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Passive and Active Transport Properties of a Gill Model, the Cultured Branchial Epithelium of the Freshwater Rainbow Trout (*Oncorhynchus mykiss*)

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ABSTRACT. Branchial epithelia of freshwater rainbow trout were cultured on permeable supports, polyethylene terephthalate membranes ("filter inserts"), starting from dispersed gill epithelial cells in primary culture. Leibowitz L-15 media plus foetal bovine serum and glutamine, with an ionic composition similar to trout extracellular fluid, was used. After 6 days of growth on the filter insert with L-15 present on both apical and basolateral surfaces, the cultured preparations exhibited stable transepithelial resistances (generally 1000–5000 Ω cm^2) typical of an electrically tight epithelium. Under these symmetrical conditions, transepithelial potential was zero, and unidirectional fluxes of Na^+ and Cl^- across the epithelium and permeability to the paracellular marker polyethylene glycol-4000 (PEG) were equal in both directions. Na^+ and Cl^- fluxes were similar to one another and linearly related to conductance (inversely related to resistance) in a manner indicative of fully conductive passive transport. Upon exposure to apical fresh water, transepithelial resistance increased greatly and a basolateral-negative transepithelial potential developed. At the same time, however, PEG permeability and unidirectional effluxes of Na^+ and Cl^- increased. Thus, total conductance fell, and ionic fluxes and paracellular permeability per unit conductance all increased greatly, consistent with a scenario whereby transcellular conductance decreases but paracellular permeability increases upon dilution of the apical medium. In apical fresh water, there was a net loss of ions from the basolateral to apical surfaces as effluxes greatly exceeded influxes. However, application of the Ussing flux ratio criterion, in two separate series involving different methods for measuring unidirectional fluxes, revealed active influx of Cl^- against the electrochemical gradient but passive movement of Na^+. The finding is surprising because the cultured epithelium appears to consist entirely of pavement-type cells. COMP BIOCHEM PHYSIOL 119A;1:87–96, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. Gill model, cell culture, epithelial cells, filter inserts, *Oncorhynchus mykiss*, transepithelial resistance, ionic fluxes, polyethylene glycol

INTRODUCTION

Mechanistic understanding of ion transport in the freshwater gill has been greatly hindered by the lack of a flat epithelial gill surrogate. Such models are well established for the seawater gill, for example, the jaw skin of the seawater-adapted goby (*Gillichthys mirabilis*) (14) and opercular epithelia of seawater-adapted killifish (*Fundulus heteroclitus*) (8) and tilapia (*Oreochromis mossambicus*) (5). These have been used to overcome the morphological complexity and fragility of the intact gill and have been subjected to rigorous electrical and radioisotopic analyses that have greatly enhanced our knowledge of active NaCl excretion mechanisms in seawater fish [reviewed by (18,33)]. To date, the search for a comparable freshwater model has proved disappointing, although the opercular epithelium of the freshwater-adapted killifish may hold some promise (18,31).

Recently, we tried an alternate *in vitro* approach to this problem and developed a method for culturing a flat reconstructed membrane of branchial epithelial cells of freshwater rainbow trout (*Oncorhynchus mykiss*) (32). The epithelium is cultured on a permeable support ("filter insert"), which allows it to be exposed to asymmetrical conditions (i.e., basolateral extracellular fluid, apical fresh water) as *in vivo*. The epithelium consists of several overlapping layers of pavement-type cells ("respiratory cells") (24); "chloride cells" appear to be absent. Our initial studies indicated that the cultured epithelium survives for at least 48 hr in apical fresh water and duplicates several of the properties of the intact gill: numerous tight junctions, comparable permeability to a paracellular marker, polyethylene glycol-4000 (PEG), a high transepithelial resistance that increases fur-

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ther upon apical exposure to fresh water, a negative transepithelial potential in fresh water and low Na^+ and Cl^- leakage rates in fresh water (32).

These initial studies raised several issues, which were objectives of the present study. The first of these was to explore the relationship(s) between transepithelial resistance, Na^+ and Cl^- movements and PEG permeability. In particular, we were interested in whether these movements were a simple function of resistance, whether resistance was a function of paracellular permeability and whether the increase in resistance upon freshwater exposure represented a decrease in paracellular permeability and/or ionic fluxes. The second objective focussed on the possible transport function(s) of the epithelium when exposed to fresh water. Our previous studies examined only net fluxes and documented small net losses of Na^+ and Cl^- after abrupt apical exposure to fresh water. Given the very large concentration gradients from the extracellular fluid (ECF)–like basolateral medium to apical fresh water, such losses are not surprising. However, it is possible that such losses may mask active influx components. Thus, our goal here was to measure unidirectional influx and efflux rates of Na^+ and Cl^- using radioisotopes and to apply the Ussing flux ratio criterion to determine whether active or passive mechanisms are involved (9). Because the cultured epithelium appears to lack "chloride cells," these data might cast some light on the current controversy as to the relative transport roles of pavement cells and chloride cells in the freshwater gill [reviewed by (6,7,13,20)].

MATERIALS AND METHODS

Preparation of Cultured Branchial Epithelia

Experiments were performed in both Uppsala, Sweden and Hamilton, Canada using locally obtained stocks of 1- to 2-year-old rainbow trout (*O. mykiss*; 15–100 g) held in fresh water at seasonal temperatures (6–16°C). Cultured branchial epithelia grown in the two laboratories exhibited very similar properties, and data from the two sources have been combined in the present report. Additional information on animal husbandry in the two laboratories is presented elsewhere (26,32).

