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DILUTE CULTURE MEDIA AS AN ENVIRONMENTAL OR PHYSIOLOGICAL SIMULANT IN CULTURED GILL EPITHELIA FROM FRESHWATER RAINBOW TROUT

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SUMMARY

The electrophysiological and ion-transporting properties of cultured gill epithelia from freshwater (FW) rainbow trout were examined in the presence of dilute cell culture media as an environmental or physiological simulant. Gill epithelia were cultured on cell culture inserts under symmetrical conditions (L15 apical–L15 basolateral) for 6–7 d. The following experiments were then conducted. (1) To mimic a gradual lowering of environmental salinity, apical L15 medium was progressively diluted with FW (first to 2/3 L15 for 8 h and then to 1/3 L15 for 6 h) before the introduction of apical FW (FW apical–L15 basolateral, analogous to a fish in a natural FW environment). Dilute apical media had no significant effect on the electrophysiological properties of preparations compared with symmetrical culture conditions, and no evidence for active Na^+ or Cl^- transport was observed. Preparations subsequently exposed to apical FW exhibited a negative transepithelial potential and evidence of active Cl^- uptake and slight Na^+ extrusion. (2) To mimic the extracellular fluid dilution that occurs in euryhaline fish after abrupt transfer from saline to FW, the osmolality or ionic strength (or both) of basolateral media was reduced by 20–40% (using either FW or FW + mannitol) while simultaneously replacing apical media with FW. Under these conditions, Na^+ and Cl^- influx rates were low compared with efflux rates, while the Ussing flux ratio analysis generally indicated active Cl^- uptake and Na^+ extrusion. The Na^+–K^+ adenosine triphosphatase activity was not affected by alterations in basolateral osmolality. Our studies indicate that cultured trout gill epithelia are tolerant of media dilution from both the apical and the basolateral direction; however, neither treatment alone appeared to increase ion influx rates or stimulate active Na^+ uptake in cultured trout gill epithelia.

Key words: ion transport; pavement cells; mitochondria-rich cells; osmolality; Na^+–K^+ ATPase.

INTRODUCTION

The fish gill is a highly dynamic and multifunctional epithelial "barrier" directly separating the external (water) and internal (blood) environment. Anatomically, it is a complex organ comprising a heterogeneous cell population of various functionally specialized cell types (for review, *see* Wilson and Laurent, 2002). This anatomical and cellular heterogeneity makes the in situ study of gill epithelial physiology technically very challenging. Consequently, much of our knowledge in this area is derived from the whole-animal studies or the innovative use of surrogate gill models derived from various parts of the fish's cranium, epithelia that are architecturally simple (flat) but essentially composed of "gill" cells (Karnaky et al., 1977; Marshall, 1977; Foskett and Scheffey, 1982). Although surrogate gill models have been very successful in illuminating the ion transport characteristics of the marine teleost gill, they have generally proven less successful in doing the same for the freshwater (FW) teleost gill (*see* Foskett et al., 1981; Wood and Marshall, 1994; Burgess et al., 1998). In particular, simultaneous active Na^+ and Cl^- transport, which is inwardly directed across FW fish gills (apical FW to basolateral extracellular fluid), has yet to be demonstrated in any FW surrogate model at rates even remotely comparable with those found in vivo.

A growing interest in the culture of gill cells has resulted in the development of several techniques that allow the in vitro study of gill cell function in a more controlled and simplified manner (McCormick and Bern, 1989; Pärt et al., 1993; Fernandes et al., 1995; Witters et al., 1996). In particular, recent developments in techniques for the primary culture of gill cells as "reconstructed" flat epithelia have provided gill epithelial models that are similar to the surrogate systems described above. These epithelia faithfully replicate the passive permeability characteristics of the intact gill and have already provided new insight into the physiology of the FW fish gill (Wood and Pärt, 1997; Gilmour et al., 1998; Wood et al., 1998; Fletcher et al., 2000; Kelly and Wood, 2001a, 2002a). Yet, despite considerable progress, limitations remain (for review, *see* Wood et al., 2002). Primarily, under asymmetrical culture conditions analogous to those found in vivo—i.e., apical FW–basolateral culture medium (L15)—cultured gill epithelial models do not yet actively transport Na^+ and Cl^- in the inward direction at rates similar to those found in vivo (a limitation also found in surrogate gill models, *see* above).

Supplementing culture media with piscine ionoregulatory hormones (e.g., cortisol and prolactin) or homologous fish serum (Kelly and Wood, 2001b, 2002a, 2002b) has allowed us to favorably ma-

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nipulate net Na^+ and Cl^- movements by reducing the efflux components. However, these treatments have not radically increased active ion uptake under asymmetrical culture conditions. An alternative approach may be to manipulate culture conditions to simulate either gradual environmental "salinity" reduction or key aspects of the physiological response to environmental dilution. For example, to simulate pseudo–in vivo conditions, our current experimental approach involves a rapid single-step replacement of apical L15 with sterile FW (i.e., FW apical–L15 basolateral; e.g., Kelly et al., 2000). Although cultured gill epithelia are able to readily tolerate this acute change in apical media composition, such a rapid changeover from an isotonic environment to FW is not likely to be commonplace in nature. Indeed, when intact fish are experimentally subjected to such an acute FW challenge, severe internal physiological consequences are evident, such as simultaneous alterations in extracellular fluid composition (Jacob and Taylor, 1983; Morgan et al., 1997; Kelly and Woo, 1999; Marshall et al., 2000).

