THE JOURNAL OF EXPERIMENTAL ZOOLOGY 281:531–545 (1998)

Permeability and Morphology of a Cultured Branchial Epithelium From the Rainbow Trout During Prolonged Apical Exposure to Fresh Water

K.M. GILMOUR,[1]* P. PÄRT,[2] P. PRUNET,[3] M. PISAM,[4] D.G. MCDONALD,[1] AND C.M. WOOD[1]

[1]*Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1*

[2]*EC Joint Research Centre, Environment Institute, TP 460, I-21020 Ispra, Italy*

[3]*Laboratoire de Physiologie des Poissons, Institut National de la Recherche Agronomique, Campus de Beaulieu, 35042 Rennes Cedex, France*

[4]*Department de Biologie Cellulaire et Moléculaire, CEA Saclay, 9119, Gif-Sur-Yvette, France*

ABSTRACT The electrical, structural, and permeability properties of primary cultures of rainbow trout gill cells on permeable supports were examined after 6 days of growth in culture medium, followed by prolonged (48 hr) apical exposure to fresh water. Permeability to the paracellular marker polyethylene glycol-4000 (PEG) increased significantly over 48 hr, indicating that paracellular permeability increased continuously throughout the freshwater exposure. The significant increases measured in the net Na^+ and Cl^- fluxes were attributed primarily to the opening up of the paracellular pathway. The elevated transepithelial resistance characteristic of the cultured branchial epithelium with fresh water present on the apical surface gradually declined during prolonged exposure, and the decrease (increase in conductance) was greater than that expected on the basis of the increasing paracellular permeability. At any given time, PEG permeability was linearly related to conductance, but PEG permeability per unit conductance decreased significantly over 48 hr. These results are suggestive of an increase in transcellular permeability in addition to that in paracellular permeability. Since transcellular permeability appears to decrease on first exposure to fresh water, the elevated permeability during prolonged exposure was interpreted as a reopening of the transcellular pathway. A morphological examination revealed evidence of damage to the superficial cell layer of the multilayered culture following 48 hr of apical freshwater exposure, accounting at least in part for the physiological changes observed. Hormonal supplementation of the basal culture medium was examined as a means of enhancing adaptation to apical fresh water. The inclusion of teleost prolactin (200 ng/L) or teleost growth hormone (200 ng/L) in the basal culture medium had no effect on either the initial values or the pattern of changes during prolonged exposure for TER, PEG permeability, and net ion fluxes. *J. Exp. Zool. 281:531–545, 1998.* © 1998 Wiley-Liss, Inc.

The fragility and structural complexity of the fish gill together with its involvement in a variety of physiological functions provide numerous difficulties with respect to the investigation of branchial ion transport mechanisms. These problems have been overcome to a large extent in the study of branchial ion transport across the seawater fish gill through the use of surrogate models consisting of flat epithelial preparations that contain many ion-transporting cells (Karnaky et al., '77; Marshall, '77; Foskett et al., '81). Comparable substitutes for freshwater fish gills have proven to be elusive (Wood and Marshall, '94; Marshall, '95), but a recently developed cultured branchial epithelium (Wood and Pärt, '97) shows indications of being a valuable new approach for the study of branchial ion transport mechanisms across the freshwater fish gill. A cultured branchial epithelium also has been used recently as a model for the seawater fish gill (Avella and Ehrenfeld, '97).

Grant sponsor: NSERC Collaborative Program.
*Correspondence to: K.M. Gilmour, Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ Scotland. E-mail: k.gilmour@bio.gla.ac.uk
Received 10 December 1997; Accepted 24 February 1998

© 1998 WILEY-LISS, INC.

In the cultured branchial epithelial model, gill epithelial cells are grown on a permeable support, which enables the in vivo situation of asymmetrical apical and basolateral conditions to be reproduced. The cultured epithelium from either sea bass, *Dicentrarchus labrax*, or freshwater rainbow trout, *Oncorhynchus mykiss*, consists of several overlapping layers of pavement cells but appears to lack chloride cells and in this latter respect differs from the epithelium of the marine or freshwater fish gill (Avella and Ehrenfeld, '97; Wood and Pärt, '97). Nonetheless, like the intact gill, the cultured epithelium in both cases contains numerous tight junctions and exhibits a high transepithelial resistance (TER) (Avella and Ehrenfeld, '97; Wood and Pärt, '97). Furthermore, the permeability of the freshwater trout cultured epithelium under symmetrical conditions (culture medium on both sides) to the paracellular marker polyethylene glycol-4000 (PEG) is similar to that of the intact gill in freshwater fish, and the Na^+ and Cl^- flux rates (corrected for gill area) across the cultured epithelium under symmetrical conditions are comparable with those measured for intact rainbow trout acclimated to an isotonic salinity (Wood et al., '97; Wood and Pärt, '97).

Cultured branchial epithelia from sea bass have to date been studied only under symmetrical conditions of culture medium on both sides (Avella and Ehrenfeld, '97). Cultured branchial epithelia from freshwater rainbow trout, *O. mykiss*, have been examined during both symmetrical and asymmetrical conditions, the latter involving exposure of the apical surface of the epithelium to fresh water while the basolateral surface is bathed in a culture medium that mimics the extracellular fluid (Wood et al., '97; Wood and Pärt, '97). It has not (yet) proven to be possible to culture branchial epithelial cells on permeable supports with fresh water on the apical surface. Instead, the cultured epithelium is grown under symmetrical conditions and is then exposed to a step change from isosmotic to freshwater conditions.

When the apical culture medium is replaced with fresh water, the cultured branchial epithelium, like the intact gill, develops a basolateral negative transepithelial potential, and both the TER and the PEG permeability increase (Wood et al., '97; Wood and Pärt, '97). The latter effects have been interpreted as reflecting an increase in paracellular permeability (as evidenced by the increase in PEG permeability) concomitant with a decrease in transcellular permeability, which must be postulated to account for the overall increase in resistance (Wood et al., '97). The cultured epithelium also displays low Na^+ and Cl^- leakage rates during these early stages (first 12 hr) of apical freshwater exposure. However, over 48 hr of apical freshwater exposure, the net Na^+ and Cl^- loss rates increase substantially, while the elevated resistance and negative TEP gradually attenuate (Wood and Pärt, '97); PEG permeability has not been measured over 48 hr of freshwater exposure. The significance of these physiological changes, i.e., whether they are normal or pathological, is as yet unclear.

The first objective of the present study was to carry out a detailed investigation of the resistance and permeability characteristics of the cultured branchial epithelium during prolonged apical exposure to fresh water. Specifically, PEG permeability together with TER and ion flux rates were assessed over 48 hr of apical freshwater exposure to determine whether the progressive fall in resistance during prolonged freshwater exposure results from further increases in paracellular permeability or whether it is associated with a reopening of the transcellular pathway. Through comparisons of Na^+ and Cl^- flux rates with resistance and PEG permeability, the cause of the increases in net ion loss rates during prolonged apical freshwater exposure also was examined.

A second objective was to evaluate the possible importance of two key osmoregulatory hormones in enhancing adaptation of the preparation to apical fresh water. The resistance and permeability characteristics of cultured branchial epithelia during prolonged apical freshwater exposure were examined both with and without teleost prolactin or teleost growth hormone in the basolateral culture medium. The transition from sea water to fresh water in euryhaline fish is accompanied by an integrated series of hormonal adjustments that includes changes in the levels of prolactin, growth hormone, cortisol, and thyroid hormones. Prolactin, in particular, appears to play an important role in freshwater acclimation, with plasma prolactin levels increasing rapidly following the transfer of euryhaline fish from sea water to fresh water and decreasing when fish are transferred in the opposite direction (Prunet et al., '85; Young et al., '89; Yada et al., '91). The replacement of the culture medium on the apical surface of a cultured branchial epithelium with fresh water resembles a transition in vivo from brackish to freshwater conditions, a situation in which circulating prolactin levels would be expected to increase.

Finally, the third objective of the present study

involved assessing the morphology of the cultured branchial epithelium under symmetrical conditions and following exposure of the apical surface to fresh water for varying periods of time. The intention of this morphological characterization was to determine whether the changes in the physiological properties (net ion fluxes, PEG permeability, TER) of the cultured branchial epithelium that occurred during prolonged apical freshwater exposure were the result of alterations in the morphology of the culture.