Initially, branchial epithelial cells obtained from tryptic digests of trout gills were grown at 18°C under sterile conditions in plastic culture flasks using Leibowitz L-15 medium (Flow Laboratories, London, U.K. or GIBCO, Burlington, Ontario, Canada) supplemented with 2 mmol/l glutamine and 5% foetal bovine serum (FBS). Methods were identical to those detailed elsewhere (24–26). Between days 6 and 9, a time when radiothymidine incorporation is greatest but full confluence has not yet been reached, the cells were harvested by retrypsination and reseeded onto Falcon cell culture inserts (Cyclopore polyethylene terephthalate, Becton Dickinson, Franklin Lakes, New Jersey, U.S.A.). Sterile conditions were again maintained. Seeding density was 500,000 cells/cm^2. The filters had a pore size of 0.45 μm, a pore density of 1.6×10^6 pores per cm^2, an effective growth surface area of 0.83 cm^2 and were housed in Falcon 12-well cell culture companion plates. These smaller filter inserts yielded identical results to the larger inserts used originally (4.30 cm^2 diameter) and were much more economical of cells. Apart from this slight difference, methods were identical to those described in detail elsewhere (32) for this stage of culture.

In brief, L-15 medium (plus glutamine and FBS) was present in both the well (basolateral surface) and insert (apical surface). The medium was replenished at 24 hr after seeding and every 48 hr thereafter. The "growth" of the epithelium, manifested as an increase in transepithelial resistance, was monitored daily, and experiments were started once a resistance plateau had been reached. This generally occurred between days 6 and 9 after reseeding onto the filter inserts. Resistances were monitored *in situ* in the culture dishes using STX-2 "chopstick" electrodes attached to an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, Florida, U.S.A.).

Experimental Methods

Experiments involved the radioisotopic measurement of unidirectional fluxes of Na^+, Cl^- and the paracellular permeability marker PEG across the cultured epithelium *in situ*, together with simultaneous measurements of transepithelial potential (TEP) and transepithelial resistance, using the chopstick electrode/EVOM system. At the start of a flux measurement, the apical (= insert side; volume 1.4 ml) and basolateral (= well side; volume 2 ml) media were renewed. When this involved a changeover to fresh water (apical side only), the compartment was rinsed four times with the new media to ensure complete changeover. Particular care was taken during aspiration of waste solution to avoid contact with and therefore damage to the cultured epithelium; rupture of the epithelium was detectable as an immediate drop in resistance to background levels. However, criteria were adopted based on stability of resistance (see below) so as to eliminate membranes possibly suffering more subtle non-penetrating damage.

When L-15 (Na^+ 155, Cl^- 143, Ca^{2+} 1.3 mmol/l, pH 7.7–7.8) was present on both apical and basolateral surfaces, unidirectional fluxes were determined by adding 1 μCi of the appropriate radioisotope (^{22}Na, ^{36}Cl, [^3H]PEG, all NEN-Dupont, Boston, Massachusetts, U.S.A.) to one side and monitoring the appearance over time of radioactivity on the other side. Apical and basolateral solutions were sampled regularly, and TEP and resistance were monitored at times of sampling. Flux periods varied in different experiments from 3 to 48 hr, the longer periods being necessary when resistances were particularly high.

When fresh water (Na^+ 0.55, Cl^- 0.70, Ca^{2+} 1.0 mmol/l, pH 7.8–8.0, sterilized) was used on the apical surface, flux periods did not exceed 12 hr. Radioisotopes were added to the basolateral side to monitor "efflux" or to the apical side

to measure "influx" (i.e., unidirectional uptake from the water). Unidirectional fluxes were recorded in the standard fashion by monitoring the appearance of radioactivity on the non-labelled side. Net fluxes were determined by measuring changes in the total concentration of Na^+ or Cl^- in the dilute apical medium. Changes in the concentrated basolateral L-15 were too small to detect reliably. For preparations in which influx was measured directly, it was possible to monitor efflux simultaneously as the difference between net flux and influx, signs considered (see below). This has been termed indirectly measured efflux and was useful in allowing flux ratio analysis with all necessary measurements (TEP, influx, efflux) determined on the same cultured epithelium.

For these flux ratio analysis experiments, two series were performed. In the first series, directly measured influxes and net fluxes and indirectly measured effluxes were determined on the same preparations. In a second series, directly measured influxes and directly measured effluxes, together with net fluxes in both, were measured on separate preparations. In both series, unidirectional and net flux measurements lasting 8 hr were started 4 hr after transfer to apical fresh water to allow initial stabilization. At the end of the 8 hr flux measurement, apical L-15 media was restored, and a final resistance measurement was taken at 24 hr. This final resistance measurement was used as index of viability to eliminate preparations that might have been damaged by the transfer and sampling procedures. Only cultured epithelia that exhibited a resistance at 24 hr no more than 50% lower than their initial resistance immediately before freshwater exposure were used in the analyses.

Analytical Methods

Transepithelial resistance and TEP were measured using STX-2 chopstick electrodes attached to an EVOM epithelial voltohmmeter that had been custom-modified by the manufacturer (World Precision Instruments) to measure resistances up to 200,000 Ω. 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. This was necessary to correct for junction potentials and for the resistance of the solutions plus the filter itself. When asymmetrical solutions were used, it was important to make these measurements immediately after setup in the blanks and immediately after changeover in the experimentals, before ionic gradients changed. It should be noted that the resistance of the filter itself is not a function of the pore size or density, because the entire filter is wetted with the experimental solutions and therefore conductive. Furthermore, the resistance of the filter plus the solutions was always less than half the total and was subtracted from the total to yield the resistance of the cultured epithelium. Transepithelial resistance (in Ω cm^2) was expressed as the product of the corrected value × filter surface area. Transepithelial conductance was expressed as mS/cm^2 (i.e., 1000 × inverse of resistance). 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.

Na^+ was measured by an Eppendorf (Hamburg, Germany) flame photometer (in Uppsala) or Varian AA1275 (Mulgrave, Australia) atomic absorption unit (in Hamilton). Cl^- was measured using a colorimetric assay (34). Radioactivity was determined by scintillation counting using either a Packard (Downers Grove, Illinois, U.S.A.) Tricarb 1900 CA (in Uppsala) or LKB (Turku, Finland) 1217 Rackbeta (in Hamilton).