Therefore, two series of experiments were conducted to investigate the potential effects of more realistic scenarios of exposure to dilute media. First, simulated gradual environmental salinity reduction was achieved by progressively diluting apical media with FW—i.e., the introduction of asymmetrical culture conditions without acute replacement of apical L15 for FW. Therefore, pseudo–in vivo culture conditions (FW apical–L15 basolateral) were introduced gradually, more akin to a natural change in environmental salinity. Second, simulated extracellular fluid dilution accompanying environmental dilution was achieved by reducing basolateral media osmolality and ionic strength, or ionic strength alone, while simultaneously introducing apical FW (i.e., direct replacement of apical media with FW). We hypothesized that dilute media may mimic natural conditions to a higher degree and, potentially, stimulate increased ion uptake across cultured gill epithelia from FW rainbow trout.

Materials and Methods

Fish and holding conditions. Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local supplier and held in dechlorinated running Hamilton tap water (composition: $[Na^+]$ = 0.55, $[Cl^-]$ = 0.70, $[Ca^{++}]$ = 1.00, $[Mg^{++}]$ = 0.15, and $[K^+]$ = 0.05 m*M*, pH 7.8–8.0). Temperature and photoperiod were allowed to vary seasonally. Gill cells were obtained from 145 to 500 g fish taken from stock tanks with water temperatures that ranged from 14.5 to 15° C.

Preparation and culture of rainbow trout gill epithelia. The procedures used for the preparation and culture of rainbow trout gill epithelia in this study have been outlined in detail by Kelly et al. (2000) and are based on those originally developed by Fletcher et al. (2000). The resulting epithelia have been demonstrated to contain approximately 85% pavement cells and 15% mitochondria-rich cells (Fletcher et al., 2000). In brief, gill cells were freshly isolated from the branchial epithelium of rainbow trout by tryptic digestion. Cells were suspended in Leibovitz L15 medium supplemented with 2 mmol/L glutamine, 5–6% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml gentamycin and seeded directly on to permeable cell culture filter inserts (Cyclopore polyethylene terephthalate "filters"; Becton Dickinson, Franklin Lakes, NJ; pore density, 1.6×10^6 pores/cm²; pore size, 0.45 μm; growth surface, 0.9 cm²). Twenty-four hours after seeding, nonadherent material (i.e., mucus, unattached cells, cellular debris) was rinsed from the inserts and a second layer of cells seeded over adherent cells from the first seeding. The second seeding allows the epithelium to comprise both pavement cells and mitochondria-rich cells, typical of the intact gill (c.f. Fletcher et al., 2000). Epithelia were cultured for 6–7 d bathed symmetrically in L15 media (L15 apical–L15 basolateral) until the formation of stable electrophysiological characteristics (c.f. Wood and Pärt, 1997; Fletcher et al., 2000; Kelly et al., 2000). All procedures were carried out in a laminar-flow hood under sterile conditions. Cell culture solutions and reagents were obtained from GIBCO-BRL (GIBCO-BRL, Canadian Life Technologies Inc., Burlington, ON, Canada). Solutions that were not of cell culture grade, such as FW (obtained from inlets to original holding tanks, *see* composition above) and FW osmotically compensated with mannitol (Sigma-Aldrich, Oakville, ON, Canada), were sterilized by filtration through a 0.2-μm filter (Falcon; Becton Dickinson, Franklin Lakes, NJ). Gill cells and epithelia were cultured in an air atmosphere at 18° C, and all experiments were carried out at this temperature.

Electrophysiological measurements. Transepithelial resistance (TER) was measured using STX-2 chopstick electrodes connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). Measured values of TER were corrected for growth area and for "background" TER by subtracting TER measured across blank inserts bathed in appropriate solutions (expressed in kΩ cm²). Transepithelial potential (TEP) measurements were obtained using agar-salt bridges (4% agar in 3 *M* KCl) connected through Ag–AgCl electrodes (World Precision Instruments) to a pH meter used as a high-impedance electrometer (pHM 84, Radiometer, Copenhagen, Denmark). All TEP measurements were expressed relative to the apical side as 0 mV after correction for junction potential (for details, *see* Kelly and Wood, 2001b).

Unidirectional ion flux measurements. Unidirectional Na^+ and Cl^- flux rates were determined using the radiotracers ^{22}Na and ^{36}Cl (NEN Dupont, Boston, MA). Approximately 1 μCi of each radioisotope was added to either the basolateral or the apical compartment, and the appearance of radioisotope was monitored on the "cold" side. Experimental methods and calculations for determining influx (apical to basolateral: positive sign), efflux (basolateral to apical: negative sign), and net flux ($J_{net} = J_{in} + J_{out}$) rates have been previously outlined by Wood et al. (1998) and Fletcher et al. (2000).

(1) Simulated environmental salinity reduction: Unidirectional Na^+ and Cl^- flux rates were determined initially under symmetrical culture conditions as outlined above. After symmetrical flux measurements, apical L15 medium was replaced with medium diluted 33% (two-thirds L15 apical), basolateral medium was replaced with fresh regular L15 medium, and unidirectional Na^+ and Cl^- flux rates were immediately determined over an 8-h period. After flux experiments under two-thirds L15 apical–L15 basolateral conditions, apical two-thirds L15 medium was replaced with medium diluted to one-third L15, basolateral medium was replaced with fresh regular L15 medium, and again unidirectional Na^+ and Cl^- flux rates were immediately determined over a 6-h period. A final switch to asymmetrical conditions (FW apical–L15 basolateral) was made immediately after flux experiments under one-third L15 apical–L15 basolateral conditions. Apical medium was replaced with sterile FW (*see* composition above), and epithelia were fluxed under asymmetrical conditions for 6 h. During unidirectional flux experiments, the electrophysiological characteristics of epithelia were monitored at regular intervals. Preparations were used for either influx or efflux measurements only and matched for determination of net flux rates and the Ussing flux ratio criterion (*see* below) using electrophysiological criteria (c.f. Fletcher et al., 2000).

