

CULTURED BRANCHIAL EPITHELIA FROM FRESHWATER FISH GILLS

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Summary

We have developed a method for the primary culture of gill epithelial cells from freshwater rainbow trout on permeable supports, polyethylene terephthalate membranes ('filter inserts'). Primary cultures of gill cells (6–9 days in Leibowitz L-15 culture medium plus foetal bovine serum and glutamine) are trypsinized and the cells seeded onto the inserts. After 6 days of growth with L-15 medium on both surfaces (approximately isotonic to trout plasma), the cells form a tight epithelium as judged from a progressive rise in transepithelial resistance which reaches a stable plateau for a further 6 days, as long as L-15 exposure is continued on both surfaces. The cultured epithelium (approximately 8 µm thick) typically consists of 2–4 overlapping cell layers organized as in the lamellae *in vivo*, with large intercellular spaces, multiple desmosomes and putative tight junctions. The cells appear to be exclusively pavement-type cells with an apical surface glycocalyx, an abundance of rough endoplasmic reticulum, no selective DASPEI staining and relatively few mitochondria. Transepithelial resistance (approximately 3.5 kΩ cm²), permeability to a paracellular marker (polyethylene glycol-4000; 0.17 × 10⁻⁶ cm s⁻¹) and

unidirectional flux of Na⁺ and Cl⁻ (approximately 300 nmol cm⁻² h⁻¹) all appear realistic because they compare well with *in vivo* values; net fluxes of Na⁺ and Cl⁻ are zero. The preparation acidifies the apical medium, which accumulates a greater concentration of ammonia. Upon exposure to apical freshwater, resistance increases six- to elevenfold and a basolateral-negative transepithelial potential (TEP) develops as *in vivo*. These responses occur even when mannitol is used to prevent changes in apical osmotic pressure. Net Na⁺ and Cl⁻ loss rates are low over the first 12 h (-125 nmol cm⁻² h⁻¹) but increase substantially by 48 h. The elevated resistance and negative TEP gradually attenuate but remain significantly higher than pre-exposure values after 48 h of apical freshwater exposure. The preparation may provide a valuable new tool for characterizing some of the mechanisms of active and passive ion transport in the pavement cells of the freshwater gill.

Key words: gills, cell culture, epithelial cells, filter inserts, *Oncorhynchus mykiss*, transepithelial potential, transepithelial resistance, ionic fluxes, rainbow trout.

Introduction

The complex three-dimensional morphology of the gill, together with its poor viability under *in vitro* conditions of perfusion and incubation, have proved a formidable barrier to understanding the mechanisms of branchial ion transport in freshwater fish. More than 60 years after the discovery of mitochondria-rich chloride cells (Keys and Willmer, 1932), physiologists are still debating what exact functions these cells perform relative to those of the more abundant pavement cells in the freshwater gill (e.g. Payan *et al.* 1984; Avella *et al.* 1987; Perry and Laurent, 1989; Wood, 1991; Goss *et al.* 1992, 1995; Lin and Randall, 1995; Morgan and Potts, 1995). In marine teleosts, the same problems long hindered understanding of branchial ion transport. However, rapid progress was made once surrogate models for the seawater gill were discovered, such as the jawskin of the seawater-adapted goby (Marshall, 1977) and the opercular epithelia of seawater-adapted killifish

(Karnaky *et al.* 1977; Zadunaisky, 1984) and tilapia (Foskett and Scheffey, 1982). These surrogate models are flat epithelial preparations which contain an abundance of ion-transporting cells. By virtue of their thin sheet-like morphology, these preparations survive well *in vitro* in the absence of blood flow, allow exposure of the apical and basolateral surfaces to asymmetrical media, as *in vivo*, and have proved amenable to rigorous electrical and radioisotopic flux approaches such as Ussing chamber analyses.

So far, the search for a comparable flat epithelial model for the freshwater gill has proved disappointing. Preparations investigated to date include the cleithral epithelium of freshwater-adapted trout (Marshall *et al.* 1992) and the opercular epithelia of freshwater-adapted killifish (Wood and Marshall, 1994; Marshall *et al.* 1995) and tilapia (Foskett *et al.* 1981; McCormick *et al.* 1992). These preparations have helped

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to localize Ca^{2+} transport to the chloride cells and have helped to reinforce the wealth of biochemical information on Ca^{2+} transport that has been discovered over the past decade (see Flik *et al.* 1995, for a review). However, these models have cast little light on the mechanisms or sites of Na^+ and Cl^- uptake in the freshwater gill.

An entirely different approach could be to reconstruct elements of the freshwater gill *in vitro* by primary culture of flat epithelial sheets. The first indication that this might be possible came when Pärt *et al.* (1993) developed a protocol for successful culture of confluent layers of gill pavement cells on plastic culture dishes. More recently, Pärt and Wood (1996) demonstrated that these cells exhibit dynamic properties of ion and acid–base regulation. However, it seems most unlikely that such cultured cells on solid supports will ever serve as a viable model for the freshwater gill, for the simple reason that they cannot be exposed to the asymmetrical conditions which occur *in vivo*; that is, freshwater on the apical surface and extracellular fluid (ECF) on the basolateral surface. Instead, they are exposed to ECF-like culture medium on the apical surface and to solid plastic on the basolateral surface. Normal *in vivo* polarity (i.e. apical transporters functioning at a freshwater interface, basolateral transporters functioning at an ECF interface) cannot exist.

However, there may be a solution to this problem. The culture of epithelial layers on permeable filters was first developed by customized techniques using filters designed for filtration, not culture (Misfeldt *et al.* 1976; Cerejido *et al.* 1978). However, this approach has now become a well-established method in mammalian and amphibian transport physiology (e.g. Ford *et al.* 1990; Rutten, 1992; Madara *et al.* 1992; Candia *et al.* 1993), thanks in part to the advent in recent years of commercially available permeable filter inserts specifically designed for tissue culture. In fish, this approach was first exploited successfully by Dickman and Renfro (1986) to study the physiology of transport in flounder renal tubules. This type of culture allows the study of electrical and transport properties when the two surfaces are exposed to asymmetrical conditions. The goal of the present study was to adapt the primary culture approach of Pärt *et al.* (1993) to allow the growth of sheets of gill pavement cells from freshwater trout on such permeable inserts. We provide details on the electrical, structural and permeability properties of the preparation under both symmetrical and asymmetrical conditions.

Materials and methods

Experimental animals

All gill cell preparations used in this project were obtained from 1- to 2-year-old rainbow trout [*Oncorhynchus mykiss* (Walbaum) 80–240 g] in non-breeding condition, from May to August 1994. The fish were held in running freshwater at 11–13 °C in tanks (1000 l) in the aquarium facility of Uppsala University. The composition of the synthetic freshwater, which was recirculated from a large filtration and ultraviolet sterilization reservoir, was as follows (in mmol l^{-1}): Na^+ , 0.06;

Cl^- , 0.05; Ca^{2+} 0.2; Mg^{2+} , 0.041; SO_4^{2-} , 0.04; HCO_3^- , 0.4; pH 6.9. Seasonal photoperiod was set to match that of Hamburg, Germany (to allow longer hours of daylight access in winter than would be provided by an Uppsala photoperiod). The fish were fed a fixed ration (1% of body mass per day) of commercial pellets (Ewos T40), but were generally killed prior to daily feeding to prevent regurgitation of stomach contents onto the gills.

Cell culture methods

Ethanol-sterilized dissecting equipment was employed. All procedures were performed under sterile conditions in a laminar flow hood. Solutions were sterilized by autoclaving and/or by passage through 0.2 μm Acrodisc syringe filters (Gelman, Ann Arbor, USA), and all containers were either purchased sterile or sterilized by autoclaving.