Calculations

Unidirectional fluxes of Na^+ and Cl^- were measured in the standard fashion. For example, influx (apical to basolateral flux, radioisotope placed on apical side):

$$J_{\text{in}}^{\text{Na+}} = \Delta[\text{Na*}]_{\text{Bl}} \times \frac{1}{\text{SA}_{\text{AP}}} \times \frac{\text{Volume}_{\text{Bl}}}{\text{Time} \cdot \text{Area}}, \tag{1}$$

where $\Delta[\text{Na*}]_{\text{Bl}}$ is the change in radioactivity on the basolateral side and SA_{Ap} is the mean specific activity on the apical side. Conversely, for efflux (basolateral to apical flux, radioisotope placed on basolateral side):

$$J_{\text{out}}^{\text{Na+}} = \Delta[\text{Na*}]_{\text{Ap}} \times \frac{1}{\text{SA}_{\text{Bl}}} \times \frac{\text{Volume}_{\text{Ap}}}{\text{Time} \cdot \text{Area}}, \tag{2}$$

where $\Delta[\text{Na*}]_{\text{Ap}}$ is the change in radioactivity on the apical side and SA_{Bl} is the mean specific activity on the basolateral side.

Net flux:

$$J_{\text{net}}^{\text{Na+}} = \Delta[\Sigma\,\text{Na}^+]_{\text{Ap}} \times \frac{\text{Volume}_{\text{Ap}}}{\text{Time} \cdot \text{Area}}, \tag{3}$$

where $\Delta[\Sigma\,\text{Na}^+]_{\text{Ap}}$ is the change in total sodium concentration on the apical side.

"Indirectly measured efflux" could be calculated from simultaneous measurements of J_{in} and J_{net} on the same preparation:

$$J_{\text{out}}^{\text{Na+}} = J_{\text{net}}^{\text{Na+}} - J_{\text{in}}^{\text{Na+}}. \tag{4}$$

The criterion used to detect the presence of active transport was disagreement of the measured flux ratio ($J_{\text{in}}/J_{\text{out}}$) with that predicted by the Ussing flux ratio equation (9):

$$\frac{J_{\text{in}}}{J_{\text{out}}} = \frac{A_{\text{Ap}}}{A_{\text{Bl}}} \cdot e^{(zFV/RT)}, \tag{5}$$

where A_{Ap} and A_{Bl} are the activities of the ion (Na^+ or Cl^-) on the apical and basolateral surfaces, z is the valency, V is the measured TEP and F, R and T have their usual thermodynamic meanings. Using an Na^+-specific micro-electrode, A^{Na} was measured to be 75% of total Na^+ concentra-

tion in L-15 media. In accordance with theory for a solution of this ionic strength (12), the same percentage was used to predict A^{Cl} from total Cl^- concentration. For fresh water, the activities were taken as equal to the measured concentrations, in view of the very low concentrations (<1 mmol/l). Values of J_{in} and J_{out} used in the calculation were either directly measured influx and indirectly measured efflux from the same preparation or directly measured influx and directly measured efflux from different preparations. In the latter case, preparations were "paired" based on various matching rules (see Results), and an average value for TEP for the two were epithelia was used.

Permeability to PEG, for example, in the efflux direction (basolateral to apical flux, radioisotope placed on basolateral side), was calculated as

$$P = \frac{\Delta[\mathrm{PEG}^*]_{\mathrm{Ap}} \cdot \mathrm{Volume}_{\mathrm{Ap}}}{[\mathrm{PEG}^*]_{\mathrm{Bl}} \cdot \mathrm{Time} \cdot 3600 \cdot \mathrm{Area}}, \quad (6)$$

where $[\mathrm{PEG}^*]_{\mathrm{Bl}}$ is mean radioactivity on the basolateral side, $\Delta[\mathrm{PEG}]_{\mathrm{Ap}}$ is the change in radioactivity on the apical side and 3600 converts time from hours to seconds.

Data are expressed as means ± SEM (*n*), where *n* represents the number of filter inserts. Regression lines were fitted by the method of least squares, and the significance of the correlation coefficient was determined. The statistical significance of differences, between means has been assessed using Student's two-tailed *t*-test, paired or unpaired as appropriate, with the Bonferroni correction procedure for multiple comparisons.

RESULTS

Resistance vs Permeability Relationships

Typically, the transepithelial resistance of the cultured gill epithelium (with L-15 on both basolateral and apical surfaces) reached a stable plateau of 1000–5000 $\Omega\,cm^2$ on days 6–9. However, there was considerable variation, and individual preparations were obtained with stable resistance values ranging from as low as 250 to as high as 27,000 $\Omega\,cm^2$ (Fig. 1A). In general, higher resistance epithelia were cultured from fish in winter months (water holding temperatures of 6–10°C) than in summer months (11–16°C). We exploited this natural variability to examine resistance vs permeability relationships.

With L-15 on both surfaces, TEP was zero, and there was no rectification of Na^+, Cl^- or PEG movements, as illustrated in Table 1 using a large, closely matched set of epithelia, all with resistances in the low range of 600–800 $\Omega\,cm^2$. Thus, under symmetrical conditions, Na^+ and Cl^- movements were passive. In view of this result, fluxes and permeabilities measured in both directions were included in the subsequent analyses.

Unidirectional fluxes of both Na^+ and Cl^- were inversely related to resistance, and measurements for both ions appeared to follow a common relationship (Fig. 1A). Transformation of these data as a plot of unidirectional flux vs conductance (mS/cm^2) revealed a more or less linear relationship between flux and conductance (Fig. 1B). At very high conductance, the relationship became more variable, and there was a tendency for higher Cl^- than Na^+ movements for a given conductance. However, it should be noted that measurement error is greatest for high conductances (low resistances) because the "blank" resistance of the filter plus solution (which must be subtracted) approaches half of the total measurement.

