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Cultured trout gill epithelia enriched in pavement cells or in mitochondria-rich cells provides insights into Na^+ and Ca^{2+} transport

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Summary The lack of a suitable flat epithelial preparation isolated directly from the freshwater fish gill has led, in recent years, to the development of cultured gill epithelia on semipermeable supports. To date, their minimal capacity to actively transport ions has limited their utility as ionoregulatory models. The current study describes a new method of culturing gill epithelia consisting either of an enriched population of pavement (PV) cells or a mixed population of PV cells and mitochondria-rich (MR) cells from the gills of adult rainbow trout. Although the cell culture approach is similar to the double-seeded insert (DSI) technique described previously, it makes use of Percoll density centrifugation to first separate populations of PV and MR cells, which are then seeded on cell culture supports in varying proportions on successive days so as to produce preparations enriched in one or the other cell types. Based on rhodamine staining, the MR cell-rich epithelia exhibited a threefold higher enrichment of MR cells compared to traditional DSI preparations. In general, MR cell-rich epithelia developed extremely high transepithelial resistances (TER; >30 kΩ cm^2) and positive transepithelial potentials (TEP) under symmetrical conditions (i.e., L15 medium on both apical and basolateral sides). Apical exposure of cell cultures to freshwater reduced TER and produced a negative TEP in all the epithelial preparations, although MR cell-rich epithelia maintained relatively high TER and negative TEP for over 2 d under these asymmetrical conditions. Measurement of unidirectional Na^+ fluxes and application of the Ussing flux ratio criterion demonstrated active Na^+ uptake in PV cell-rich and MR cell-rich epithelia under both symmetrical and asymmetrical conditions. In comparison, Ca^{2+} uptake and Na^+/K^+-ATPase activity were significantly elevated in MR cell-rich preparations relative to the traditional DSI or PV cell-rich cultures under symmetrical conditions. This new methodology enhances our ability to tailor cultured gill epithelia on semipermeable supports with different proportions of PV cells and MR cells, thereby illuminating the ionoregulatory functions of the two cell types.

Keywords Mitochondria-rich cells · Pavement cells · Ussing flux criterion · Transepithelial resistance · Transepithelial potential

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Introduction

The gill epithelium of freshwater and seawater fish is a structurally complex organ with numerous cell types implicated in ion and acid–base regulation, nitrogenous waste excretion, gas exchange, and barrier properties (Goss et al. 2001; Galvez et al. 2002). Solute regulation has been classically associated with two principal cell types in the gill epithelium (Evans et al. 2005). The dominant cell type, the pavement (PV) cell, accounts for greater than 80% of all gill epithelial cells. The second cell type, termed the mitochondria-rich (MR) cell, comprises roughly 5–15% of the cells in the gill (Perry and Laurent 1993; Perry and Walsh 1989). It is generally accepted that solute regulation is mediated mainly by the MR cells (Evans et al. 2005), although a number of studies have also implicated the PV

cells in this role (Goss et al. 1992; Lin et al. 1994; Sullivan et al. 1995; Goss et al. 1998). This uncertainty about the relative roles of these two cell types stems from the lack of an established model system to test their functional roles.

Isolation of epithelia from extrabranchial regions from seawater-adapted fish, including the opercular membranes of killifish (*Fundulus heteroclitus*; Wood and Marshall 1994) and tilapia (Foskett et al. 1982; Scheffey et al. 1983), or the jaw skin of the goby (Marshall 1977) have provided valuable insights into the active and passive movements of osmolytes. These flat membranes have a cellular composition similar to that of the gill epithelium but, in contrast, are amenable to mounting in Ussing chambers, where detailed electrophysiological characterization of ion transport can be performed. Unfortunately, a comparable surrogate model of the freshwater gill epithelium has not emerged, with most of these preparations having only a limited capacity for active ion transport under asymmetrical conditions (apical freshwater and basolateral physiological medium; Burgess et al. 1998; Marshall et al. 1997).

Recent advancements in gill cell culture provide an alternate model system to study the freshwater fish gill epithelium. Unlike traditional culture of gill epithelial cells on solid supports (Part et al. 1993; Fernandes et al. 1995; Leguen et al. 2001), more recent work has managed to grow polarized branchial epithelia from freshwater rainbow trout on permeable cell culture inserts. Wood and Pärt (1997) first used the single-seeded insert (SSI) technique to produce epithelia enriched in PV cells derived from a single animal. The SSI method requires an initial culture in flasks, during which PV cells alone are "selected" by the culture. Fletcher et al. (2000) were able to integrate MR cells within the PV cell layers, in a procedure they termed the double-seeded insert (DSI) technique. The DSI method involves sequential seeding from two animals on successive days. A third method, the single direct-seeded insert technique (SDSI), involving a one-time-only direct seeding from a single fish, was developed by Wood et al. (2002a) and more recent modifications by Leguen et al. (2007). Of these, the DSI preparation appears to be the most robust and the one which has been employed most frequently. All three methods ultimately result in confluent gill epithelia grown on permeable cell culture inserts, which are amenable to experimental alteration of ion activities on both sides of the epithelia and simultaneous monitoring electrophysiological parameters such as transepithelial resistance (TER) and transepithelial potential (TEP). Although these reconstructed gill epithelia are able to withstand prolonged apical exposure to freshwater and show many physiological and morphological characteristics similar to those of the gill epithelium in vivo (Gilmour et al. 1998; Kelly et al. 2000; Kelly and Wood 2002a,b), attempts to stimulate active Na^+ and Ca^{2+} uptake (apical to basolateral transport) have only produced modest effects, especially in cultures under asymmetrical conditions where the apical solution is freshwater (Wood et al. 1998; Fletcher et al. 2000; Kelly and Wood 2001a,b, 2002a,b; Zhou et al. 2003; Zhou et al. 2004; Kelly and Wood 2008). As such, many of the cellular and molecular underpinnings of ion regulation in freshwater fish remain poorly described.

The overall aim of this study was to investigate new methods of culturing the freshwater fish gill, which better mimic its physiological properties in vivo. As a course of action, we focused on a recently developed technique utilizing Percoll density centrifugation to isolate enriched populations of MR cells and PV cells from freshwater rainbow trout (Goss et al. 2001; Galvez et al. 2002; Galvez et al. 2006). It was hoped that this methodology would provide a means of producing "designer" epithelia with different ratios of PV and MR cell subtypes on cell culture inserts. This study contrasts the electrical and ion transport properties of PV cell-rich and MR cell-rich gill epithelia with the traditional DSI preparation.