(2) Simulated extracellular fluid dilution: Unidirectional flux experiments under conditions of reduced basolateral media osmolality and ionic strength, or under reduced ionic strength only, plus simultaneous introduction of apical FW were conducted as follows. "Dilute" media (this time designated for bathing epithelia on the basolateral side of the cell culture insert) were prepared by diluting regular L15 (osmolality = 307.3 ± 0.6 mOsm; n = 15) media with sterile FW (by 20 or 40%) or sterile FW (also by 20 or 40%) osmotically compensated with mannitol back to the same value as regular L15. Epithelia were cultured under symmetrical conditions (L15 apical–L15 basolateral) for 6–7 d until ready for experimentation (TER = 35.53 ± 0.38 kΩ cm²; TEP = +19.9 ± 1.0 mV; n = 88). After this period of symmetrical culture, apical medium was removed and replaced with sterile FW (after several rinses to ensure complete removal of media). Basolateral medium was replaced with L15 media diluted to (1) 80% L15 (osmolality = 248.5 ± 0.5 mOsm; n = 10), (2) 80% L15 osmotically compensated with mannitol (osmolality = 305.5 ± 0.8 mOsm; n = 10), (3) 60% L15 (osmolality = 198.2 ± 0.6 mOsm; n = 5), or (4) 60% L15 osmotically compensated with mannitol (osmolality = 307.0 ± 1.2 mOsm; n = 5). Unidirectional Na^+ and Cl^- fluxes were conducted as outlined above. For all treatments, unidirectional flux experiments were conducted for a 6-h period immediately after the introduction of new media conditions (T 0–6 h). In preparations exposed

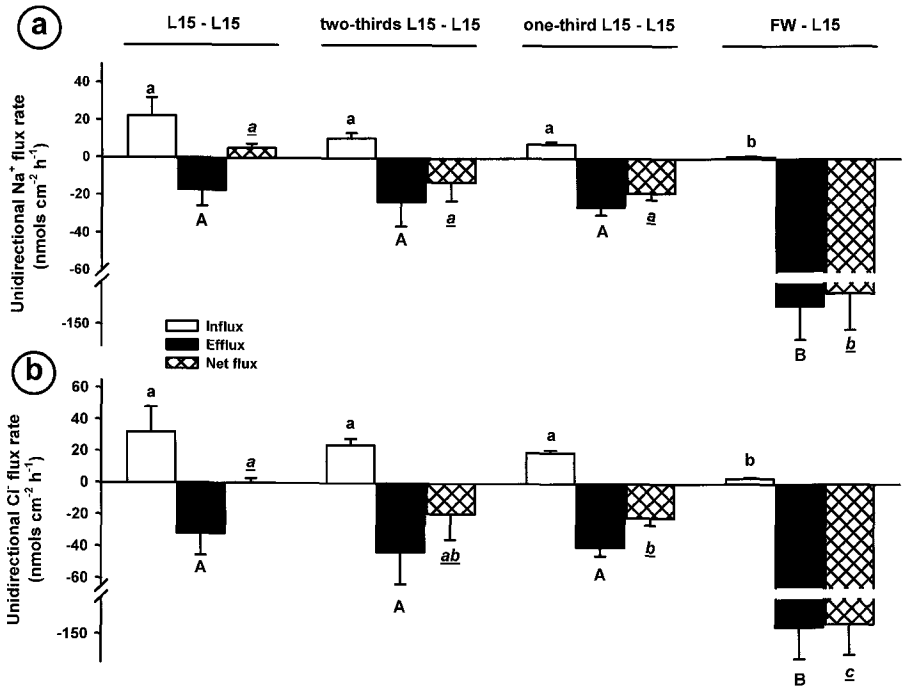

FIG. 1. Unidirectional (*a*) Na^+ and (*b*) Cl^- flux rates across cultured gill epithelia subjected to a progressive dilution of apical media from L15 to freshwater (FW). Fluxes were measured under L15 apical–L15 basolateral, two-thirds L15 apical–L15 basolateral, one-third L15 apical–L15 basolateral, and FW apical–L15 basolateral conditions. *Open, solid,* and *hatched bars* represent influx, efflux, and net flux, respectively. Apical media were diluted with sterile FW. Data are expressed as mean ± SEM (n = 9–7 matched inserts per treatment). Significant differences between specific flux rates from different apical media treatments are denoted by different *letters*.

to FW apical–80% L15 basolateral conditions (both with and without osmotic compensation), a second 6-h flux was conducted after epithelia had been exposed to these conditions for 18 h (T 18–24 h). As before, epithelia were used for either influx or efflux measurements only and matched for determination of net flux rates and the Ussing flux ratio criterion using the electrophysiological criteria outlined by Fletcher et al. (2000). In preparations designated for exposure to basolateral media diluted with FW osmotically compensated with mannitol, the osmolality of dilute solutions were matched to in situ basolateral L15 osmolality immediately before commencing the experiment.

Ussing flux ratio. As an indication of active transport, disagreement of the measured flux ratio (J_{in}/J_{out}) with the prediction of the Ussing flux ratio for Na^+ and Cl^- was used (Kirschner, 1970). The predicted Ussing flux ratio was calculated as

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

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 all media and in dilute media solutions, A_{Na} and A_{Cl} were measured using ion-specific electrodes and were used accordingly. A_{Na} and A_{Cl} in apical FW were taken as equal to the measured concentrations.

