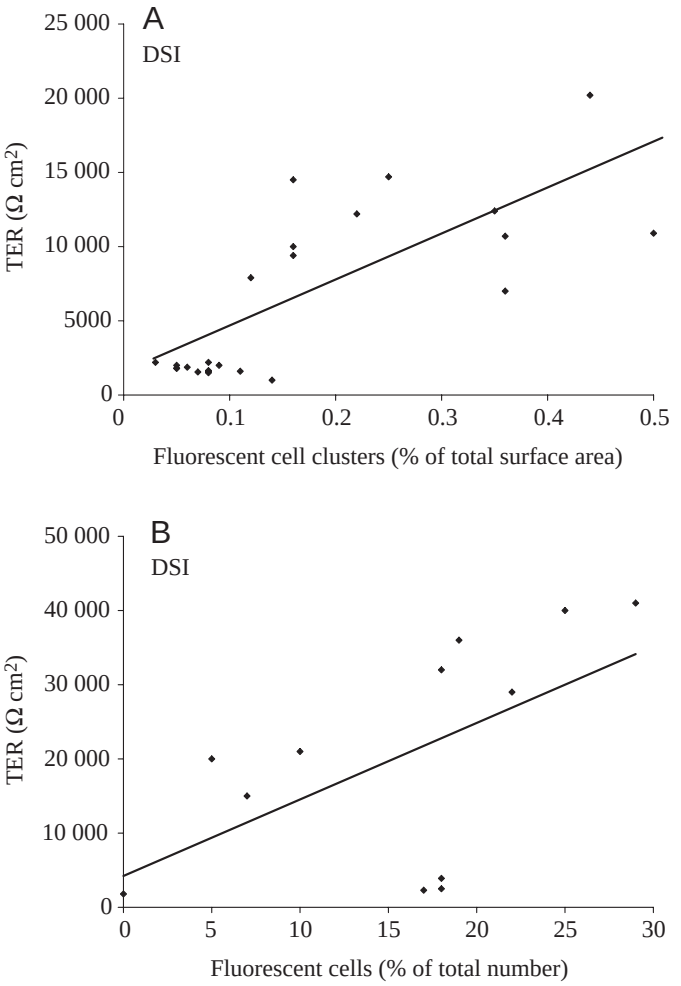

Fig. 4. Relationships between transepithelial resistance (TER) and the presence of mitochondria-rich cells in double-seeded insert (DSI) membranes as indicated by Rhodamine 123 fluorescence. Counts and surface area were measured under symmetrical conditions. (A) The surface area of fluorescing clusters expressed as a percentage of total area *versus* TER (in $\Omega\,cm^2$). The regression equation is y=31 388x+1439, r=0.763, N=24, P<0.0001. (B) The number of fluorescing cells expressed as a percentage of total cell numbers *versus* TER (in $\Omega\,cm^2$). The regression equation is y=1031x+4217, r=0.577, N=12, P<0.01.