Materials and Methods

Fish holding and animal care. Adult rainbow trout (*Oncorhynchus mykiss*, ~100 g) of both sexes were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and acclimated to laboratory conditions for at least 2 wk prior to experimentation. Fish were held under continuous flow-through conditions (~1–2 L/min) in 400-L polyethylene tanks supplied with Lake Ontario dechlorinated tap water (hardness 140 mg/L as $CaCO_3$; and in mM: 1.0 Ca^{2+}, 0.2 Mg^{2+}, 0.6 Na^+, 0.2 K^+, 0.7 Cl^-, and pH 8.0) at a constant temperature of 12°C. Fish were kept at an initial density of approximately 10 g/L H_2O and fed fish chow (Silver Cup Fish Feed, South Murray, UT) at a ration of ~1–2% of their body weight per day. Photoperiod was kept similar to the natural light/dark cycle of Hamilton, Canada (43°15′00″ N–79° 51′ 00″ W).

Preparation of cultured branchial epithelia. Gill cells were obtained using aseptic techniques under a laminar flow hood as described previously (Fletcher et al. 2000; Kelly et al. 2000), with additional modifications as outlined in Galvez et al. (2002) and Galvez et al. (2006). In brief, fish were killed by decapitation, gill baskets were excised, and the filaments were washed three times in phosphate-buffered saline (PBS) containing antibiotics (see *below*). Macerated tissue was taken through two consecutive cycles of tryptic digestion (0.05% trypsin containing 5.5 mM ethylenediamine tetraacetic acid [EDTA] Na_2) at 300 rpm for 8 min. Dispersed gill cells were passed through a 100-μm cell strainer, before being resuspended in ~3 mL culture

medium (Leibovitz's L15 medium supplemented with 2 mM glutamine, 5% fetal bovine serum [FBS], and antibiotics [0.1 mg/mL gentamicin, 0.02 *v*/*v* penicillin–streptomycin, 0.003 *v*/*v* fungizone; Gibco). Antibiotics and FBS were kept in the L15 medium at all times to prevent contamination. Approximately 20% of the dispersed cells were collected at this stage, diluted approximately fivefold in L15 medium, and counted using a hemocytometer. The remaining dispersed gill cells were layered on a discontinuous Percoll gradient (1.03 and 1.05 g/mL; Sigma, St. Louis, MO). Mucus and cellular debris distributing to the top of the 1.03 g/mL layer were discarded. Cells fractionating to the 1.03–1.05 g/mL interface (PV cells), and to the bottom of the 1.05 g/mL Percoll fraction (predominantly MR cells) were collected, pelleted in PBS by centrifugation, and resuspended in approximately 2–5 mL L15 medium. All density centrifugations were performed using a swing-type rotor at 500×*g* for 10 min. Aliquots of total dispersed gill cells and of each isolated gill cell population were subjected to trypan blue dye exclusion test to identify live cells. Cell viability was always between 90% and 95% of the total cells. Cell counts for each of the collected fractions was obtained after Percoll density centrifugation using a hemocytometer.

Three distinct preparations of cultured gill epithelia were developed on semipermeable cell culture inserts (Falcon, pore density=1.6×10^6 pores per cm^2, pore size=0.4 μm), which were housed in Multiwell™ cell culture insert plates (Falcon). In one preparation, epithelial cultures were prepared using the original DSI technique, as outlined by Fletcher et al. (2000). In this method, dispersed gill cells (without Percoll separation) were seeded directly on cell culture inserts. These preparations produced a mixed population of PV cells and MR cells, following seeding of total dispersed gill cells collected from two separate fish on back-to-back days. The other two cultured epithelia were prepared from dispersed cells following Percoll density centrifugation. One preparation, termed PV cell-rich, was prepared using only cells collected from the 1.03–1.05-g/mL Percoll fraction, once again using successive seedings from two different fish on consecutive days. The other cultured gill epithelia, termed MR cell-rich, was seeded using a ratio of PV cells/MR cells of 85/15% for the first seeding and 0/100% for the second seeding. Thus, all three types of cultured epithelia (DSI, PV cell-rich, and MR cell-rich) were prepared by seeding gill cells from two fish at 24-h intervals, using a density of $1.0–3.0\times10^6$ viable cells per 0.9 cm^2 insert for each seeding. This seeding density is typical for the DSI technique described by Fletcher et al. (2000). The production of cultured gill epithelia was typically limited by the abundance of MR cells that could reliably be obtained. In general, 155×10^6 PV cells, 15×10^6–20×10^6 MR cells, and 260×10^6 total gill cells were obtained per fish. Cultured gill epithelia were incubated under an air atmosphere at 18°C. During the initial 2 d of culture, the apical side of the membrane contained a final volume of 0.8 mL L15 media, whereas the basolateral side contained 1.0 mL L15 medium. Before the second batch of cells were seeded, cell culture inserts were washed once with PBS, and fresh L15 medium was added to the apical and basolateral sides. Inserts were washed at the end the second day, and 1.5 and 2.0 mL L15 medium were added to the apical and basolateral sides, respectively. The preparations were subsequently cultured for 8–14 d under these conditions, with both apical and basolateral media changed every 48 h. All experiments were performed on mature epithelia, which were characterized by the establishment of a stable TER. Phase-contrast microscopy and electrophysiology (see *below*) were used to monitor the development of gill epithelial preparations over time.

Electrophysiological measurements. Beginning on day 2, and every 24 h afterwards, the development of the cultured gill epithelia was monitored by analysis of TER, and on every second day, cell culture inserts were replenished with fresh L15 media. On these days, electrophysiological measurements were taken immediately after the media change. TER was monitored using STX-2 chopstick electrodes connected to a specially modified 200-kΩ EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). The TER of blank inserts (cell culture inserts alone with no gill cells) bathed basolaterally with L15 media and apically with appropriate solution (L15 media or dechlorinated fresh water, the latter in asymmetrical tests) was subtracted from the TER values obtained for cultured gill epithelia. These blank TERs were consistently between 0.1 and 0.15 kΩ, which represented less than 1% of the TER of gill epithelia. The TER values (in kΩ) provided by the voltohmmeter were normalized to the surface area of the cell culture insert by multiplying the blank-corrected TER values by 0.9 cm^2 (the area of each insert). TEP was measured using agar (4%) salt bridges in 3 M KCl, connected to a high impedance voltmeter (Radiometer pHM 84, Copenhagen, Denmark) through Ag/AgCl electrodes (World Precision Instruments). TEP measurements were expressed relative to the apical side as 0 mV after correction for junction potentials.