Analytical analysis. Methods for measurement of the activity of Na^+–K^+ adenosine triphosphatase (ATPase) in individual cultured gill epithelia have been described in detail by Kelly and Wood (2001b). All enzyme activities are expressed as protein-specific activity after measuring the protein content of supernatants using the Bradford method (Sigma Chemical Co., Oakville, ON, Canada). Osmolality was determined using a Wescor 5100C vapor pressure osmometer.

Statistical analysis. All data are expressed as mean ± standard error (n), where n represents the number of matched filter inserts. For comparisons among varying groups, data were either subjected to a one-way or a two-way analysis of variance (Sigmastat software, SPSS Inc., Chicago, IL) as appropriate. Significant differences ($P \leq 0.05$) among groups were detected using either a Student–Newman–Keuls test or Student's unpaired or paired *t*-tests as appropriate (Sigmastat software).

RESULTS

Simulated environmental salinity reduction by apical media dilution. During progressive dilution of apical media from regular L15 through two-thirds L15, one-third L15 to FW, ion influx rates generally decreased proportionally as apical ion concentrations decreased (Fig. 1). This pattern of response was evident for both Na^+ and Cl^- influx rates, but absolute rates did not become significantly different ($P < 0.05$) between treatments until the condition FW apical–L15 basolateral was introduced. Similarly, Na^+ and Cl^- efflux rates also did not exhibit a statistically significant difference until the same condition (apical FW) was introduced (Fig. 1). Na^+ and Cl^- net flux rates became negative after the introduction of two-thirds L15 to the apical side of the preparations and continued to become increasingly negative as the apical environment became increasingly dilute (Fig. 1). Net flux rates increased significantly to their highest rate (movement in the outward direction, basolateral to apical, carrying a negative sign) under asymmetrical FW apical–L15 basolateral conditions.

When either media or dilute media (two-thirds L15 and one-third L15) were present on the apical side of the cultured preparations, TER varied only slightly between ~27 and 24 kΩ cm^2 (Table 1). After the introduction of apical FW, TER decreased to ~18 kΩ cm^2, but the change was not significant. In the presence of regular apical media or dilute media, all TEP values were positive and ranged from ~+4.6 to +3.7 mV (Table 1). In contrast, in the presence of apical FW, TEP became negative and was significantly different from all other values (Table 1).

Under symmetrical culture conditions, the Ussing flux ratio analysis indicated passive Cl^- movement because there was no significant difference between the predicted and the observed flux ratios (Table 1). These observations are in accord with those of Fletcher et al. (2000) and Kelly and Wood (2002b). In contrast, the Ussing flux ratio analysis indicated active, inwardly directed (apical to basolateral) Na^+ movement under the same conditions (Table 1). Inwardly directed Na^+ transport has been previously reported under symmetrical conditions (Kelly and Wood, 2002b). When the apical media were diluted to either two-thirds L15 or one-third L15, the

TABLE 1

COMPARISON BETWEEN USSING FLUX RATIOS (J_{in}/J_{out}) FOR Na^+ AND Cl^- AND ELECTROPHYSIOLOGICAL CHARACTERISTICS OF CULTURED TROUT EPITHELIA (*FIRST COLUMN*). PREPARATIONS WERE BATHED IN APICAL L15 MEDIUM THAT WAS GRADUALLY DILUTED WITH FW (TWO-THIRDS L15 APICAL–L15 BASOLATERAL AND THEN ONE-THIRD L15 APICAL–L15 BASOLATERAL) BEFORE FW APICAL–L15 BASOLATERAL CULTURE CONDITIONS BEING INTRODUCED[a,b]

	Flux ratio[c]							
	L15–L15[d]		Two-thirds L15–L15[e]		One-third L15–L15[f]		FW–L15	
	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted ($\times 10^{-3}$)	Observed ($\times 10^{-3}$)
Na^+	0.87 ± 0.03	1.54 ± 0.26*	0.53 ± 0.02	0.64 ± 0.13	0.29 ± 0.01	0.29 ± 0.05	15.70 ± 0.56	9.64 ± 1.48*
Cl^-	1.17 ± 0.05	0.89 ± 0.10	0.72 ± 0.03	0.83 ± 0.10	0.48 ± 0.02	0.50 ± 0.04	12.30 ± 0.61	23.80 ± 1.64*
TER ($k\Omega$ cm^2)	25.23 ± 3.90		27.05 ± 2.60		23.98 ± 2.18		18.00 ± 3.41	
TEP (mV)	+3.81 ± 0.96[a]		+4.56 ± 1.11[a]		+3.70 ± 0.91[a]		−6.00 ± 0.40[b]	
	(n = 8)		(n = 9)		(n = 9)		(n = 7)	

[a] Abbreviations: TEP, transepithelial potential; TER, transepithelial resistance; FW, freshwater.

[b] Data are expressed as mean ± SEM (n = number of matched epithelia).

[c] Predicted flux ratio = $A_{Ap} \cdot e^{-(zFV/RT)}/A_{Bl}$; observed flux ratio = J_{in}/J_{out}.

[d] L15 = Leibovitz L15 medium supplemented with 2 mmol/L glutamine and 5–6% foetal bovine serum.

[e] 2/3 L15 = L15 diluted to 2/3 strength with FW.

[f] 1/3 L15 = L15 diluted to 1/3 strength with FW.

*Denotes significant ($P < 0.05$) difference between predicted and observed flux ratio within a treatment. TEP values with different *superscripts* are significantly different ($P < 0.05$).

Ussing flux ratio analysis indicated passive Na^+ and Cl^- movement (Table 1). Under asymmetrical FW apical–L15 basolateral conditions, our observations of Na^+ and Cl^- transport by the Ussing flux ratio criterion were consistent with all previous reports (for review, *see* Wood et al., 2002). That is, Cl^- movement was active and inwardly directed (apical to basolateral), whereas Na^+ movement was active and in the outward (basolateral to apical) direction (Table 1).

