Prolactin effects on cultured pavement cell epithelia and pavement cell plus mitochondria-rich cell epithelia from freshwater rainbow trout gills

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Abstract

The physiological effects of ovine prolactin (oPRL) and recombinant rainbow trout prolactin (rbtPRL) on cultured gill epithelia derived from freshwater rainbow trout were assessed. Epithelia composed of either pavement cells only (single seeded inserts, SSI) or both pavement and mitochondria-rich cells (double seeded inserts, DSI) were cultured in media, supplemented with doses of oPRL ranging from 10 to 100 ng/ml. Under symmetrical culture conditions (L15 media apical/L15 media basolateral), oPRL had no effect on transepithelial resistance, paracellular permeability (assessed with PEG-4000), or Na^+ and Cl^- transport across both preparations of cultured gill epithelia. Under asymmetrical conditions (freshwater apical/L15 media basolateral), SSI epithelia treated with oPRL (10 and 50 ng/ml), in comparison to comparably treated epithelia receiving no oPRL, exhibited a greater increase in the transepithelial resistance, particularly during the first 12 h of freshwater exposure, no difference in paracellular permeability and Na^+–K^+-ATPase activity, and lowered net Na^+ flux rates (i.e., reduced basolateral to apical loss rates). These reflected reduced unidirectional efflux rates. The PRL effect appeared to be mainly a reduction in transcellular permeability. SSI epithelia treated with rbtPRL (10 ng/ml) exhibited similar patterns of response to those treated with oPRL. Na^+–K^+-ATPase activity increased in DSI epithelia treated with oPRL; however, oPRL did not stimulate ion uptake across either SSI or DSI epithelial preparations. The data demonstrated that, as the sole hormone supplement for cultured gill epithelia, PRL did not promote active ion uptake. However, the observed PRL-induced alterations in cultured gill epithelial physiology were consistent with the in vivo actions of PRL on the gills of freshwater teleost fish. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Prolactin; Gill; Cell culture; Pavement cell; Mitochondria-rich cell; Permeability; Ion transport; Na^+–K^+-ATPase

1. Introduction

Prolactin (PRL) has a wide spectrum of activity in teleost fish, but is principally considered to affect epithelial barriers that promote ion retention and prevent blood and tissue hydration in hyperosmoregulating fish (Hirano, 1986). These observations were first reported by Pickford and Phillips (1959). In response to elevated PRL levels, osmoregulatory surfaces have been demonstrated to undergo physiological, morphological, and biochemical alterations that facilitate reduced ion loss and water loading. In the intact fish, PRL reduces passive branchial Na^+ loss (Hirano, 1986), Na^+–K^+-ATPase activity (Kelly et al., 1999; Madsen and Bern, 1992; Pickford et al., 1970), chloride cell numbers and/or chloride cell exposure to the external environment (Herndon et al., 1991; Kelly et al., 1999), and osmotic water permeability (Ogasawara and Hirano, 1984). PRL has also been reported to reduce active Cl^- extrusion across isolated opercular membranes from seawater-adapted fish (Foskett et al., 1982). To date, however, few studies have investigated the direct action of PRL on the physiology of branchial epithelia or related tissues.

The development of surrogate gill models for marine fish has enhanced our understanding of ion transport across seawater branchial epithelia (Foskett and Scheffey, 1982; Karnaky et al., 1977; Marshall, 1977; Zadunaisky,

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1984). In contrast, comparable models for the freshwater fish gill have met with limited success (Burgess et al., 1998; Foskett et al., 1981; Marshall, 1995; Marshall et al., 1992; Wood and Marshall, 1994). Based on a variety of techniques, currently popular models of Na^+ and Cl^- uptake across the gills of freshwater fish incorporate two gill cell-types, the pavement cell and the chloride cell (for review see Perry, 1997). Although debated, Na^+, at least in part, is thought to move across pavement cells in association with the electrogenic actions of a vacuolar-type H^+-ATPase while evidence suggests that Cl^- moves across the chloride cells in association with a Cl^-/HCO_3^- exchanger (for review see Perry, 1997). However, models of ion transport across freshwater fish gills are likely to remain controversial until suitable direct techniques can be found to investigate the physiological processes associated with gill cell function.

Recently developed novel techniques for the preparation and culture of "reconstructed" branchial epithelia from freshwater rainbow trout have provided a promising new approach toward the development of an appropriate model for the freshwater fish gill. One model is composed solely of branchial pavement cells (termed SSI epithelia) (Gilmour et al., 1998; Wood and Pärt, 1997; Wood et al., 1998) and a second is composed of both pavement and mitochondria-rich (MR) cells (termed DSI epithelia) (Fletcher et al., 2000). In the latter of these preparations, the MR cells exhibit all the ultrastructural characteristics of branchial chloride cells (Fletcher et al., 2000). Both preparations mimic the electrophysiological and passive transport characteristics of the freshwater fish gill faithfully, but the active transport component is at best far less than in vivo. Currently, we are investigating the potential use of hormonal supplements to promote active ion transport across cultured gill epithelia with some degree of success (Kelly and Wood, 2001a,b). Given the important role ascribed to PRL in freshwater fish osmoregulation, an evaluation of its use in these models is warranted.

The present study was undertaken to evaluate the potential use and actions of PRL as the sole hormone supplement in cultured gill epithelia from freshwater rainbow trout. In addition to the reasons cited above, the question of PRL's potential action on the gill tissue of rainbow trout is a particularly interesting one. First, while PRL levels are reported to elevate in a number of salmonid species, including rainbow trout, adapted to freshwater (Prunet et al., 1985, 1990), hypophysectomised rainbow trout are able to survive in freshwater (Donaldson and McBride, 1967; Komourdjian and Idler, 1977). Second, current literature suggests that the role of PRL in salmonid fish osmoregulation is a complex issue (for reviews see Bern and Madsen, 1992; McCormick, 1995). A good example of this is the reported response of gill $Na^+–K^+$-ATPase to PRL treatment. PRL has been reported to increase (Boeuf et al.,

1994), reduce (Madsen and Bern, 1992), and have no effect (McCormick et al., 1991) on the activity of this enzyme in the gill tissues of various salmonid species. These differences in response have been suggested to stem from the use of heterologous hormones, species differences or developmental differences (McCormick, 1995). Therefore, conflicting results highlight a pressing need to evaluate the direct actions of PRL on the physiology of osmoregulatory surfaces of salmonid fish.

2. Materials and methods

2.1. Preparation of cultured branchial epithelia

All gill cell cultures were derived from locally obtained stocks of rainbow trout (*Oncorhynchus mykiss*) (80–175 g) held in dechlorinated running tap water (composition: $[Na^+] = 0.55$, $[Cl^-] = 0.70$, $[Ca^{2+}] = 1.00$, $[Mg^{2+}] = 0.15$, and $[K^+] = 0.05$ mM, pH 7.8–8.0) at seasonal temperatures (13–17 °C). Procedures for gill cell isolation were conducted using sterile techniques according to methods originally developed by Pärt et al. (1993). Methods for single seeded insert (SSI) preparations composed of pavement cells only were based on those originally described by Wood and Pärt (1997) while double seeded insert (DSI) preparations composed of both pavement and mitochondria-rich cells were prepared according to methods originally outlined by Fletcher et al. (2000). Full details and methodology for the preparation and culture of both types of epithelia can be found in Kelly et al. (2000). Cultures were maintained and all experiments were performed at 18 °C. In brief, SSI preparations were first grown in flask culture for 6–7 days, which eliminated MR cells, then trypsinated, and reseeded onto filter inserts for a further 6–8 days. DSI preparations were grown by seeding freshly isolated cells directly onto filter inserts on two successive days, from two separate fish. This approach allowed MR cells to be incorporated into the epithelia, which were again allowed to grow for 6–8 days.