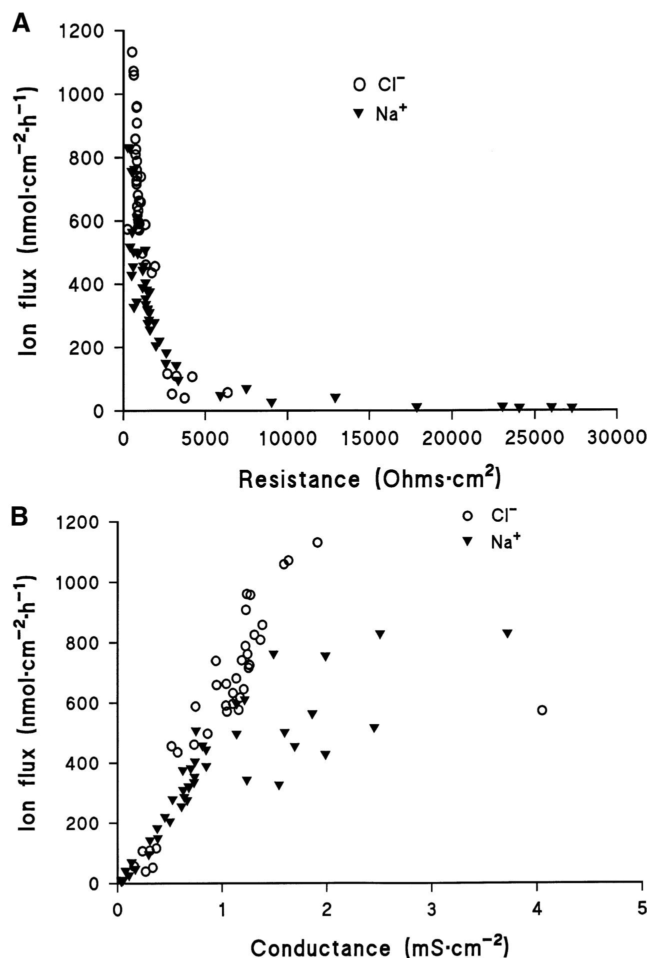

FIG. 1. (A) The relationship between unidirectional ion flux (Na^+ or Cl^-) and transepithelial resistance in 80 cultured trout branchial epithelia with L-15 media on both apical and basolateral surfaces. Fluxes measured in either direction are included. (B) The relationship between unidirectional ion flux and transepithelial conductance, using the same data set as A.

PEG permeability data were obtained only up to a transepithelial resistance of about 6300 $\Omega\,cm^2$. PEG permeability was negatively related to resistance (Fig. 2). The relationship was somewhat more variable than for Na^+ and Cl^- fluxes.

Transepithelial resistance increases greatly and TEP becomes negative (relative to apical side) when the apical sur-

TABLE 1. Na^+, Cl^- and PEG movements, measured radioisotopically, from apical-to-basolateral surfaces and from basolateral-to-apical surfaces in a matched set of cultured branchial epithelia, all with transepithelial resistances in the range of 600–800 Ω cm²

	Apical → Basolateral	Basolateral → Apical	P*
Unidirectional Na^+ flux	550 ± 60	610 ± 50	NS
(nmol/cm²/hr)	(10)	(10)	
Unidirectional Cl^-	730 ± 60	790 ± 40	NS
(nmol/cm²/hr)	(16)	(16)	
PEG permeability	0.18 ± 0.01	0.20 ± 0.01	NS
(cm/sec × 10^{-6})	(25)	(25)	

L-15 media was present on both surfaces (symmetrical conditions). Values are means ± SEM, with n in parentheses.
*$P \geq 0.05$; NS, not significant.

face of the cultured epithelium is exposed to fresh water. Earlier, we showed that for a freshwater exposure of 2.5 hr, the resistance change is stable and completely reversible upon return to apical L-15 (32). However, longer exposures induced irreversible changes. For example, in the present 12-hr exposure to apical fresh water, resistance increased initially but dropped markedly in the second 6-hr period (Fig. 3, top). However, resistance remained significantly elevated relative to the starting value in symmetrical L-15 media. Upon return to apical L-15, resistance fell greatly and remained significantly depressed relative to this initial value.

Despite the initial increase in resistance, simultaneous measurements of PEG permeability (isotope added to the basolateral side) demonstrated that it approximately doubled during the first 6 hr of freshwater exposure and tripled during the second 12 hr of freshwater exposure (Fig. 3, bottom). Upon return to apical L-15 after 12 hr, PEG permeability remained at this elevated value despite the 80% drop in resistance.

Unidirectional fluxes of both Na^+ and Cl^- ("effluxes"; isotope added to the basolateral side) did not change during the first 6 hr of freshwater exposure but approximately doubled during the second 6 hr (Fig. 4). Unlike PEG, these unidirectional effluxes exhibited a further significant in-

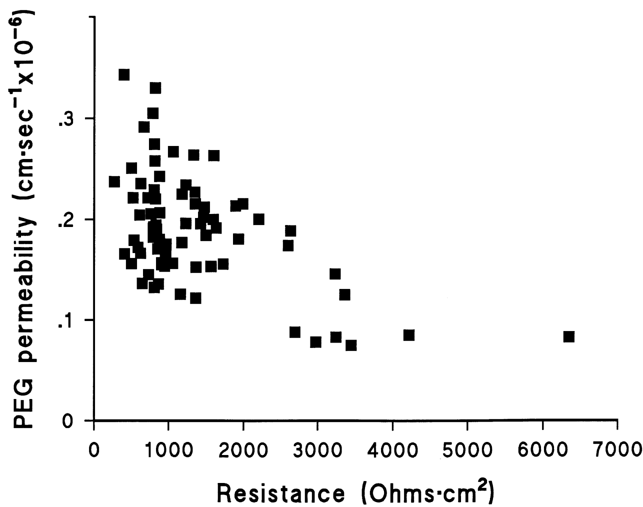

FIG. 2. The relationship between PEG permeability and transepithelial resistance in 74 cultured trout branchial epithelia with L-15 media on both apical and basolateral surfaces. Permeabilities measured in either direction are included.