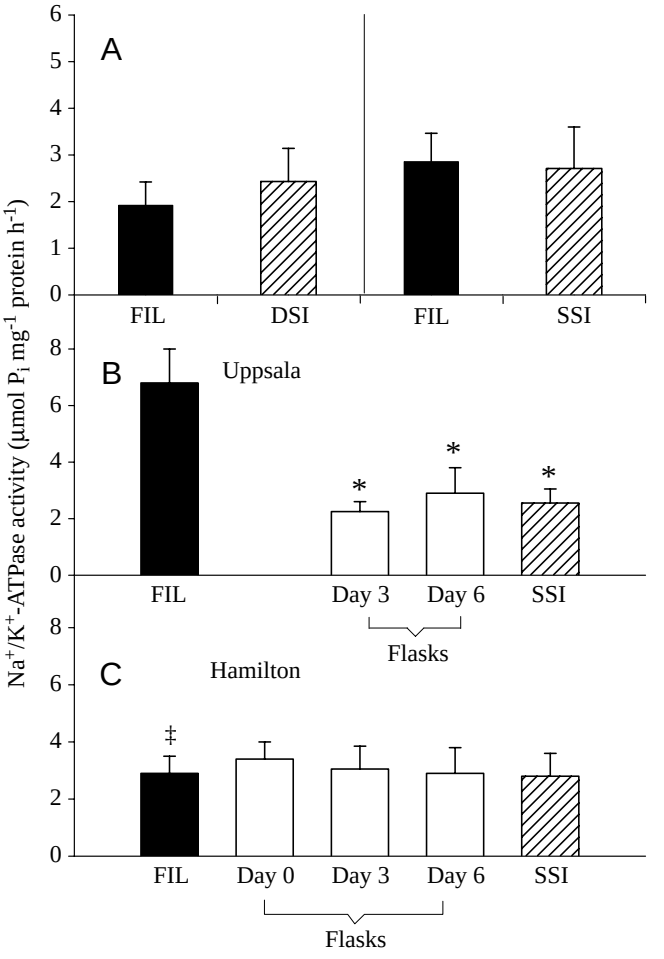

Fig. 5. Na^+/K^+-ATPase activity levels of branchial cells at various times in culture. (A) Comparison of double-seeded insert (DSI) (N=6) and single-seeded insert (SSI) epithelia (N=6) cultured in Hamilton, and the filaments (FIL) (N=10) from which they were derived. There were no significant differences (P>0.05). (B) Cells cultured in Uppsala according to SSI procedures. Enzyme activity was measured in whole filaments (N=10), at day 3 (N=4) and day 6 (N=4) in primary culture in flasks, and in cultured SSI epithelia 6 days (N=5) after seeding onto inserts. (C) Cells cultured in Hamilton according to SSI procedures. Enzyme activity was measured in whole filaments (N=10), in freshly dispersed cells at day 0 at the time of initial seeding into flasks (N=8), at day 3 (N=4) and day 6 (N=4) in primary culture in flasks, and in cultured SSI epithelia at 6–7 days (N=6) after seeding onto inserts. Asterisks (*) indicate significant differences between cultured cells and filaments (P<0.05) from the same set of fish. A double dagger (‡) indicates a significant difference between preparations in Uppsala and Hamilton at the same stage (P<0.05). Values are expressed as means +1 S.E.M. (N is the number of samples, and each sample contains approximately 2×10^6 cultured cells or a whole gill filament).

(N=124) at days 7–9, which was significantly different from zero (P<0.01). This positive TEP, which ranged from 0 to 8 mV, exhibited a positive linear correlation with TER (r=0.87, P<0.0001, N=124; Fig. 6A). The slope of the TEP *versus* TER relationship gives an estimate of the mean current across DSI membranes as $0.4\,\mu A\,cm^{-2}$ (Fig. 6A). A net transepithelial flux of $14.9\,nequiv\,h^{-1}\,cm^{-2}$ would be necessary to account for this current. In contrast, SSI membranes, also cultured in Hamilton, exhibited a mean TEP of +0.4±0.2 mV (N=8) at day 7, which was not significantly different from zero (P=0.087).

When DSI epithelia were exposed to apical fresh water for 4 h, they exhibited a negative mean TEP of −10.3±0.2 mV (N=88). TEP measurements were not made on SSI epithelia exposed to apical fresh water in the present study, but Wood et al. (1998) reported that SSI preparations exhibited similarly negative TEPs (−10.5±0.7 and −11.1±0.8 mV in two series). In the present DSI preparations, individual values ranged from −6 to −15 mV, and there was an overall linear negative correlation with TER, with an intercept at approximately −8.6 mV

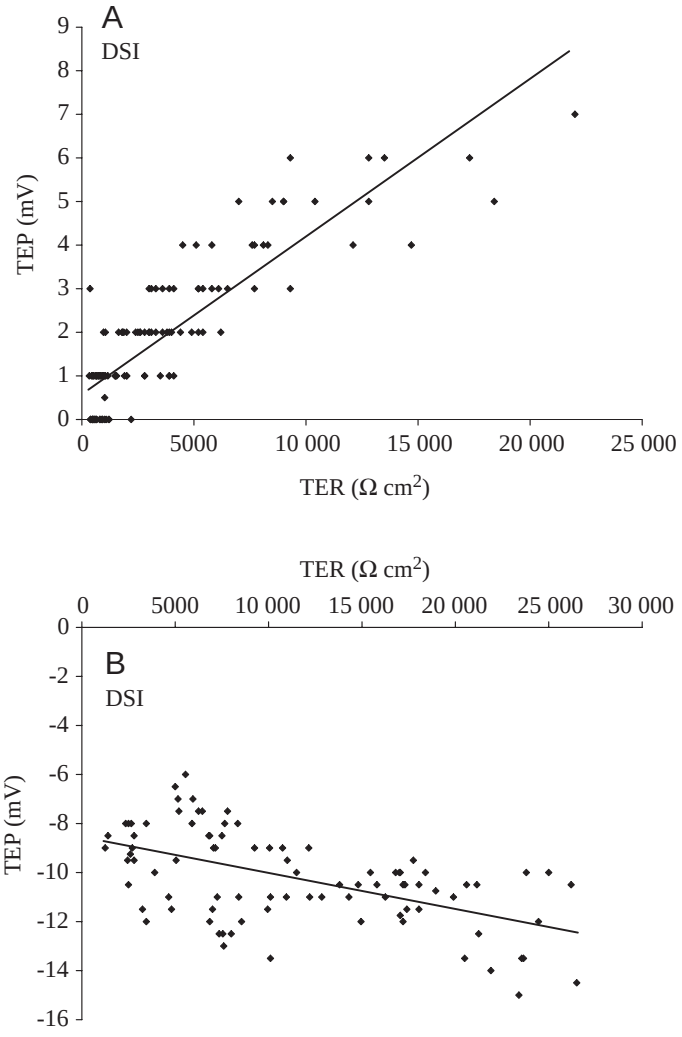

Fig. 6. Relationships between transepithelial resistance (TER, in $\Omega\,cm^2$) and transepithelial potential (TEP, in mV) in cultured double-seeded insert (DSI) membranes. (A) Measurements made under symmetrical conditions. The regression equation is $y=0.0004x+0.589$, $r=0.869$, $N=124$, $P<0.0001$. (B) Measurements made under asymmetrical conditions after 4 h of freshwater treatment on the apical side. The regression equation is $y=-0.0001x-8.599$, $r=0.540$, $N=89$, $P<0.0001$.