MATERIALS AND METHODS

Preparation of cultured branchial epithelia

Cultured branchial epithelia were prepared from the gills of 1- to 2-year-old rainbow trout, *O. mykiss* (15–60 g), obtained locally and held in dechlorinated Hamilton tap water at seasonal summer temperatures (17–20°C). The composition of the tap water was (mmol/L) [Na^+] = 0.55, [Cl^-] = 0.70, [Ca^{2+}] = 1.0, pH = 7.8–8.0; identical fresh water was used in the experiments with cultured epithelia. The methods used in the preparation of cultured branchial epithelia were very similar to those described elsewhere (Pärt et al., '93; Pärt and Bergström, '95; Pärt and Wood, '96; Wood et al., '97; Wood and Pärt, '97). Briefly, branchial epithelial cells obtained from tryptic digests of trout gills were grown initially at 18°C in an air atmosphere under sterile conditions in plastic culture flasks. The culture medium consisted of Leibowitz L-15 medium supplemented with 2 mmol/L glutamine, 5% foetal bovine serum (FBS), and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, 200 µg/ml gentamicin, and 0.5 µg/ml fungizone); all constituents were obtained from Canadian Life Technologies (Burlington, ON). The use of antibiotics throughout the culturing procedure was a departure from previous work in which antibiotics were excluded from the culture medium after day 4. Continuous use of antibiotics in the culture medium was employed to avoid contamination of the cultures by microorganisms, a more frequent problem during the hot summer months. The use of antibiotics did not appear to affect the growth or subsequent performance of the cultures; transepithelial resistances (TERs) measured under either symmetrical or asymmetrical conditions were within the range of values observed in previous studies (Table 1 versus Wood et al., '97; Wood and Pärt, '97), and the net ionic fluxes (J_{net}^{Na+} and J_{net}^{Cl-}) of cultures in the present study did not differ significantly from those of cultures in which antibiotics were excluded from the culture medium after day 4 (two-sample *t* tests on combined data for all treatments at a given time for J_{net}^{Na+} or J_{net}^{Cl-} from Table 3 versus data from Fig. 7 of Wood and Pärt, '97).

Cells were harvested by retrypsination between days 6 and 8, the time of greatest mitotic activity (Pärt et al., '93), and were reseeded onto Falcon cell culture inserts (Cyclopore polyethylene terephthalate, Becton Dickinson, Franklin Lakes, NJ) at a seeding density of 600,000 cells per centimeter squared. The filters, which were housed in Falcon cell culture companion plates, had a pore size of 0.45 µm, a pore density of 1.6×10^6 pores per centimeter squared, and an effective growth surface area of 0.83 cm^2. Cells were grown on the inserts with L-15 culture medium supplemented with FBS and antibiotics (as above) present in both the insert (apical surface) and well of the culture plate (basolateral surface) for 6 to 8 days before use. The hormone prolactin (200 ng/L; rainbow trout prolactin purified according to the protocol of Prunet et al., '84) also was included in the culture medium from day 2 after reseeding in some experiments to evaluate the impact on the culture during apical water exposure of long-term exposure to this hormone. During the 6- to 8-day culture period, the TER of the epithelium was monitored every 48 hr in situ in the culture dish using STX-2 "chopstick" electrodes connected to an EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). Measurement of the TER enabled the growth of the culture to be assessed; cultures were deemed ready for experiments when a resistance plateau was observed.

Experimental protocol

Experiments involved the comparison, during exposure of the apical side of a cultured epithelium to fresh water for 48 hr, of control epithelia and epithelia "supported" with hormones known to be important in freshwater adaptation in fish. The hormone support treatment consisted of the inclusion of either rainbow trout prolactin (200 ng/L) or growth hormone (200 ng/L; recombinant rainbow trout growth hormone prepared according to Boeuf et al. ['94] and kindly provided by J. Smal, Eurogentec, Liège, Belgium) in the culture medium bathing the basolateral side of the epithelia during the apical freshwater exposure. Cultured epithelia were characterized during the freshwater exposure by measurement of the TER, net ionic fluxes (J_{net}^{Na+} and J_{net}^{Cl-}), and movement of the paracellular permeability marker polyethylene gly-

col-4000 (PEG) across the cultured epithelium. Net ionic fluxes were assessed by determination of changes in the total concentration of Na^+ or Cl^- in the dilute apical medium, whereas PEG permeability was evaluated by adding [^{3}H]PEG to the basolateral medium and monitoring the appearance of radioactivity on the apical side.

The 48-hr freshwater exposure was divided into eight consecutive 6-hr flux periods. The apical and basolateral media were renewed at the beginning of each flux period; 1.8 ml of sterilized fresh water (composition as above) was added to the apical (insert) side, whereas 2.0 ml of the appropriate (i.e., hormone-supplemented or control) culture medium containing 0.5 µCi [^{3}H]PEG (NEN-Dupont, Boston, MA) was added to the basolateral (well) side. To ensure complete changeover, the apical compartment was rinsed four times with sterilized fresh water before the final freshwater medium was added. Resistance was measured immediately following solution renewal. Apical and basolateral solutions were sampled at the end of each flux period, following mixing of the solutions to ensure that a representative sample was obtained.

Microscopy

To describe changes in the morphology of cultured epithelia following short- and long-term apical freshwater exposure, a separate group of filters was exposed to fresh water for 30 min, 24 hr, or 48 hr and then fixed for electron microscopy together with a control group in which the cultured epithelia were not water-exposed but rather maintained with L-15 medium on the apical surface. For fixation, apical and basolateral media were replaced with 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) and left overnight at 4°C. The following day, the filters were rinsed thoroughly (three times) with 0.1 mol/L sodium cacodylate buffer (pH 7.4) containing 0.01% sodium azide and then stored in the same buffer for shipment to France, where the morphological study was carried out.

To better visualize intracytoplasmic membranous systems, the glutaraldehyde-fixed cultures were postfixed for 1 hr at room temperature in a 1:1 mixture of 2% aqueous osmium tetroxide and 3% aqueous potassium ferrocyanide according to the method of Karnovsky ('71). After dehydration in increasing concentrations of ethanol, cultures were embedded in Epon 812. Thin sections obtained with an automatic Reichert ultramicrotome were contrasted for 2 min with lead citrate and examined at 80 kV using a Philips CM12 electron microscope.

Analytical procedures

Solution ^{3}H activity was assessed by liquid scintillation counting (LKB 1217 Rackbeta) with a commercial fluor. Flame-emission spectrophotometry (Varian AA1275 atomic absorption unit) was used to determine Na^+ concentrations, whereas Cl^- concentrations were assayed by means of a mercuric thiocyanate spectrophotometric method (Zall et al., '56). An EVOM epithelial voltohmmeter, custom-modified by the manufacturer (World Precision Instruments) to measure resistances of up to 200,000 ohms and STX-2 "chopstick" electrodes were used to determine TER; the "chopstick" electrodes can be transferred rapidly between filter inserts such that resistance measurements can be made in situ without disturbance to the cultured epithelium. Resistances were determined immediately following solution renewal, a procedure that is necessary with asymmetrical solutions to ensure that TER is measured before the ionic gradients change. To correct for junction potentials and for the resistance of the solutions and filter insert itself, the resistance of an identical blank filter insert set up with the same apical and basolateral solutions was determined at the time of cultured epithelium TER measurements. The value for this blank filter was subtracted from the measured TER of the cultured epithelium, and the corrected value for the cultured epithelium was then multiplied by the filter effective growth surface area to yield the final value for the transepithelial resistance (in ohms · cm^{-2}). Transepithelial conductance was calculated as the inverse of TER and expressed in milliSiemens per centimeter squared.

Calculations and statistical analysis

Efflux was defined as movement from the basolateral (culture medium) to the apical (freshwater) solution, and influx, vice versa. Permeability to PEG (in centimeters per second), which was determined in the efflux direction by the addition of radioisotope to the basolateral solution, was calculated as

$$P = \frac{\Delta[\text{PEG*}]_{Ap} \cdot \text{volume}_{Ap}}{[\text{PEG*}]_{Bl} \cdot \text{time} \cdot 3600 \cdot \text{area}}$$

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

Net ionic fluxes were calculated using standard methods; the net sodium flux (in micromoles per square centimeter per hour), for example, was calculated as

$$J_{net}^{Na^+} = \Delta[Na^+]_{Ap} \cdot \frac{volume_{Ap}}{time \cdot area}$$

where $\Delta[Na^+]_{Ap}$ is the change in total sodium concentration in the apical solution. It should be noted that under asymmetrical conditions with fresh water on the apical surface, the influx component of the net flux is very small (Wood et al., '97), and therefore, the net flux is comparable with the unidirectional efflux.

Data are presented as means ± 1 SEM (*N*), where SEM refers to the *standard error of the mean* and *N* represents the number of filter inserts. The least-squares method was used to fit regression lines, with determination of the significance of the correlation coefficient. The slopes of regression lines were compared using a Student's *t* test. Statistical differences among control and hormone support treatments were determined by one-way analysis of variance or the Student's two-tailed unpaired *t* test, as appropriate. Statistical differences within the pooled data sets over time were assessed using one-way repeated-measures analysis of variance followed by Bonferroni *t* tests for multiple comparisons. The fiducial limit of significance in all analyses was 5%.