All eight gill arches were excised from trout which had first been isolated for 0.5 h in bacteria-free water then killed by decapitation. Gills from each fish were processed separately, and cells from different fish were never mixed. Gill epithelial cells were collected from the filaments by several cycles of tryptic digestion (Gibco BRL Life Technologies no. 043-05090H trypsin, 2.5% stock, diluted to 0.05% in phosphate-buffered saline, PBS, with 5.5 mmol l^{-1} EDTA). The cells were then resuspended in culture medium (Leibowitz L-15 supplemented with 2 mmol l^{-1} glutamine and 5% foetal bovine serum, FBS, plus antibiotics as below) and finally plated into flasks for initial culture using the procedures developed by Pärt *et al.* (1993). Leibowitz L-15 medium (Flow Laboratories, UK; measured $[\text{Na}^+]=155$, $[\text{Cl}^-]=143$, $[\text{K}^+]=6.0 \text{ mmol l}^{-1}$) essentially duplicates the ionic composition of trout extracellular fluid plus amino acids and vitamins. Full details on methods, composition of other solutions and sources of chemicals are presented in both Pärt *et al.* (1993) and Pärt and Bergstrom (1995). Only key points and important modifications will be noted here.

To ensure cell viability, we found it important to maintain temperature at 20 °C or below throughout the isolation procedure, which necessitated chilling the various solutions on ice; these brief cold shocks had no apparent ill effects on the preparations. For initial culture, the cells were seeded at a density of 520 000 cm^{-2} (determined using a haemocytometer) into 25 cm^2 or 75 cm^2 flasks (Falcon or Nunc) and kept at 18 °C in an air atmosphere. Non-adherent cells were removed by changing the medium at 24 h and again at 96 h. Until this time, the media contained antibiotics: 100 i.u. ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 200 $\mu\text{g ml}^{-1}$ gentamycin. Beyond day 4, antibiotic-free medium (L-15 plus 2 mmol l^{-1} glutamine and 5% FBS) was used. The medium was changed every 3 days thereafter until the cells were harvested for culture on filters.

For harvesting, the medium was removed, the flask was rinsed with PBS, and 0.7 ml (per 25 cm^2 flask) of trypsin solution (composition as above) was then added, and the flask was mechanically agitated. The trypsinization process was observed visually using a phase contrast microscope and

terminated once most of the cells had detached, which generally took approximately 3–6 min at 20 °C or up to 15 min at a colder temperature (10 °C). Termination was achieved by adding FBS to the flask (final concentration 10 %) and then filtering the eluate, plus several rinses of the flask, through an 80 µm nylon filter into additional 10 % FBS in PBS. The eluates from all flasks from one fish were combined. Cells were pelleted by centrifugation at 200 g for 10 min and then resuspended in antibiotic-free medium as above. At this point, the cell density was determined using a haemocytometer and adjusted as appropriate for seeding onto filter inserts.

The permeable filters used were Falcon cell culture inserts, 0.45 µm pore size (Cyclopore polyethylene terephthalate; Becton Dickinson, Franklin Lakes, New Jersey, USA). Filter inserts of both low pore density (1.6×10^6 pores cm^{-2}) and high pore density (10^8 pores cm^{-2}), and three different sizes (effective growth surface areas 4.30, 0.83 and 0.31 cm^2 for 6, 12 and 24 Falcon cell culture companion plates, respectively) were used successfully. However, the majority of methods development and experiments were performed with the 4.3 cm^2 size, low-pore-density filters. The filter inserts sit inside wells in the covered culture plates. The cells grow on the upper surface of the permeable filter; culture medium is placed in both the insert (upper surface) and the well (lower surface).

Prior to seeding, each membrane was wetted for 2–3 h with antibiotic-free medium. At seeding, the appropriate number of cells was pipetted into the centre of the insert membrane and distributed by gentle swirling. We found that it was critical to maintain the fluid level in the insert equal to or higher than that in the well at all times (i.e. a positive hydrostatic gradient) to avoid lifting the cells off the filter by reversing the gradient. In the 4.3 cm^2 size/six-well plate, for example, this was achieved by first putting 1.5 ml of cell suspension in the insert, then 2.5 ml of medium in the well. At 24 h after seeding, and every 48 h thereafter, these volumes were replaced with 3.5 ml in the insert and 4.5 ml in the well, the greater volumes being required to accommodate the ‘chopstick’ electrodes for transepithelial resistance and potential measurements (see below). Medium replacement was achieved by aspirating first the medium from the well and then the medium from the insert. The media were then quickly replaced on the two sides, in stages, ensuring that the level in the insert remained greater than or equal to the level in the well. In experiments where the composition of the apical medium was altered, the procedure was repeated three times to ensure complete replacement. Filter culture was performed at 18 °C in an air atmosphere.

Microscopy

Phase contrast microscopy was routinely used to view the cells during culture in the flasks and on the filter inserts. However, on low-pore-density filters, it was difficult to get a clear view of the cells near the edge of the insert under phase contrast and impossible to see the cells on high-pore-density filters because of the refraction of light by the pores. This could be overcome by adding fluorescein diacetate (Molecular Probes, Eugene, Oregon, USA) to the apical medium in the

inserts (final concentration 10–100 $\mu\text{mol l}^{-1}$ in 0.1 % dimethylsulphoxide), washing out after 15 min, and viewing under epifluorescence. Fluorescence microscopy was similarly used to check for the possible presence of mitochondria-rich cells; DASPEI [2-(4-dimethylaminostyryl)-*N*-ethylpyridinium iodide; Molecular Probes] was added to both apical and basolateral media for 45 min (final concentration 25 $\mu\text{mol l}^{-1}$), then washed out prior to viewing. Transepithelial resistance invariably exhibited a decline after fluorescence microscopy, so these procedures were considered terminal for the preparations. Experiments in which fluorescent dyes were loaded into preparations but not excited by fluorescent illumination demonstrated that it was the illumination, and not the dye alone, which caused the deterioration. For example, Fig. 2A, taken under regular phase contrast after epifluorescent illumination, provides visual evidence of this excitation damage to the cells. Of course, prolonged illumination under epifluorescence also caused probe fading, a separate phenomenon.

Selected preparations cultured on filters were fixed for transmission electron microscopy. Procedures were performed at 4 °C. Apical and basolateral media were completely replaced first with HCO_3^- -free Cortland saline (composition as above), and then with 2.5 % glutaraldehyde in 0.1 mol l^{-1} sodium cacodylate buffer, pH 7.5, and left overnight for fixation. Subsequent procedures followed those laid out in Falcon Technical Bulletin 406 (available from Becton Dickinson Europe, BP 37-38241 Meylan Cedex, France). The preparations were dehydrated in ethanol and embedded in resin (Agar 100, Agar Scientific Ltd, UK); sections (50 nm) were cut on an ultramicrotome and stained in lead citrate/uranyl acetate. Sections were examined in a Philips CM10 transmission electron microscope.

Experimental methods

In experiments which evaluated the influence of changing the NaCl content of the apical medium without alterations in osmotic activity, a modified HCO_3^- -free Cortland saline based on the formulation of Wolf (1963) was employed. The composition was as follows (in mmol l^{-1}): NaCl 136, KCl 5.1, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.9, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 3.0, glucose 5.6; pH 7.8. A second, osmotically compensated saline (with minimal Na^+ and Cl^- content) was made by replacing the NaCl component with 250 mmol l^{-1} mannitol. The two salines were mixed in various proportions to achieve variations in NaCl content without variations in osmotic pressure.