Simulated extracellular fluid dilution by basolateral media dilution. The replacement of symmetrical culture conditions (L15 apical–L15 basolateral) with apical FW and either 60% L15 basolateral or 60% L15 osmotically compensated with mannitol (osmolality = regular L15) basolateral media conditions revealed Na^+ and Cl^- flux rate patterns similar to those seen under "typical" asymmetrical conditions (FW apical–L15 basolateral). That is, Na^+ and Cl^- influx rates were low relative to efflux rates (Fig. 2 c.f. Fig. 1). Consequently, net flux rates were in the outward direction (basolateral to apical) and not significantly different from efflux rates (Fig. 2). No significant difference in Na^+ influx, efflux, or net flux rates was observed between preparations bathed in apical 60% L15 with or without osmotic compensation (Fig. 2*a*). This was also apparent for Cl^- flux rates (Fig. 2*b*). However, *note* that Cl^- efflux and net flux rates were considerably lower than Na^+ efflux and net flux rates (Fig. 2*b*). In preparations where 60% L15 was introduced on the basolateral side without osmotic compensation, TER values increased slightly (but significantly) relative to values observed under symmetrical conditions (L15–L15 TER = 37.36 ± 0.19 $k\Omega$ cm^2, FW–60% L15 TER = 39.79 ± 0.53 $k\Omega$ cm^2, $P < 0.05$). Where osmotically compensated 60% L15 media were introduced basolaterally, no difference in TER, relative to TER values observed under symmetrical conditions, was observed (L15–L15 TER = 37.67 ± 0.21 $k\Omega$ cm^2, FW–60% L15 + mannitol TER = 37.92 ± 0.58 $k\Omega$ cm^2, $P > 0.05$), but the value was significantly lower than in the absence of osmotic compensation (39.79 ± 0.53 $k\Omega$ cm^2, $P < 0.05$). In both treatments, TEP fell dramatically from symmetrical values ranging from +27 to +32 mV to new values that were not significantly different from 0 mV (Table 2).

In both circumstances, the Ussing flux ratio criterion indicated active Na^+ extrusion across preparations bathed on the basolateral side diluted to 60% L15 (Table 2). In contrast, Cl^- movement appeared to be passive across epithelia bathed on the basolateral side with 60% L15 alone and active in the inward direction (apical to basolateral) across preparations bathed on the basolateral side with 60% L15 osmotically compensated with mannitol (Table 2).

After the immediate introduction (T 0–6 h) of FW apical–80% L15 basolateral or FW apical–80% L15 + mannitol basolateral conditions to cultured epithelia, ion flux rate patterns resembled those described above in this subsection. Na^+ and Cl^- influx rates were low relative to efflux rates, resulting in net flux rates that were outwardly directed and not significantly different from efflux rates (Fig. 3). No significant difference in ion flux rates could be detected between groups treated with 80% L15 basolateral in the presence or absence of osmotic compensation. This trend was also apparent after epithelia had been exposed to these conditions for longer time periods (Fig. 3, T 18- to 24-h flux rates). However, flux rates tended to be greater across all epithelia during the flux period T 18–24 h when compared with those measured during the period T 0–6 h (Fig. 3). One notable exception to this trend was Na^+ efflux (and consequently net flux) across preparations bathed on the basolateral side with 80% L15, which did not increase significantly between the two time periods (Fig. 3).

The TER across epithelia held in FW apical–80% L15 basolateral conditions was not significantly different from TER values observed under symmetrical conditions before commencing flux experiments (L15–L15 TER = 34.44 ± 0.70 $k\Omega$ cm^2, FW–80% L15 TER = 35.22 ± 0.83 $k\Omega$ cm^2, $P > 0.05$). This was also evident in epithelia bathed in 80% L15 + mannitol on the basolateral side (L15–L15 TER = 34.56 ± 0.78 $k\Omega$ cm^2, FW–80% L15 + mannitol TER = 31.90 ± 1.78 $k\Omega$ cm^2, $P > 0.05$). Between treatments at this time period (T 0–6 h), TER was significantly different. After longer exposure periods (T 18–24 h) to both environments, TER decreased significantly by 35–50% (Table 2), but there was no significant difference in TER between treatments. Under symmetrical