Unidirectional sodium fluxes. Unidirectional Na^+ flux measurements were performed on cultured gill epithelia under symmetrical (L15 media on apical surface) or asymmetrical conditions (dechlorinated fresh water on apical surface) using radioactive $^{22}Na^+$ (NEN-Dupont, Boston, MA) following the protocol of Wood et al. (1998). $^{22}Na^+$ influx rates were measured by adding radioactivity to either the L15

medium or dechlorinated fresh water on the apical side and determining the rate of its appearance on the basolateral side. For $^{22}Na^+$ flux measurements on symmetrical gill cultures, both sides of each cultured epithelium were rinsed with 1 mL L15 medium; the wells were then replenished with 1.5 and 2.0 mL of fresh L15 medium on the apical and basolateral sides, respectively. $^{22}Na^+$ (0.5 μCi mL^{-1}) was added to the apical L15 medium, and the appearance of radioactivity on the basolateral side of the membrane was monitored after at least 6 h incubation. At the start and end of a flux period, 0.1 mL L15 medium was removed from the apical side, and at the end of a flux period, 1.0 mL medium was removed from the basolateral side of each preparation for analyses of total Na^+ concentration and $^{22}Na^+$ radioactivity. Na^+ concentrations were measured by atomic absorption spectrophotometry (Varian AA220FS, Mulgrave, Australia), and radioactivity was determined using an automated γ-counter (Perkin Elmer). Apical radioactivity was calculated from the average specific activities before and after fluxing, whereas the terminal basolateral sample was used to assess the appearance of radioactivity on the basolateral side.

Following analysis of unidirectional $^{22}Na^+$ influx, each insert was washed three times with PBS to remove radioactivity. Inserts were kept in a fourth rinse for an additional 2–3 h to allow for $^{22}Na^+$ depuration from the epithelia. Unidirectional $^{22}Na^+$ efflux (basolateral to apical movement) rates were then measured under symmetrical conditions by adding $^{22}Na^+$ to the basolateral side and monitoring its appearance on the apical side, as described previously. This approach made it possible to measure unidirectional $^{22}Na^+$ influx and $^{22}Na^+$ efflux in a single preparation and to subsequently calculate net Na^+ flux rates, as well as the Ussing flux ratio for each preparation (see below).

Direct estimates of unidirectional influx (J_{in}) and efflux (J_{out}) of $^{22}Na^+$ under symmetrical conditions were expressed in nmol cm^{-2} per hour according to Eqs. 1 and 2:

$$J_{in}^{Na^+} = \Delta[Na^*]_{bl} \times \frac{1}{SA_{ap}} \times \frac{Volume_{bl}}{Time \times Area} \quad (1)$$

$$J_{out}^{Na^+} = \Delta[Na^*]_{ap} \times \frac{1}{SA_{bl}} \times \frac{Volume_{ap}}{Time \cdot Area} \quad (2)$$

Where $\Delta[Na^*]$ is the change in radioactivity (cpm mL^{-1}) and SA is the mean specific activity (cpm $nmol^{-1}$ Na^+) in the apical (ap) or basolateral (bl) sides. Net flux (J_{net}) was calculated according to Eq. 3:

$$J_{net} = J_{in} + J_{out} \quad (3)$$

For $^{22}Na^+$ flux experiments in cultures under asymmetrical conditions, inserts were washed as described before, except that dechlorinated fresh water (ionic composition same as acclimation water) was used to rinse the apical side of the membrane. $^{22}Na^+$ (0.5 μCi mL^{-1}) was added to dechlorinated fresh water on the apical side only. Unidirectional influx (J_{in}) rates for $^{22}Na^+$ were performed in cultured inserts under asymmetrical conditions as described before and calculated using Eq. 1. In this set of fluxes, net flux rates were calculated based on the differences in total Na^+ concentration of the fresh water in the apical side over time. Accordingly, J_{out} was calculated using Eq. 3; this method did not necessitate the use of unidirectional $^{22}Na^+$ efflux experiments.

Passive (diffusional) Na^+ transport was predicted using the Ussing flux ratio criterion according to Eq. 4:

$$\text{Using flux ratio}: \frac{J_{in}}{J_{out}} = \frac{A_{ap}}{A_{bl}}\, e(-zFV/RT) \quad (4)$$

where A represents the Na^+ activities in the apical and basolateral solutions, respectively, V is the measured TEP in volts (See Tables *1* and, *2*), z is the valence, and F, R, and T are thermodynamic constants. Na^+ activities (A) in L15 media were estimated at 75% of the total Na^+ concentrations and in fresh water as 100%, as described by Wood et al. (1998). Statistically significant disagreement in the measured ratio of J_{in} (Eq. 1) to J_{out} (Eq. 2) to that predicted by the Ussing ratio (Eq.4) was used as the criterion for the presence of active Na^+ transport.

Calcium binding and unidirectional influx. Cultured gill epithelia were washed three times with L15 medium prior to measurements of Ca^{2+} fluxes, as described above. Briefly, $^{45}Ca^{2+}$ (as $^{45}CaCl_2$; NEN-Dupont) was added to the apical sides of cultured epithelia at a concentration of 7.5 μCi mL^{-1} in 1.5 mL L15 medium. The appearance of radioactive $^{45}Ca^{2+}$ on the basolateral side after a 3-h period

Table 1. Comparison between the predicted and the observed (J_{in}/J_{out}) flux ratios for Na^+ flux rates and the measured TEP used in the calculation of the predicted ratio for cultured DSI, PV cell-rich, and MR cell-rich gill epithelia under symmetrical conditions (apical L15/basolateral L15 exposure)

Epithelia	Flux ratios		
	Predicted	Observed	TEP (mV)
DSI	0.92±0.015	9.20±1.98*	2.05±0.28a
PVC	0.93±0.007	26.93±0.93*	1.91±0.17a
MRC	0.38±0.134	13.66±1.52*	24.10±3.15b

Values are expressed as means±SE (n=8)
DSI Double-seeded inserts, *PVC* pavement cells, *MRC* mitochondria-rich cells, *L15* Leibovitz's L15 medium, *TEP* transepithelial potential
*p<0.05, represents a statistically significant difference between the observed and the predicted flux ratio for each treatment. Significant differences (p<0.05) between TEP of different treatment wherever common letters are not shared.