RESULTS

Effect of hormonal support

A total of 61 cultured branchial epithelia were used in the present study, with TER values under symmetrical conditions, immediately prior to the commencement of apical freshwater exposure, ranging from approximately 500 to greater than 13,000 ohms · cm^{-2} with an overall mean of 3,137 ± 434 ohms · cm^{-2}. As in previous studies (Wood et al., '97; Wood and Pärt, '97), the transepithelial resistance increased significantly on replacement of the apical culture medium with fresh water, but the initial increase was not maintained during prolonged apical freshwater exposure (Table 1). In parallel with the attenuation of the elevated TER, PEG permeability (Table 2) and net Na^+ and Cl^- fluxes (J_{net}^{Na+} and J_{net}^{Cl-}; Table 3) increased over the course of the apical freshwater exposure period.

The potential impact on these trends of supporting the cultured epithelium with hormones known to be important in freshwater acclimation in vivo in euryhaline fish was investigated by a comparison of cultured epithelia in which 200 ng/L prolactin or growth hormone was included in the basolateral culture medium during the apical freshwater exposure with cultured epithelia lacking this hormonal supplementation (control); a concentration of 20 ng/L prolactin also was used in a few cases (*N* = 4), but the data were similar to those for 200 ng/L and therefore have not been reported. In one series of experiments, 200 ng/L prolactin also was included in the culture medium during growth of the culture on the filter insert (long-term prolactin). Hormonal supplementation of the basolateral culture medium had no significant effect at any time on TER, PEG permeability, J_{net}^{Na+}, or J_{net}^{Cl-} (see Tables 1 to 3). Therefore, the results from all treatments (control, prolactin, long-term prolactin, and growth hormone) have been combined for subsequent analyses of

TABLE 1. Effect of hormone support treatments on TER (ohms · cm^{-2}) during apical freshwater exposure[1]

Time (hr)	Control (*N* = 27)	Prolactin (*N* = 17)	Long–term prolactin (*N* = 7)	Growth hormone (*N* = 6)	Pooled data (*N* = 61)
0	9043 ± 1117	9330 ± 1755	7473 ± 1464	13404 ± 2403	9368 ± 773*
6	6840 ± 1017	7870 ± 1566	5422 ± 1037	3872 ± 881	6939 ± 706*
12	5045 ± 616	6431 ± 1309	4719 ± 802	3692 ± 933	5412 ± 518*
18	5020 ± 767	5292 ± 1081	1779 ± 295	5853 ± 1482	5134 ± 583
24	4245 ± 809	4449 ± 1201	2273 ± 321	5411 ± 1658	4490 ± 561
30	3960 ± 702	3429 ± 768	2711 ± 431	2549 ± 728	3681 ± 424
36	2825 ± 624	1768 ± 472	1815 ± 416	2413 ± 1027	2495 ± 362
42	1761 ± 373	1451 ± 369	1296 ± 366	534 ± 147	1667 ± 260*
48	856 ± 197	645 ± 222	692 ± 158	180 ± 76	882 ± 191*

[1]No significant differences existed among treatments (control, prolactin, long-term prolactin, and growth hormone) at any time (one-way ANOVA, $P > 0.05$). Note that the mean TER in L-15/L-15, before replacement of the apical culture medium with fresh water, was 3137 ± 434 (61).

*Indicates a significant difference within the pooled data set only from the initial TER in L-15/L-15 (one-way repeated-measures ANOVA followed by Bonferroni *t* tests, $P < 0.05$).

TABLE 2. Effect of hormone support treatments on PEG permeability (cm/sec × 10^{-6}) during apical freshwater exposure[1]

Time (hr)	Control (N = 27)	Prolactin (N = 17)	Long-term prolactin (N = 7)	Growth hormone (N = 6)	Pooled data (N = 61)
0–6	0.20 ± 0.03	0.21 ± 0.04	0.17 ± 0.03	0.11 ± 0.01	0.19 ± 0.02
6–12	0.28 ± 0.04	0.34 ± 0.07	0.23 ± 0.04	0.19 ± 0.03	0.27 ± 0.03
12–18	0.27 ± 0.04	0.34 ± 0.08	0.19 ± 0.02	0.32 ± 0.11	0.27 ± 0.03
18–24	0.30 ± 0.04	0.38 ± 0.09	0.20 ± 0.02	0.31 ± 0.13	0.31 ± 0.03
24–30	0.56 ± 0.13	0.74 ± 0.23	0.29 ± 0.05	0.35 ± 0.15	0.55 ± 0.09*
30–36	0.57 ± 0.13	0.80 ± 0.23	0.31 ± 0.06	0.52 ± 0.23	0.58 ± 0.09*
36–42	0.95 ± 0.18	1.14 ± 0.24	0.52 ± 0.11	1.03 ± 0.28	0.95 ± 0.11*
42–48	1.09 ± 0.17	1.20 ± 0.24	0.77 ± 0.14	1.10 ± 0.23	1.09 ± 0.11*

[1]No significant differences existed among treatments (control, prolactin, long-term prolactin, and growth hormone) at any time (one-way ANOVA, $P > 0.05$).
*Indicates a significant difference within the pooled data set only from the PEG permeability for the 0–6 hr time period (one-way repeated-measures ANOVA followed by Bonferroni t tests, $P < 0.05$).

the effects of apical water exposure on resistance and permeability relationships.

Permeability versus conductance relationships

The elevated transepithelial resistance characteristic of the cultured branchial epithelium during apical freshwater exposure gradually declined (pooled data; see Table 1) over 48 hr. At the same time, PEG permeability (pooled data; see Table 2) and net Na^+ and Cl^- fluxes (pooled data; see Table 3) increased significantly. However, TER decreased (or conductance increased) by a factor of approximately 10 over the 48-hr freshwater exposure, whereas the ion fluxes or PEG permeability increased only four- to sixfold, suggesting that the flux or permeability versus conductance relationships changed over time.

To further analyze these changes, the relationships between net ion fluxes or PEG permeability and conductance were compared for the first (0–6 hr) and last (42–48 hr) flux periods. For the net Na^+ and Cl^- fluxes, linear relationships with conductance were obtained for both time periods (Fig. 1), but the slope of the regression decreased significantly from 0 to 6 hr to 42 to 48 hr, reflecting the difference in the factors by which mean values for J_{net}^{Na+} or J_{net}^{Cl-} and TER changed over time during the apical freshwater exposure. In addition, the y-intercept of the regression increased over the exposure period from very close to zero at 0 to 6 hr to significantly different from zero at 42 to 48 hr. Increases in the net ion fluxes over time during apical freshwater exposure could reflect increases in the paracellular and/or transcellular permeabilities, and therefore, the relationship between conductance and the permeability of the cultured epithelium to the paracellular marker PEG during apical freshwater exposure was examined to distinguish between these possibilities. PEG permeability was linearly related to conduc-

TABLE 3. Effect of hormone support treatments on J_{net}^{Na+} and J_{net}^{Cl-} (µmol /cm²/hr) during apical freshwater exposure[1]

Time (hr)	Control J_{net}^{Na+} (N = 27)	Control J_{net}^{Cl-} (N = 11)	Prolactin J_{net}^{Na+} (N = 17)	Prolactin J_{net}^{Cl-} (N = 11)	Long-term prolactin J_{net}^{Na+} (N = 7)	Growth hormone J_{net}^{Na+} (N = 6)	Pooled data J_{net}^{Na+} (N = 61)	Pooled data J_{net}^{Cl-} (N = 26)
0 – 6	0.30 ± 0.06	0.63 ± 0.22	0.33 ± 0.08	0.48 ± 0.11	0.24 ± 0.03	0.14 ± 0.02	0.28 ± 0.03	0.51 ± 0.11
6 – 12	0.40 ± 0.07	0.56 ± 0.15	0.42 ± 0.11	0.56 ± 0.17	0.27 ± 0.03	0.30 ± 0.06	0.36 ± 0.05	0.52 ± 0.10
12 – 18	0.48 ± 0.11	0.76 ± 0.23	0.50 ± 0.16	0.74 ± 0.24	0.29 ± 0.04	0.56 ± 0.27	0.45 ± 0.07	0.68 ± 0.14
18 – 24	0.58 ± 0.12	0.71 ± 0.20	0.57 ± 0.15	0.73 ± 0.24	0.40 ± 0.06	0.73 ± 0.40	0.56 ± 0.08	0.66 ± 0.13
24 – 30	0.96 ± 0.22	1.50 ± 0.38	1.18 ± 0.31	1.26 ± 0.41	0.58 ± 0.15	0.80 ± 0.45	0.96 ± 0.14*	1.34 ± 0.25*
30 – 36	1.13 ± 0.23	1.79 ± 0.66	1.42 ± 0.29	1.21 ± 0.25	0.70 ± 0.16	1.24 ± 0.61	1.17 ± 0.15*	1.62 ± 0.38*
36 – 42	1.70 ± 0.28	2.65 ± 0.51	1.97 ± 0.32	2.26 ± 0.43	1.21 ± 0.27	2.28 ± 0.63	1.75 ± 0.18*	2.35 ± 0.32*
42 – 48	1.93 ± 0.27	2.04 ± 0.43	2.04 ± 0.32	2.01 ± 0.35	2.02 ± 0.39	2.61 ± 0.52	1.99 ± 0.17*	1.91 ± 0.25*

[1]No significant differences existed among treatments (control, prolactin, long-term prolactin, and growth hormone) for J_{net}^{Na+} or between control and prolactin-treated epithelia for J_{net}^{Cl-} at any time (one-way ANOVA and unpaired Student's t test, respectively, $P > 0.05$).
*Indicates a significant difference within the pooled data set only from the ion flux for the 0–6 hr time period (one-way repeated-measures ANOVA followed by Bonferroni t tests, $P < 0.05$).