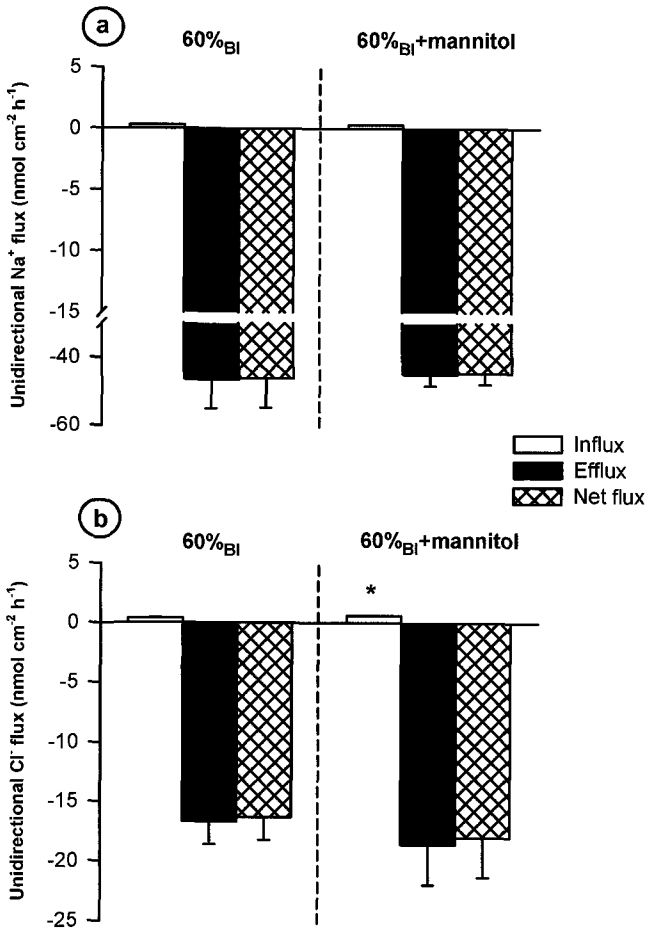

FIG. 2. Unidirectional (*a*) Na^+ and (*b*) Cl^- flux rates across cultured gill epithelia bathed on the apical side with freshwater (FW) and on the basolateral side with 60% L15 ($60\%_{BI}$) or 60% L15 osmotically compensated with mannitol ($60\%_{BI}$ + mannitol, osmolality = 100% L15). *Open, solid,* and *hatched bars* represent influx, efflux, and net flux, respectively. Fluxes were determined over a 6-h period immediately after the introduction of apical FW. Data are expressed as mean ± SEM (n = 7 matched preparations per treatment). An *asterisk* denotes significant difference between treatments.

conditions before experimental manipulation, the TEP across epithelia subsequently exposed to FW apical and 80% L15 or 80% L15 + mannitol basolateral ranged from +13 to +15 mV. After the introduction of experimental conditions, TEP in both treatments became negative (Table 2). No significant difference in TEP was detected between treatments or after a longer culture period of T 18–24 h (Table 2).

The Ussing flux ratio analysis indicated that epithelia treated with either 80% L15 or 80% L15 + mannitol on the basolateral side and FW on the apical side exhibited active Cl^- uptake (apical to basolateral) and Na^+ extrusion (basolateral to apical). This pattern of active transport was apparent during both the flux periods T 0–6 h and T 18–24 h (Table 2).

The activity of Na^+–K^+ ATPase was not significantly altered by the presence of mannitol as an osmotic "clamp" in dilute basolateral media (Fig. 4). There was also no significant difference in the activity of Na^+–K^+ ATPase in preparations bathed basolaterally with either 60 or 80% dilute media at T 0–6 h. However, the activity of Na^+–K^+ ATPase was significantly reduced after 24 h of exposure to apical FW and either 80% L15 alone or 80% L15 + mannitol (Fig. 4).

DISCUSSION

Overview. The results of this study demonstrate that cultured rainbow trout epithelia are tolerant of dilute media manipulation from both the apical and the basolateral direction. Although tolerance of apical media dilution is entirely consistent with all previous reports of apical FW exposure (for review, *see* Wood et al., 2002), epithelial tolerance of reduced basolateral osmolality and ionic strength is a new and promising avenue for further study in these preparations. This tolerance of the cultured epithelium is consistent with the ability of rainbow trout, and euryhaline fish in general, to endure ionoregulatory perturbation (e.g., extracellular fluid dilution) after rapid salinity transfer. Furthermore, the limits of cultured epithelia tolerance to dilute basolateral conditions seem likely to reflect realistic conditions because Fletcher (1997) has demonstrated that even brief basolateral exposure (3 h) of cultured gill epithelia to FW results in irreversibly compromised epithelial integrity. This is clearly not the case with moderate basolateral media dilution in the current study. Taken together, these data emphasize functional polarity in cultured gill preparations (for further discussion, *see* Wood et al., 2002) and demonstrate that the use of dilute media is a valid experimental approach for introducing more realistic conditions to gill epithelia culture.

However, it is clear from the present studies that simulating gradual environmental salinity reduction or extracellular fluid dilution solely by diluting culture media does not greatly alter the pattern of ion uptake across cultured rainbow trout preparations exposed to apical FW. Therefore, we can reject our hypothesis that dilute media as a sole environmental or physiological simulant will increase the magnitude of Na^+ and Cl^- uptake from FW in cultured trout gill epithelia.

Apical media dilution. Progressively diluting apical media had very little effect on the electrophysiological properties of cultured rainbow trout gill epithelia until apical FW was introduced (FW apical–L15 basolateral). Furthermore, progressive apical media dilution had little effect on the eventual response of cultured preparations to asymmetrical conditions. The Ussing flux ratio criterion indicated passive Na^+ and Cl^- transport during the dilution phase of the experiment (two-thirds L15 and one-third L15 apical), followed by low rates of active Cl^- uptake and Na^+ extrusion across preparations bathed in apical FW. Cl^- uptake and Na^+ extrusion have been previously observed in identically prepared cultured rainbow trout gill epithelia after the rapid introduction of asymmetrical conditions (Fletcher et al., 2000; Wood et al., 2002). Hence, there appeared to be no beneficial effects of the dilution protocol in promoting the "normal" pattern of both active Na^+ and active Cl^- uptake from apical FW. In future studies, it would be interesting to examine the additional effect of supplementary hormones in combination with gradual apical media dilution. We have already demonstrated beneficial effects of cortisol as well as prolactin on passive permeability properties of the preparation when bathed in apical FW (Kelly and Wood, 2001b, 2001c, 2002b).