2.2. Hormone treatment

Single use aliquots of stock hormone solutions were prepared by dissolving either ovine prolactin (oPRL, Sigma) or recombinant rainbow trout prolactin (rbtPRL, supplied by F. Rentier-Delrue, Université de Liège, Belgium) in PBS (pH 7.7) to achieve a final concentration of 0.2–0.5 mg/ml. Aliquots of stock hormone solutions were stored at −70 °C until use. Prior to addition to epithelial preparations, stock hormone solutions were thawed and diluted in Leibowitz's L15 media (L15 plus 2 mmol/L glutamine, 6% FBS with or without antibiotics as appropriate cf. Kelly et al., 2000) so as to be added fresh on each media change. Thus,

PRL was renewed every 24–48 h. In SSI epithelia, three concentrations of oPRL (10, 50, and 100 ng/ml) and two concentrations of rbtPRL (1 and 10 ng/ml) were assessed. Hormone treatment commenced immediately after first seeding the cells into culture flasks and was continued during culture on filter inserts. Based on our observations from SSI epithelia, one dose of oPRL (50 ng/ml) was used in DSI preparations and media were supplemented with oPRL upon first seeding cells onto filter inserts. Therefore, cells comprising SSI epithelia were treated with oPRL for 12–15 days, prior to experimental manipulation whereas cells comprising DSI epithelia were treated for 8–10 days, prior to experimental manipulation. In both SSI epithelia, after cells were reseeded onto filter inserts, and DSI epithelia, PRL was supplemented on the basolateral side of the preparation only. The media on the apical side contained no PRL. Cells and epithelia bathed in media without the addition of supplemental PRL were assigned as controls (0 ng/ml oPRL or rbtPRL). All doses of PRL were selected based on reported levels of circulating PRL in freshwater rainbow trout (10–30 ng/ml, Prunet et al., 1985). Only the highest dose of 100 ng/ml oPRL greatly exceeded physiological levels for freshwater rainbow trout. The biological activity of rbtPRL has previously been reported (Le Rouzic et al., 2001).

2.3. Electrophysiological measurements

Measurements of transepithelial resistance (TER) were recorded using chopstick electrodes (STX-2) connected to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL, USA) and multiplied by epithelial surface area (0.9 cm^2) after correcting for "background" TER using vacant inserts containing appropriate solutions. After reseeding cells onto filter inserts, measurements of TER under symmetrical conditions (i.e., with culture media on both sides) were recorded daily from 48 h onwards. Under asymmetrical conditions (i.e., freshwater added to the apical side) TER and/or TEP measurements were conducted at appropriate time intervals (see Section 3). Measurements of transepithelial potential (TEP) were conducted using agar-salt bridges (3 M KCl in 4% agar) connected through Ag/AgCl electrodes (World Precision Instruments) to a high impedance electrometer (Radiometer pHM 84, Copenhagen, Denmark). After correction for junction potential, TEP measurements were expressed relative to the apical side as 0 mV.

2.4. [³H]PEG-4000 permeability

The paracellular permeability of the cultured epithelia was measured using a paracellular permeability marker, [³H]polyethylene glycol (molecular mass 4000 Da; 'PEG-4000', NEN-Dupont) according to the methods and equations previously outlined by Wood et al. (1998). Paracellular permeability was determined in the efflux (basolateral to apical) direction only, after the addition of 1 μCi [³H]PEG-4000 to basolateral culture media. The appearance of PEG-4000 in the apical compartment was determined at time intervals outlined in Section 3, and during [³H]PEG flux periods, TER was closely monitored at appropriate time intervals.

2.5. Net ion flux measurements

The effect of PRL treatment on net Na$^+$ flux rates under asymmetrical conditions (directly measured $J_{net}^{Na^+}$ without the use of isotopes) was assessed by measuring the net Na$^+$ appearance in the apical compartment using several series of epithelia over 6–24 h periods, with methods and calculations identical to those outlined by Wood et al. (1998). By convention, a net flux from basolateral to apical compartments ("equivalent" to a net loss from an intact animal) carries a negative sign.

2.6. Unidirectional ion flux measurements

In several series of control (no hormone treatment) and PRL treated epithelia, unidirectional ion flux rates (employing radiotracers) were determined according to the methods and calculations of Wood et al. (1998). For both influx (apical to basolateral: positive sign) and efflux (basolateral to apical: negative sign) studies, 1 μCi isotope (²²Na or ³⁶Cl, NEN-Dupont) was added to the apical or basolateral side of the culture and the appearance of radioactivity was monitored on the "cold" side. Under both symmetrical and asymmetrical conditions, preparations were used for either influx or efflux measurements only and matched for calculations of the Ussing flux ratio criterion based on criteria of electrophysiological similarity (see Fletcher et al., 2000). Each insert was used only once in the pairing procedure. Measurements of TER and TEP were taken at the beginning, middle (3 h), and end of each flux period (6 h).

2.7. Ussing flux ratio criterion

Disagreement of the measured flux ratio (J_{in}/J_{out}) with the prediction of the Ussing flux ratio was employed as an indication of active transport (Kirschner, 1970). The predicted Ussing flux ratio was calculated as

$$\frac{J_{in}}{J_{out}} = \frac{A_{Ap}\, e^{-(zFV/RT)}}{A_{Bl}}, \tag{1}$$

where A_{Ap} and A_{Bl} are the activities of the ions (Na$^+$ and Cl$^-$) on the apical and basolateral sides, z is the ionic valence, V is the measured TEP in volts (average for matched inserts), and F, R, and T have their usual thermodynamic values. In media, A_{Na} and A_{Cl} were 75% of measured concentrations (cf. Wood et al., 1998).

Under asymmetrical conditions, A_{Na} and A_{Cl} in apical freshwater were taken as equal to the measured concentrations.

2.8. Na^+–K^+-ATPase activity

Na^+–K^+-ATPase activity in individual inserts was measured as described by McCormick (1993), with slight modifications. After mild trypsination (≈ 2 min) to obtain a cell suspension, trypsination was terminated using a "stop" solution (10% fetal bovine serum in phosphate-buffered saline, pH 7.7) and the cell suspension was centrifuged (Beckman J-21C, 0–4 °C) for 10 min at $500g$ to obtain a cell pellet. The cell pellet was resuspended, washed in phosphate-buffered saline (PBS, pH 7.7), and centrifuged again for a further 10 min at $500g$. After removing the PBS, SEI buffer (150 mM sucrose, 10 mM EDTA, and 50 mM imidazole: pH 7.3, 0–4 °C) was added to the cell pellet and the preparation was quick-frozen in liquid nitrogen. Cells were stored at -70 °C until further analysis.