In experiments which evaluated the effects of changing the apical medium to freshwater, a synthetic freshwater was used consisting of the following (in mmol l^{-1}): $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.45, $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ 0.09, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.45 and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.05, thereby providing a ‘typical’ freshwater (pH 7.5) with 1 mmol l^{-1} Na^+ , 1 mmol l^{-1} Cl^- , sufficient hardness cations and buffering capacity.

In experiments in which resistances and transepithelial potentials (TEPs) were recorded after replacement of the apical medium (either with the same composition or an acutely

altered composition), measurements were first made approximately 10 min after medium replacement.

In experiments in which fluxes were measured, samples from the appropriate side (apical, from the insert; basolateral, from the well) were removed for analysis at the beginning and end of the flux period. The initial volumes on each side were adjusted so that the correct volumes would remain after the initial samples had been taken. Net fluxes were measured in the conventional fashion by factoring the change in composition by the known volume of the compartment, the time and the filter surface area, and were expressed as $\text{nmol cm}^{-2} \text{h}^{-1}$. Unidirectional fluxes of Na^+ and Cl^- were monitored by adding 37 kBq ($1 \mu\text{Ci}$) of the appropriate radioisotope (^{22}Na , ^{36}Cl ; NEN) to one side and monitoring the appearance over time of radioactivity on the other side. Radioisotope regulations did not allow us to run radioactive solutions through the available analytical equipment (flame photometer, chloridometer), so unidirectional fluxes of Na^+ and Cl^- were measured only under symmetrical conditions (L-15 medium on both sides) where the 'cold' concentrations, and thus the relevant specific activities, were known. The flux of [^3H]polyethylene glycol (molecular mass 4000 Da; 'PEG-4000', NEN, 37 kBq= $1 \mu\text{Ci}$ per well or insert) was measured as an index of paracellular permeability.

Analytical methods

An EVOM epithelial voltohmmeter with STX-2 'chopstick' electrodes (WPI, Sarasota, Florida, USA) was used to monitor transepithelial resistance and transepithelial potential (TEP). The electrodes can be quickly moved from one filter insert to the next without disturbing the cultured membranes. At the same time, the TEP and resistance values for an identical blank filter insert with the same apical and basolateral solutions were recorded and subtracted from the measured values in order to correct for junction potentials and for the resistance of the solutions plus the filter membrane itself. When asymmetrical solutions were used, it was important to make these measurements immediately after set-up in the blanks and immediately after medium replacement in the experimentals (in practice, approximately 10 min after because three exchanges were performed), before ionic gradients changed. TEP (in mV) was expressed as the corrected potential on the basolateral surface (well side) relative to the apical surface (insert side) as 0 mV. Transepithelial resistance (in $\text{k}\Omega \text{cm}^2$) was expressed as the product of the corrected value and filter surface area.

Na^+ and K^+ concentrations were measured using an Eppendorf flame photometer, Cl^- concentration by coulometric titration (Radiometer CMT-10), radioactivity by scintillation counting (Packard Tricarb 1900 CA) and total ammonia concentration (T_{Amm}) by the enzymatic method of Mondzac *et al.* (1965) using a Sigma kit. For pH measurements, small samples of apical and basolateral media were drawn into a 100 μl gas-tight Hamilton syringe and injected into a Radiometer E5021 capillary microelectrode

assembly and pHM 84 M at the experimental temperature. Appropriate constants (pK_{Amm} and α_{NH_3}) and equations from the work of Cameron and Heisler (1983) were used to calculate P_{NH_3} gradients based on measurements of total ammonia and pH.

Data have been expressed as means ± 1 S.E.M. (N), where N represents the number of filter inserts. The entire study is based on 155 inserts from 72 fish. The statistical significance of differences ($P \leq 0.05$) has been assessed using Student's two-tailed t -test, paired or unpaired as appropriate, with the Bonferroni correction procedure for multiple comparisons.

Results

The cultured epithelium

On the basis of tests with cultures ranging in age from 0 to 21 days, re-seeding onto filter inserts was most successful on days 6–9. In general, this corresponded to the period several days before the cells reached confluence in flask culture. Seeding densities between 50 000 and 1 000 000 cells cm^{-2} on the filter inserts were evaluated. A minimum density of approximately 300 000 cells cm^{-2} was required to achieve confluent membranes. In practice, we therefore routinely seeded approximately 500 000 cells cm^{-2} , or 2 150 000 cells on each 4.3 cm^2 filter insert, after days 6–9 days of flask culture (optimal conditions). No attachment occurred when freshly prepared cells (directly isolated from gills) were seeded onto filters; some attachment was usually seen with preparations trypsinized and re-seeded on filters at days 3–5 and after day 9, or in preparations seeded at lower density, but full coverage of the filters did not occur. Initially, we had greatest success with the largest filter size (4.3 cm^2), probably because it was easiest to work with mechanically, but later found that, with care, successful confluent cultures with identical characteristics could also be achieved on the smaller inserts (0.83 and 0.31 cm^2). In general, we had greater success with low-pore-density filters (1.6×10^6 pores cm^{-2}), reflecting better initial attachment, but those preparations that did grow on high-pore-density filters (10^8 pores cm^{-2}) exhibited essentially identical electrical properties (data not shown).

Measurements of transepithelial resistance were used to follow changes in electrical tightness of the cultured epithelia on the filter inserts (Fig. 1). At 24 h after seeding under optimal conditions, the resistance of the preparation was significantly above background, and by 48 h resistance started to increase rapidly. Visualization with fluorescein at this time demonstrated that confluence to the edge of the filter insert was complete. The resistance curve was sigmoidal against time, approaching a plateau at approximately 3.5 $\text{k}\Omega \text{cm}^2$ by day 6 (i.e. after approximately 14 days of culture in total). Experiments were routinely started at this time. Resistance was stable ($\pm 15\%$) for the next 6 days (not illustrated).

Under phase contrast microscopy, the cells seemed to become more tightly packed over the period from day 2 to day 6 (Fig. 2A), during which resistance increased tenfold.

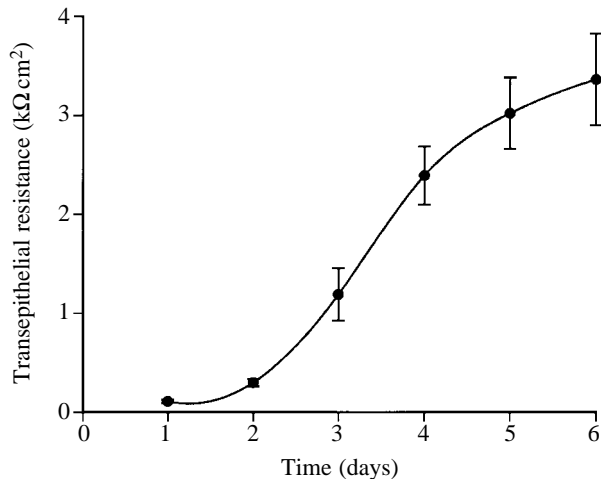


Fig. 1. Changes in transepithelial resistance of cultured branchial epithelia over time with L-15 culture medium on both apical and basolateral surfaces. Cells were re-seeded onto filter inserts at day 0, after 6–9 days of initial culture in flasks. Means \pm 1 S.E.M. ($N=14$).