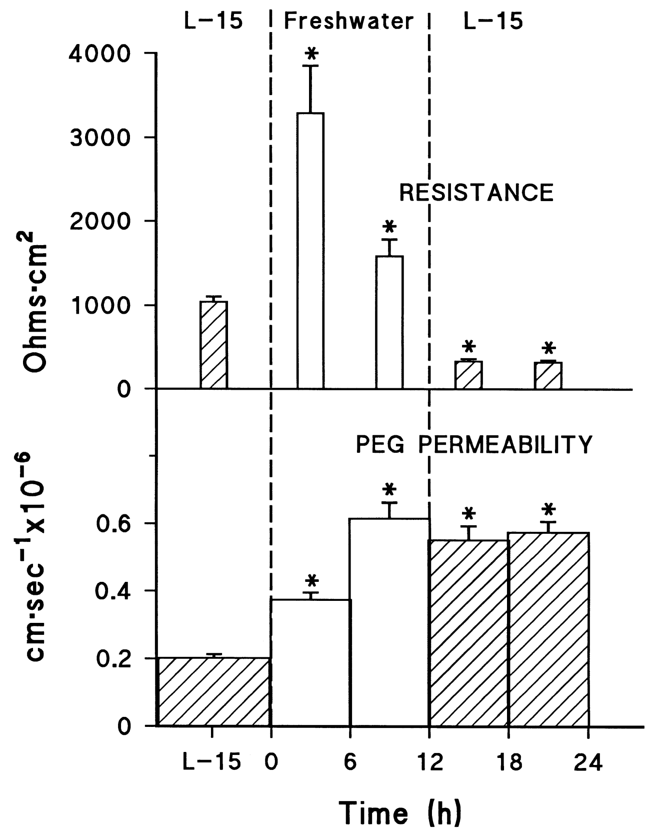

FIG. 3. Changes in (top) transepithelial resistance and (bottom) PEG permeability in cultured trout branchial epithelia exposed to apical L-15 media for 12 hr, followed by return to apical L-15 for a further 12 hr. Means ± SEM (n = 19). * Means significantly different ($P \leq 0.05$) from the original L-15 value.

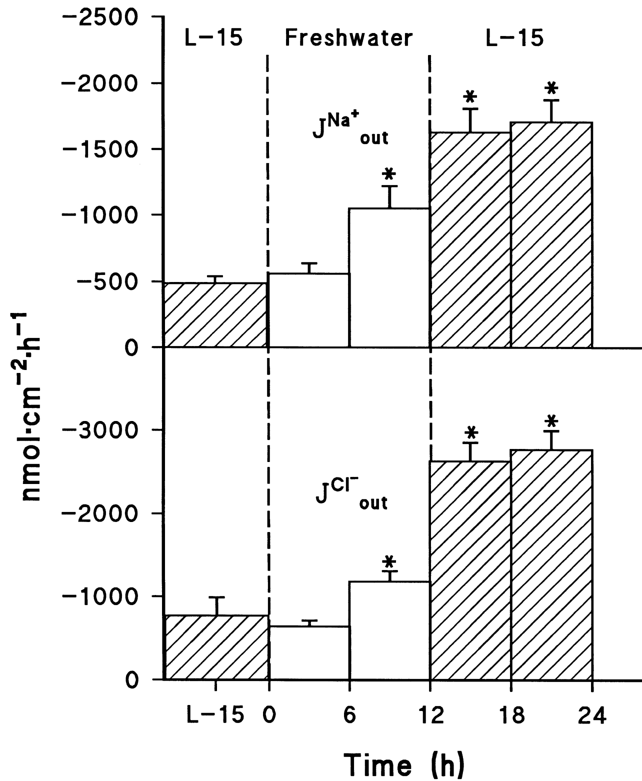

FIG. 4. Changes in (top) unidirectional efflux of Na^+ and (bottom) unidirectional efflux of Cl^- (i.e., basolateral to apical fluxes) in cultured trout branchial epithelia exposed to apical L-15 media for 12 hr, followed by return to apical L-15 for a further 12 hr. Means ± SEM (n = 9 for Na^+, n = 10 for Cl^-). * Means significantly different ($P \leq 0.05$) from the original L-15 value.

crease followed by stabilization upon return to apical L-15 (Fig. 4). These results suggested that the relationships between resistance and fluxes or permeability are altered upon exposure to fresh water.

To explore this more fully, the relationship between resistance and unidirectional effluxes of Na^+ and Cl^- was examined using all preparations that met the resistance stability criterion in freshwater tests (see Materials and Methods). Because of the elevation of resistance associated with apical fresh water, the general range of resistances in these preparations (4000–15000 Ω cm^2) tended to be higher than in preparations in apical L-15 (typically 1000–6000 Ω cm^2). Nevertheless, there was considerable overlap, allowing comparison, and the relationships between conductance and unidirectional efflux for both Na^+ and Cl^- were linear (Fig. 5A). When filters of similar resistances were compared (Fig. 5A), unidirectional Na^+ and Cl^- effluxes per unit conductance were 3.5-fold higher to apical fresh water than to apical L-15.

When a similar analysis was performed for PEG permeability, the relationships were clearly more variable (Fig. 5B). Nevertheless, it was again clear that PEG permeability per unit conductance was again higher to apical fresh water than to apical L-15.