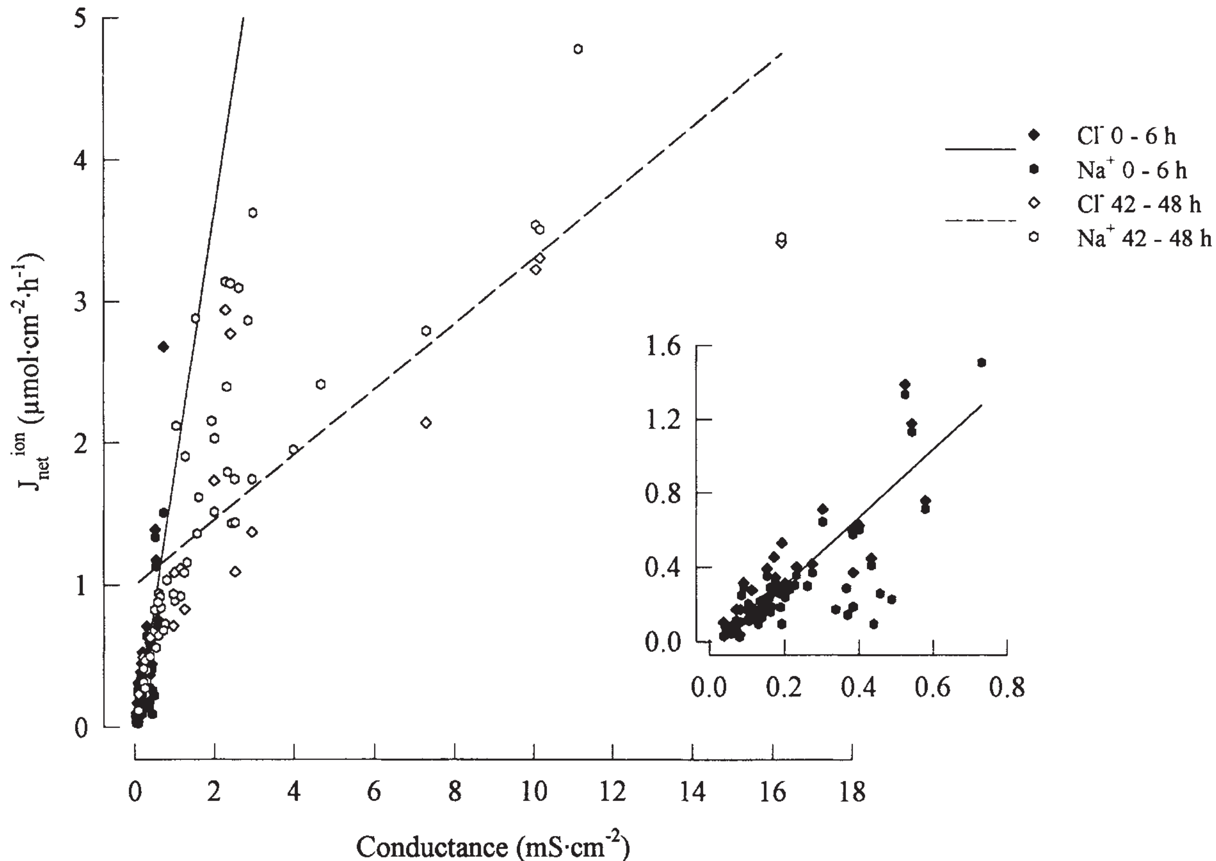

Fig. 1. The relationship between net ion fluxes ($J_{net}^{Cl^-}$ and $J_{net}^{Na^+}$) and transepithelial conductance for cultured trout branchial epithelia during 0 to 6 hr (filled-in symbols) and 42 to 48 hr (open symbols) of apical freshwater exposure. In the inset plot, the ranges for the *x*- and *y*-axes have been modified to more clearly present the data for 0 to 6 hr. The regression equations, which have significantly different slopes (Student's *t* test, $P < 0.05$) are 0–6 hr (solid line): $J_{net}^{ions} = -0.069 + 1.84$ (conductance), $r = 0.78$, $N = 87$, $P < 0.0001$; and 42–48 hr (dashed line): $J_{net}^{ions} = 1.00 + 0.23$ (conductance), $r = 0.75$, $N = 67$, $P < 0.0001$.

tance, and the slope of the regression equation decreased significantly from 0 to 6 hr to 42 to 48 hr (Fig. 2), results similar to those obtained for $J_{net}^{Na^+}$ and $J_{net}^{Cl^-}$. As was also the case for the net ion flux data, the *y*-intercept of the regression was significantly different from zero for 42 to 48 hr but not for the 0- to 6-hr regression. Thus plots of the net ion fluxes against PEG permeability over all flux periods revealed a significant linear relationship with a *y*-intercept that was significantly different from zero (Fig. 3).

Morphological effects of apical freshwater exposure

Examination of the cultures by transmission electron microscopy revealed that the cultures exhibited a variable number (four to seven) of cell layers (Fig. 4A–C). The cells making up the most superficial layer appeared to be flattened and were separated from one another by apical tight junctions (see Fig. 4D–F). In all cell layers, the cells contained an ovoid nucleus, mitochondria, and bundles of filaments, whereas cisternae of endoplasmic reticulum were particularly numerous in the cells of the deepest layer. The intercellular spaces were slightly dilated. These ultrastructural features were similar in both control (see Fig. 4A) and freshwater-exposed (see Fig. 4B, C) cultures and did not appear to be modified by freshwater exposure. In cell cultures exposed to apical fresh water for 48 hr, however, some cells of the superficial layer were altered, in that holes could be observed in the cytoplasm and the cytoplasm appeared to contain a reduced number of cell organelles (see Fig. 4C). The cells present in the deepest layers, in contrast, were never damaged by prolonged apical freshwater exposure.

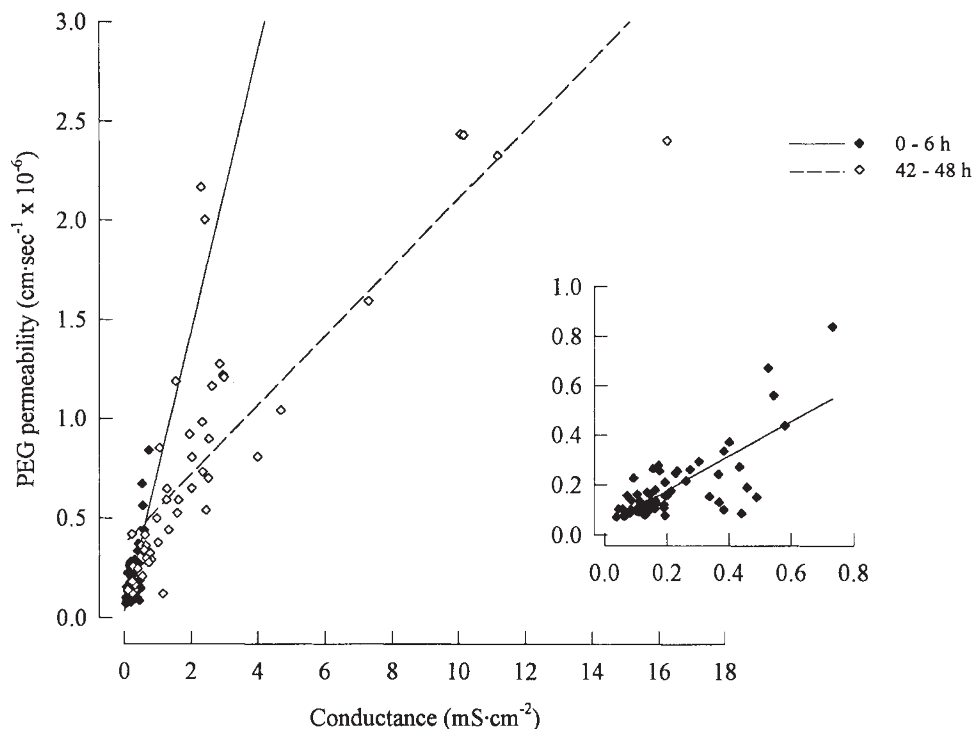

Fig. 2. Data illustrating the relationship between the permeability to the paracellular marker PEG and transepithelial conductance for cultured trout branchial epithelia over 0 to 6 hr (♦) and 42 to 48 hr (◇) of apical freshwater exposure. The data for 0 to 6 hr only are presented in the inset plot, in which the ranges for both the *x*- and *y*-axes have been modified for clarity. The regression equations, which have significantly different slopes (Student's *t* test, $P < 0.05$) are 0–6 hr (solid line): PEG permeability = 0.036 + 0.70 (conductance), $r = 0.76$, $N = 61$, $P < 0.0001$; and 42–48 hr (dashed line): PEG permeability = 0.38 + 0.17 (conductance), $r = 0.84$, $N = 49$, $P < 0.0001$.