Prior to analysis, samples were thawed and a volume of SEID buffer (0.5 g sodium deoxycholate in 100 ml SEI) was added, resulting in a final SEI to SEID ratio of 4:1. Cells were sonicated (Branson, Sonifier 450) on ice for 30 s centrifuged (Eppendorf 5415C, 0–4 °C) at $5000g$ for 1 min and the resulting supernatant was immediately analysed for Na^+–K^+-ATPase activity. Due to the low amount of protein available, the assay was run for 40 min at 24 °C, however, the enzyme activity was linear throughout this period. The protein content of supernatants was measured by the Bradford method (Sigma) using bovine serum albumin (Sigma) as a standard. All enzyme activities are expressed as protein-specific activities.

2.9. Statistical analysis

All data are expressed as mean values $\pm$ SE (n), where n represents the number of filter inserts. For comparisons between varying groups, data were either subjected to repeated One-Way Analysis of Variance or a Two-Way Analysis of Variance (Sigmastat software, Jandel Scientific) as appropriate. Significant differences ($P \leqslant 0.05$) between groups were detected using either a Student–Newman–Keuls test or Student unpaired or paired t tests as appropriate (Sigmastat software, Jandel Scientific).

3. Results

3.1. Treatment of pavement cell epithelia (SSI epithelia) with varying doses of oPRL

After 6 days in culture, the transepithelial resistance (TER) of SSI epithelia treated with varying doses of oPRL under symmetrical culture conditions (L15 apical/

L15 basolateral) did not significantly differ with a mean value for all preparations of 1.00 ± 0.08 kΩ cm^2 ($n = 40$). In the same preparations, PEG-4000 permeability, determined over a 12-h flux period, also did not significantly vary between different oPRL dose levels, with a mean value of 5.18 ± 0.29 cm s^{-1} $\times$ 10^{-7} ($n = 40$).

Immediately after the introduction of asymmetrical culture conditions (freshwater apical/L15 basolateral), all epithelia exhibited a rapid large increase in TER (Fig. 1). A peak in TER (10–22 $\times$ symmetrical levels) was observed in preparations 3 h after exposure to apical freshwater. This peak was greatest in the order of 50 > 10 > 100 > 0 ng/ml oPRL; however, only preparations treated with 10 and 50 ng/ml oPRL exhibited significantly greater ($P < 0.05$) TER than control groups (Fig. 1). After 12-h exposure to apical freshwater, TER had declined substantially to 3–4 $\times$ symmetrical levels and was more or less stable through 24 h. At 12 h, no significant difference could be observed between groups; however, after 24 h, epithelia treated with 10 and 50 ng/ml oPRL exhibited a slight but significantly elevated TER when compared to both control and 100 ng/ml oPRL-treated preparations. When measured over the 24 h exposure period to apical freshwater, there were no significant differences in paracellular permeability (as measured with PEG-4000), which averaged 6.01 ± 0.19 cm s^{-1} $\times$ 10^{-7} ($n = 40$), a small but significant ($P < 0.05$) increase from the mean value under symmetrical conditions (Fig. 2a). However, net Na^+ flux was lower in 10 and 50 ng/ml oPRL-treated epithelia (Fig. 2b).
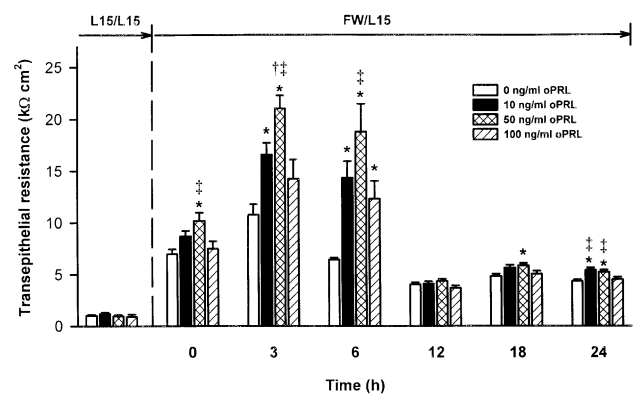

Fig. 1. Effect of ovine prolactin (oPRL) on changes in transepithelial resistance (TER) of pavement cell (SSI) epithelia over time under asymmetrical (freshwater apical/L15 basolateral) culture conditions. Data are expressed as mean values $\pm$ SE ($n = 9$–11). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng/ml oPRL) and oPRL-treated epithelia. † denotes significant difference ($P < 0.05$) between epithelia treated with either 50 or 10 ng/ml oPRL and ‡ denotes significant difference ($P < 0.05$) between epithelia treated with either 50 or 100 ng/ml oPRL. All increases in TER throughout the experiment (freshwater apical/L15 basolateral) were significant ($P < 0.05$), relative to the pre-exposure condition (L15/L15).
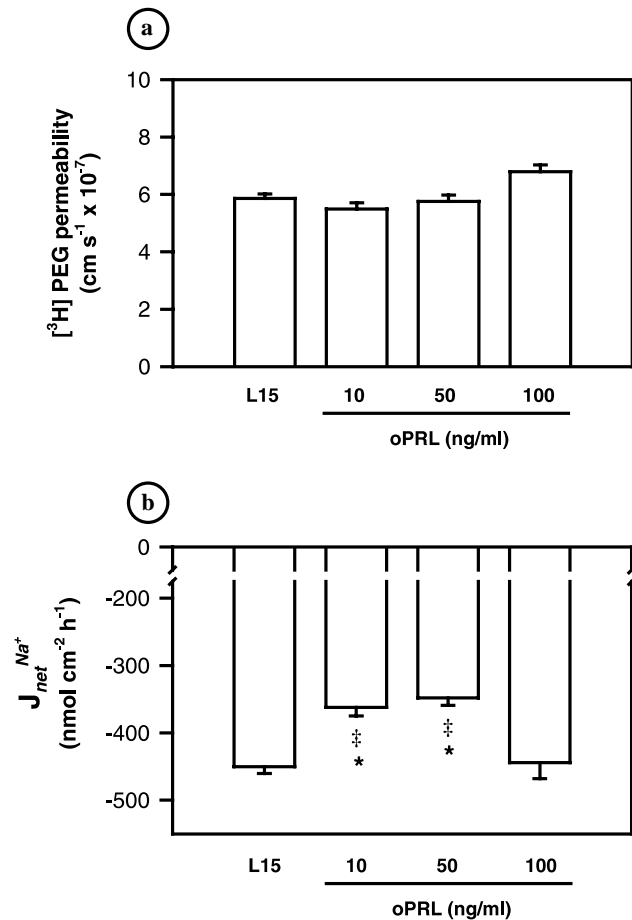
Fig. 2. Effect of ovine prolactin (oPRL) on (a) [^{3}H]PEG-4000 permeablility and (b) net Na$^+$ flux (basolateral to apical, over 24 h) in pavement cell (SSI) epithelia under asymmetrical culture conditions. Data are expressed as mean values ± SE ($n = 9$–11). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng/ml oPRL) and oPRL-treated epithelia while ‡ denotes significant difference ($P < 0.05$) between 10 and 50 ng/ml oPRL-treated epithelia and 100 ng/ml oPRL-treated epithelia.

3.2. Treatment of pavement cell (SSI) epithelia with oPRL or rbtPRL and short-term exposure to apical freshwater

Based on our observations from the above experiment, a second set of experiments were conducted to establish (1) whether the absence of an oPRL effect on paracellular permeability was due to prolonged exposure (24 h) to apical freshwater (i.e., whether high paracellular permeability during the final 12 h of freshwater exposure in the former experiment may have masked any observable effect occurring during the first 12 h of exposure when TER differences were at their greatest) and (2) whether recombinant rainbow trout PRL (rbtPRL) could elicit the same response as oPRL in cultured pavement cells.