Basolateral media dilution. In these experiments, we used basolateral media dilutions that ranged from 20 to 40%, a reduction in basolateral osmolality from regular media levels of ~307 to ~250 mOsm/kg (80% L15) or ~200 mOsm/kg (60% L15). These values

TABLE 2

COMPARISON BETWEEN USSING FLUX RATIOS (J_{in}/J_{out}) FOR Na^+ AND Cl^- AND ELECTROPHYSIOLOGICAL CHARACTERISTICS OF CULTURED TROUT GILL EPITHELIA (*FIRST COLUMN*). PREPARATIONS WERE BATHED IN APICAL FRESHWATER AND ON THE BASOLATERAL SIDE WITH EITHER DILUTE MEDIA (60 OR 80% L15) OR DILUTE MEDIA (60 OR 80% L15) OSMOTICALLY COMPENSATED WITH MANNITOL (OSMOLALITY BACK TO SAME VALUE AS REGULAR L15)[a,b]

	Flux ratio[c]			
	Bl media diluted with FW		Bl media diluted with FW + mannitol (osmolality same as 100% L15 media)[d]	
	Predicted ratio ($\times 10^{-3}$)	Observed ratio ($\times 10^{-3}$)	Predicted ratio ($\times 10^{-3}$)	Observed ratio ($\times 10^{-3}$)
$60\%_{Bl}$ T 0–6 h				
Na^+	19.70 ± 0.53	7.51 ± 1.46*	17.60 ± 0.26	6.75 ± 0.76*
Cl^-	17.10 ± 0.76	24.70 ± 3.60	15.80 ± 0.46	30.10 ± 0.77*
TEP (mV)	zero		zero	
TER (kΩ cm²)	39.79 ± 0.53		37.92 ± 0.58[e]	
	(n = 7)	(n = 7)	(n = 7)	(n = 7)
$80\%_{Bl}$ T 0–6 h				
Na^+	18.60 ± 0.27	11.60 ± 2.01*	18.00 ± 0.69	13.30 ± 1.49*
Cl^-	11.40 ± 0.37	16.40 ± 1.66*	11.60 ± 0.49	20.40 ± 2.41*
TEP (mV)	−5.80 ± 0.33		−5.60 ± 0.42	
TER (kΩ cm²)	35.22 ± 0.83		31.90 ± 1.78[e]	
	(n = 14)	(n = 14)	(n = 12)	(n = 12)
$80\%_{Bl}$ T 18–24 h				
Na^+	20.40 ± 0.31	9.00 ± 0.92*	18.00 ± 0.38	10.10 ± 1.55*
Cl^-	11.50 ± 0.11	19.70 ± 1.13*	12.30 ± 0.58	23.80 ± 3.26*
TEP (mV)	−6.90 ± 0.14		−6.30 ± 0.28	
TER (kΩ cm²)	22.57 ± 1.56		16.64 ± 2.67	
	(n = 5)	(n = 5)	(n = 5)	(n = 5)

[a] Abbreviations: TEP, transepithelial potential; TER, transepithelial resistance; FW, freshwater.
[b] Data are expressed as mean ± SEM (n = number of matched epithelia).
[c] Predicted flux ratio = $A_{Ap} \cdot e^{-(zFV/RT)}/A_{Bl}$; observed flux ratio = J_{in}/J_{out}.
[d] L15 = Leibovitz L15 medium supplemented with 2 mmol/L glutamine and 5–6% foetal bovine serum.
[e] Denotes significant difference between TER values for epithelia within a treatment group.
*Denotes significant difference between predicted and observed flux ratio.

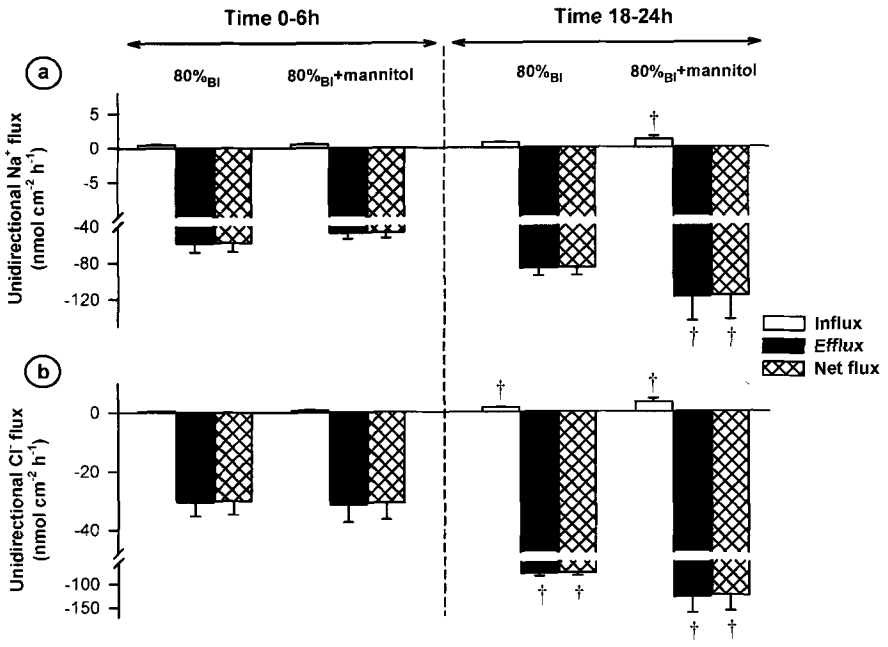

FIG. 3. Unidirectional (*a*) Na^+ and (*b*) Cl^- flux rates across cultured gill epithelia bathed on the apical side with freshwater (FW) and on the basolateral side with 80% L15 ($80\%_{Bl}$) or 80% L15 osmotically compensated with mannitol ($80\%_{Bl}$ + mannitol, osmolality = 100% L15). Fluxes were determined over a 6-h period immediately after the introduction of apical FW (T 0–6 h) and then repeated over a second 6-h period after 18 h of exposure to FW (T 18–24 h). *Open, solid,* and *hatched bars* represent *influx*, efflux, and net flux, respectively. Data are expressed as mean ± SEM (n = 5–14 matched preparations per treatment). †, significant difference between treatments T 0–6 h and T 18–24 h.