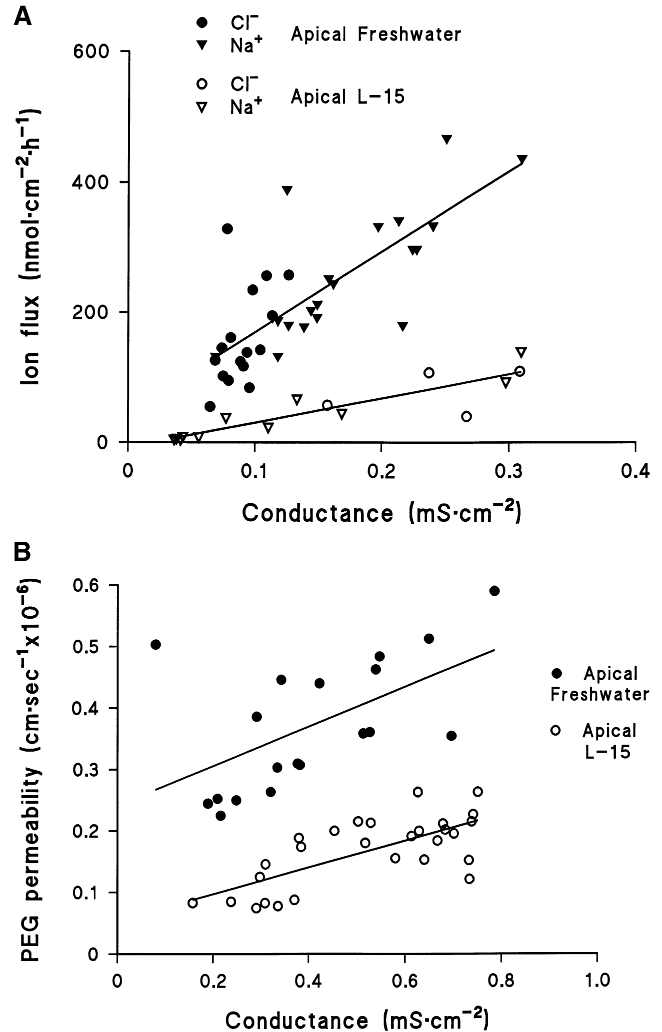

FIG. 5. (A) The relationship between unidirectional Na^+ and Cl^- effluxes (i.e., basolateral to apical fluxes) and transepithelial conductance in cultured trout branchial epithelia exposed to either apical L-15 medium or apical fresh water. The data were selected to cover a common range of conductances in the L-15 and freshwater experiments. The regression equations are as follows: ion flux = 1243 (conductance) + 45, r = 0.76, n = 37, $P < 0.001$ in apical fresh water; ion flux = 372 (conductance) − 7, r = 0.89, n = 16, $P < 0.001$ in apical L-15. (B) The relationship between PEG permeability and transepithelial conductance in cultured trout branchial epithelia exposed to either apical L-15 medium or apical fresh water. The data were selected to cover a common range of conductances in the L-15 and freshwater experiments. The regression equations are as follows: PEG perm = 0.323 (conductance) + 0.241, r = 0.56, n = 19, $P < 0.01$ in apical fresh water; PEG perm = 0.218 (conductance) + 0.053, r = 0.71, n = 29, $P < 0.001$ in apical L-15.

Unidirectional Ion Fluxes in Apical Fresh Water—Active or Passive?

Two series of unidirectional flux experiments were performed; in one, efflux was measured indirectly as the difference between net flux and influx and in the other, efflux was measured directly (isotope addition to basolateral side).

TABLE 2. Flux ratio analysis of cultured branchial epithelia exposed to apical fresh water

	Na^+		Cl^-	
J_{in} (nmol/cm^2/hr)	1.43 ± 0.16 (13)		1.76 ± 0.16 (10)	
J_{out} (nmol/cm^2/hr)	−263.9 ± 29.9 (13)		−116.6 ± 9.4* (10)	
J_{net} (nmol/cm^2/hr)	−262.5 ± 29.8 (13)		−114.8 ± 9.3* (10)	
TEP (mV)	−10.5 ± 0.7 (13)		−11.1 ± 0.8 (10)	
Observed flux ratio (×10^{-3})	5.81 ± 0.79 (13)		15.40 ± 1.31* (10)	
		P = NS		$P < 0.0001$
Predicted flux ratio (×10^{-3})	7.59 ± 0.40 (13)		5.79 ± 0.17* (10)	

Influx and net flux were both measured directly and efflux calculated indirectly, all in the same preparations. Values are means ± SEM, with n in parentheses.
*Significant difference ($P \leq 0.05$) between Na^+ and Cl^- values.

In both, influx was measured directly (isotope addition to apical side). Only epithelia that met the resistance stability criterion were used. In the first series, initial resistance in apical L-15 was 2562 ± 371 and the 24-hr resistance in apical L-15 was 2151 ± 174 Ω cm^2 (n = 23), whereas in the second initial resistance was 2447 ± 292 and the 24-hr resistance was 2013 ± 148 Ω cm^2 (n = 28).

In the first series, Na^+ and Cl^- influxes from fresh water (apical to basolateral transport) were approximately two orders of magnitude lower than indirectly measured effluxes (Table 2). Despite the basolateral negative TEP, Cl^- influx was slightly higher than Na^+ influx, but the difference was not significant. However, the negative Cl^- net flux was less than 50% of Na^+ net flux, so the indirectly determined effluxes were also substantially different. For Na^+, the observed flux ratio was not significantly different from that predicted by the Ussing flux ratio criterion, indicating that Na^+ movements were passive (Table 2). However, for Cl^-, the observed flux ratio was 2.7-fold higher than that predicted by the Ussing criterion, indicating active Cl^- uptake from the apical fresh water to basolateral L-15 against the opposing electrical and concentration gradients.