The effect of oPRL-treatment (50 ng/ml) in a second series of epithelia was similar to those previously

observed. After 6 days of growth, no differences were observed between the TER (control = $0.98 \pm 0.09\,k\Omega$ cm^2, $n = 3$; oPRL-treated = $1.09 \pm 0.23\,k\Omega$ cm^2, $n = 4$) or paracellular permeability (control = 4.79 ± 0.28 cm s$^{-1} \times 10^{-7}$, $n = 3$; oPRL-treated = 4.84 ± 0.49 cm s$^{-1} \times 10^{-7}$, $n = 4$) of preparations under symmetrical culture conditions. Upon exposure to apical freshwater, epithelia treated with 50 ng/ml oPRL exhibited a significantly greater increase in TER and lower net Na$^+$ flux (basolateral to apical; control = -244.6 ± 7.4 nmol cm^{-2} h^{-1}, $n = 3$; oPRL-treated = -197.1 ± 2.3 nmol cm^{-2} h^{-1}, $n = 4$) than did untreated epithelia, but no difference in paracellular permeability, which was slightly elevated in both groups (Fig. 3). After 12 h exposure to apical freshwater, epithelia were returned to symmetrical culture conditions; however, again, no differences in TER or paracellular permeability could be observed between control and oPRL-treated preparations (Fig. 3).

As with oPRL, treatment of epithelia with 1 ng/ml rbtPRL had no significant effect on TER or paracellular permeability under symmetrical culture conditions (Fig. 4a). However, 1 ng/ml rbtPRL treatment resulted in no significant differences in TER, paracellular permeability or net Na$^+$ flux (basolateral to apical; control = -221.5 ± 33.2 nmol cm^{-2} h^{-1}, $n = 4$; 1 ng/ml rbtPRL-treated = -208.8 ± 11.6 nmol cm^{-2} h^{-1}, $n = 4$) over the 12 h exposure period to apical freshwater (Fig. 4a). The absence of any significant effect of rbtPRL
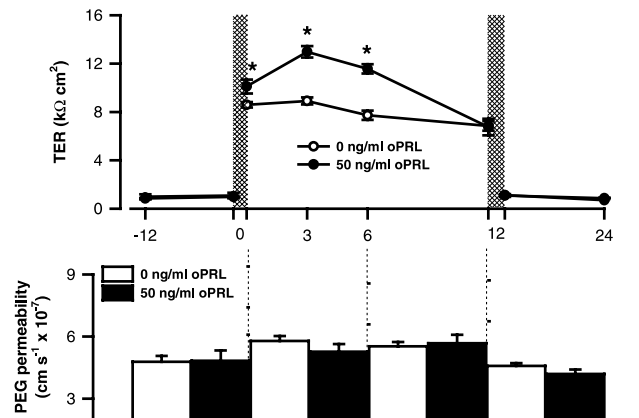

Fig. 3. Effect of ovine prolactin (oPRL) and short-term exposure to asymmetrical culture conditions on (top panel) transepithelial resistance (TER) and (bottom panel) corresponding [^{3}H]PEG-4000 permeability in pavement cell (SSI) epithelia. Data are expressed as mean values ± SE ($n = 3$–4). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng/ml oPRL) and oPRL-treated (50 ng/ml) epithelia. Relative to pre-exposure conditions, all increases in TER during apical freshwater exposure were significant ($P < 0.05$) but changes in PEG-4000 permeability were not. Under asymmetrical conditions, net Na$^+$ flux rates (basolateral to apical) were significantly different ($P < 0.05$); (control = -244.6 ± 7.4, oPRL = -197.1 ± 2.3 nmol cm^{-2} h^{-1}).
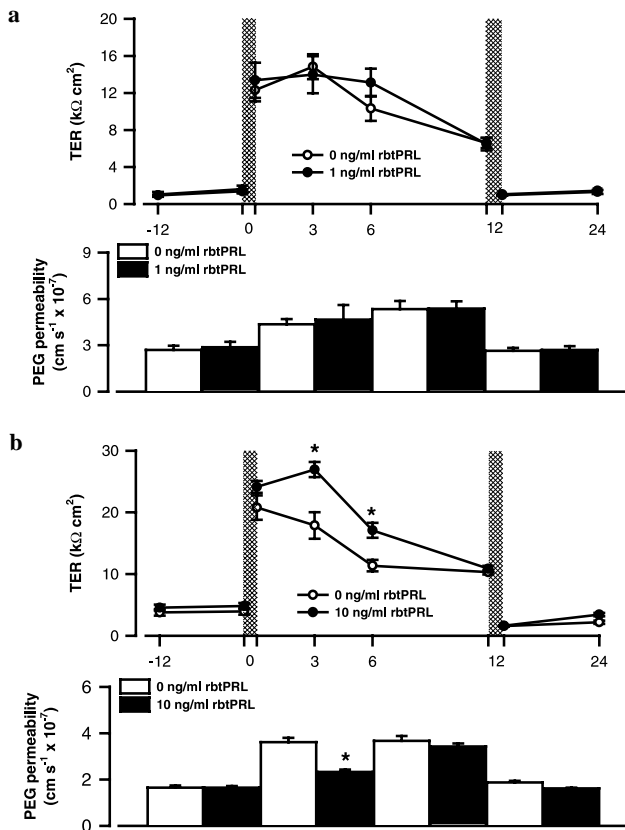
Fig. 4. Effect of (a) 1 ng/ml and (b) 10 ng/ml rainbow trout prolactin (rbtPRL) and short-term exposure to asymmetrical culture conditions on (top panels a and b) transepithelial resistance (TER) and (bottom panels a and b) corresponding [^{3}H]PEG-4000 permeability in pavement cell (SSI) epithelia. Data are expressed as mean values $\pm$ SE ($n = 4$–6). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng/ml oPRL) and rbtPRL-treated (1 or 10 ng/ml) epithelia. Relative to the pre-exposure conditions, all increases in TER were significant ($P < 0.05$) while all increases in PEG-4000 permeability, with the exception of 0–6 h 1 ng/ml rbtPRL, were also significant ($P < 0.05$). Under asymmetrical conditions, net Na$^+$ flux rates (basolateral to apical) for (a) were not significantly different (control = -221.5 ± 33.2, 1 ng/ml rbtPRL = -208.8 ± 11.6 nmol cm^{-2} h^{-1}) while net Na$^+$ flux rates (basolateral to apical) for (b) were significantly different ($P < 0.05$) (control = -204.9 ± 9.1, rbtPRL = -169.4 ± 4.3 nmol cm^{-2} h^{-1}).

under symmetrical culture conditions could also be observed in preparations treated with 10 ng/ml rbtPRL. However, the response of 10 ng/ml rbtPRL-treated epithelia to freshwater was similar to that observed in epithelia treated with oPRL (Fig. 4b). That is, TER was significantly greater, paracellular permeability remained unaltered at least during the 6–12 h period of exposure and net Na$^+$ flux was significantly reduced (basolateral to apical; control = -204.9 ± 9.1 nmol cm^{-2} h^{-1}, $n = 6$; 10 ng/ml rbtPRL-treated = -169.4 ± 4.3 nmol cm^{-2} h^{-1}, $n = 6$). During the 0–6 h period of exposure to apical freshwater, paracellular permeability was significantly lower in 10 ng/ml rbtPRL-treated inserts (Fig. 4b).