On the basis of transmission electron microscopy (TEM) (performed only at day 6 or later), we attribute the large rise in resistance to the formation of junctional complexes between overlapping cell layers (Fig. 2B,C). These consisted of areas of tight membrane-to-membrane contact of approximately $0.5\ \mu\text{m}$ in length (putative tight junctions or *zona occludentes*) and numerous desmosomes. Typically, there were 2–4 cell layers in most sections (occasionally five or six), often with prominent intercellular spaces, and the overall thickness of the cultured epithelium was $7.74\pm 1.07\ \mu\text{m}$ (mean \pm S.E.M., $N=4$). On the basis of nuclear counts under phase contrast, cell density on the filter inserts at days 6–9 was $275\ 100\pm 30\ 900\ \text{cells cm}^{-2}$ ($N=6$), which may be compared with approximately $80\ 000\ \text{cells cm}^{-2}$ at confluence in flask culture (where they appear to form monolayers only, though this has not yet been confirmed by TEM). On the inserts, TEM revealed a relatively, simple uniform structure to the cells; mitochondria were present but were not especially abundant and rough endoplasmic reticulum was prominent (Fig. 2B,C). Some ridges or projections and a prominent glycocalyx were present on the apical surface. DASPEI staining revealed no intensely fluorescent cells even with the relatively high concentration used, $25\ \mu\text{mol l}^{-1}$ (in comparison with obvious staining seen in chloride cells of freshly dispersed gill preparations, even at much lower DASPEI concentrations of 2.5 and $10\ \mu\text{mol l}^{-1}$). Our interpretation is that the cultured epithelium consists of several interdigitating layers of pavement-type cells only.

Experimental characterization of the cultured epithelium

In epithelia with L-15 culture medium on both sides, examined between days 6 and 9, there was no detectable net flux of Na^+ or Cl^- between apical and basolateral surfaces in flux periods of up to 48 h. However, in these same tests, there

was a consistent slight but significant acidification of the apical medium relative to the basolateral medium and a polarity to the distribution of ammonia after 48 h, such that T_{Amm} was significantly higher in the apical medium than in the basolateral medium (Table 1). This should not necessarily be interpreted as a flux from one side to the other, as the cells clearly produced ammonia and may also have served as a barrier to ammonia movement, resulting in an asymmetrical distribution. In the experiment shown in Table 1, the starting T_{Amm} concentration in the medium on both sides was $438\ \mu\text{mol l}^{-1}$, whereas the final concentrations at 48 h were 1.4- to 1.8-fold higher. Interestingly, P_{NH_3} was significantly higher in the apical medium after 48 h, so this asymmetrical distribution of T_{Amm} occurred against an apparent P_{NH_3} gradient.

Under these same conditions, unidirectional fluxes of Na^+ and Cl^- , measured radioisotopically, were both approximately $300\ \text{nmol cm}^{-2}\ \text{h}^{-1}$ (Fig. 3). The basolateral-to-apical flux tended to be higher than the apical-to-basolateral flux, a difference that was significant for Na^+ only. However, inasmuch as a net flux could not be detected, and since the unidirectional fluxes in the two directions were measured in different preparations, it is questionable whether the difference is biologically significant. To evaluate this point further, an experiment was performed in which preparations were incubated for 48 h with L-15 medium of identical specific activities (^{22}Na , ^{36}Cl) on the two surfaces for 48 h. The final apical-to-basolateral activity ratios were 0.997 ± 0.010 ($N=11$) for ^{22}Na and 1.004 ± 0.002 ($N=9$) for ^{36}Cl ; neither is significantly different from 1.0. We conclude that no net transport of Na^+ or Cl^- occurred across the cultured epithelium under symmetrical conditions with L-15 present on both sides. PEG-4000 permeability was approximately $0.17\times 10^{-6}\ \text{cm s}^{-1}$ and identical in either direction (Fig. 3) under these same conditions.

With L-15 medium on both surfaces of the cultured epithelium, TEP was not significantly different from 0 mV (Fig. 4A). When the apical medium was changed to Cortland saline, which represented a small reduction in $[\text{NaCl}]$, there was no significant change in either TEP (Fig. 4A) or resistance (Fig. 4B). This situation was maintained when

Table 1. Differences in pH, total ammonia concentration (T_{Amm}) and the partial pressure of ammonia (P_{NH_3}) between basolateral and apical media (both L-15) of cultured branchial epithelial membranes after 48 h of incubation (on days 6–9 of culture)

	Basolateral	Apical
pH	7.880 ± 0.04	$7.823\pm 0.006^*$
T_{Amm} ($\mu\text{mol l}^{-1}$)	623 ± 11	$806\pm 10^*$
P_{NH_3} (Torr)	260 ± 5	$296\pm 7^*$

Values are means \pm S.E.M., $N=14$.

*Significant difference between basolateral and apical values, $P<0.05$.