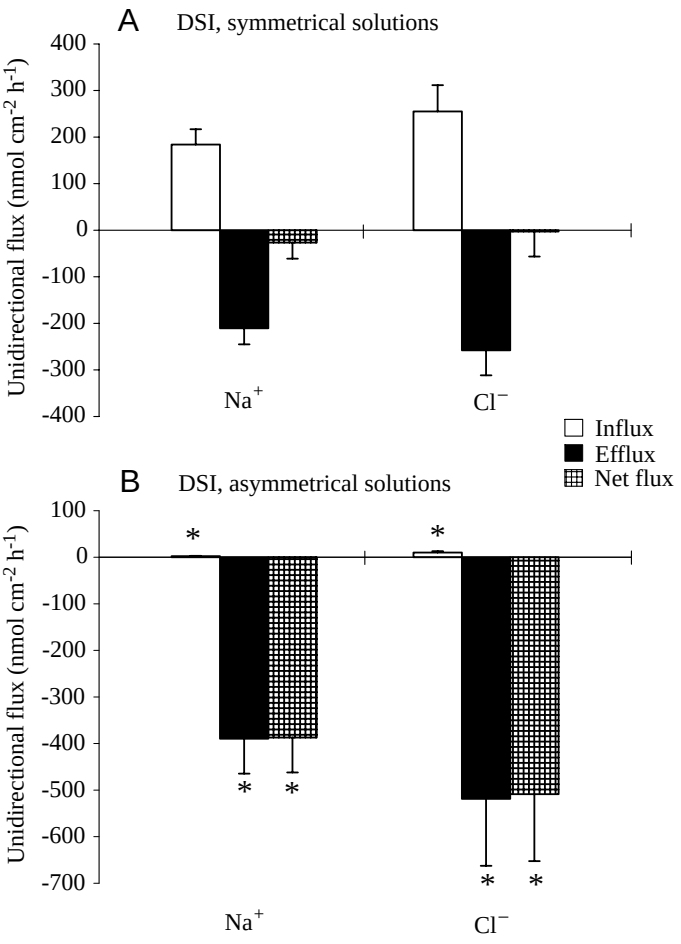

Fig. 7. (A) Unidirectional Na^+ and Cl^- influx (apical to basolateral; open columns), efflux (basolateral to apical; filled columns) and net flux (the difference between influx and efflux; checked columns) in double-seeded insert (DSI) cultured membranes with culture medium on both the apical and basolateral sides (symmetrical conditions). No significant differences existed between influx and efflux values for the same ion or between corresponding fluxes for different ions ($N=15$ for Na^+, $N=14$ for Cl^-). (B) Unidirectional Na^+ and Cl^- influx, efflux and net flux in DSI membranes under asymmetrical conditions (fresh water in the apical compartment, culture medium in the basolateral compartment) ($N=13$ for Na^+, $N=12$ for Cl^-). Values are expressed as means ±1 S.E.M. Asterisks (*) indicate that mean values are significantly different ($P<0.05$) from mean values measured under symmetrical conditions.

(Fig. 6B; $r=0.54$, $P<0.0001$, $N=89$). The slope of this relationship (current $0.1\,\mu A\,cm^{-2}$) was only approximately 25% of that in symmetrical media, indicating that a net transepithelial flux of less than $4\,nequiv\,cm^{-2}\,h^{-1}$ would be needed to explain the current.

PEG permeability

PEG permeability, measured only in DSI preparations in the present study, was low ($<10^{-7}\,cm\,s^{-1}$ in symmetrical media) relative to previous measurements on SSI epithelia (10^{-7} to $5\times10^{-7}\,cm\,s^{-1}$; Wood and Pärt, 1997; Wood et al., 1998), in general accord with the higher TER values in DSI epithelia. In a paired comparison ($N=12$), PEG permeability was twice as high in DSI membranes with fresh water on the apical surface ($1.01\times10^{-7}\pm0.09\times10^{-7}\,cm\,s^{-1}$) than when the same preparations were bathed with culture medium on both surfaces ($0.47\times10^{-7}\pm0.03\times10^{-7}\,cm\,s^{-1}$).

Unidirectional Na^+ and Cl^- fluxes

In DSI preparations used for Na^+ and Cl^- flux measurements with culture medium on both the apical and basolateral surfaces, mean TER values were $8.51\pm1.44\,k\Omega\,cm^2$ ($N=28$) and $7.90\pm1.37\,k\Omega\,cm^2$ ($N=30$), respectively, and corresponding TEPs were +2.9±0.5 mV ($N=28$) and +2.6±0.6 mV ($N=30$). Under these symmetrical conditions, unidirectional Na^+ and

Cl^- flux rates were around 200 nmol cm^{-2} h^{-1} and in balance, with no significant differences between absolute influx and efflux values for the same ion (Fig. 7A). Unidirectional Cl^- fluxes tended to be higher than unidirectional Na^+ fluxes, but these differences were not significant. Previous flux studies with SSI membranes under symmetrical conditions demonstrated very similar patterns (Wood and Pärt, 1997; Wood et al., 1998).