Table 2. Comparison between the predicted and the observed (J_{in}/J_{out}) flux ratios for Na^+ flux rates and the measured TEP used in the calculation of the predicted ratio for cultured DSI, PV cell-rich, and MR cell-rich gill epithelia under asymmetrical conditions (apical freshwater/basolateral L15 exposure)

Epithelia	Flux ratios ($\times 10^{-3}$)		
	Predicted	Observed	TEP (mV)
DSI	7.4±0.1	58.2±14.3	−1.1±0.5a
PVC	7.4±0.1	287.7±64.3*	−3.1±0.2 b
MRC	9.4±0.3	40.7±6.1*	−9.3±0.6c

Values are expressed as means±SE (n=8)
DSI Double-seeded inserts, *PVC* pavement cells, *MRC* mitochondria-rich cells, *L15* Leibovitz's L15 medium, *TEP* transepithelial potential
*$p<0.05$, represents a statistically significant difference between the observed and the predicted flux ratio for each treatment. Significant differences ($p<0.05$) between TEP of different treatment wherever common letters are not shared.

was used as an index of the unidirectional influx of $^{45}Ca^{2+}$. Ca^{2+} influx rates were calculated in a similar fashion to Na^+ influx rate as described in Eq. 1 and expressed in nmol cm^{-2} h^{-1}. Inserts were then washed four times to remove external $^{45}Ca^{2+}$, and membranes were digested overnight in 1 mL HNO_3 acid. The samples of L15 medium and the membrane digests were transferred into 5–10 mL Ultima Gold scintillation fluor (Perkin Elmer, Downers Grove, IL) for beta counting (Tri-carb 2900TR Liquid Scintillation Counter, Perkin Elmer). Internal standardization demonstrated that quench was both constant and negligible, so no correction for counting efficiency was necessary. Ca^{2+} binding was expressed in pmol cm^{-2}.

Na^+/K^+-ATPase activity. Na^+/K^+-ATPase activity was measured using an enzymatically coupled reaction converting each mole of adenosine triphosphate (ATP) hydrolysis to NADH oxidation as described by McCormick (1993). Gill cells were obtained from the cultured epithelial preparations following a brief trypsin digestion (~2–3 min). Trypsination was terminated by the addition of 10% FBS in PBS (pH 7.7). Resuspended cells were centrifuged at 500×g for 5 min, washed once in PBS, and resuspended in 100 μL ice-cold SEI buffer (150 mM sucrose, 10 mM Na_2 EDTA, 50 mM imidazole, pH 7.3). Cells were frozen at −80°C awaiting further processing. Immediately prior to analyses, samples were thawed, and 25 μL SEID buffer (0.5 g sodium deoxycholate dissolved in SEI buffer) was added. Cells were homogenized by passing ten times through a 20-G, 0.5-in. needle, and the resulting homogenate was centrifuged at 5,000×g for 2 min. Na^+/K^+-ATPase activity in the supernatant was calculated based on the difference in ATP hydrolysis between reactions performed in the absence and presence of ouabain (500 μM; Sigma). The rate of NADH oxidation was monitored by the change in UV absorbance at 340 nm at 15-s intervals over a period of at least 10 min. These rates were normalized to total protein as measured by the Bradford assay using a commercial dye reagent (Sigma) and bovine serum albumin (Sigma) as a standard.

Visualization of mitochondria-rich cells. Rhodamine-123 (Sigma-Aldrich) was added to both the apical and basolateral culture media at a final concentration of 26 μM and was allowed to incubate for 1.5 h at 18°C. At the end of the incubation, the rhodamine solution was removed, and the cultures were washed twice with PBS for 5 min. The cultures were then observed with an inverted fluorescence microscope (Model IM35, Zeiss, West Germany). MR cells were seen as brightly stained cells. Five images (0.4 mm^2 per image) were randomly selected per cell culture insert (n =6 inserts per treatment) and used to calculate the number of cells staining positively with rhodamine-123.

Statistical analyses. All data are expressed as mean values ±standard error of the mean (n=6–8). The total number of replicates for any analyses was obtained from two or more separate preparations. Statistical differences between means were tested using one-way analysis of variance and the Student Newman–Keuls test for multiple comparisons. All tests assumed a two-tailed probability, except for the statistics for Na^+/K^+-ATPase activity, which assumed a one-tail probability because higher activity levels were predicted a priori in the MR cell-rich preparations. A paired t test was used for Ussing flux ratio criteria comparisons. A fiducial limit of $p<0.05$ was considered statistically significant throughout.

Results

Electrophysiology of cultured gill epithelia under symmetrical and asymmetrical conditions. TER in DSI, PV cell-rich, and MR cell-rich gill epithelia increased over time, showing a sigmoidal growth pattern in each of the preparations (Fig. 1*A*). MR cell-rich epithelia generally reached the highest plateau levels, with TER values routinely greater than 30 kΩ cm^2. TERs of DSI and PV cell-rich preparations would routinely plateau at lower values (typically 20–30 and 10–25 kΩ cm^2, respectively). As early as day 3, TER was significantly elevated in MR cell-rich epithelia compared to DSI and PV cell-rich preparations. DSI and PV cell-rich epithelia had similar TER values during the early stages of growth, although PV cell-rich cultures showed significantly lower TER than DSI cultures by day 7. TER of DSI and PV cell-rich preparations would occasionally plateau near values