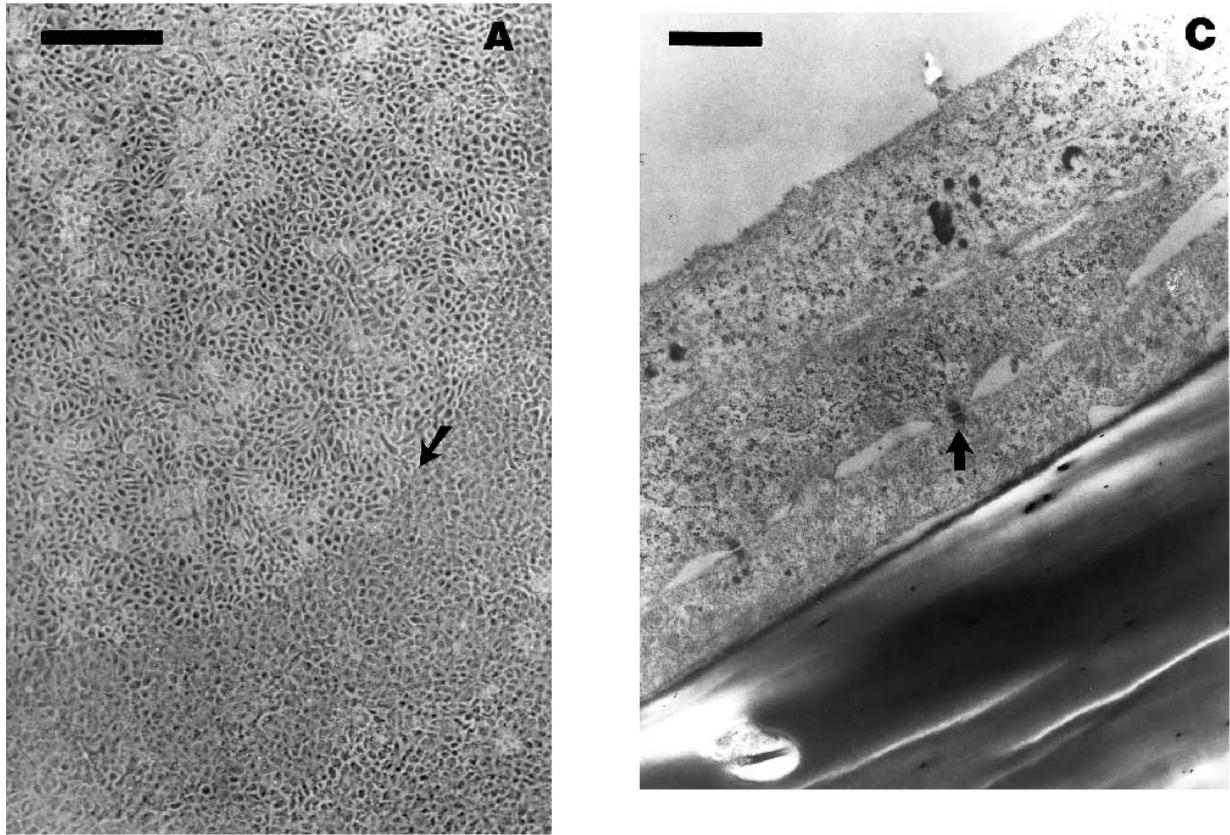


Fig. 2. (A) Phase contrast photomicrograph of a cultured branchial epithelium (day 6, with L-15 medium on both surfaces) on a filter insert. The preparation had earlier been stained with fluorescein diacetate ($10\mu\text{mol l}^{-1}$) and viewed under epifluorescence. The arrow points to the circular demarcation line between the paler area to the upper left, which had been viewed under epifluorescence and suffered excitation damage, and the darker area to the lower right which had not been exposed. Scale bar, $200\mu\text{m}$. (B) Transmission electron micrograph of a cultured branchial epithelium on a filter insert. The preparation had been grown for 7 days with L-15 medium on both surfaces and was never exposed to freshwater. The light area on the upper left represents the region which would be occupied by L-15 medium on the apical surface. The light area on the lower right is the polyethylene terephthalate filter underlying the basolateral surface. Note the prominent glycocalyx on the apical surface, the projections on the apical surface, the multiple overlapping cell layers (five in this section), the large intercellular space (S), the abundance of rough endoplasmic reticulum (R) but scarcity of mitochondria (M), the putative tight junctions (filled arrows) and the desmosomes (open arrow). Scale bar, $0.5\mu\text{m}$. (C) Transmission electron micrograph of a cultured branchial epithelium on a filter insert which had been grown for 6 days with L-15 medium on both surfaces and then incubated with freshwater on the apical surface for a further 48 h. The light area on the upper left represents the region which would be occupied by freshwater on the apical surface; the dark area on the lower right is the polyethylene terephthalate filter underlying the basolateral surface. Multiple overlapping cell layers (three in this section) can be seen. The filled arrow indicates a desmosome. Scale bar, $2\mu\text{m}$. Other details as in B.

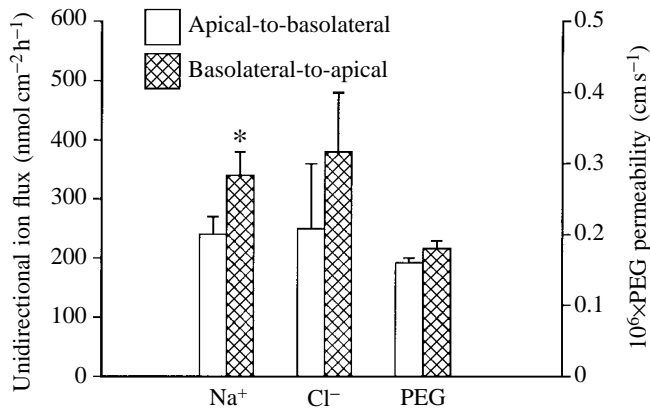


Fig. 3. Unidirectional fluxes of Na⁺ ($N=10$) and Cl⁻ ($N=10$) and permeability to PEG-4000 ($N=20$), measured radioisotopically, in cultured branchial epithelia with L-15 culture medium on both apical and basolateral surfaces. Measurements were made during days 6–9 of culture on filter inserts. The asterisk indicates a significant difference ($P<0.05$) between measurements in the apical-to-basolateral direction *versus* the basolateral-to-apical direction. Means + 1 s.e.m. Transepithelial resistance, 2–3 k Ω cm².

the apical [NaCl] was subsequently reduced by approximately 50%, with osmotic compensation by mannitol. However, further reductions in [NaCl] (again osmotically compensated), first to 23 mmol l⁻¹ and finally to 3 mmol l⁻¹, induced a highly negative TEP (Fig. 4A) and substantial increases in resistance (Fig. 4B). Values were approximately -17 mV and 12 k Ω cm², respectively, in the most dilute apical solution. Upon return of the apical medium to Cortland saline, these alterations in TEP were partially reversed, while those in resistance were fully reversed. All of these values were recorded at 10 min of exposure and were stable up to 30 min.

The tolerance of the cultured membrane to synthetic freshwater (not osmotically compensated) on the apical surface was assessed initially by exposure for 2.5 h during which resistance alone was followed (Fig. 5). Resistance increased more than fivefold to approximately 14 k Ω cm² by 0.5 h, and thereafter was more or less stable until 2.5 h. Upon return to L-15 on the apical surface, the resistance decreased precipitously to a level significantly below the initial value in the first 10 min, but thereafter recovered to the control value by 0.5 h, and remained stable over the subsequent 3 h.

On the basis of this encouraging result, a more extensive exposure to freshwater for 48 h was conducted, with replacements of apical media at 12 h intervals for electrical recording (Fig. 6) and flux measurements (Fig. 7). TEP immediately became highly negative (approximately -35 mV) and remained more or less stable over the first 24 h (Fig. 6A). This potential deteriorated during the second 24 h of freshwater exposure to approximately -15 mV. Resistance exhibited a somewhat different pattern, increasing initially to approximately 30 k Ω cm² and thereafter declining in a quasi-exponential fashion (Fig. 6B). However, even after 48 h in

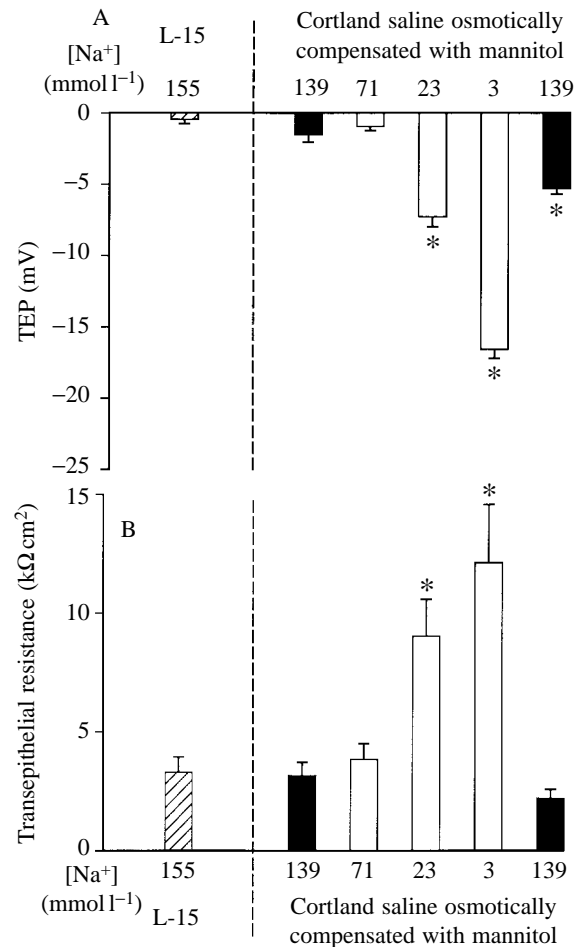


Fig. 4. The influence of changing the [NaCl] of the apical medium without altering the concentrations of other ions or osmotic pressure on (A) the transepithelial potential (TEP) and (B) the transepithelial resistance of cultured branchial epithelia. Measurements were made during days 6–9 of culture on filter inserts. The basolateral medium was L-15 for all measurements. The apical medium was changed from L-15 (hatched bar) to Cortland saline (left-hand filled bar), then to Cortland saline with successive reductions in [NaCl] osmotically compensated with mannitol (open bars), and finally back to Cortland saline (right-hand filled bar). The measured concentration of Na⁺ in each apical medium is shown. Asterisks indicate significant differences ($P<0.05$) from the first Cortland saline exposure (left-hand filled bar). Means + 1 s.e.m. ($N=9$).

freshwater, resistance remained significantly elevated to more than twice the initial value. TEM performed on cultured epithelia fixed at this time (Fig. 2C) revealed intact cells with no obvious signs of abnormal pathology, though the glycocalyx was less prominent.