Unidirectional Na^+ and Cl^- fluxes were also measured across DSI epithelia when fresh water was placed in the apical compartment. TER values were somewhat greater [12.36±1.38 kΩ cm^2 (*N*=30) for Na^+ and 11.17±1.22 kΩ cm^2 (*N*=32) for Cl^- fluxes], and TEP became negative [−10.9±0.3 mV (*N*=30) for Na^+ and −10.7±0.3 mV (*N*=32) for Cl^- fluxes]. Under these asymmetrical conditions, unidirectional Na^+ and Cl^- efflux rates were significantly elevated by approximately twofold, and unidirectional influx rates became extremely small (a few nmol cm^{-2} h^{-1}), resulting in net flux rates that were only slightly lower than the unidirectional efflux rates (Fig. 7B). Again, unidirectional Cl^- flux rates tended to be higher than unidirectional Na^+ flux rates, but the difference was significant only for the twofold higher Cl^- influx rate. Previous flux studies with SSI membranes under the same asymmetrical conditions demonstrated similar patterns (Wood et al., 1998).

In DSI epithelia under symmetrical conditions, the Ussing flux ratio criterion indicated that the movements of both Na^+ and Cl^- were passive (Table 1). In contrast, when fresh water was present on the apical surface, the measured flux ratio for Cl^- was significantly greater than the predicted ratio, whereas the measured ratio for Na^+ was significantly smaller than the predicted ratio. These results indicate active Cl^- uptake from apical fresh water to basolateral culture medium and active Na^+ extrusion in the opposite direction. Wood et al. (1998) reported very similar patterns for SSI preparations. In the latter, only the measured ratio for Cl^- was significantly different from the predicted ratio (i.e. greater), but again there was a trend for a lower measured Na^+ ratio.

Table 1. *Comparisons between measured and predicted Ussing flux ratios for Na^+ and Cl^- across cultured DSI epithelia under symmetrical (culture medium on both surfaces) and asymmetrical conditions (fresh water on apical surface, culture medium on basolateral surface)*

	Predicted ratio	Measured ratio
Symmetrical		
Na^+	0.904±0.013 (15)	1.095±0.196 (15)
Cl^-	1.105±0.016 (14)	1.086±0.145 (14)
Asymmetrical		
Na^+	0.031±0.0001 (13)	0.009±0.003* (13)
Cl^-	0.018±0.0001 (12)	0.023±0.003* (12)

Values are means ± 1 S.E.M. (*N* is the number of membrane pairs).
*Significant difference between measured and predicted ratio (*P*<0.05).

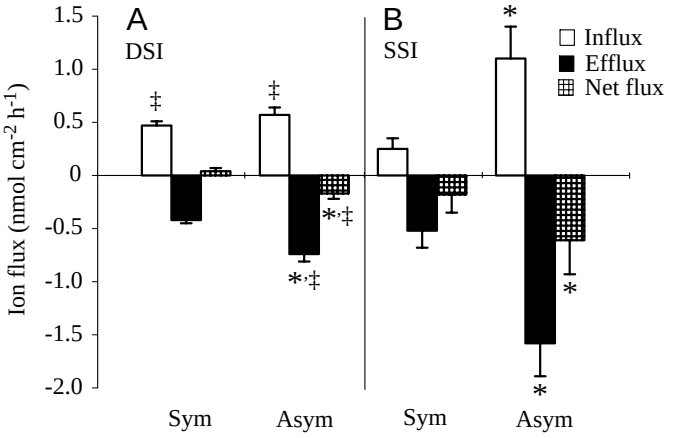

Fig. 8. Unidirectional Ca^{2+} influx (apical to basolateral; open columns), efflux (basolateral to apical; filled columns) and net flux (difference between influx and efflux; checked columns) for (A) double-seeded insert (DSI) and (B) single-seeded insert (SSI) cultured membranes under symmetrical (Sym) and asymmetrical (Asym) conditions. Values are means ±1 S.E.M. (*N*=10 for DSI symmetrical, *N*=11 for DSI asymmetrical; *N*=14 for SSI symmetrical, *N*=19 for SSI asymmetrical). An asterisk (*) indicates a significant difference (*P*<0.05) between flux values for the same preparation under different conditions (asymmetrical *versus* symmetrical), and a double dagger (‡) indicates a significant difference (*P*<0.05) between DSI *versus* SSI preparations under the same conditions.

Unidirectional Ca^{2+} fluxes

Unidirectional Ca^{2+} fluxes were measured for the first time across cultured gill epithelia, employing DSI membranes (Fig. 8A) grown in Hamilton and SSI membranes (Fig. 8B) grown in Uppsala. In both preparations, unidirectional Ca^{2+} flux rates were small (<2 nmol cm^{-2} h^{-1}) in absolute terms compared with unidirectional Na^+ and Cl^- flux rates.