3.3. Unidirectional Na$^+$ flux and Ussing flux ratios in PRL-treated pavement cell epithelia (SSI epithelia)

Unidirectional Na$^+$ flux experiments were conducted on pavement cell epithelial preparations treated either with 50 ng/ml oPRL or 10 ng/ml rbtPRL. Under symmetrical culture conditions, the electrophysiological characteristics of pavement cell epithelia treated with oPRL were consistent with those observed in previous experiments (Table 1). In both control and oPRL-treated preparations, Na$^+$ movement was approximately balanced in both directions so the net flux was not significantly different from zero. The Ussing flux ratio criterion revealed that neither control or oPRL-treated epithelia exhibited active Na$^+$ transport (Table 1).

Under asymmetrical conditions, unidirectional Na$^+$ flux experiments were conducted over two 6 h time periods. The first was immediately after the introduction of apical freshwater and the second, after epithelia had been exposed to asymmetrical conditions for 18 h. During these apical freshwater exposures, unidirectional influx rates were reduced to about 1% of those occurring in symmetrical media (reflecting the decrease in apical Na$^+$ concentration), while unidirectional efflux rates were modestly elevated relative to those in symmetrical media. As a result, net flux became negative and was approximately equal to the unidirectional efflux rate. During the 0–6 h flux period, oPRL-treated epithelia again exhibited a significantly elevated TER ($P < 0.05$) with a decrease in the Na$^+$ efflux and net flux rate, although this effect escaped significance ($P = 0.08$; Table 1). There was no stimulatory effect of oPRL on unidirectional Na$^+$ influx rates. The Ussing flux ratio criterion revealed no difference in the pattern of Na$^+$ movement with both control and oPRL-treated preparations exhibiting J_{in}/J_{out} ratios significantly less than that predicted, suggestive of active Na$^+$ extrusion. Exposing epithelia for longer time periods to asymmetrical conditions (18–24 h) did not alter this pattern of Na$^+$ movement (Table 1). However, Na$^+$ influx, efflux, and net flux rates increased slightly in both preparations relative to rates at 0–6 h, which were in line with a general decrease in TER. Again, Na$^+$ efflux and net flux rates tended to be lower in oPRL-treated epithelia (Table 1).

Epithelia treated with rbtPRL (10 ng/ml) exhibited similar results over a 6-h flux period under asymmetrical conditions with predicted and observed flux ratios ($\times 10^{-3}$) of 14.49 ± 0.57 and 5.04 ± 0.60 ($P < 0.05$), respectively (TER = 14.37 ± 1.51, TEP = -11.40 ± 0.60, $n = 5$). These values were consistent with those observed for epithelia treated with oPRL and indicated that the pattern of Na$^+$ movement in rbtPRL-treated epithelia was identical to that observed in oPRL-treated epithelia.

Table 1
Comparison between Na^+ flux rates, Ussing flux ratios (J_{in}/J_{out}) and electrophysiological characteristics of control and oPRL-treated SSI (pavement cell) epithelia under symmetrical (L15 apical/L15 basolateral) and asymmetrical (freshwater apical/L15 basolateral) culture conditions

	Control	oPRL (50 ng/ml)
Symmetrical (L15/L15)		
Influx ($nmol\,cm^{-2}\,h^{-1}$)	129.63 ± 11.76	118.23 ± 7.66
Efflux ($nmol\,cm^{-2}\,h^{-1}$)	-137.66 ± 4.88	-117.53 ± 10.31
Net flux ($nmol\,cm^{-2}\,h^{-1}$)	-2.46 ± 11.01	0.71 ± 6.64
Flux ratio		
Predicted	0.993 ± 0.002	1.012 ± 0.004
Observed	0.980 ± 0.082	1.028 ± 0.065
TER ($k\Omega\,cm^2$)	3.97 ± 0.17	4.17 ± 0.28
TEP (mV)	0 ± 0	0 ± 0
Asymmetrical (FW/L15) T0–6 h		
Influx ($nmol\,cm^{-2}\,h^{-1}$)	0.85 ± 0.05	0.65 ± 0.06
Efflux ($nmol\,cm^{-2}\,h^{-1}$)	-181.78 ± 23.30	-131.19 ± 13.10
Net flux ($nmol\,cm^{-2}\,h^{-1}$)	-180.94 ± 23.31	-130.54 ± 13.06
Flux ratio ($\times 10^{-3}$)		
Predicted	19.90 ± 0.46	17.37 ± 0.24
Observed	$4.90 \pm 0.42^*$	$6.72 \pm 0.56^*$
TER ($k\Omega\,cm^2$)	15.28 ± 1.15	19.46 ± 1.52†
TEP (mV)	-9.25 ± 0.38	-9.69 ± 0.44
Asymmetrical (FW/L15) T18–24 h		
Influx ($nmol\,cm^{-2}\,h^{-1}$)	1.54 ± 0.07	1.23 ± 0.09
Efflux ($nmol\,cm^{-2}\,h^{-1}$)	-235.17 ± 24.52	-190.99 ± 5.19
Net flux ($nmol\,cm^{-2}\,h^{-1}$)	-234.17 ± 24.50	-188.75 ± 4.25
Flux ratio ($\times 10^{-3}$)		
Predicted	23.47 ± 0.33	21.06 ± 0.52
Observed	$6.81 \pm 0.59^*$	$6.46 \pm 0.39^*$
TER ($k\Omega\,cm^2$)	10.55 ± 0.50	11.42 ± 0.46
TEP (mV)	-8.31 ± 0.17	-6.53 ± 0.11

†Denotes significant difference ($P < 0.05$) between control and oPRL-treated epithelia while * denotes significant difference ($P < 0.05$) between predicted and observed flux ratio. All data are expressed as mean values $\pm$ SE ($n = 7$ for all groups).

3.4. Unidirectional Na^+ and Cl^- flux and Ussing flux ratios in oPRL-treated DSI epithelia containing pavement and mitochondria-rich (MR) cells

Under symmetrical culture conditions, oPRL had no significant effect on TER, TEP, or Na^+ or Cl^- flux rates in DSI epithelia (Table 2, Fig. 5). Relative to SSI epithelia, all DSI epithelia exhibited a characteristically much higher TER (28–30 kΩ cm^2) and a basolateral positive TEP (+9 to +11 mV; cf. Fletcher et al., 2000; Kelly et al., 2000). Consistent with the development of a

high TER were the much lower Na^+ and Cl^- unidirectional flux rates observed under symmetrical culture conditions (Fig. 5 versus Table 1). Net Na^+ flux rates were approximately zero, while net Cl^- flux rates were significantly different ($P < 0.05$) from zero in the inward direction (apical to basolateral). However, in light of the positive potential on the basolateral side, the Ussing flux ratio criterion indicated that both control and oPRL-treated preparations exhibited passive Cl^- movement while Na^+ was actively transported in the inward direction (Table 2).