In this same experiment, net fluxes of Na⁺, Cl⁻ and K⁺, while slightly negative, were extremely low during the first 12 h of apical freshwater exposure (Fig. 7). Indeed, net loss rates of Na⁺ and Cl⁻ remained equal to or smaller than the unidirectional fluxes measured with radiotracer in other preparations when L-15 was present on both surfaces (see

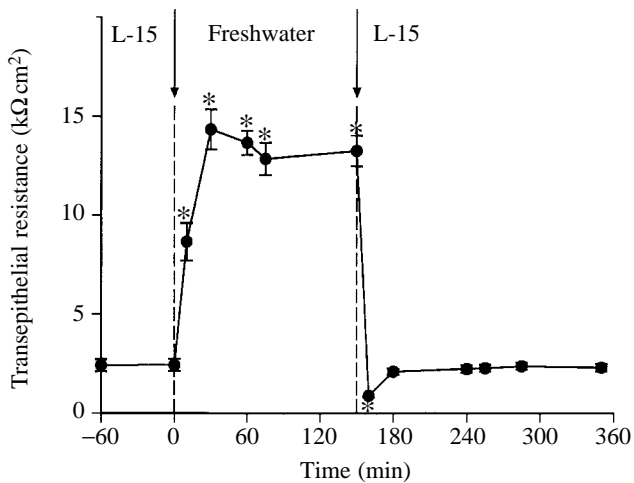


Fig. 5. The influence of changing the apical medium from L-15 to freshwater for 150 min, and then back to L-15, on the transepithelial resistance of cultured branchial epithelia. Measurements were made during days 6–9 of culture on filter inserts. The basolateral medium was L-15 for all measurements. Asterisks indicate significant differences ($P < 0.05$) from the initial values prior to freshwater exposure. Means \pm 1 S.E.M. ($N=5$).

Fig. 3). These rates increased substantially over the second 24 h period. Net Na^+ and Cl^- losses were equimolar in all flux periods (Fig. 7A,B), while K^+ losses were approximately 20-fold lower (Fig. 7C).

Discussion

While several groups have now achieved successful primary cultures of gill epithelial cells (Naito and Ishikawa, 1980; Ku and Chen, 1991; Pärt *et al.* 1993; Bols *et al.* 1994; Avella *et al.* 1994; Fernandes *et al.* 1995; Witters *et al.* 1996), to our knowledge, this is the first report of cultured epithelia of trout gill pavement cells on permeable supports. Intact frog skin exposed to synthetic freshwater on the mucosal surface and ECF-like medium on the serosal surface has been kept alive for several days in organ culture (Goudeau *et al.* 1979). However, the present preparation appears to be the only epithelium constructed from single cells in primary culture to survive prolonged exposure to freshwater on the apical surface and ECF-like medium on the basolateral surface. Resistances and TEP were still significantly elevated (Fig. 6) after 48 h of exposure to freshwater, and cell morphology under TEM still appeared healthy (Fig. 2C). These results alone suggest that the preparation exhibits properties similar to the epithelium of the freshwater gill; most other cultured cell types would have burst and died. It is true that the cultured epithelium shows some physiological evidence of change over time as freshwater exposure is continued (decreases in resistance and TEP, increases in ionic permeability; Figs 6, 7), but it is unclear which of these changes is 'normal' and which is pathological. Certainly, the preparation exhibits electrical stability over the first few hours of exposure (Fig. 5), and ion leakage rates are

extremely low during the first 12 h of exposure (Fig. 7). This stability is at least comparable to that of most dissected epithelial preparations used as possible gill surrogates (see Introduction); such epithelia typically maintain stable resistances for 2–5 h *in vitro* and exhibit a more negative ionic balance than the cultured epithelium.

This relative stability was seen in the present experiments despite the fact that the apical (external) surface of the cultured epithelium was exposed to a step change from iso-osmotic conditions (L-15 medium) to freshwater and that no hormonal support was provided on the basolateral surface. *In vivo*, when euryhaline fish move from seawater or brackish water into freshwater, they usually do so gradually, and a complex suite of hormonal changes occurs (involving cortisol, growth hormone, thyroid hormones and prolactin), the most important of which appears to be an increased secretion of prolactin (reviewed by McCormick, 1995). We have found that cultured epithelia cannot be grown with freshwater in the insert; however, future experiments should test the efficacy of gradually reducing the apical salinity over time either prior to or after the plateau phase of resistance development (Fig. 1) has been reached. Elevating the Ca^{2+} concentration in synthetic freshwater to equal that in the L-15 medium (1.6 mmol l^{-1}) in order to avoid Ca^{2+} shock is another possible strategy. Furthermore, it is possible that the combination of L-15 and FBS alone does not supply the specific factors needed for the cells to adapt fully to apical freshwater. Hormonal and

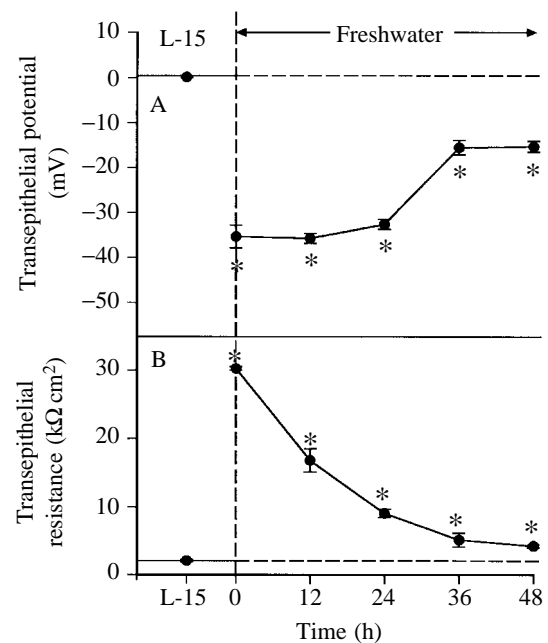


Fig. 6. The influence of changing the apical medium from L-15 to freshwater for 48 h on (A) the transepithelial potential and (B) the transepithelial resistance of cultured branchial epithelia. Measurements were made during days 6–9 of culture on filter inserts. The basolateral medium was L-15 for all measurements. Asterisks indicate significant differences ($P < 0.05$) from the initial values prior to freshwater exposure. Means \pm 1 S.E.M. ($N=4$).

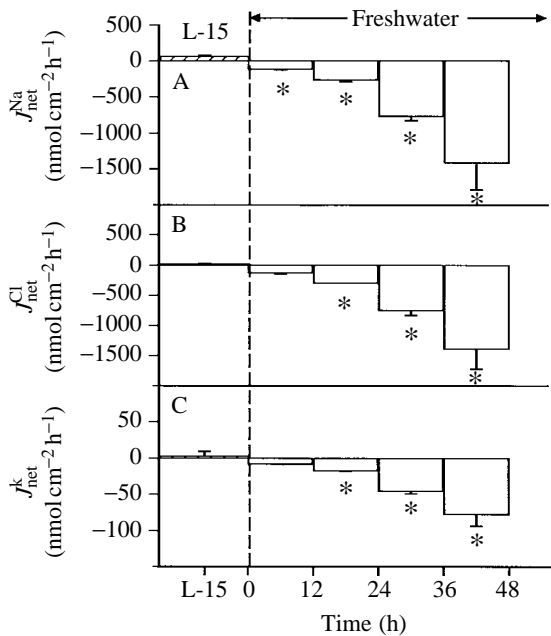


Fig. 7. The influence of changing the apical medium of the cultured branchial epithelium from L-15 to freshwater, for 48h, on the net fluxes (J_{net}) of (A) Na^+ , (B) Cl^- and (C) K^+ . Measurements were made during days 6–9 of culture on filter inserts. The basolateral medium was L-15 for all measurements. Negative fluxes represent net losses to the apical medium. Asterisks indicate significant differences ($P < 0.05$) from the initial values prior to freshwater exposure. Means ± 1 S.E.M. ($N=4$).

nutritional supplementation in the basolateral medium should also be evaluated; prolactin in particular (see above) should be tested.