The TER and TEP values for the DSI membranes used for these measurements were 9.85±0.99 kΩ cm^2 (*N*=24) and 4.3±0.3 mV (*N*=24) in symmetrical conditions, and 25.72±1.34 kΩ cm^2 (*N*=22) and −12.3±0.3 mV (*N*=22) in asymmetrical conditions. Under symmetrical conditions, unidirectional influx and efflux rates were similar, although the balance was in favour of a small net uptake from the apical medium (Fig. 8A). Upon exposure to apical fresh water, Ca^{2+} influx was unaltered, but Ca^{2+} efflux increased significantly so that the net flux became negative.

SSI membranes used in these measurements had a TER of 9.62±1.45 kΩ cm^2 (*N*=24) in symmetrical conditions and 18.25±0.63 kΩ cm^2 (*N*=38) in asymmetrical conditions. TEP was not measured. Under both conditions, there was a small net loss of Ca^{2+} to the apical medium, the loss rate being significantly greater in asymmetrical solutions (Fig. 8B). In fact, all three parameters, influx, efflux and net flux rates, were significantly increased with apical freshwater exposure.

Table 2. *Comparisons between measured and predicted Ussing flux ratios for Ca^{2+} across cultured DSI and SSI epithelia under symmetrical (culture medium on both surfaces) and asymmetrical conditions (fresh water on apical surface, culture medium on basolateral surface)*

	Predicted ratio	Measured ratio
SSI epithelia		
Symmetrical	1.000‡	0.792±0.117 (12)
Asymmetrical	1.331§	0.864±0.199 (19)
DSI epithelia		
Symmetrical	0.701±0.008 (10)	2.157±0.086* (10)
Asymmetrical	2.153±0.037 (11)	0.764±0.036* (11)

Values are means ± 1 S.E.M. (*N* is the number of membrane pairs).

*Significant difference between measured and predicted ratio ($P<0.05$).

‡Assuming mean TEP=0 mV (Wood et al., 1998); §assuming mean TEP=−10.8 mV (Wood et al., 1998).

Ussing flux ratio criterion calculations (Table 2) employed measured TEP values for DSI epithelia; for SSI epithelia, values of 0 mV (symmetrical) and −10.8 mV (apical fresh water) were assumed on the basis of previous SSI measurements in Uppsala (Wood et al., 1998). Note that, in all cases, these calculations were based on the individual flux values of matched epithelia, and the mean observed flux ratios in Table 2 were therefore somewhat different from the value that might be estimated from the overall mean influx and efflux values of Fig. 8. For DSI membranes under symmetrical conditions, the actual ratio was more than three times higher than the predicted ratio, indicating active uptake of Ca^{2+} (apical to basolateral transport). However, the measured ratio was approximately three times lower than the predicted ratio when fresh water was present on the apical surface, indicating active extrusion of Ca^{2+} (basolateral to apical transport). In contrast, for SSI membranes, Ca^{2+} movements were apparently passive with either culture medium or fresh water on the apical side.

Discussion

Mitochondria-rich cells in DSI epithelia

The idea behind the double-seeding approach is that it is first necessary to have pavement cells attach to the substratum to create the correct environment for settlement and attachment of MR cells. Initially, we tested the double-seeding idea in flask cultures and found that it worked, although most of the MR cells were located in clusters that welled up in domes on the solid plastic substratum. The protocol was then transferred to filter culture, initially using pavement cells harvested from flask culture for the first seeding on filters and cells freshly dispersed from gill filaments for the second. However, we soon found that this could be circumvented, and preparations could be produced more quickly and reliably using double-seeding of freshly dispersed cells directly onto filter inserts. Earlier, we had reported that epithelia would not grow when branchial cells freshly dispersed from gill filaments were seeded directly onto filters (Pärt et al., 1993; Pärt and Bergstrom, 1995; Wood and Pärt, 1997). However, up until then, we had not tried the double-seeding approach, nor had we used the very high seeding densities that were found to be necessary in the current study (2×10^6–2.5×10^6 viable cells cm^{-2} on each of 2 days). Since the final cell density of mature epithelia on filters inserts at days 6–9 is approximately 3×10^5 cells cm^{-2}, it is clear that only a small percentage of the initially seeded cells actually attach and survive.

To our knowledge, the present DSI preparations constitute the first incorporation of MR cells into a cultured branchial epithelium on a filter support, a goal that has proved elusive since our first report of trout gill cells in primary culture (Pärt et al., 1993). At present, we refrain from calling these cultured cells ‘chloride cells’, because it remains unclear to what extent they duplicate the functions of true ‘chloride cells’ in the intact freshwater gill *in vivo*. Certainly, their discrete sequestration of Rhodamine 123 (also of DASPEI and DASPMI, fluorescent probes for mitochondria) validates the term ‘mitochondria-rich cells’ (Bereiter-Hahn, 1976; Shinomiya et al., 1992), and they exhibit morphological similarity at the transmission electron microscope level. Furthermore, their numerical density in the DSI epithelia (approximately 15 %) is very typical of that in the intact gill (Perry and Walsh, 1989; Perry and Laurent, 1993). However, as outlined below, the transport properties of the DSI epithelia are not the same as those of the intact gill.