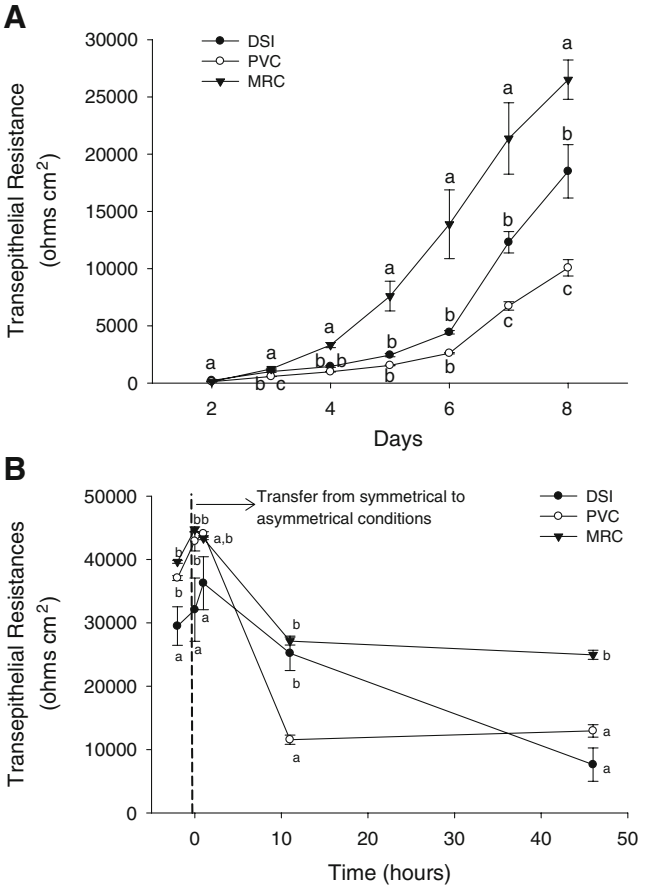

Figure 1. The TER (in Ω cm^2) of cultured gill epithelia developed using the standard or a modified version of the double-seeded insert technique, and kept under *A* symmetrical conditions or after transfer to *B* asymmetrical conditions. Standard cultures are identified as DSI, whereas modified cultures are identified as PV cells or MR cells. The modified gill cultures were established by first enriching for pavement cells (*PVC*) and mitochondria-rich cells (*MRC*) using Percoll density centrifugation based on the methodology of Galvez et al. (2002). The PV cell-rich cultures involved seeding PV cells from two fish on succeeding days. The MR cultures involved seeding PV cells and MR cells at ratio of 85:15, respectively, on day 1 and 100% MR cells on day 2. Values are means±SE (n=6). Significant differences between treatments at a specific time exist (p<0.05) wherever common letters are not shared.

approaching those MR cell-rich epithelia (e.g., Fig. 1*B*), although in these circumstances, it would take approximately 2–3 d longer for all preparations to have similar TER values. Once plateau-level TER had been reached, cultured gill epithelia were transferred from symmetrical to asymmetrical conditions (Fig. 1*B*). Initial exposure of epithelia to apical freshwater elicited a transient increase (within the first 3 h) in TER. However, by 12-h freshwater transfer, TERs were lowered significantly from pretransfer values, although MR cell-rich preparations stabilized at a TER at least twofold higher than PV cell-rich or DSI epithelia (Fig. 1*B*).

The TEP of MR cell-rich epithelia was also significantly higher than measured in DSI and PV cell-rich cultures by day 8 (Fig. 2*A*). Under symmetrical conditions, TEP was +23.3±3.5 mV for MR cell-rich epithelia, compared to +5.4±0.7 mV and +2.0±0 mV for DSI and PV cell-rich preparations, respectively. Freshwater transfer led to an immediate reversal of TEP to negative values (Fig. 2*B*), followed by a partial recovery (return toward 0 mV) in all treatments, in particular for those of DSI and PV cell-rich cultures. Nonetheless, all treatments had negative TEP values even after 48 h in fresh water.

Unidirectional sodium fluxes under symmetrical and asymmetrical conditions. Unidirectional Na^+ fluxes were measured using radioisotopic $^{22}Na^+$ under symmetrical and asymmetrical conditions. Unidirectional Na^+ influx (apical to basolateral flux) rates were not significantly different

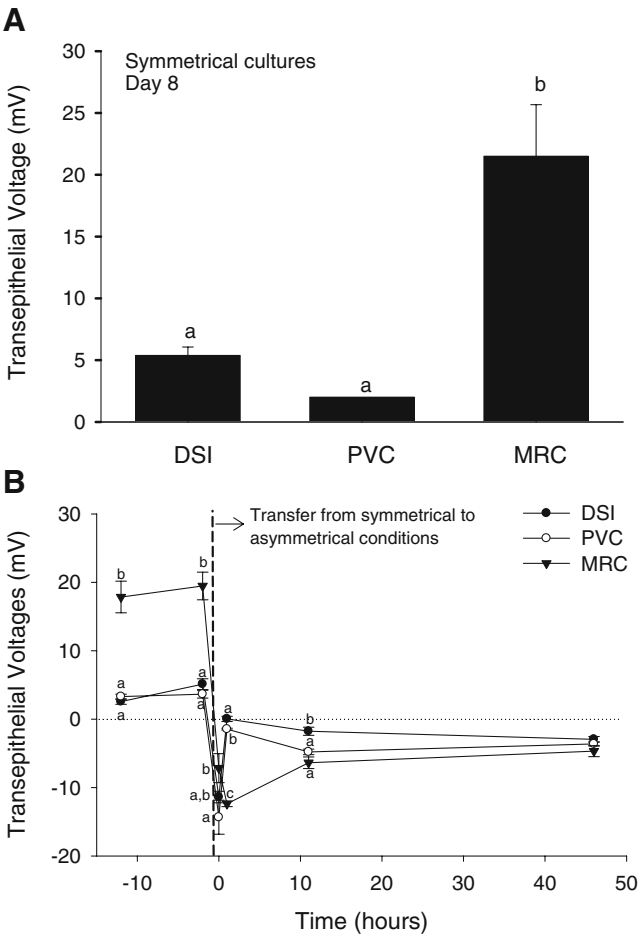

Figure 2. The transepithelial potential (in mV) across 8-d cultured gill epithelia developed using the standard or a modified version of the double-seeded insert technique and kept under *A* symmetrical conditions or after transfer to *B* asymmetrical conditions. See *legend of Fig. 1* for additional information. Values are means±SE (n=6). Significant differences between treatments at a specific time exist (p< 0.05) wherever common letters are not shared.

from one another, ranging between 162.5±13.4 and 198.8±18.8 nmol cm^{-2} h^{-1} (Fig. 3*A*). Additionally, unidirectional Na^+ effluxes were significantly lower than their respective influxes for all treatments (Fig. 3*A*). However, Na^+ efflux was significantly lower in the PV cell-rich epithelia (6.1±0.4 nmol cm^{-2} h^{-1}, compared to the DSI (21.4±3.5 nmol cm^{-2} h^{-1}) and MR cell-rich (26.2±1.4 nmol cm^{-2} h^{-1}) cultures, which in the latter cases did not vary significantly from each other. The calculated net fluxes revealed large net movements of Na^+ in the apical-to-basolateral directions across the three types of epithelia under symmetrical conditions. These positive net fluxes were approximately equal in the three different types of preparations. During fluxes, TEP remained relatively constant through time. Application of the Ussing flux ratio criterion showed a large active transport of Na^+ in the inward direction in the DSI, PV cell-rich, and MR cell-rich gill epithelia (Table 1). However, the greatest deviation between the observed ratio and the predicted flux ratio was found in the MR cell-rich preparations, showing a 36-fold deviation, compared to a 29-fold deviation in the PV cell-rich preparations and only a tenfold difference between these ratios in the DSI preparations.