In the second series, the TEPs and the Na^+ and Cl^- influxes were similar to those of the first series (Table 3). However, in this set, Cl^- influx was significantly greater than Na^+ influx. In addition, whereas directly measured Na^+ efflux was very similar to the indirect determination, directly measured Cl^- efflux was substantially greater than the indirectly calculated value (*cf*. Tables 2 and 3). Indeed, directly measured Cl^- efflux was also higher than the net flux measured in these preparations. Regardless, the conclusion of the Ussing flux ratio analysis was identical to that of the first series. Thus, Cl^- was actively transported in the direction of uptake, whereas Na^+ movements were again passive (Table 3). It should be noted that this conclusion is robust. For the purposes of the flux ratio analysis, influx and efflux preparations were matched on the basis of most similar initial resistances in L-15 immediately before freshwater exposure. However, when other matching criteria were used (e.g.,

TABLE 3. Flux ratio analysis of cultured branchial epithelia exposed to apical fresh water

	Na^+		Cl^-	
J_{in} (nmol/cm^2/hr)	1.41 ± 0.17 (7)		1.92 ± 0.14* (8)	
J_{out} (nmol/cm^2/hr)	−249.3 ± 34.8 (7)		−220.1 ± 22.1 (6)	
J_{net} (nmol/cm^2/hr)	−205.5 ± 7.9 (14)		−140.9 ± 13.3* (14)	
TEP (mV)	−11.6 ± 0.7 (14)		−10.9 ± 0.6 (14)	
Observed flux ratio (×10^{-3})	6.50 ± 1.28 (7)		9.74 ± 1.31* (8)	
		P = NS		$P < 0.02$
Predicted flux ratio (×10^{-3})	8.82 ± 0.10 (7)		5.74 ± 0.18* (8)	

Influx and efflux were both measured directly on separate preparations. Net flux was measured directly in all preparations. Values are means ± SEM, with n in parentheses.
*Significant difference ($P \leq 0.05$) between Na^+ and Cl^- values.

most similar resistances or TEP values in water at various times), the same conclusion as to active Cl^- and passive Na^+ transport was reached.

DISCUSSION

Passive Permeability Relationships

Under symmetrical conditions (isotonic L-15 media on both surfaces, zero TEP), unidirectional fluxes of Na^+ and Cl^- were equal in both directions (Table 1) and followed a simple passive function of resistance (Fig. 1A). Fluxes per unit conductance were similar for Na^+ and Cl^- and approximately linear through origin (Fig. 1B). If the slight tendency for greater Cl^- than Na^+ flux at higher conductances is real, it likely reflects the higher absolute mobility of the Cl^- ion in free solution (4). As noted earlier (32), the magnitude of these unidirectional fluxes, when translated to a standard gill area, is comparable with that seen in an intact rainbow trout acclimated to an isotonic salinity (2). Fluxes per unit conductance, approximately 450 nmol/hr/mS for Na^+ and Cl^- separately, were essentially identical to those measured in the opercular epithelium of the brook trout (15), an electrically "tight" epithelium with a similar resistance (1170 $\Omega\,cm^2$) and a similar lack of TEP (0 mV) when incubated under symmetrical isotonic conditions. At 18°C, under symmetrical conditions, the conversion factor from flux of a monovalent ion in $nmol/cm^2/hr$ to conductance in mS/cm^2 is approximately 0.001, so the sum of Na^+ and Cl^- fluxes ($900\ nmol/cm^2/hr = 0.9\ mS/cm^2$ at a conductance of $1.0\ mS/cm^2$; *cf*. Fig. 1b) could essentially account for all measured conductance. This indicates that all measured ion flow is conductive and the contribution of other ions is negligible. Marshall (15) reached a similar conclusion in the brook trout opercular epithelium and concluded that it served as a useful model of the passive transport properties of the intact gill. The same appears true of the cultured rainbow trout branchial epithelium.

Marshall (15) noted that resistance went up markedly and a negative TEP developed when the brook trout opercular epithelium was exposed to apical fresh water, just as in the present study (see also ref. 32). Marshall (15) did not investigate the cause(s) of these responses, but in other experiments done only in apical fresh water, he concluded that the ionic fluxes were largely paracellular, as changes in ion flux paralleled changes in mannitol permeability. In the present study, we used PEG as a paracellular permeability marker rather than mannitol. Our choice was conservative, based on findings that mannitol may penetrate cells and may be metabolized by certain trout tissues, including the gills, whereas this is much less of a problem with PEG (3,10,21,23). The disadvantage of PEG is that because of its large size, its mobility in free solution will be far less than those of Na^+ and Cl^- and thus its fluxes will be much lower. This likely contributed to the greater variability seen in the PEG permeability data in the present study. Nevertheless, it is clear that PEG permeability was positively related to conductance (Figs. 2 and 5B), although this does not prove that all ion flux is necessarily paracellular.

The PEG data do, however, clarify the nature of the resistance and permeability changes occurring upon transfer to apical fresh water. Clearly, the marked increase in resistance that takes place at this time is not due to a decrease in paracellular permeability, because PEG permeability increases significantly rather than decreases (Fig. 3). This is further supported by the marked upward shift in the PEG permeability *vs* conductance relationship (Fig. 5B) seen in epithelia incubated in apical fresh water. Thus, conductance decreases, paracellular permeability increases and paracellular permeability per unit conductance increases greatly. These findings agree with our earlier observation that ionic dilution of the apical medium (reduction of Na^+ and Cl^-) *in the absence of osmotic reduction* causes a similar increase in transepithelial resistance (32). The changes occurring are much more complex than a simple cell swelling, leading to a mechanical tightening of the cell junctions.

In light of these results, the most likely explanation appears to be that the paracellular permeability actually increases upon freshwater exposure (as shown by the increase in PEG permeability; Figs. 3, bottom, and 5B), but its effect on resistance (conductance) is more than compensated by a decrease in transcellular permeability. How this might occur is unclear, although there is abundant evidence in many transport epithelia that external dilution may close apical channels (11,31,33). The observation that PEG permeability remains elevated upon return to isosmotic media after 12 hr in freshwater (Fig. 3, bottom), whereas resistance falls below original levels (Fig. 3, top), suggests that the increase in paracellular permeability is permanent, whereas the decrease in transcellular conductance is reversible.