DISCUSSION

Despite the key role that regulation of passive permeability of the gill must play during adaptation to salinity changes, studies of such adaptation effects both in vivo (see review by Wood and Marshall, '94) and in vitro (see review by Marshall, '95) generally have focused on the role of active ion uptake. The freshwater cultured branchial epithelium, in contrast, appears to be an excellent preparation for the study of passive permeability under either symmetrical or asymmetrical conditions. Under symmetrical conditions, i.e., with L-15 medium on both surfaces of the epithelium and zero TEP, the measured fluxes of Na^+ and Cl^-, when converted to conductance, have been found to fully account for the total transepithelial conductance of the freshwater cultured branchial epithelium, indicating that all the ion flux measured is conductive and that the contribution of ions other than Na^+ and Cl^- is negligible (Wood et al., '97). Similar results have been reported for the opercular epithelium of brook trout (Marshall, '85), and in both cases it was concluded that the epithelium served as a useful model of the passive transport properties of the intact gill.

Replacement of the apical medium with fresh water resulted, in both the cultured epithelium and the brook trout opercular epithelium, in significant increases in the TER and the development of a negative (relative to the apical side) TEP (Marshall, '85; Wood et al., '97; Wood and Pärt, '97). The origin of these responses was investigated in the rainbow trout cultured branchial epithelium through measurements of ion fluxes and permeability to the paracellular tracer PEG under both symmetrical and asymmetrical conditions (Wood et al., '97). The observation of simultaneous increases in PEG permeability and TER following exposure of the apical surface of the cultured epithelium to fresh water led to the conclusion that paracellular permeability increases on freshwater exposure but that the effect of this increase on resistance is counteracted by a decrease in the transcellular permeability. The gradual increases measured in Na^+ and Cl^- effluxes over the course of the 12-hr exposure period were attributed to the opening up of the paracellular pathway, with the elevated ion flows remaining

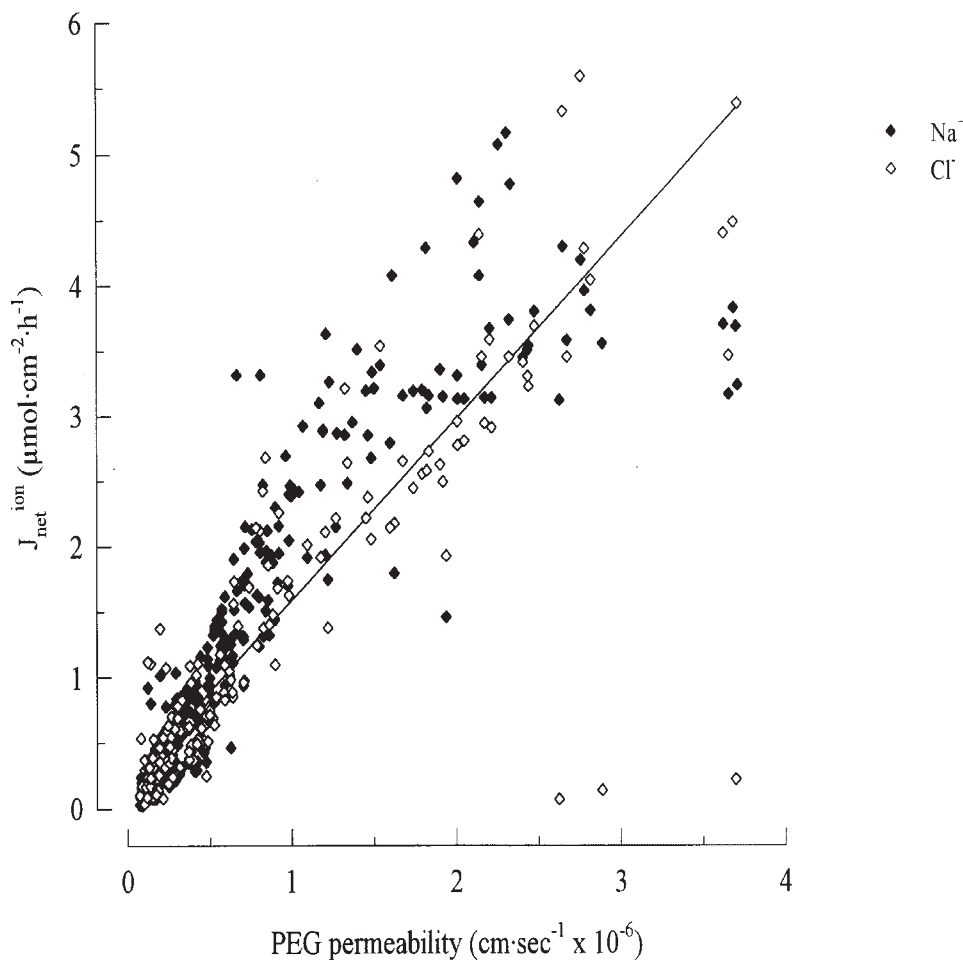

Fig. 3. The relationship between the net ion fluxes ($J_{net}^{Cl^-}$ and J_{net}^{Na+}) and the permeability to the paracellular marker PEG during apical freshwater exposure for 61 cultured trout branchial epithelia followed over 48 hr (net Cl^- flux measurements were carried out on only 26 of the 61 filters). Ion fluxes and PEG permeability were measured every 6 hr. The regression equation is J_{net}^{ions} = 0.19 + 1.39 (PEG permeability), r = 0.85, N = 685, P < 0.0001.

largely conductive, accounting for more than 80% of the measured conductance (Wood et al., '97). The very different passive permeability relationships for the cultured branchial epithelium under symmetrical versus asymmetrical conditions (first 12 hr of freshwater exposure only) are illustrated in Fig. 5, in which net ion fluxes and PEG permeability are plotted as a function of TER.

Over prolonged apical freshwater exposure (48 hr rather than 12 hr), however, the elevated TER and TEP were found to gradually decrease, and at the same time, the net ion fluxes increased (Wood and Pärt, '97). Similar responses were observed in the present study; net Na^+ and Cl^- fluxes increased by a factor of 4 to 7, while TER declined approximately 10-fold over the 48-hr exposure period (see Tables 1 and 3). Measurement of PEG permeability over the 48-hr freshwater exposure in the present study provided further insight into the causes of the changes observed. PEG permeability increased approximately 6-fold over 48 hr (see Table 2), an increase that paralleled that observed for the net ion fluxes (see Fig. 3). Thus the opening up of the paracellular pathway, which appears to commence very soon after the apical culture medium is replaced with fresh water (Wood et al., '97), continues throughout the exposure period. The difference in size, and therefore mobility, between PEG and Na^+ or Cl^- precludes a direct comparison of flux rates for PEG and ions such as that carried out by Marshall ('85) on the extracellular tracer mannitol and Na^+ or Cl^- flux rates for the brook trout opercular epithelium under asymmetrical conditions. Nevertheless, the similarity of the increases in PEG permeability and net ion flux rates suggests that