Upon transfer to asymmetrical conditions, the unidirectional Na^+ influxes decreased significantly, whereas the unidirectional Na^+ effluxes rates were increased, leading to net Na^+ losses in all the treatments (Fig. 3*B*). The only apparent difference between the treatments under asymmetrical conditions was a lower unidirectional Na^+ efflux rate in the MR cell-rich gill cultures.

However, application of the Ussing flux ratio criterion revealed evidence of significant active uptake of Na^+ from apical fresh water in the PV cell-rich preparations, with an observed flux ratio 39-fold higher than predicted for passive transport alone (Table 2). Much lower differences for MR cell-rich (fourfold higher than predicted) and DSI cultures (eightfold higher) were observed. However, of these two treatments, only the former was statistically significant, indicating active Na^+ uptake in MR cell-rich preparations.

Na^+/K^+-ATPase activity. The activity of branchial Na^+/K^+-ATPase was approximately 2.5-fold higher in the MR cell-rich preparations compared to the PV cell-rich epithelia (Fig. 4). Although DSI and PV cell-rich epithelia had similar gill Na^+/K^+-ATPase activities, DSI and MR cell-rich preparations were not significantly different from one another.

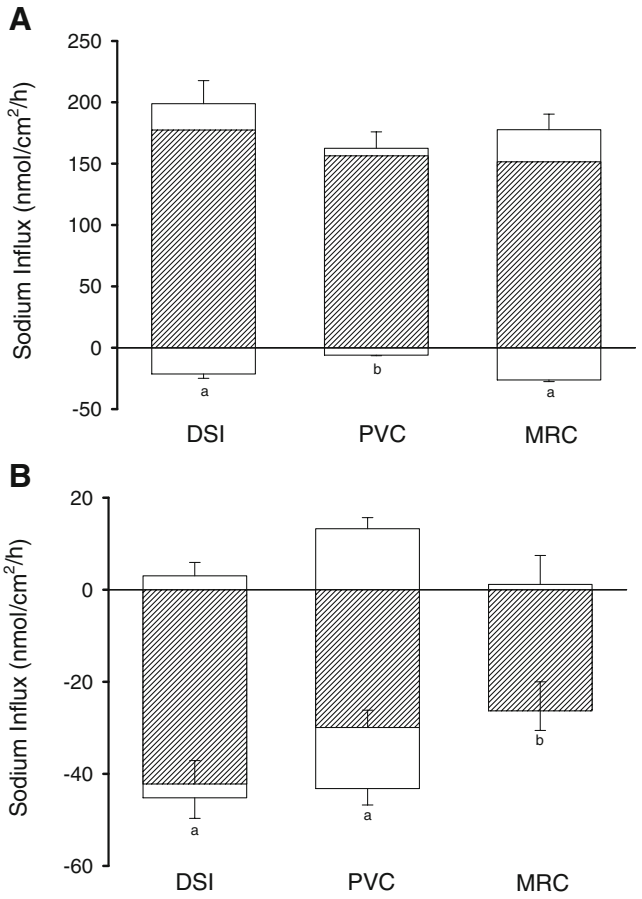

Figure 3. Na^+ influx (*upward bars*), efflux (*downward bars*), and net flux (*hatched bars*) rates in cultured gill epithelia, developed using the standard (DSI) or a modified version of the double-seeded insert technique (PV cell-rich or MR cell-rich), and kept under *A* symmetrical conditions, or after transfer to *B* asymmetrical conditions. See *legend of Fig. 1* for additional details. Values are means±SE (n= 8). Significant differences in Na^+ efflux rates between treatments at any specific time exist (p<0.05) wherever common letters are not shared. There were no significant differences in Na^+ influx or net Na^+ flux rates between treatments.

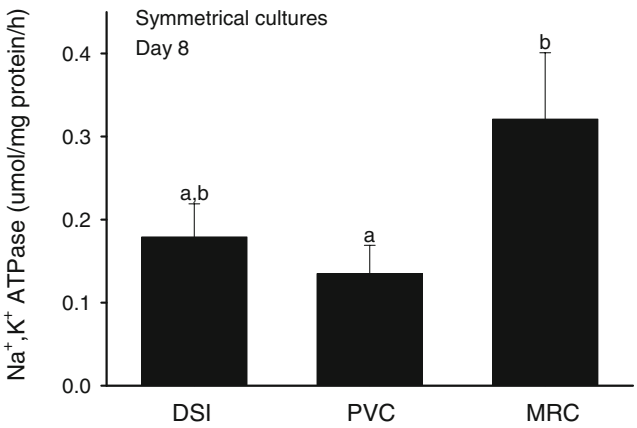

Figure 4. Na^+/K^+-ATPase activity in 8-d cultured gill epithelia developed using the standard or a modified version of the double-seeded insert technique and kept under symmetrical conditions. See *legend of Fig. 1* for additional information. Values are means±SE (n= 6). Significant differences exist (p<0.05) wherever common letters are not shared.

Unidirectional Ca^{2+} influx and epithelial Ca^{2+} binding under symmetrical conditions. The apical to basolateral flux of radioisotopic $^{45}Ca^{2+}$ was used to monitor the unidirectional influx of Ca^{2+} in the cultured gill epithelia under symmetrical conditions. MR cell-rich gill cultures exhibited Ca^{2+} influx rates approximately fourfold higher than measured in the both the standard DSI cultures and PV cell-rich cultures (Fig. 5*A*). Similarly, $^{45}Ca^{2+}$ binding to the MR cell-rich cultures after 3-h incubation was approximately sixfold higher than measured in DSI and PV cell-rich gill cultures (Fig. 5*B*). The DSI and PV cell-rich preparations had similar rates of unidirectional Ca^{2+} influx and $^{45}Ca^{2+}$ binding (Fig. 5*A*, *B*).

Visualization of mitochondria-rich cells. Cultured gill epithelia were stained with rhodamine-123 to assess MR cell density on culture inserts. MR cell-rich epithelia had a MR cell density of 113.2±10.0 cells per 0.4 mm^2, which was approximately threefold higher than that of DSI cultures (40.8±1.6 cells per 0.4 mm^2). There was no significant rhodamine-123 staining in PV cell-rich cultures. Five images per cell culture insert (n=6 inserts per treatment) were used in the analyses.