Under asymmetrical conditions, unidirectional flux measurements were, again, conducted over two 6-h time periods as previously described for SSI epithelia. Characteristic of these high resistance DSI preparations (cf. Fletcher et al., 2000), TER increased slightly (but significantly, $P < 0.05$) upon apical freshwater exposure at 0–6 h and subsequently declined below pre-exposure levels at 18–24 h (Table 2). TEP changed from positive values (+9 to +11 mV) under symmetrical conditions to negative values (−4 to −5 mV) under asymmetrical conditions at both measurement periods. During the 0–6 h flux period, ion efflux rates increased relative to rates found under symmetrical conditions while influx rates diminished to just a few percent of those previously observed (Fig. 6). Resulting Na^+ and Cl^- net flux rates were not significantly different from unidirectional efflux rates. While no significant effect of oPRL was observed on Na^+ efflux or net flux rates, Cl^- efflux and net flux rates were significantly lower in oPRL-treated epithelia during this time period. The Ussing flux ratio criterion indicated active Cl^- transport in the inward direction in both control and oPRL-treated DSI epithelia while Na^+ flux tended toward active transport in the outward direction (Table 2).

During the 18–24 h time period of asymmetrical exposure, outwardly directed Na^+ and Cl^- flux rates increased (Fig. 6). This was consistent with a general decrease in TER in both control and oPRL-treated epithelia (Table 2); however, the pattern of ion movement as revealed by the Ussing flux ratio criterion did not alter: active Cl^- uptake and Na^+ extrusion (Table 2). During this time period, no significant difference was observed between control and oPRL-treated preparations (Fig. 6).

3.5. Na^+–K^+-ATPase activity in cultured epithelia

The activity of Na^+–K^+-ATPase in cultured SSI epithelia (pavement cells only) was unaltered by the presence of oPRL. In all groups of pavement cell epithelia, activity levels were around 0.5 µmol ADP $mg^{-1}\,h^{-1}$ and this was not significantly different from that found in control DSI epithelia (Fig. 7). However, treatment of DSI epithelia (containing pavement cells plus MR cells) with 50 ng/ml oPRL resulted in a

Table 2
Comparison between Ussing flux ratios (J_{in}/J_{out}) for Na^+ and Cl^- and electrophysiological characteristics of control and oPRL-treated DSI epithelia under symmetrical (L15 apical/L15 basolateral) and asymmetrical (freshwater apical/L15 basolateral) culture conditions

	Control		oPRL (50 ng/ml)	
Symmetrical (L15/L15)				
Flux ratio	Predicted	Observed	Predicted	Observed
Na^+	0.71 ± 0.03	$1.00 \pm 0.07^*$	0.66 ± 0.03	$1.04 \pm 0.08^*$
Cl^-	1.44 ± 0.06	1.49 ± 0.14	1.60 ± 0.10	1.56 ± 0.23
TER ($k\Omega\ cm^2$)	28.60 ± 1.41		30.15 ± 1.03	
TEP (mV)	$+9.05 \pm 1.04$		$+10.88 \pm 1.38$	
Asymmetrical (FW/L15) T0–6 h				
Flux ratio	Predicted ($\times 10^{-3}$)	Observed ($\times 10^{-3}$)	Predicted ($\times 10^{-3}$)	Observed ($\times 10^{-3}$)
Na^+	13.90 ± 0.16	10.95 ± 1.58	13.19 ± 0.26	$8.19 \pm 0.72^*$
Cl^-	12.38 ± 0.18	$16.64 \pm 1.70^*$	12.75 ± 0.16	$17.72 \pm 1.81^*$
TER ($k\Omega\ cm^2$)	32.67 ± 0.62†		32.52 ± 0.64	
TEP (mV)	-4.69 ± 0.31		-4.13 ± 0.32	
Asymmetrical (FW/L15) T18–24 h				
Na^+	14.54 ± 0.21	$9.08 \pm 0.58^*$	14.67 ± 0.15	$8.07 \pm 0.32^*$
Cl^-	13.00 ± 0.17	$23.22 \pm 1.56^*$	13.64 ± 0.28	$22.49 \pm 0.82^*$
TER ($k\Omega\ cm^2$)	20.14 ± 0.59 † ‡		18.71 ± 0.97 † ‡	
TEP (mV)	-4.01 ± 0.41		-4.19 ± 0.23	

*Denotes significant difference ($P < 0.05$) between predicted and observed flux ratio. †Denotes significant difference ($P < 0.05$) between symmetrical and asymmetrical conditions while ‡ denotes significant difference ($P < 0.05$) between 0–6 h and 18–24 h asymmetrical conditions. All data are expressed as mean values ± SE ($n = 14$ for all groups).

significant increase in Na^+–K^+-ATPase activity when compared to both control DSI epithelia and SSI epithelia treated with 50 ng/ml oPRL (Fig. 7). Epithelial protein levels (μg epithelia^{-1}) in SSI preparations were as follows; L15 control = 68.9 ± 3.6 ($n = 11$), 10 ng/ml oPRL = 72.7 ± 4.8 ($n = 9$), 50 ng/ml = 84.8 ± 2.9 ($n = 9$), and 100 ng/ml = 78.80 ± 2.2 ($n = 11$). The protein content of SSI epithelia treated with 50 ng/ml oPRL was significantly different from control values. No other significant differences could be detected between SSI preparations. In DSI epithelia, protein levels (μg epithelia^{-1}) were L15 control = 55.4 ± 2.1 ($n = 24$) and oPRL (50 ng/ml) = 60.6 ± 1.3 ($n = 24$), respectively. DSI protein values were significantly different.

4. Discussion

Despite the important role PRL is thought to have in teleost fish osmoregulation and the reported presence of PRL receptors in the gill tissue of selected fish species (Dauder et al., 1990; Prunet and Auperin, 1994), few studies have examined the in vitro effects of prolactin (PRL) on the physiology of freshwater gill tissue or freshwater surrogate gill models. Furthermore, in vivo studies have generally focused on the potential effects of PRL on plasma composition and/or chloride cells and associated biochemical correlates (Herndon et al., 1991; Kelly et al., 1999; Madsen and Bern, 1992) while no study has attempted to examine the potential effect of PRL exclusively on pavement cell function.

In the current study, we were unable to observe any effect of PRL on epithelia cultured under symmetrical conditions. This is in contrast to the reported effects of other hormones such as cortisol and 3,5',3'-triiodo-L-thyronine, which alter the physiological status of cultured gill epithelia under similar conditions (Kelly and Wood, 2001a,b). In other ion transporting epithelia, such as those derived from mouse mammary cells, PRL (together with supplemental bovine serum and insulin) has also been reported to have no effect on the electrical characteristics of preparations grown symmetrically on permeable filter supports (Zettl et al., 1992). However, PRL has been reported to alter the electrical characteristics and Na^+ transporting properties of confluent monolayers of mouse mammary epithelia (symmetrically bathed with Hanks' solution) when added in addition to supplemental insulin and cortisol (Bisbee et al., 1979; Bisbee, 1981).