Morphological comparison of DSI and SSI epithelia

The morphology of pavement cells in DSI epithelia was virtually identical to that previously described in SSI preparations by Wood and Pärt (1997) and Gilmour et al. (1998b). In short, the bulk of the epithelium was composed of multiple (two or more layers) overlapping pavement cells linked by tight junctions. Pavement cells contained few mitochondria but abundant rough endoplasmic reticulum, numerous projections (villi or microridges) and a prominent glycocalyx on the apical surface of the outer layer. Taken together, all these characteristics are typical features of pavement cells found in the intact gill (Laurent and Perry, 1991; Goss et al., 1995). However, unique to DSI preparations was the presence of MR cells with morphological characteristics very similar to the ‘chloride cells’ of the intact gill. The MR cells of the DSI preparation were open apically to the ‘external’ environment, contained a branching tubular system and numerous mitochondria (Fig. 3A,B) and formed deep-type junctions with neighbouring pavement cells (Fig. 3C). No ‘accessory cells’ or very shallow-type junctions typical of seawater ‘chloride cells’ (see Laurent and Perry, 1991; Perry and Laurent, 1993) were seen.

Na^+/K^+-ATPase activities

Comparison of DSI and SSI epithelia cultured in Hamilton (Fig. 5A) showed that there was no additional expression of Na^+/K^+-ATPase activity in DSI epithelia, despite an obvious

incorporation of MR cells into the preparations. *In vivo*, Na^+/K^+-ATPase is much more strongly expressed in chloride cells than in pavement cells (Perry and Walsh, 1989; McCormick, 1995). This indicates that the conditions under which the DSI epithelia were cultured are inadequate to allow the MR cells to function as true freshwater chloride cells, a conclusion that is further supported by the ion transport data (see below). The fact that the DSI epithelia are grown under symmetrical isotonic conditions is one possible explanation; others include an absence of key hormonal or nutritional support. Clearly, the next step in the development of a realistic cultured gill epithelium is to solve this problem. In this regard, the demonstration (Avella and Ehrenfeld, 1997; Avella et al., 1999) that serum of fish origin rather than bovine origin promoted normal morphology and superior transport and electrical characteristics in the cultured sea bass gill epithelium is of considerable interest.

The much higher Na^+/K^+-ATPase activities in the gill filaments of Uppsala trout (Fig. 5B,C) were probably caused by the acclimation of these fish to a synthetic soft water, much lower in Na^+, Cl^- and Ca^{2+} concentrations than in Hamilton. Chloride cell proliferation is a well-documented response to softwater acclimation (for reviews, see Laurent and Perry, 1991; Perry and Laurent, 1993; Goss et al., 1995; Perry, 1997). We interpret the rapid loss of activity during culture, and the similarity of final activities in SSI epithelia grown in Uppsala and Hamilton, as a disappearance of functional chloride cells during SSI culture, and an absence of MR cells was, indeed, documented from day 3 of flask culture onwards. The consistent activity levels of approximately 2–3 $\mu mol\ P_i\ mg^{-1}$ protein h^{-1} in cultured cells were presumably representative of those in pavement cells alone.

Transepithelial resistance of DSI and SSI epithelia

The increase in TER over time in filter culture (Fig. 1) probably reflects the formation of junctions of increasing 'tightness' between the cells (Cereijido et al., 1981), with those in the outermost cell layer providing the greatest resistive barrier (Klyce, 1972; Nagel, 1978). The formation of desmosomes and the addition of multiple cell layers may also make a minor contribution. In general, the number of tight junctions between cells correlates with TER (Cereijido et al., 1981), particularly paracellular resistance (Lewis, 1997). These junctions have now been clearly seen in transmission electron micrographs of both DSI (Fig. 3) and SSI (Gilmour et al., 1998b) epithelia and especially between cells in the outermost layer on the apical surface.

As discussed previously (Wood and Pärt, 1997; Wood et al., 1998; Gilmour et al., 1998b), TER values of cultured branchial epithelia (as high as 34 $k\Omega\,cm^2$ in symmetrical media) are in the very highest range of reported resistances for cultured epithelia and are indicative of an epithelium that is very 'tight' electrically. While the TER of the intact freshwater gill has never been measured, on the basis of the permeability and morphological criteria it is thought to be a very 'tight' epithelium electrically (Sardet, 1980; Isaia, 1984). Various dissected epithelia that have been tested as surrogates for the freshwater gill exhibited TER values in the range 0.50–2.00 $k\Omega\,cm^2$ with symmetrical media, which increased to as much as 12.0 $k\Omega\,cm^2$ with apical fresh water (Foskett et al., 1981; Marshall, 1985; McCormick et al., 1992; Marshall et al., 1992, 1995, 1997; Wood and Marshall, 1994; Burgess et al., 1998). Recently, Avella and Ehrenfeld (1997) and Avella et al. (1999) reported TER values of 4.0–12.0 $k\Omega\,cm^2$ in symmetrical media for an epithelium grown in filter culture which was composed solely of branchial pavement cells of a marine fish, the seabass.