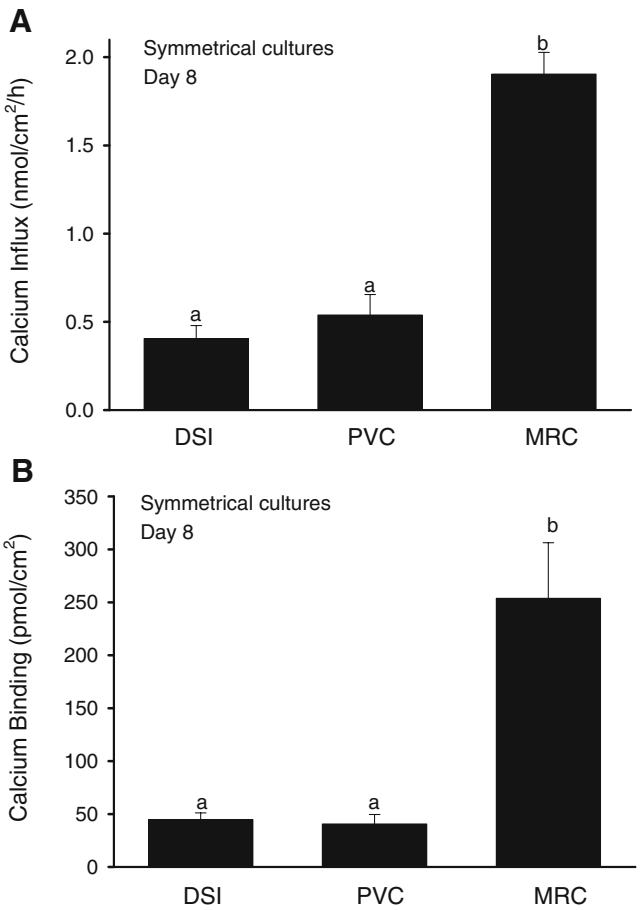

Figure 5. *A* Ca^{2+} binding (in pmol/cm^2) and unidirectional Ca^{2+} influx rates (in nmol cm^{-2} h^{-1}) in cultured gill epithelia, developed using the standard (DSI) or a modified version of the double-seeded insert technique (PV cell-rich) or (MR cell-rich), and kept under symmetrical conditions. See *legend of Fig. 1* for additional details. Values are means±SE (n=6). Significant differences exist (p<0.05) wherever common letters are not shared.

Discussion

This study highlights a new method for producing cultured epithelia consisting either of an enriched population of PV cells or a mixed population of PV cells and MR cells from the gills of adult rainbow trout. Although this method is similar in many respects to the traditional DSI technique, it utilizes Percoll density centrifugation to purify PV cells and MR cells from a dispersed gill cell population prior to culture (Goss et al. 2001; Galvez et al. 2002; Galvez et al. 2006). This approach provides several notable advantages over the standard SSI, DSI, and SDSI methods (see "Introduction"). First, even though the SSI technique can reliably establish PV cell-rich gill epithelia, it requires dispersed cells to be preseeded in culture flasks for 6–9 d and then retrypsinated, before PV cell-rich epithelia can be established on semipermeable membranes (Wood and Part 1997). Our new PV cell-rich method allows direct seeding of pure populations of PV cells onto culture inserts, which expedites their development. Alternatively, MR cell-rich cultures can be prepared with an average threefold higher enrichment of MR cells over the traditional DSI method. One inherent advantage is that the same pair of fish can be used to establish PV cell-rich and MR cell-rich epithelia, allowing them to be maintained under similar conditions throughout their culture, and tested in parallel experiments. Preliminary results also suggest that cultured gill epithelia can be developed having different proportions of MR cells to PV cells and with different electrophysiological characteristics (data not shown). Ultimately, the method provides an excellent platform for more careful examination of PV cell and MR cell functions in an epithelial system.

MR cell-rich gill epithelia showed several unique electrophysiological and ion transport characteristics likely associated with the threefold higher level of MR cell incorporation. Na^+/K^+-ATPase activity of MR cell-enriched gill epithelia was similarly increased by about 2.5-fold over either DSI cultures or PV cell-rich gill epithelia, demonstrating an improved integration of MR cells into epithelia using this new technique (Fig. 4). Another important factor is that Percoll centrifugation allows us to remove mucus before seeding of cells onto semipermeable membranes. Based on our experience, mucus causes aggregation of gill cells, particularly MR cells, tending to reduce their

integration within cultured epithelia. There is anecdotal evidence that the DSI and SSI cultures are more prone to failure during the summer months, when fish tend to have higher levels of mucus on the gills (Kelly et al. 2000).

We noted that under symmetrical conditions, the TEP values of MR cell-rich epithelia were highly positive (Fig. 2*A*) and their TER values were significantly elevated (Fig. 1*A*) compared to the other two epithelial preparations. Even under circumstances when the TER of PV cell-rich and DSI cultures rose to levels approaching those of MR cell-rich preparations, they required significantly more time to reach a stable TER. These results are qualitatively similar to those observed previously for SSI and DSI cultures, where SSI epithelia (PV cells only) had consistently lower values of both TER and TEP (Fletcher et al. 2000; Kelly and Wood 2001b, 2002a, b; Wood et al. 2002b). Fletcher et al. (2000) found a significant positive relationship between MR cell density and TER values in DSI preparations, which they proposed was associated with the presence of increased tight junction proteins between PV cells and MR cells. There was no relationship of TER with absolute cell numbers or protein content in the epithelia (Wood et al. 2002a). Replacement of the apical L15 medium with fresh water led to significant reductions in TER in all the preparations; however, the effect on epithelial integrity was least pronounced in MR cell-rich epithelia.

An explanation may be found in the fact that several studies have demonstrated a direct influence of Na^+/K^+-ATPase activity on the permeability of tight junction proteins. Inhibition of Na^+/K^+-ATPase was found to reduce the phosphorylation of specific residues on occludins and claudins, which represent important constituents of tight junctions in transporting epithelia (Violette et al. 2006; Rajasekaran et al. 2007). Although the control of epithelial integrity by tight junction proteins is poorly understood in teleost fish, a similar role of Na^+/K^+-ATPase in fish gill is probable considering the highly conserved nature of these proteins in transporting epithelia.