This interpretation is supported by the unidirectional Na^+ and Cl^- flux data. Ionic effluxes gradually increased upon freshwater exposure, explicable by an opening up of the paracellular pathway, and remained elevated upon return to L-15 (Fig. 4). In fresh water, the unidirectional effluxes per unit conductance increased more than 3-fold, although a linear relationship was maintained (Fig. 5A). Taking into account the negative TEP and neglecting the very small influx components under these assymetrical conditions, the sum of Na^+ and Cl^- effluxes still accounted for more than 80% of the measured conductance, so the elevated ion flows remained largely conductive. In this respect, the present results differ partially from those on the opercular epithelium (15), where the ionic fluxes remained fully conductive but fell rather than increased in fresh water as paracellular permeability decreased. Further analysis of this problem will require determination of current-voltage relationships, such as the Ussing chamber voltage clamping experiments performed by Marshall (16), to sort out a similar problem in brook trout urinary bladder.

Active Transport Relationships in Fresh Water

The cultured branchial epithelium exhibited a net loss rather than a net uptake of Na^+ and Cl^- when exposed to apical fresh water (Fig. 4, Tables 2 and 3). In this respect, it was similar to all other preparations evaluated as possible models for the freshwater gill: tilapia opercular epithelium (5), brook trout opercular epithelium (18), rainbow trout cleithrum epithelium (17), goby jawskin epithelium (31) and killifish opercular epithelium (31), all from animals acclimated to dilute solution or fresh water. However, in contrast to these preparations, the cultured epithelium was acutely exposed to fresh water, allowed at most 4 hr to "acclimate" before flux measurements began and provided with no hormonal support. In this respect, it is remarkable that net ion loss rates remained low and that the preparation survived at least 48 hr in apical fresh water. Future experiments should evaluate whether the net ionic balance is improved by gradual adaptation to dilute apical media and/or by supplementation of the basolateral media with hormones such as prolactin known to be involved in freshwater adaptation (20).

Two experimental designs were used to evaluate whether ionic exchanges in apical fresh water involved active transport, as assessed by the Ussing flux ratio criterion. The first involved direct measurements of influx and indirect measurements of efflux on the same preparation (Table 2), an approach that was preferable for statistical reasons. However, a potential drawback of this approach has been seen in preparations such as the trout cleithrum epithelium (17) where the net flux rate of Na^+, and therefore the indirectly calculated efflux rate, were artifactually elevated 2- to 4-fold above the true transepithelial unidirectional efflux rate due to leaching of Na^+ from the epithelial suface. Cl^- efflux was not affected. In view of the negative finding with respect to Na^+ transport in Table 2, the experiment was repeated using direct measurements of both influx and efflux in different but suitably matched preparations (Table 3). The results demonstrated that the indirectly measured Na^+ efflux was in fact reliable but that the indirectly measured Cl^- efflux actually underestimated the true efflux. The reason for this difference is unknown. Nevertheless, the overall conclusion of the flux ratio analysis was confirmed; Cl^- was actively transported from apical fresh water to the basolateral surface, whereas Na^+ movements were passive.

In view of current models of ion transport in the freshwater gill, this result was most surprising. The cultured branchial epithelium is thought to consist entirely of pavement ("respiratory") cells (24,25,32). Various theories place Na^+ uptake on the pavement cells and Cl^- uptake on the chloride cells (6,7,13,22,25) both processes on the chloride cells (1,28,30) or both processes on the pavement cells (27). To our knowledge, no theory places only Cl^- uptake on the pavement cells. Possible explanations would be that the cultured epithelium retains a few undetected chloride cells

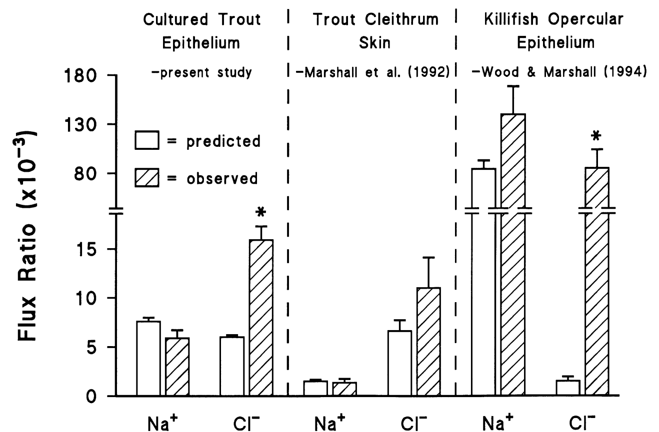

FIG. 6. A comparison of the results of Ussing flux ratio analysis for the present study on cultured trout epithelium (data from Table 2) with the results of similar analyses on two intact epithelial preparations evaluated as models for the freshwater gill, the rainbow trout cleithrum skin (17) and the killifish opercular epithelium (31). Means ± SEM. All three preparations were exposed to apical fresh water. Note that none of the preparations exhibit evidence for the active inward transport of Na^+, whereas the cultured trout epithelium and the killifish opercular epithelium both exhibit active inward transport of Cl^-. A similar trend is apparent but not significant in the trout cleithrum skin.

or that the cultured epithelial cells have differentiated (or even dedifferentiated) so as express different transport characteristics than *in vivo*. Alternately, the observation may be representative of the intact gill, which would suggest that current theories are flawed. Based on the evidence at hand, we cannot distinguish between these possibilities. However, an obvious goal for future research is to attempt to incorporate chloride cells into the cultured epithelium and to evaluate whether the transport characteristics change. In this regard, Witters *et al.* (29) recently reported that chloride cells may survive in primary cultures of trout gill cells started from explants.

It is interesting that this same pattern, suggesting active Cl^- uptake and passive Na^+ movement, has been seen in two intact epithelia examined as potential models for the freshwater gill (Fig. 6). In the opercular epithelium of the killifish, the evidence for active Cl^- uptake was highly significant (31), whereas in the rainbow trout cleithrum skin, the effect was just below statistical significance (17). Both preparations have abundant chloride cells and actively take up Ca^{2+} from the water, apparently through these cells (17,19). It may therefore be very instructive to examine whether the cultured trout gill epithelium also exhibits active uptake of Ca^{2+} from apical fresh water.

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