The tendency for higher TER values in DSI compared with SSI epithelia may be associated with the positive correlations between TER and MR cell numbers and/or cluster surface area (Fig. 4A,B). *A priori*, these relationships may seem counterintuitive since MR cells are well-known to form shallow 'leaky' junctions with adjacent accessory cells in seawater gills, a pattern that is essential for paracellular Na^+ efflux as part of the mechanism of active NaCl extrusion (Laurent and Perry, 1991; Perry and Laurent, 1993). However, these accessory cells appear to be absent from freshwater fish and, indeed, were never seen in the present cultured epithelia. In current models for active ion uptake in fresh water, there is no role for paracellular flux, and the reduction of paracellular permeability would seem to be adaptive in minimizing diffusive ion losses. Therefore, it is possible that the junctions between mitochondria-rich cells and pavement cells are in fact 'tighter' than those between adjacent pavement cells. The lower PEG-4000 permeabilities in DSI compared with SSI epithelia are in accord with this argument.

Transepithelial potentials of DSI and SSI epithelia

DSI epithelia in symmetrical media exhibited a significantly positive TEP (+1.9±0.2 mV) with some individual values as high as +8 mV (Fig. 6A). This is a clear difference from SSI epithelia, in which TEP was not significantly different from zero in three studies (present study; Wood and Pärt, 1997; Wood et al., 1998) and significantly negative in a fourth (–2.3±0.2 mV; Gilmour et al., 1998a). The difference was probably associated with the presence of MR cells in DSI epithelia. Both TEP (Fig. 6A) and MR cell abundance (Fig. 4) increased with TER. The origin of this potential was not investigated but, since it occurs under symmetrical conditions, it almost certainly arises from electrogenic ionic transport. This positive TEP was similar to measurements across the intact gill *in vivo* for freshwater fish placed in symmetrical media (Potts, 1984), and for at least one other gill surrogate *in vitro* which is known to contain MR cells, the opercular epithelium of the freshwater-acclimated killifish (Marshall et al., 1995, 1997). Much larger basolateral positive TEPs have been reported in the cultured epithelium from seawater-adapted bass (+13 to +30 mV) by Avella and Ehrenfeld (1997) and Avella et al. (1999), who clearly demonstrated that they were due to a high net rate of electrogenic Cl^- extrusion (60–130 nequiv $h^{-1}\,cm^{-2}$), a surprising observation for a reportedly pure pavement cell epithelium. However, the net ion

transport (approximately 15 nequiv h^{-1} cm^{-2} of Cl^- extrusion or Na^+ uptake) needed to explain the much smaller TEP in the present DSI preparations would be difficult to detect against the high background of unidirectional fluxes (see Fig. 7). The Ussing flux ratio analysis did not detect active Na^+ or Cl^- transport (Table 1). In contrast, Ca^{2+} uptake, while apparently active (Table 2), would appear to be too small (Fig. 8) to explain the observed TEP.

In vivo, the TEP across the gills is usually blood-side negative when fish are in fresh water (for a review, see Potts, 1984), with typical values in trout (e.g. McWilliams and Potts, 1978; Perry and Wood, 1985; Perry and Flik, 1988) very similar to the −10 mV seen in both SSI and DSI epithelia exposed to apical fresh water. This is thought to be largely a diffusion potential due to the differential permeability of the tight junctions to Na^+ compared with Cl^-. The significant negative relationship between TEP and TER seen in DSI epithelia bathed with apical fresh water (Fig. 6B) suggests that a small negative electrogenic component may be superimposed on a large negative diffusion potential (i.e. the intercept, approximately −8.6 mV). This conclusion is in accord with the Ussing flux ratio analysis (Table 1), suggesting active uptake of Cl^- and efflux of Na^+ in these preparations (see below).

Unidirectional Na^+ and Cl^- fluxes of DSI and SSI epithelia

In the present study, Na^+ and Cl^- exchanges were measured only in DSI epithelia, but the overall patterns were similar to those documented previously in SSI membranes (Wood and Pärt, 1997; Wood et al., 1998). Thus, in symmetrical media, fluxes were approximately equal in both directions (Fig. 7A); there was no evidence of rectification or active transport (Table 1). This agrees with the absence of net uptake reported in most other surrogates for the freshwater gill when mounted under symmetrical conditions *in vitro* (e.g. Burgess et al., 1998), although the killifish opercular epithelium is a notable exception (Marshall et al., 1997). Absolute flux rates, when extrapolated to estimates of total gill surface area (Hughes and Morgan, 1973), were approximately equal to those seen *in vivo* in salmonids acclimated to isotonic media (Bath and Eddy, 1979; Wood and Pärt, 1997). With apical fresh water, net Na^+ and Cl^- balance became highly negative, reflecting greatly reduced influxes and moderately increased effluxes (Fig. 7B). The latter were probably caused by elevated paracellular permeability, as demonstrated by the doubling of PEG-4000 permeability. Cl^- fluxes in asymmetrical conditions tended to be larger than Na^+ fluxes, possibly reflecting the higher mobility of Cl^- compared with Na^+ (Potts, 1984). Overall, the unidirectional flux rates compare favourably with other *in vitro* models for the freshwater gill such as the tilapia opercular epithelium (Burgess et al., 1998) and the cleithrum skin of rainbow trout (Marshall et al., 1992).