Under symmetrical conditions, the positive TEP values in all three types of cultured gill epithelia were possibly caused by an electrogenic Cl^- extrusion as in seawater fish (Potts 1984; Wood and Marshall 1994) and/or by an electrogenic Na^+ uptake across the epithelia. Certainly, in the present study, clear active uptakes of Na^+ were observed preparations with influxes greatly exceeding effluxes in all three (Fig. 3*A*; Table 1). Transfer of all three types of cultured gill epithelia from symmetrical to asymmetrical conditions (apical fresh water and basolateral L15) resulted in an instantaneous reversal of TEP to negative values (Fig. 2*B*), each eventually stabilizing at similar levels. These TEP reversals were likely associated with large negative Na^+ diffusion potentials in freshwater, which outweigh any small electrogenic components and reflect a greater passive permeability of the epithelial junctions to Na^+ than to Cl^- (Potts 1984). The exact mechanism controlling these diffusive processes is unclear. Evidence is now emerging that tight junction proteins function much like ion channels with a high degree of specificity. Claudins represent a large family of proteins believed to control the selectivity of the paracellular pathway to ions (Loh et al. 2004; Van Itallie and Anderson 2006). An added consequence is that diffusion potentials, which are dictated by the relative permeability of paracellular channels to cations versus anions, will be altered, helping to explain the highly negative TEP of MR cell-rich epithelia under asymmetrical conditions.

Using the Ussing flux ratio criterion by which a significant discrepancy between observed and predicted values signifies active transport, we were able to demonstrate active Na^+ uptake in each of the gill epithelial preparations under symmetrical conditions (Table 1, Fig. 3*A*) or in the PV cell-rich and MR cell-rich epithelia under asymmetrical conditions (Table 2, Fig. 3*B*). This has been observed in some previous studies under symmetrical conditions (e.g., Kelly and Wood 2001a; Zhou et al. 2003) and not in others (Fletcher et al. 2000; Kelly and Wood 2001b, 2002a,b; Zhou et al. 2003; Zhou et al. 2004) but only in DSI epithelia with hormonal supplementation under asymmetrical conditions (Zhou et al. 2003). It has never previously been seen under asymmetrical conditions in SSI preparations, which are essentially composed of PV cells and devoid of MR cells (Wood et al. 1998; Kelly and Wood 2001a,b). Recent work with SSI epithelia has largely found Na^+ transport to be either passive (Wood and Part 1997; Wood et al. 1998) or active in the efflux direction (Kelly and Wood 2001a, 2002a,b). Attempts to stimulate non-diffusional sodium uptake with cortisol (Kelly and Wood 2001a), prolactin (Kelly and Wood 2002b), and homologous serum supplementation (Kelly and Wood 2002a) led to only modest effects in SSI cultures.

Clearly, the PV cell-rich cultures of the present study differ from the SSI epithelia of earlier studies, for the first time showing that PV cells are capable of active Na^+ uptake from both freshwater and isotonic media. Possibly in the SSI technique, the PV cells lose their active Na^+ transport function as a result of the prolonged culture in flasks and retrypsinization prior to epithelial formation, whereas this is avoided with the present PV cell-rich cultures. Our results therefore support previous studies based on more circumstantial evidence, which have proposed a role for PV cells in unidirectional Na^+ uptake in the freshwater trout gill (Goss et al. 1992; Lin et al. 1994; Sullivan et al. 1995; Goss et al. 1998). In contrast, the active Na^+ uptake in the MR cell-rich preparations suggests also a role of MR cells in this ion transport. However, it can also be argued that the

somewhat attenuated Na^+ active transport in the MR cell-rich epithelia, in comparison to the PV cell-rich epithelia, may be due to the lower number of PV cells seeded on the cultured inserts of the latter. Clearly, more work is needed to further elucidate the relative roles of PV cells and MR cells in active Na^+ transport.

The abilities of these different epithelial preparations to transport and/or bind Ca^{2+} were also assessed under symmetrical conditions (Fig. 5). Binding in fact reflects both internal and surface accumulation of $^{45}Ca^{2+}$; these measurements were taken because of our interest in biotic ligand modeling of metal (including Ca^{2+}) binding to cultured gill epithelia, which involve 3-h binding assays (Zhou et al. 2005). Previous studies have found strong correlative evidence for the role of MR cells in the unidirectional Ca^{2+} uptake in the freshwater fish gill (Perry and Wood 1985; Perry et al. 1992; Marshall et al. 1995), describing a clear relationship between apical MR cell exposure and density and ion transport. For the most part, molecular studies have corroborated these findings, demonstrating a relationship between epithelial calcium channel (ECaC) expression, MR cell density, and unidirectional Ca^{2+} uptake (Qiu and Hogstrand 2004; Pan et al. 2005; Hwang and Lee 2007). However, the situation is confounded by recent reports that ECaC expression also occurs in PV cells in freshwater rainbow trout and that changes here also correlate with changes in unidirectional Ca^{2+} uptake in the intact organism (Shahsavarani et al. 2006; Shahsavarani and Perry 2006). Results from the present study argue for the preferential involvement of MR cells in Ca^{2+} transport, demonstrating a fourfold higher unidirectional Ca^{2+} uptake (Fig. 5*A*) and more than sixfold higher Ca^{2+} accumulation (Fig. 5*B*) in MR cell-rich epithelia under symmetrical conditions, compared to PV cell-rich and DSI cultures. Fletcher et al. (2000) first demonstrated active Ca^{2+} uptake in DSI cultures under symmetrical conditions and reported that it was absent in SSI epithelia. This was confirmed by Kelly and Wood (2008) who further showed that cortisol, which is known to promote MR cell function (Perry and Wood 1985; Shahsavarani and Perry 2006), stimulated Ca^{2+} uptake in DSI preparations. The present results suggest that the MR cell-rich epithelium, either due to its high MR cell density or some other factor, is more conducive to transporting Ca^{2+}, than is the DSI preparation. In this regard, we recently demonstrated a threefold higher accumulation of $^{45}Ca^{2+}$ in a subpopulation of MR cells (the so-called peanut lectin agglutinin positive or PNA^+ cells), compared to PV cells and PNA-MR cells (Galvez et al. 2006). Future studies should attempt to investigate the influence of differential incorporation of MR cell subtypes into gill epithelial preparations to better ascertain their roles in transepithelial transport.

Conclusions

Our understanding of the mechanistic basis of ion regulation in freshwater fish is complicated by the lack of a suitable model of the freshwater fish gill. Seeding of cell culture inserts with PV cells and MR cells following Percoll density gradient separation of the two cell types allows us to "tailor"-make gill epithelia with different proportions of MR cells and PV cells, thereby illuminating their respective functions.

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