Over 6 days of culture with L-15 medium present on both sides, the electrical resistance of the epithelium increased in a sigmoidal fashion (Fig. 1), eventually reaching a stable plateau. Such curves are characteristic of many epithelia in culture (e.g. Cerejido *et al.* 1981). As first shown by Cerejido *et al.* (1981), this increase in resistance correlates with the formation of tight junctions between the cells. These are believed to be the most important sites of paracellular electrical resistance; the greater the number of strands in the junctions, the greater the resistance. Multi-stranded tight junctions are abundant in the intact gill epithelium (Sardet, 1980; Laurent, 1984), so it is likely that the same explanation applies in the present preparation. However, freeze–fracture studies (e.g. Ernst *et al.* 1980; Sardet, 1980; Cerejido *et al.* 1981) will be required to characterize completely the regions of tight membrane-to-membrane contact (approximately $0.5\ \mu\text{m}$ in length; Fig. 2B) in the cultured epithelium, which we have tentatively identified as tight junctions. The numerous desmosomes which are clearly seen in the cultured epithelium (Fig. 2B,C) are characteristic of epithelial cell layers, such as the pavement cells of fish gills, and serve as spot-like snap-buttons to keep the cells together. However, they are thought to make a minimal contribution to electrical resistance. In general, in intact epithelia, it is the outer cell layer which is the

major resistive barrier and not the sublayers that are connected by numerous desmosome junctions (Klyce, 1972; Nagel, 1978).

The resistance of the cultured branchial epithelium reached a mean plateau of approximately $3.5\ \text{k}\Omega\ \text{cm}^2$ (Fig. 1). Comparison with values for other cultured epithelia illustrates that the current preparation has a much higher resistance than typical ‘leaky’ epithelia: for example, flounder kidney cells ($23\ \Omega\ \text{cm}^2$; Dickman and Renfro, 1986), dog kidney MDCK cells ($100\ \Omega\ \text{cm}^2$; Cerejido *et al.* 1981), CaCo-2 intestinal cells ($258\ \Omega\ \text{cm}^2$; Artursson, 1990) and guinea pig gastric mucosa cells ($429\ \Omega\ \text{cm}^2$; Rutten, 1992). Indeed, $3.5\ \text{k}\Omega\ \text{cm}^2$ is typical of ‘tight’ epithelia such as rat alveolar cells ($2.3\ \text{k}\Omega\ \text{cm}^2$; Cheek *et al.* 1989) and toad A6 kidney cells (1.6 – $5.0\ \text{k}\Omega\ \text{cm}^2$; Steele *et al.* 1992; Candia *et al.* 1993). To our knowledge, the electrical resistance of the intact gill *in vivo* has never been reported; however, the present measurements support the commonly held assumption, based on morphological and permeability criteria, that the freshwater gill is a ‘tight’ epithelium (e.g. Sardet, 1980; Isaia, 1984).

Upon exposure to freshwater on the apical surface, the already high resistance increased six- to elevenfold within a few minutes to 10 – $30\ \text{k}\Omega\ \text{cm}^2$ (Figs 5, 6). This remarkable increase brought it up to or above the values reported for two dissected epithelial preparations which have been tested *in vitro* as possible surrogates for the freshwater gill – the cleithral epithelium of freshwater-adapted trout (approximately $11\ \text{k}\Omega\ \text{cm}^2$; Marshall *et al.* 1992) and the

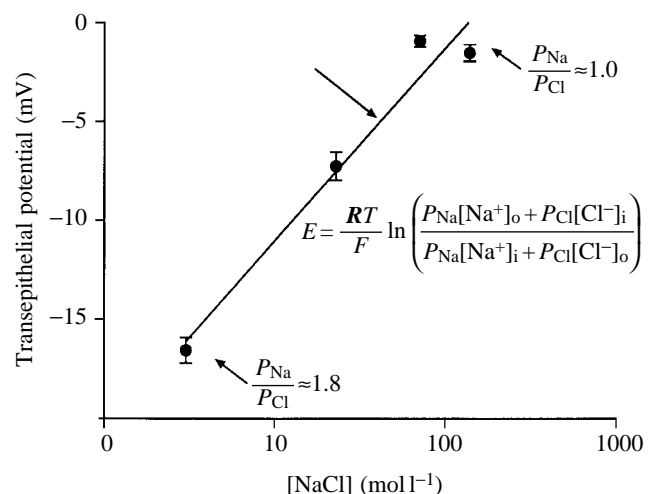


Fig. 8. Nernstian analysis of changes in the ratio of diffusive permeabilities to Na^+ and Cl^- of the cultured branchial epithelium needed to explain the transepithelial potential data of Fig. 4A when the NaCl concentration of the apical medium was experimentally altered. In the equation, E is the transepithelial potential, R is the gas constant, T is the absolute temperature, F is Faraday’s constant, P_{Na} is permeability to Na^+ , P_{Cl} is permeability to Cl^- , and the subscripts o and i refer to concentrations on the outside (apical) and inside (basolateral) surfaces, respectively, of the cultured epithelium relative to its situation in an intact fish. Means ± 1 S.E.M. ($N=9$). The line has a slope of $9.8\ \text{mV}$ per decade change in $[\text{NaCl}]$.

opercular epithelium of freshwater-adapted tilapia (approximately $3.7 \text{ k}\Omega \text{ cm}^2$; Foskett *et al.* 1981; McCormick *et al.* 1992). By way of contrast, the opercular epithelium of the seawater-adapted killifish is particularly 'leaky' ($132 \Omega \text{ cm}^2$; Ernst *et al.* 1980) but becomes much 'tighter' after freshwater adaptation ($645 \Omega \text{ cm}^2$; Wood and Marshall, 1994). These data support the commonly held view that the permeability of the gill to ions decreases greatly when euryhaline fish move into freshwater (Maetz, 1970; Potts, 1984; Marshall, 1995). The presence of large numbers of chloride cells in the opercular epithelium of the killifish may account for its lower resistance relative to the present cultured epithelium, which lacks these ionocytes.

A possible explanation for the rapid increase in resistance upon apical freshwater exposure is a rapid cell swelling which in some way mechanically tightens the cell junctions. However, this possibility is largely discounted by the fact that a similar increase in resistance was seen when the osmotic pressure of the apical medium was 'clamped' with mannitol, and only the Na^+ and Cl^- levels were lowered to freshwater values (Fig. 4B). More likely, the effect involves rapid modulation of transcellular conductance caused by the reduction of external salinity (e.g. by closure of apical Cl^- channels; see Kirschner, 1996) and/or a change in paracellular conductance (tight junctions, intercellular spaces) mediated by the action of intracellular messengers (e.g. Duffey *et al.* 1981; Kottra and Fromter, 1993). Tests following the movement of PEG-4000, a paracellular permeability marker, upon apical exposure to freshwater may be useful in separating these two possibilities.