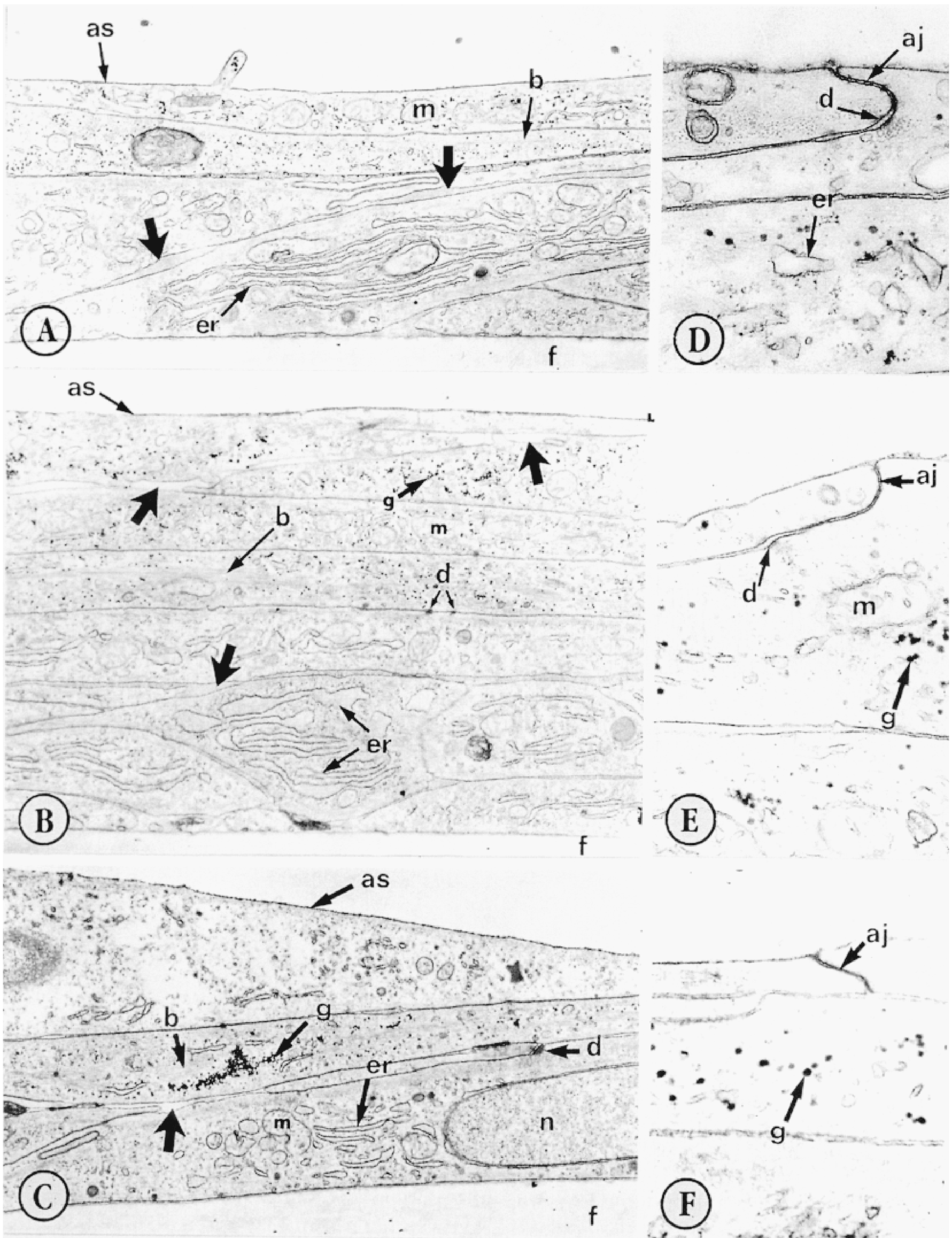

Figure 4.

the gradual elevation in the net ionic flux is largely a paracellular phenomenon, presumably the result of a loosening of the tight junctions between the cells (see also below).

During the first 12 hr of freshwater exposure, the measured rates of $J_{net}^{Na^+}$ and $J_{net}^{Cl^-}$ (approximately 0.4 μmol/cm^2/hr) across the cultured epithelium would translate to about 800 μmol/kg/hr for a whole trout using the nonspecific total gill area tabulated by Hughes and Morgan ('73). The effect of the increase in paracellular permeability during prolonged apical freshwater exposure is that by the end of the exposure period, the ion fluxes (approximately 2 μmol/cm^2/hr) become equivalent to approximately 4000 μmol/kg/hr for the whole trout, substantially higher than those typical of intact freshwater trout (300 μmol/kg/hr; Goss et al., '95).

The contribution of the Na^+ and Cl^- fluxes to the measured conductance can be determined for the 0- to 6-hr and 42- to 48-hr flux periods, taking into consideration the negative TEP and neglecting the very low ion influx under asymmetrical conditions, as outlined by Wood et al. ('97). During the early stages of apical freshwater exposure, the ionic fluxes are largely conductive, providing more than half the measured conductance, but by the 42- to 48-hr flux period, the sum of the Na^+ and Cl^- effluxes contributes less than 30% of the measured conductance. This change over time is reflected in the significant reduction in the net ion flux per unit conductance over the exposure period (see Fig. 1). Leakage via the paracellular pathway of ions other than Na^+ and Cl^- may be one source of the difference between the measured conductance and the magnitude of the measured conductive ion flow. Alternatively, the permeability-conductance relationships for the cultured branchial epithelium under asymmetrical conditions are more complex than our current understanding.

Although the gradual increases in net ion fluxes observed during apical freshwater exposure probably can be explained largely by the rising paracellular permeability, additional factors must be invoked to account for the significantly greater increase in conductance over the exposure period. The approximately 10-fold increase in conductance (see Table 1) was greater than that which could be attributed solely to the elevated paracellular permeability, which exhibited only a 6-fold increase (see Table 2) over 48 hr; PEG permeability per unit conductance decreased by a factor of approximately 4 (see Fig. 2). On replacement of the apical culture medium with fresh water, transcellular conductance was hypothesized to decrease considerably to rationalize the marked increase in TER in the face of a simultaneous increase in paracellular permeability (Wood et al., '97). It seems likely that a reopening of the transcellular pathway during prolonged apical freshwater exposure contributes to the substantial increases in conductance measured under these conditions.

The morphological component of the present study provided additional insight into the effects of prolonged apical freshwater exposure on the cultured branchial epithelium. The ultrastructural features of the cultured cells are typical of pavement cells (Laurent and Dunel, '80; Laurent, '84), and the multiple layers and prominent intercellular spaces of the cultured branchial epithelium (see Fig. 4) correspond well with the organization of the lamellar epithelium in vivo; the lamellar epithelium characteristically consists of two or more layers of pavement cells that are separated by an intercellular space (Laurent and Dunel, '80; Laurent, '84). Apical exposure to fresh water for 30 min had no apparent effect on the morphology of the cultured branchial epithelium, illustrating the capacity of the cultured cells to regulate their volume when subjected to a continuous hyposmotic shock. Even after 48 hr of apical exposure to fresh water, the integrity of the cultured branchial epithelium was in large part maintained. However, cells of the most superficial layer exhibited some evidence of deterioration after 48 hr of exposure to fresh water, including a reduction in the number of cell organelles and the appearance of holes in the cytoplasm. In intact epithelia, the outer cell layer is thought to be the major resistive barrier (Klyce, '72; Nagel, '78), and thus progressive degradation of the superficial cell layer of the cultured bran-

Fig. 4. Electron micrographs of cultured branchial epithelia under control conditions (**A, D**) and following apical exposure to fresh water for 30 min (**B, E**) or 48 hr (**C, F**). In A–C, the multilayered nature of the cultured epithelium is apparent. Elongated, flattened cisternae of endoplasmic reticulum (er) may be observed, and the intercellular spaces (arrow) are slightly dilated in both control and freshwater-exposed cultures. 48 hr of apical freshwater exposure resulted in some alteration of the superficial layer of cells (F), including a reduction in the number of cell organelles and the appearance of holes in the cytoplasm. Apical junctions (aj) between cells of the superficial layer are shown in D–F (as, apical surface; m, mitochondria; b, bundles of filaments; d, desmosomes; g, glycogen particles; f, filter) (A–C: ×11,000; D–F: ×40,000).