Under asymmetrical conditions, the response of cultured pavement cell epithelia to PRL treatment appeared to be more consistent with the reported effects of PRL on the osmoregulatory surfaces of intact teleost fish in vivo (see Hirano, 1986). That is, pavement cell epithelia exhibited an increase in TER, a reduction in permeability, and thus, a limitation of net Na^+ movement from the basolateral to apical side of the preparation. This reduction in permeability appeared to result from a decline in transcellular and not paracellular permeability as PEG-4000 permeability was not affected. PRL-induced reductions in the permeability of epithelia in higher vertebrates are generally associated with a
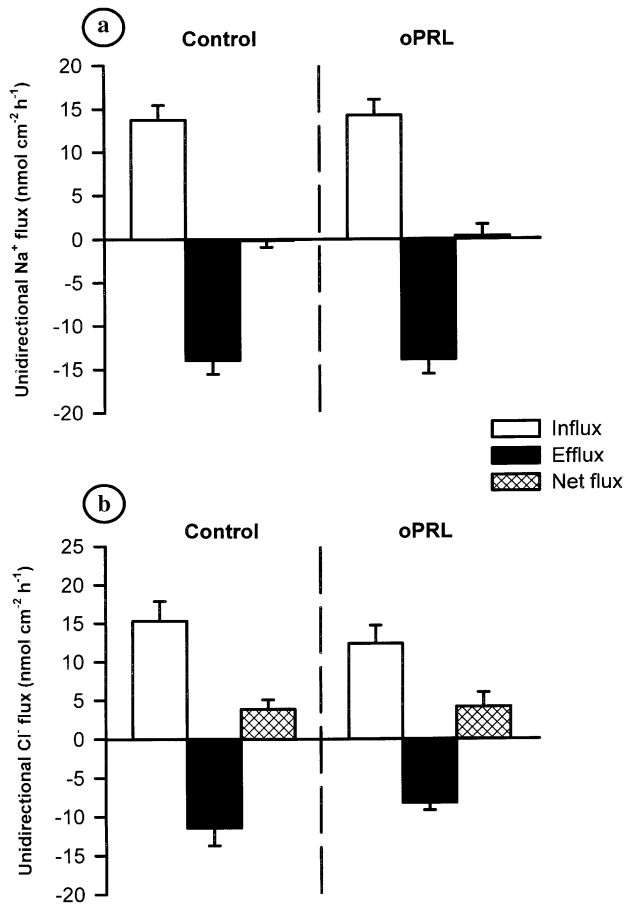
Fig. 5. Unidirectional fluxes of (a) Na^+ and (b) Cl^- in control (0 ng/ml oPRL) and oPRL-treated (50 ng/ml) DSI epithelia under symmetrical culture conditions (L15 apical/L15 basolateral). Open, solids and hatched bars represent ion influx, efflux, and net flux, respectively. Data are expressed as mean values $\pm$ SE ($n = 14$). No significant difference ($P > 0.05$) was found between treatments.

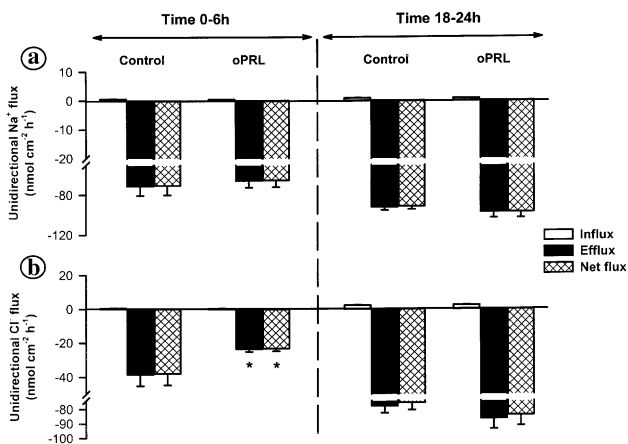Fig. 6. Unidirectional fluxes of (a) Na^+ and (b) Cl^- in control (0 ng/ml oPRL) and oPRL-treated (50 ng/ml) DSI epithelia under asymmetrical culture conditions (freshwater apical/L15 basolateral). Open, solid, and hatched bars represent ion influx, efflux, and net flux, respectively. Data are expressed as mean values $\pm$ SE ($n = 14$). An asterisk denotes significant difference ($P < 0.05$) between control and oPRL-treated epithelia.

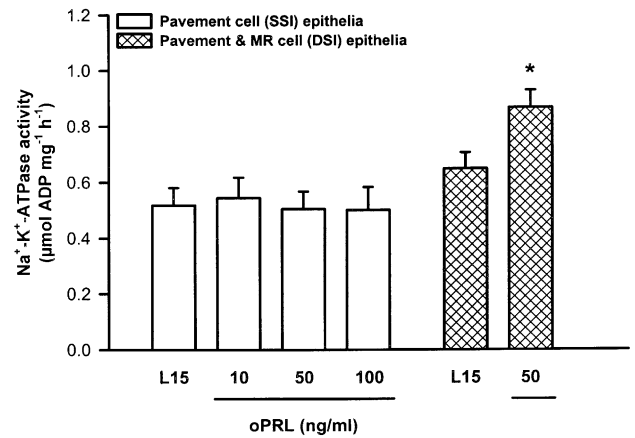Fig. 7. Effect of oPRL on Na^+–K^+-ATPase activity in cultured SSI and DSI gill epithelia after 24h asymmetrical (freshwater apical/L15 basolateral) exposure. Data are expressed as mean values $\pm$ SE ($n = 9$–24). An asterisk denotes significant difference ($P < 0.05$) between control (0 ng/ml oPRL) and oPRL-treated DSI epithelia.

reduction in the paracellular permeability in association with decreased tight junction permeability (Linzell et al., 1975; Stelwagen et al., 1999). Nevertheless, despite being able to limit passive movement of Na^+ in the "outward" direction, PRL was unable to alter the influx component or to alter the situation of apparent slight active extrusion of Na^+ observed in these preparations under asymmetrical culture conditions. There are two probable reasons for this lack of effect of PRL on active transport. First, the general role of PRL in hyperosmoregulating fish is thought to involve a reduction in passive ion and water permeability and not altered ion uptake (Bern and Madsen, 1992; Hirano, 1986). Second, many of the actions of PRL in teleost fish have been suggested to be permissive, requiring the presence of other hormones, especially cortisol (Hirano and Mayer-Gostan, 1978). In cultured gill epithelia, cortisol has been demonstrated to increase epithelial "tightness" principally by reducing paracellular permeability, presumably via the formation of tight junctions (Kelly and Wood, 2001a). Corticosteroid-induced formation of tight junctions in mouse mammary epithelia has also been reported (Zettl et al., 1992) and may, in part, explain the ability of PRL to increase the net Na^+ absorption in the presence of cortisol in these kind of preparations as described by Bisbee et al. (1979) and Bisbee (1981). In teleost fish, few studies have examined the potential actions of PRL and cortisol together on hyperosmoregulatory ability. Recently, however, Eckert et al. (2001) have suggested that both PRL and cortisol may be involved synergistically in the ion uptake in stenohaline freshwater catfish.

The specific reasons for a PRL-induced alteration in the physiological response of epithelia under asymmetrical culture conditions (specifically during the initial 12 h of exposure to FW and then later during the

18–24 h time period), and not under symmetrical conditions, are not clear. Most studies investigating the actions of PRL on gill tissue and overall osmoregulatory response involve a relatively long period of PRL treatment. While this is also the case in the current experiments, no previous studies have examined the immediate response of PRL-treated gill tissues to ionic/osmotic challenge in a manner similar to that employed with our cultured gill epithelial models. A rapid switch from symmetrical to asymmetrical culture conditions undoubtedly results in acute modulation of ion channels (e.g., by modulation of pavement cell Cl^- channels; see O'Donnell et al., 2001), transporters and, as has previously been demonstrated, paracellular permeability (Glimour et al., 1998; Wood et al., 1998). As paracellular permeability does not appear to be affected by PRL, in these experiments at least, it seems likely that under asymmetrical conditions PRL is exerting its effects on transcellular permeability. If these effects are related to changes in the activity or presence of channels and/or transporters that are osmotically/ionically sensitive than under isoosmotic conditions, we may not expect to see differences between PRL-treated and control (untreated) epithelia. However, the fact that PRL-induced differences can be seen only during a relatively short time period (i.e., 0–12 h after the introduction of apical freshwater) suggests that other factors may be needed to sustain cellular and epithelial integrity over longer periods of time. For example, cortisol is a hormone that potentially complements PRL-induced changes in transcellular permeability with changes in paracellular permeability (Kelly and Wood, 2001a).