In the present experiments, PEG-4000 permeability of the cultured epithelium was measured only with L-15 medium on both surfaces, where it averaged approximately $0.17 \times 10^{-6} \text{ cm s}^{-1}$ and was the same in both directions. This value is very similar to the value of $0.16 \times 10^{-6} \text{ cm s}^{-1}$ which can be calculated from the mass-specific total gill area tabulated by Hughes and Morgan (1973) and the branchial clearance data of Curtis and Wood (1991) for PEG-4000 in intact unanaesthetized rainbow trout in freshwater. The value also falls within the range (0.05×10^{-6} to $0.35 \times 10^{-6} \text{ cm s}^{-1}$) which can be calculated from the earlier branchial clearance data of Kirschner (1980) for several other paracellular permeability markers in freshwater trout.

Na^+ and Cl^- fluxes across the cultured epithelium also correspond well to *in vivo* rates. Unidirectional fluxes for both ions in the presence of L-15 on both surfaces were approximately $300 \text{ nmol cm}^{-2} \text{ h}^{-1}$ (Fig. 3). Upon exposure to apical freshwater, only net fluxes were measured; the preparation went from no significant net flux under iso-osmotic conditions to net losses of approximately $125 \text{ nmol cm}^{-2} \text{ h}^{-1}$ for both ions in the first 12 h of apical freshwater exposure (Fig. 7). We do not know whether there was any active ion uptake from the water in these experiments. If we estimate conservatively that there was none, then unidirectional effluxes could be no greater than the measured net loss rates. Again using the gill area data of Hughes and Morgan (1973), unidirectional

effluxes of Na^+ and Cl^- across the cultured epithelium would translate to approximately $250 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ (in apical freshwater) to approximately $600 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ (in apical iso-osmotic media) in the whole trout. Branchial unidirectional fluxes of Na^+ and Cl^- in intact freshwater trout are typically approximately $250 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ (e.g. Wood, 1988; Goss *et al.* 1995) and increase approximately threefold after adaptation to 33% seawater (Bath and Eddy, 1979), close to the composition of the present iso-osmotic medium.

Upon exposure to more dilute media (Fig. 4A) or freshwater (Fig. 6), the cultured epithelium developed a basolateral-negative TEP. This effect is typical of the gills of euryhaline fish, such as the rainbow trout, and is generally interpreted as a diffusion potential reflecting the differential permeability of the epithelium to Na^+ over Cl^- (Potts, 1984; Kirschner, 1996). In the experiment of Fig. 4A, only Na^+ and Cl^- concentrations were changed, and no other factors known to affect gill permeability such as $[\text{Ca}^{2+}]$, $[\text{Mg}^{2+}]$ or osmotic pressure. From these data, using the Goldman equation, we can model the observed TEP as a diffusion potential assuming that the permeability ratio of Na^+ relative to Cl^- changed from approximately 1.0 under symmetrical conditions to approximately 1.8 under dilute conditions (Fig. 8). This range is quite reasonable for salmonids (McWilliams and Potts, 1978; Potts, 1984). Upon exposure to true freshwater, the TEP was even more negative (Fig. 6A), at least in part due to the lower apical Ca^{2+} concentration, which is known to raise the permeability ratio even further (Eddy, 1975; McWilliams and Potts, 1978; Kirschner, 1996).

It is highly probable that the cultured epithelium consists of a pure pavement cell population. This conclusion is based on the fact that the 'starting' population of cells re-trypsinized on day 6 of flask culture, in order to seed the filter, exhibits morphological properties typical of pavement cells only (Pärt *et al.* 1993; Pärt and Bergstrom, 1995). The chloride cells do not attach and therefore do not survive in flask culture. Furthermore, none of the cells on the cultured epithelium appears to take up the mitochondrial stain DASPEI in the manner of freshly isolated chloride cells. Morphologically, the cells appear uniform under phase contrast, and under TEM they have a simple flattened structure with multiple desmosomes, abundant rough endoplasmic reticulum, a low abundance of mitochondria and a surface glycocalyx, all features typical of pavement cells (Laurent and Dunel, 1980; Laurent, 1984; Laurent and Hebibi, 1989; Goss *et al.* 1995). The surface bears projections but it is not clear whether these are villi or microridges (both of which may be seen on pavement cell surfaces *in vivo*) or different structures. Clearly, it would be desirable in future studies to examine the surface morphology by using scanning electron microscopy. When this approach was applied to the same cells in flask culture, 'fingerprint' microridges typical of some pavement cells (Laurent, 1984) were observed (Pärt *et al.* 1993; Fernandes *et al.* 1995). It will also be useful to develop specific enzyme markers and immunocytochemical probes to distinguish the various types of cells in the gills.

The multi-layered structure of the cultured epithelium, with prominent intercellular spaces, is characteristic of pavement cell organization on intact lamellae (Laurent, 1984; Laurent and Hebibi, 1989). *In vivo*, the lamellar epithelium is not a monolayer. Rather, it usually consists of two or more pavement cell layers, the outer water-facing layer being more differentiated and having a more decorated surface than the inner layer lying on the basal lamina. Often the layers separate with a prominent 'lymphatic space' in between. If we are correct in our conclusion that only pavement cells are present in the cultured epithelium, the preparation offers an excellent opportunity to settle the current debate about the cellular localization of ion-transport processes in the freshwater gill (see Introduction). If, for example, subsequent isotopic flux analyses using the Ussing flux-ratio criterion show that the preparation actively takes up Na^+ and not Cl^- from the water, this would support current ideas that the pavement cell is the site of Na^+ uptake (Goss *et al.* 1992, 1995; Morgan and Potts, 1995; Lin and Randall, 1995). If neither Na^+ nor Cl^- is taken up, it would support the more traditional view that both processes are instead localized to the chloride cells (Avella *et al.* 1987; Perry and Laurent, 1989; Wood, 1991). Finally, if both Na^+ and Cl^- are actively taken up, it would revive the now less popular idea that the pavement cells are the dominant site for both transport pathways (Payan *et al.* 1984). Unidirectional Ca^{2+} flux measurements will also be very informative, in the light of the present consensus (Flik *et al.* 1995) that Ca^{2+} uptake is predominantly *via* chloride cells, not pavement cells.

In all these theories, there is a general belief that Na^+ uptake is in some way coupled directly to H^+ excretion (by an Na^+/H^+ exchange and/or by an H^+ -ATPase Na^+ channel system; see Lin and Randall, 1995; Kirschner, 1996) and at least indirectly to ammonia excretion (by diffusion trapping and/or by $\text{Na}^+/\text{NH}_4^+$ exchange; see Wilson *et al.* 1994; Kirschner, 1996). Recently, we have demonstrated that these same cells when cultured on solid supports in L-15 medium exhibit an active Na^+/H^+ exchanger responsive to intracellular acidosis (Pärt and Wood, 1996). It is therefore interesting that the cultured epithelium acidifies the apical medium and creates an asymmetrical distribution of ammonia (Table 1). The preparation may also serve as a useful model to settle the current controversy as to the mechanisms of branchial ammonia excretion (Wilson *et al.* 1994).

To conclude, it would appear that the cultured gill epithelium may serve as a model for the freshwater fish gill, but we emphasize that the potential has not yet been realized. The present study indicates that the cultured epithelium duplicates the passive electrical and flux properties of the intact gill quite well, but provides no information about its active ion transport properties. Furthermore, in containing only pavement cells, the present preparation offers a useful tool for studying pavement cell function in isolation, but does not illuminate the integrated function of the multiple cell types present in the branchial epithelium *in vivo*. Clearly, to meet this objective, it will be necessary to incorporate chloride cells into the cultured epithelium.

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