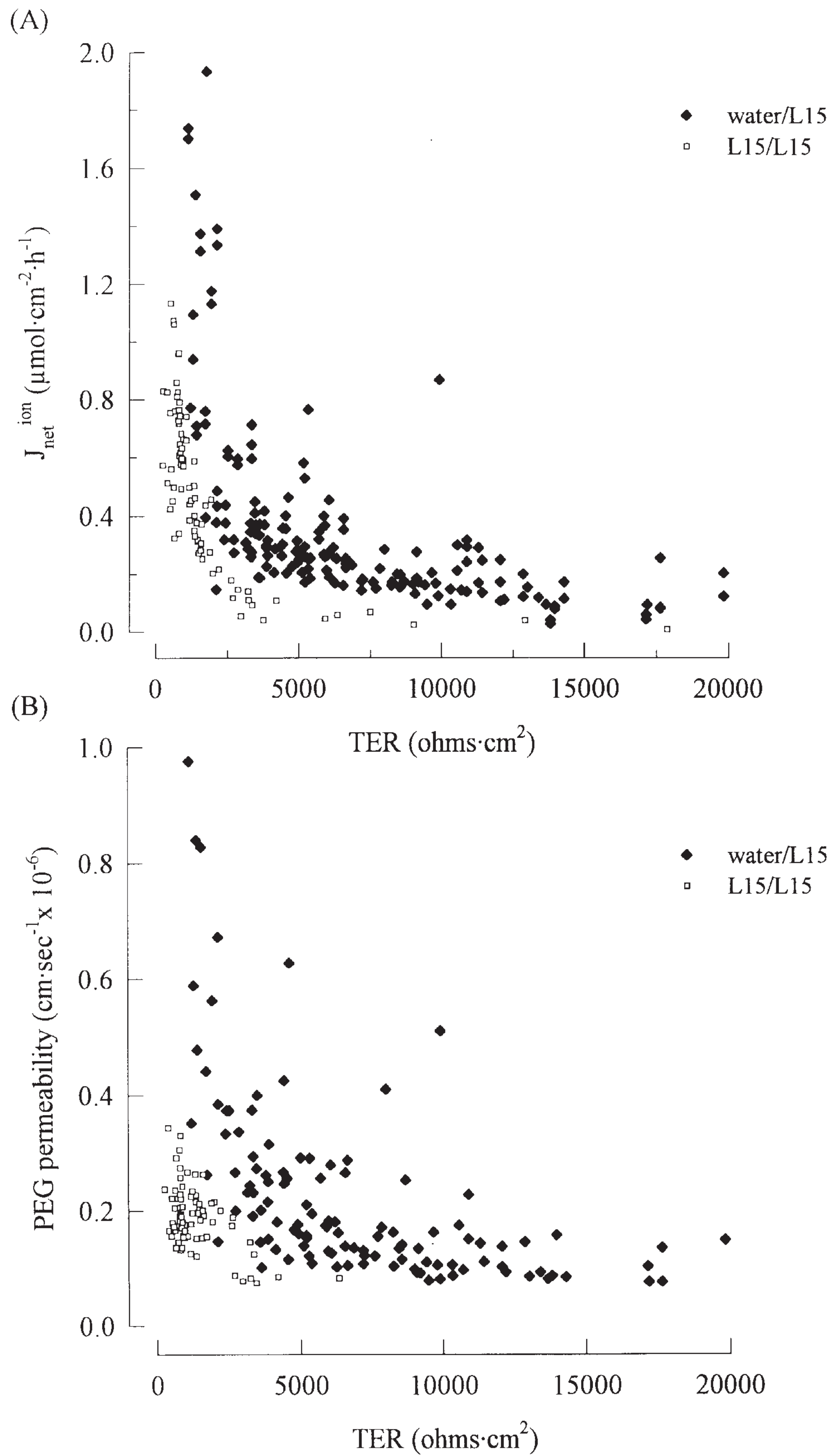

Figure 5.

chial epithelium may contribute to the physiological changes (decreases in TER, increased permeability to PEG and ions) observed during prolonged apical freshwater exposure.

Taken together, the experimental evidence available to date on the properties of the cultured branchial epithelium during apical freshwater exposure suggests that immediately following replacement of the apical culture medium with fresh water, paracellular permeability increases and transcellular permeability decreases, resulting in a marked decrease in the transepithelial conductance (Wood et al., '97; Wood and Pärt, '97). Prolonged exposure to fresh water, however, leads to continuing increases in paracellular permeability coupled with a reopening of the transcellular pathway, probably due at least in part to deterioration of the superficial cell layer of the cultured branchial epithelium. It is useful to compare these responses with those observed in fish in vivo during freshwater acclimation. The acclimation of a seawater-adapted euryhaline fish to fresh water is a two-stage process (reviewed by Wood and Marshall, '94) involving, first, immediate reductions in ion influx and efflux rates, a response that is probably controlled by direct regulatory effects of external salinity on the transcellular and paracellular conductances. In the second phase, which is initiated 30 min after transfer to fresh water and requires 24 to 48 hr to reach completion, slower, progressive reductions in ion efflux rates are observed. The immediate decrease in transepithelial conductance for the cultured branchial epithelium when the apical surface is exposed to fresh water parallels that observed in the intact fish, whereas the failure of the cultured epithelium to reduce ion efflux rates during prolonged apical freshwater exposure is reminiscent of the situation in stenohaline fish species, which lack the delayed component of the acclimation response.

The second phase in the acclimation to fresh water in vivo is controlled by endocrine mechanisms, specifically an increase in the secretion of prolactin. Plasma prolactin levels increase following transfer to fresh water (e.g., Prunet et al., '85; Potts et al., '89; Ogasawara et al., '96). While the mechanism through which prolactin effects a decrease in ion efflux rates remains uncertain, direct reductions of epithelial permeability and structural changes in the tight junctions are likely possibilities (Burden, '56; Pickford et al., '66; Ernst et al., '80; see also reviews by Foskett et al., '83; Hirano, '86). Prolactin also may exert direct effects on the transcellular and paracellular conductances of the gills, since injections of prolactin in vivo resulted in reduced NaCl secretion in vitro by the opercular epithelium of seawater-adapted tilapia (Foskett et al., '82, '83). Supplementation of the basal medium of the cultured branchial epithelium with prolactin was therefore assessed as a means of improving the stability of the cultured epithelium during prolonged apical freshwater exposure.

Despite the considerable evidence supporting an essential role for prolactin in the adaptation of euryhaline fish to fresh water, inclusion of prolactin in the basal culture medium during apical freshwater exposure and/or prior to the exposure period had no effect on the responses of the cultured epithelium to the apical freshwater exposure. Net Na^+ and Cl^- fluxes, permeability to the paracellular marker PEG, and transepithelial conductance (see Tables 1 to 3) for the hormone-supplemented cultures at the beginning of the exposure period (0–6 hr) were not significantly different from the control values, nor was the pattern of changes measured in the variables over 48 hr influenced by prolactin treatment. Potential explanations for this lack of effect fall into three categories. One possibility lies in the homogeneous cell composition of the cultured epithelium in comparison with that of the fish gill; the epithelium may require other cell types, such as mucous cells and/or chloride cells, in addition to pavement cells, to maintain stability during apical freshwater exposure. The absence of chloride cells, in particular, may be critical given the role of chloride cells in ion uptake in vivo (see review by Goss et al., '95) and the demonstrated effects of prolactin on chloride cell morphology and number in vivo (Herndon et al., '91; Pisam et al., '93).

Alternatively, other hormones may be involved in establishing a stable condition during apical freshwater exposure. Cortisol, growth hormone, insulin-like growth factor I, and thyroid hormones have been implicated in the control of the osmoregulatory changes that occur on transfer of freshwater-acclimated fish to sea water (Foskett et al.,

Fig. 5. Data illustrating the changes in the relationships between (**A**) net ion fluxes or (**B**) PEG permeability and TER under symmetrical and asymmetrical conditions. Data for asymmetrical conditions are from the present study (0–12 hr) whereas data for symmetrical conditions were taken from Wood et al. ('97).

'83; Sakamoto et al., '93; McCormick, '95), and the presence of one or more of these hormones, alone or in combination, may be required for stability of the cultured branchial epithelium during apical freshwater exposure. Although inclusion of growth hormone in the culture medium during apical freshwater exposure did not have any influence on net ion fluxes, PEG permeability, or TER in the present study (see Tables 1 to 3), the effectiveness of hormone combinations remains to be tested. The lack of effect of either prolactin or growth hormone on the properties of the cultured epithelium during freshwater exposure also could be due to the absence of appropriate receptors in the cultured gill cells. The detection of a high-affinity prolactin receptor in gill membranes isolated from tilapia (Auperin et al., '94, '95) and the observation that preincubation of isolated gills in saline containing prolactin decreased osmotic water permeability on transfer to deionized water (Ogawa et al., '73) might suggest that prolactin could have an effect on gill cells in culture. However, the direct addition of prolactin to a number of transporting epithelia from fish in vitro has not been found to elicit changes in water or ion movement across the epithelium (Bern et al., '81), whereas epithelia removed from fish treated with prolactin in vivo have displayed differences from untreated controls (Hirano et al., '73; Marshall and Bern, '80; Foskett et al., '82). This apparent difference between in vitro and in vivo prolactin administration might arise from prolactin-induced effects on water or ion movements requiring a longer term to become measurable, a problem that can be overcome in a culture situation. In any case, examination of the cultured branchial epithelium of rainbow trout for the presence of prolactin receptors would appear to be warranted.

In summary, the cultured branchial epithelium of the rainbow trout under asymmetrical conditions is a useful model of the freshwater fish gill provided that the length of the apical freshwater exposure is limited; in this respect, the cultured epithelium is comparable with a number of dissected epithelial preparations used as possible gill surrogates (e.g. Foskett et al., '81; Marshall, '85; Marshall et al., '92, '95). During prolonged freshwater exposure, however, both paracellular and transcellular permeabilities appear to increase significantly, resulting in ion flux rates that are substantially greater than corresponding values for intact fish. Supplementation of the culture medium with prolactin or growth hormone during the apical freshwater exposure did not affect the changes observed during prolonged exposure. Absence of one or more cell types from the cultured epithelium, a requirement for different hormones or a combination of hormones and the absence of appropriate receptors may all have contributed to the lack of responsiveness of the cultured branchial epithelium to hormonal supplementation of the basal medium.

ACKNOWLEDGMENTS

This study was supported by a grant from the NSERC Collaborative Program to D.G.M. (P.I.), C.M.W., P.P., and P.P. Mary Fletcher and Vandy Thomas are thanked for excellent technical assistance.

LITERATURE CITED

Auperin, B., F. Rentier-Delrue, J. Martial, and P. Prunet (1994) Characterization of a single prolactin (PRL) receptor in tilapia (*Oreochromis niloticus*) which binds both PRL_I and PRL_{II}. J. Mol. Endocrinol., *13:*241–251.

Auperin, B., F. Rentier-Delrue, J. Martial, and P. Prunet (1995) Regulation of gill prolactin receptors in tilapia (*Oreochromis niloticus*) after a change in salinity or hypophysectomy. J. Endocrinol., *145:*213–220.