In cultured pavement cell epithelia, $Na^+–K^+$-ATPase activity was unaltered by oPRL, suggesting that the oPRL-induced alterations in the physiology of these preparations did not involve modulation of the principal ionomotive enzyme of fish gills. These results are consistent with the observations of McCormick et al. (1991) who found that in vitro exposure of coho salmon gill tissue to PRL had no significant effect on the $Na^+–K^+$-ATPase activity. However, coho salmon gill tissue was undoubtedly composed of a heterogeneous cell population so it would appear that this is the first report of the effects (i.e., absence of action) of PRL solely on pavement cell $Na^+–K^+$-ATPase activity. We have previously demonstrated that treatment of cultured trout pavement cell epithelia with cortisol had no effect on the $Na^+–K^+$-ATPase activity, despite marked changes in the physiological status of the epithelia (Kelly and Wood, 2001a) whereas 3,5′,3′-triiodo-L-thyronine stimulated pavement cell $Na^+–K^+$-ATPase activity, a phenomenon that was further enhanced in the presence of cortisol (Kelly and Wood, 2001b). The activity of $Na^+–K^+$-ATPase in pavement cell epithelia tends to be low compared to most values reported for gill tissue (McCormick, 1995; McCormick and Bern, 1989). However, the present

values are consistent with our previous observations (Fletcher et al., 2000; Kelly and Wood, 2001a,b) and with values expected for an epithelium without MR cells (Kültz and Jürss, 1993; Sargent et al., 1975).

In contrast to the response of pavement cell epithelia (SSI) to oPRL, a definite increase in $Na^+–K^+$-ATPase activity was found in oPRL-treated DSI epithelia containing both pavement and MR cells, suggesting that the responsiveness is attributable to the MR cells. An increase in the $Na^+–K^+$-ATPase of gill tissues has previously been reported to occur in intact coho salmon treated with PRL (Boeuf et al., 1994); however, this is the first report to demonstrate a PRL-induced increase in gill tissue in vitro. Despite these observations, an increase in $Na^+–K^+$-ATPase activity did not appear to enhance transepithelial Na^+ or Cl^- transport.

In epithelia composed of pavement and MR cells, $Na^+–K^+$-ATPase activity was not significantly different from the activity found in epithelia composed of pavement cells only, consistent with the observations of Fletcher et al. (2000). This may seem counterintuitive, since $Na^+–K^+$-ATPase activity is normally higher in MR cells than in pavement cells (McCormick, 1995). However, few studies have examined $Na^+–K^+$-ATPase activity in surrogate gill models and those that have suggest that the relationship between MR cell presence and $Na^+–K^+$-ATPase activity in surrogate gill models may not be so simple. For example, Kültz et al. (1992) demonstrated that increasing environmental salinity (freshwater up to hypersaline conditions) increased MR cell numbers in the opercular epithelium of tilapia, yet no change in operculum $Na^+–K^+$-ATPase activity could be observed. Fish used in the same study also exhibited an increase in gill MR cell numbers in response to elevated environmental salinity, which was associated with an increase in gill $Na^+–K^+$-ATPase activity (Kültz et al., 1992).

The use of heterologous PRL for experiments in fish is often marred by potential heterosomatotropic effects; however, the Na^+ retaining effect of oPRL is clear in a number of teleost species, albeit at higher doses than those of homologous teleost hormones (Hasegawa et al., 1986). In the current study, the response of cultured epithelia to both oPRL and rbtPRL resulted in a similar physiological response and, interestingly, both oPRL and rbtPRL had effects on cultured gill epithelia at similar doses. In contrast, in vivo studies on rainbow trout adapted to 50% seawater have reported salmon PRL to be approximately 5–10 times more effective than oPRL at increasing plasma Na^+ levels (Hasegawa et al., 1986; Kawauchi et al., 1983). In the current study, oPRL doses (10–50 ng/ml) eliciting a physiological response consistent with the in vivo actions of PRL were not dissimilar to reported levels of circulating PRL in freshwater rainbow trout (approximately 10–30 ng/ml; Prunet et al., 1985). Furthermore, we were able to

observe a decrease in the efficacy of oPRL at higher doses (100 ng/ml), a phenomenon that is in line with the parabolic dose–response of a number of other teleost species to oPRL in vivo, although notably not rainbow trout (Hasegawa et al., 1986).

In cultured epithelia composed of both pavement and MR cells, almost no difference was observed in the movement of Na^+ across epithelia between control and oPRL-treated preparations during apical freshwater exposure. This contrasts with the effects of PRL on pavement cell epithelia and may seem surprising, given that the majority of the former are made up of pavement cells (cf. Fletcher et al., 2000). However, a clear distinction between the two preparations is the degree of epithelial "tightness" exhibited, prior to experimental manipulation. While pavement cell epithelia have a resting TER of approximately 1–4 kΩ cm^2 after 6–7 days in symmetrical culture conditions, DSI epithelia develop a much higher TER of approximately 30 kΩ cm^2, which does not increase upon apical freshwater exposure. These values are among some of the highest reported for lower vertebrate ion transporting epithelia (Reuss, 1992). Given such conditions, the effect of PRL may be masked by already low epithelial permeability. This suggests that the effect of PRL is one that is seen when epithelia exhibiting greater permeability are exposed to environmental perturbation. This would certainly be in line with the "freshwater adapting" effects of PRL as higher circulating levels in fish adapted/transferred from seawater to freshwater would facilitate a reduction in the permeability of "leaky" seawater gill epithelia. Foskett et al. (1982) reported that opercular tissue conductance (the inverse correlate of tissue resistance) in seawater adapted tilapia was reduced after PRL treatment.

In the current study, we have examined the direct action of PRL on the physiology of gill tissue derived from a fish species that has been demonstrated responsive to PRL treatment in vivo (Hasegawa et al., 1986; Kawauchi et al., 1983). However, the rainbow trout is by no means the most sensitive of teleosts to this hormone. It would therefore be particularly interesting to examine the effects of PRL in other more sensitive species such as *Fundulus* spp., given the inability of *Fundulus* to survive in freshwater without PRL (in contrast to rainbow trout, which are able to survive in freshwater after hypophysectomy). In addition, the potential effect of PRL on cultured gill epithelia in combination with other hormones, particularly cortisol, warrants further investigation. This avenue of research may be extremely fruitful given that; (1) cultured gill epithelia have been demonstrated to be very sensitive to cortisol treatment; (2) cultured gill epithelia respond to corticosteroid treatment in a manner very similar to models used for investigating the effect of PRL on ion transporting epithelia from higher vertebrates; (3) the actions of PRL appear to be exerted principally via transcellular pathways while cortisol has been demonstrated to strongly affect paracellular permeability and; (4) PRL has proven to modulate the electrical and ion transporting properties of ion transporting epithelia from higher vertebrates when combined with cortisol treatment.

Acknowledgments

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