The Ussing flux ratio analysis indicated a small but significant active uptake of Cl^- and active extrusion of Na^+ when DSI epithelia were bathed with apical fresh water (Table 1). Active uptake of Cl^- has been demonstrated previously using the same criterion in SSI epithelia under asymmetrical conditions (Wood et al., 1998), so the phenomenon would appear to be a function of the pavement cells. Active Cl^- uptake by pavement cells certainly does not fit any of our current 'models' for freshwater gill ion transport (Goss et al., 1995; Perry, 1997; Kirschner, 1997; Claiborne, 1997). However, by the same token, active extrusion of Cl^- by pavement cells in the cultured epithelium of the seabass (Avella and Ehrenfeld, 1997; Avella et al., 1999) certainly does not fit accepted ideas about the seawater gill (Zadunaisky, 1984; Wood and Marshall, 1994; Marshall, 1995). Active extrusion of Na^+ (also Ca^{2+}, Table 2) into the apical fresh water by DSI membranes is an even more puzzling result, yet a similar but non-significant tendency was seen in SSI membranes (Wood et al., 1998). In future, studies with metabolic blockers may be helpful in strengthening these conclusions which, at present, are based solely on the Ussing flux ratio criterion. These data suggest either that current theories are incomplete or that branchial cells cultured under symmetrical conditions differentiate or dedifferentiate so as to produce altered transport characteristics. The lack of Na^+/K^+-ATPase enrichment in DSI compared with SSI epithelia (Fig. 5A) is in accord with this interpretation, as are the kinetics of Na^+/H^+ exchange in cultured trout pavement cells (Pärt and Wood, 1996).

Unidirectional Ca^{2+} fluxes of DSI and SSI epithelia

Rates of Ca^{2+} influx and efflux (Fig. 8), which have never been measured previously in cultured branchial epithelia, were approximately 0.5–1 % of unidirectional Na^+ and Cl^- flux rates (Fig. 7). This is similar but not identical to the situation *in vivo*, where unidirectional Ca^{2+} fluxes across the gills of trout are normally approximately 2–10 % of Na^+ and Cl^- fluxes (Perry and Wood, 1985; Perry and Flik, 1988). Absolute values (0.2–1.5 nmol cm^{-2} h^{-1}) were comparable with those in the isolated cleithral skin of the rainbow trout (Marshall et al., 1992), but much lower than those in some other dissected epithelial preparations from freshwater fish that are known to transport Ca^{2+} actively (McCormick et al., 1992; Marshall et al., 1995; Burgess et al., 1998). The very high flux rates (30–70 nmol cm^{-2} h^{-1}) in the killifish preparation, in particular, have been attributed to the abundance of chloride cells (Marshall et al., 1995), and there is general acceptance that the major route of Ca^{2+} transport through the gills of freshwater fish is *via* the chloride cells (Flik et al., 1995; Perry, 1997). Unidirectional Ca^{2+} flux rates were no higher in DSI than in SSI epithelia (Fig. 8), but the more important finding was the occurrence of apparent active Ca^{2+} transport in DSI epithelia (which contained MR cells) and its absence from SSI epithelia (which did not) according to the Ussing flux ratio criterion (Table 2). *In vivo*, Ca^{2+} is thought to enter chloride cells through a lanthanum-sensitive apical channel and then to exit into the blood either through a Ca^{2+}-ATPase or through a Na^+/Ca^{2+} exchanger (Perry and Flik, 1988; Verbost et al., 1994; Marshall et al., 1995; Flik et al., 1995; Perry, 1997). Both basolateral extrusion mechanisms may play a role in Ca^{2+} uptake *in vivo*, and their relative importance is still under

debate. It is not surprising that active Ca^{2+} transport was absent from SSI epithelia because these cultured preparations contain only pavement cells, and cultured pavement cells appear to lack a lanthanum-sensitive apical Ca^{2+} channel (Block and Pärt, 1992). DSI membranes with culture medium on both sides (symmetrical conditions) behaved like an intact gill by demonstrating greater influx than efflux (Fig. 8) and active Ca^{2+} uptake from the apical to the basolateral side (Table 2). However, it is surprising that with fresh water on the apical surface (asymmetrical conditions), apparent active extrusion of Ca^{2+} occurred. Again, this is evidence that not all the correct cues for transport of the correct polarity are present. Additional hormonal support, nutritional supplementation or other improvements in the conditions of culture will be needed before the mitochondria-rich cells of the DSI epithelium can be considered to be true freshwater 'chloride cells'.

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