Avella, M., and J. Ehrenfeld (1997) Fish gill respiratory cells in culture: A new model for Cl^--secreting epithelia. J. Membrane Biol. *156:*87–97.

Bern, H.A., C.A. Bisbee, N.L. Collie, J.K. Foskett, B. Hughes, C.A. Lorez, and W.S. Marshall (1981) Failure of ovine prolactin to elicit rapid responses by osmoregulatory surfaces. Gen. Comp. Endocrinol., *44:*128–130.

Boeuf, G., A.M. Marc, P. Prunet, P.Y. Le Bail, and J. Smal (1994) Stimulation of parr-smolt transformation by hormonal treatment in Atlantic salmon (*Salmo salar* L.). Aquaculture, *121:*195–208.

Burden, C.E. (1956) The failure of hypophysectomized *Fundulus heteroclitus* to survive in fresh water. Biol. Bull., *110:*8–28.

Ernst, S.A., W.C. Dodson, and K.J. Karnaky, Jr. (1980) Structural diversity of occluding junctions in the low-resistance chloride-secreting opercular epithelium of seawater-adapted killifish (*Fundulus heteroclitus*). J. Cell Biol., *87:*488–497.

Foskett, J.K., C.D. Logsdon, T. Turner, T.E. Machen, and H.A. Bern (1981) Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. J. Exp. Biol., *93:*209–224.

Foskett, J.K., T.E. Machen, and H.A. Bern (1982) Chloride secretion and conductance of teleost opercular membrane: Effects of prolactin. Am. J. Physiol., *242:*R380–R389.

Foskett, J.K., H.A. Bern, T.E. Machen, and M. Conner (1983) Chloride cells and the hormonal control of teleost fish osmoregulation. J. Exp. Biol., *106:*255–281.

Goss, G.G., S.F. Perry, and P. Laurent (1995) Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. In: Cellular and Molecular Approaches to Fish Ionic Regulation. C.M. Wood and T.J. Shuttleworth, eds. Academic Press, San Diego, pp. 257–284.

Herndon, T.M., S.D. McCormick, and H.A. Bern (1991) Effects of prolactin on chloride cells in opercular membrane of seawater-adapted tilapia. Gen. Comp. Endocrinol., *83:*283–289.

Hirano, T. (1986) The spectrum of prolactin action in teleosts. In: Comparative Endocrinology: Developments and Directions. C.L. Ralph, ed. Alan R. Liss, New York, pp. 53–74.

Hirano, T., D.W. Johnson, H.A. Bern, and S. Utida (1973) Studies on water and ion movements in the isolated urinary bladder of selected freshwater, marine and euryhaline teleosts. Comp. Biochem. Physiol., *45A:*529–540.

Hughes, G.M., and M. Morgan (1973) The structure of fish gills in relation to their respiratory function. Biol. Rev., *48:*419–475.

Karnaky, K.J. Jr., K.J. Degnan, and J.A. Zadunaisky (1977) Chloride transport across isolated opercular epithelium of killifish: A membrane rich in chloride cells. Science, *195:*203–205.

Karnovsky, M.J. (1971) Use of ferrocyanide reduced osmium tetroxide in electron microscopy. Proc. 11th Am. Soc. Cell Biol. Abstract, 284:146.

Klyce, S.D. (1972) Electrical profiles in the corneal epithelium. J. Physiol., *226:*407–429.

Laurent, P. (1984) Gill internal morphology. In: Fish Physiology. W.S. Hoar and D.J. Randall, eds. Academic Press, London, pp. 73–183.

Laurent, P., and S. Dunel (1980) Morphology of gill epithelia in fish. Am. J. Physiol., *238:*R147–R159.

Marshall, W.S. (1977) Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae), acclimated to 5% and 100% seawater. J. Comp. Physiol., *114:*157–165.

Marshall, W.S. (1985) Paracellular ion transport in trout opercular epithelium models osmoregulatory effects of acid precipitation. Can. J. Zool., *63:*1816–1822.

Marshall, W.S. (1995) Transport processes in isolated teleost epithelia: Opercular epithelium and urinary bladder. In: Cellular and Molecular Approaches to Fish Ionic Regulation. C.M. Wood and T.J. Shuttleworth, eds. Academic Press, San Diego, pp. 1–23.

Marshall, W.S., and H.A. Bern (1980) Ion transport across the isolated skin of the teleost, *Gillichthys mirabilis*. In: Epithelial Transport in the Lower Vertebrates. B. Lahlou, ed. Cambridge University Press, New York, pp. 337–350.

Marshall, W.S., S.E. Bryson, and C.M. Wood (1992) Calcium transport by isolated skin of rainbow trout. J. Exp. Biol., *166:*297–316.

Marshall, W.S., S.E. Bryson, J.S. Burghardt, and P.M. Verbost (1995) Ca^{2+} transport by opercular epithelium of the fresh water adapted euryhaline teleost, *Fundulus heteroclitus*. J. Comp. Physiol. [B], *165:*268–277.

McCormick, S.D. (1995) Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. In: Cellular and Molecular Approaches to Fish Ionic Regulation. C.M. Wood and T.J. Shuttleworth, eds. Academic Press, San Diego, pp. 285–315.

Nagel, W. (1978) Effects of antidiuretic hormone upon electrical potential and resistance of apical and basolateral membranes of frog skin. J. Membrane Biol. *42:*99–122.

Ogasawara, T., T. Sakamoto, and T. Hirano (1996) Prolactin kinetics during freshwater adaptation of mature chum salmon, *Oncorhynchus keta*. Zool. Sci., *13:*443–447.

Ogawa, M., M. Yagasaki, and F. Yamazaki (1973) The effect of prolactin on water influx in isolated gills of the goldfish, *Carassius auratus* L. Comp. Biochem. Physiol., *44A:*1177–1183.

Pärt, P., and E. Bergström (1995) Primary cultures of teleost branchial epithelial cells. In: Cellular and Molecular Approaches to Fish Ionic Regulation. C.M. Wood and T.J. Shuttleworth, eds. Academic Press, San Diego, pp. 207–227.

Pärt, P., and C.M. Wood (1996) Na/H exchange in cultured epithelial cells from fish gills. J. Comp. Physiol. B, *166:*37–45.

Pärt, P., L. Norrgren, E. Bergström, and P. Sjöberg (1993) Primary cultures of epithelial cells from rainbow trout gills. J. Exp. Biol., *175:*219–232.

Pickford, G.E., P.K.T. Pang, and W.H. Sawyer (1966) Prolactin and serum osmolality of hypophysectomized killifish, *Fundulus heteroclitus*, in fresh-water. Nature, *209:* 1040–1041.

Pisam, M., B. Auperin, P. Prunet, F. Rentier-Delrue, J. Martial, and A. Rambourg (1993) Effects of prolactin on a and b chloride cells in the gill epithelium of the saltwater adapted tilapia *Oreochromis niloticus*. Anat. Rec., *235:*275–284.

Potts, W.T.W., C. Talbot, F.B. Eddy, D.R.N. Primmett, P. Prunet, and M. Williams (1989) Sodium balance in adult Atlantic salmon (*Salmo salar* L.) during migration into neutral and acid fresh water. Comp. Biochem. Physiol., *92A:*247–253.

Prunet, P., and L.M. Houdebine (1984) Purification and biological characterization of Chinook salmon prolactin. Gen. Comp. Endocrinol., *53:*49–57.

Prunet, P., G. Boeuf, and L.M. Houdebine (1985) Plasma and pituitary prolactin levels in rainbow trout during adaptation to different salinities. J. Exp. Zool., *235:*187–196.

Sakamoto, T., S.D. McCormick, and T. Hirano (1993) Osmoregulatory actions of growth hormone and its mode of action in salmonids: A review. Fish Physiol. Biochem., *11:*155–164.

Wood, C.M., and W.S. Marshall (1994) Ion balance, acid-base regulation, and chloride cell function in the common killifish, *Fundulus heteroclitus:* A euryhaline estuarine teleost. Estuaries, *17:*34–52.

Wood, C.M., and P. Pärt (1997) Cultured branchial epithelia from freshwater fish gills. J. Exp. Biol., *200:*1047–1059.

Wood, C.M., K.M. Gilmour, and P. Pärt (1998) Passive and active transport properties of a gill model, the cultured branchial epithelium of the freshwater rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol., *119A:*87–96.

Yada, T., K. Takahashi, and T. Hirano (1991) Seasonal changes in seawater adaptability and plasma levels of prolactin and growth hormone in landlocked sockeye salmon (*Oncorhynchus nerka*) and amago salmon (*O. rhodurus*). Gen. Comp. Endocrinol., *82:*33–44.

Young, G., B.T. Björnsson, P. Prunet, R.J. Lin, and H.A. Bern (1989) Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*): Plasma prolactin, growth hormone, thyroid hormones, and cortisol. Gen. Comp. Endocrinol., *74:*335–345.

Zall, D.M., D. Fisher, and M.Q. Garner (1956) Photometric determination of chlorides in water. Analyt. Chem., *28:*1665–1678.