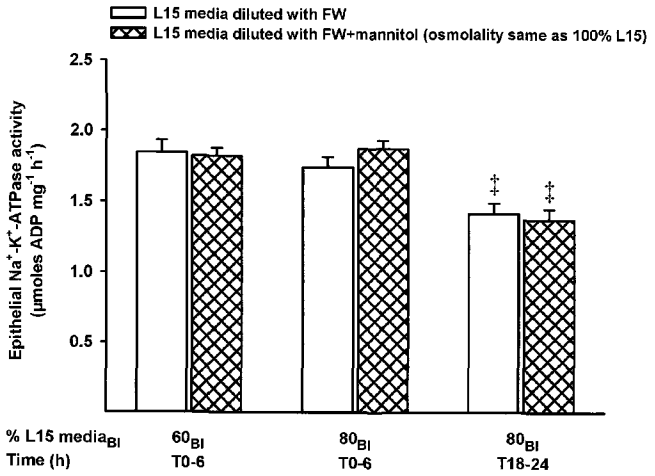

FIG. 4. Epithelial Na^+–K^+ adenosine triphosphatase activity in preparations bathed apically with freshwater and basolaterally with either 60% L15 media (60_{Bl}) or 80% L15 media (80_{Bl}). *Open bars* represent preparations treated with dilute basolateral media, whereas *hatched bars* represent preparations treated with dilute basolateral media osmotically compensated with mannitol (osmolality = 100% L15). Epithelial cells were harvested for enzyme analysis at the time periods indicated below each treatment. Data are expressed as mean ± SEM (n = 12 preparations per treatment). ‡, significant difference between treatments T 0–6 h and T 18–24 h 80_{Bl}.

accurately reflect changes that occur in the blood osmolality of euryhaline fish on rapid exposure from high environmental salinity to FW. For example, Marshall et al. (2000) reported a reduction in plasma Na^+ of ~29% 6 h after the transfer of *Fundulus heteroclitus* from seawater to FW and calculated a fall in plasma osmolality that would take values from the normal range of 290–308 mOsm/kg to 196–214 mOsm/kg.

In cultured trout gill epithelia, the introduction of apical FW (FW apical–L15 basolateral) results in active Cl^- uptake and Na^+ extrusion (Fletcher et al., 2000; Table 2). In general, reduced basolateral osmolality or ion levels (or both) did not alter this pattern and had little effect on Cl^- uptake rates from apical FW or the tendency for Na^+ to be actively extruded. A notable exception to this trend was observed in epithelia treated with 60% L15 on the basolateral side, where Cl^- transport appeared to be passive rather than active in the inward direction, an effect opposite to that desired. Very little is known about the direct effects of low extracellular fluid osmolality on ion transport across fish gills. Recently, Marshall et al. (2000) demonstrated that basolateral hypotonicity inhibited short-circuit current (I_{SC}) across opercular epithelia isolated from seawater *F. heteroclitus* in a dose-dependent and reversible manner. Because I_{SC} was equivalent to the rate of Cl^- secretion across *F. heteroclitus* opercular membranes and a reduced basolateral osmolality would mimic hydromineral imbalance on rapid entry to FW, a reduction in Cl^- transport rates would be beneficially adaptive. However, in our case, we see a maladaptive reduction in the active uptake of Cl^-. In line with the observations of Marshall et al. (2000), this can also be directly attributed to reduced basolateral osmolality and not to reduced ion levels. That is, active Cl^- uptake became passive in preparations bathed on the basolateral side with 60% L15 media but persisted (remained active in the uptake direction) across epithelia bathed basolaterally with 60% L15 media osmotically compensated with mannitol (osmolality = regular L15).

Osmolality had no observable short-term effect on the activity of Na^+–K^+ ATPase in cultured epithelia. These observations are interesting given that a reduced basolateral osmolality will likely result in cultured gill cells undergoing hypotonic shock and cell swelling. Because Na^+–K^+ ATPase is a major determinant in cellular housekeeping, controlling cell volume by maintaining low intracellular $[Na^+]$ and high intracellular $[K^+]$ and providing the driving force for the transport of other solutes, we might have expected to see osmolality-induced changes in its activity. Nevertheless, Na^+–K^+ ATPase activity did decrease after a longer time period (T 18–24 h) of exposure to FW apical–80% L15 basolateral (with or without mannitol) conditions. This decrease may reflect either adaptation to new media conditions or a decrease in enzyme activity that would occur normally under asymmetrical conditions (FW apical) in the presence of either dilute or regular basolateral media.

Future perspectives. Although the use of a simplified surrogate experimental system has numerous advantages for the researcher, a balance must be met between simplification and the provision of "realistic" conditions. In cultured gill epithelia, we are moving closer to this situation, and the results of these studies form a positive foundation for further examination of dilute media as an environmental or physiological simulant. In particular, it would be interesting to examine the additional effect(s) that ionoregulatory hormones (e.g., cortisol, prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones) have under such conditions. For example, the physiological challenge of rapid salinity transfer not only causes alterations in extracellular fluid and electrolyte composition but also invokes a complex neuroendocrine response. Therefore, the examination of key ionoregulatory hormones, hormone combinations, or even homologous serum supplements (obtained from fish during rapid salinity transfer), in association with dilute media treatment, would undoubtedly provide further insight and a more authentic in vitro environment for cultured gill epithelia.

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