Permeability properties of the teleost gill epithelium under ion-poor conditions

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Chasiotis H, Kolosov D, Kelly SP. Permeability properties of the teleost gill epithelium under ion-poor conditions. Am J Physiol Regul Integr Comp Physiol 302: R727–R739, 2012. First published December 28, 2011; doi:10.1152/ajpregu.00577.2011.—Permeability properties of the goldfish gill epithelium were examined in vivo and in vitro following exposure to ion-poor water (IPW) conditions. In gill tissue of IPW-acclimated goldfish, transcript abundance of tight junction (TJ) proteins occludin, claudin-b, -d, -e, -h, -7, and -8d increased, whereas ZO-1 and claudin 12 mRNA decreased and claudin-c was unaltered. In association with these changes, TJ depth increased among gill pavement cells (PVCs) and gill PVCs and mitochondria-rich cells (MRCs). PVC and MRC gill cell fractions were isolated using Percoll. Transcripts encoding for occludin, claudin-b, -c, -d, -e, -h, -7, -8d, -12, and ZO-1 were present in both fractions. After IPW acclimation, occludin, claudin-b and -e, and ZO-1 mRNA abundance increased in both fractions. In contrast, claudin-8d mRNA abundance increased in PVCs only while claudin-h decreased in MRCs. Gill permeability was examined using primary cultured goldfish PVC epithelia supplemented with serum derived from IPW-acclimated goldfish. IPW serum supplementation increased transepithelial resistance, reduced [3H]PEG-4000 permeability, and enhanced epithelial integrity during in vitro IPW exposure. IPW serum increased mRNA abundance of occludin, claudin-8d and -e in vitro. Using small interfering RNA, we found that occludin abundance was decreased in cultured gill epithelia, resulting in an increase in [3H]PEG-4000 flux. As occludin increased in the gills of IPW-acclimated fish as well as cultured gill epithelium exposed to IPW serum, results suggest that occludin is a barrier-forming TJ protein in fish gill epithelia. These studies support the idea that TJ proteins play an important role in regulating gill permeability in IPW.

paracellular permeability; tight junction; claudin; occludin; ZO-1; pavement cell; mitochondria-rich cell

THE TELEOST FISH GILL is an architecturally complex multifunctional organ composed of a heterogeneous epithelium overlaying a rich vasculature (42). The gill is directly exposed to the surrounding medium of water and presents a large surface area across which gas exchange, ion and acid/base balance, as well as nitrogenous waste elimination takes place. The fish gill epithelium functions as a complex and pivotal transport site and has evolved so that the requirements of a broad-spectrum transporting tissue are balanced with the need to maintain large concentration/electrochemical gradients between fluid "compartments" of enormously different composition, i.e., blood versus water. In fishes that reside in freshwater (FW), the gill epithelium contributes to the maintenance of hydromineral balance by actively acquiring ions from the surrounding water and restricting passive (obligatory) ion loss. Mechanisms of ion acquisition are complex, occur through the transepithelial pathway, and have been the focal point of numerous studies (for review see Refs. 18 and 23). In contrast, considerably less is known about the mechanisms that control passive ion loss across the gill epithelium of FW fishes. However, it is broadly acknowledged that ion loss across the FW fish gill occurs through the paracellular pathway and that this process is held in check by the properties and components of the epithelial tight junction (TJ) complex (18, 33).

The TJ complex forms the principal barrier to paracellular solute movement across vertebrate epithelia (22). It is composed of transmembrane and cytoplasmic TJ proteins; however, the overall composition of transmembrane components appears to play a crucial role in determining the permeability properties of the paracellular pathway. Variations in the composition of TJ complexes occur primarily due to heterogeneity in the distribution and abundance of claudin transmembrane TJ proteins. In mammals there are ~24 claudin proteins (30, 40) and in fishes, as many as 56 genes encoding for claudin proteins have been described (32). Furthermore, select claudins are reported to be barrier forming, whereas others seem to possess pore-forming characteristics (30). In contrast to most claudins, the transmembrane TJ protein occludin is ubiquitously expressed in vertebrate epithelia, and its abundance can vary considerably between tissues (19, 37). Given that increased occludin abundance is strongly associated with decreased epithelium permeability (for review see Ref. 19), it would seem that this protein also plays an important role in controlling the permeability properties of the vertebrate epithelium.

A role for TJ proteins in modulating the permeability properties of the fish gill epithelium has recently been proposed. For example, alterations in the mRNA abundance of select claudin isoforms have been reported in whole gill tissue taken from euryhaline fishes acclimated to FW or seawater (SW) (4, 5, 17, 38, 41), as well as fish exposed to acidic water (31). Furthermore, hormone-induced alterations in occludin and claudin transcript/protein abundance have been described in association with alterations in the paracellular permeability of primary cultured gill epithelia (12, 15, 25). However, to date, no study has attempted to directly relate measured changes in permeability and TJ molecular physiology of gill epithelia in vitro with the molecular physiology of TJs within the gill epithelium in vivo. To address this, a series of experiments were conducted to examine the properties and molecular physiology of the gill epithelium TJ complex in goldfish following exposure to ion-poor conditions in vivo and in vitro. Acclimation of goldfish (and other fish species) to ion-poor water (IPW) has been reported to result in a very significant reduction in ion efflux rates across the gills (16, 36). This presumably occurs, at least in part, by means of a TJ-mediated reduction in...
paracellular permeability. Reduced gill permeability in ion-poor conditions would curb ion loss in surroundings that not only limit ion acquisition but also significantly increase ion concentration gradients between the internal and external environment. In this regard, recent studies provide evidence to support the idea that gill TJ proteins perform an important physiological role in the regulation of ionoregulatory homeostasis in IPW. More specifically, occludin protein abundance is significantly increased in goldfish gill tissue following IPW acclimation (14), and transcript encoding for claudin-3a, -3c, and -27a have been reported to elevate in the gills of a FW species of puffer fish (Tetraodon biocellatus) following acclimation to IPW (17). In this latter study, changes in the molecular components of the TJ complex occurred in association with increased TJ depth between gill epithelial cells (17). Therefore, by directly comparing the effects of ion-poor conditions on the molecular physiology of gill TJs in vivo and in vitro, significant insight into the importance of TJ proteins in gill tissue and ionoregulatory homeostasis in FW teleost fishes can be attained.

MATERIALS AND METHODS

Experimental Animals

Common goldfish (Carassius auratus, 25–30 g) were obtained from a local supplier (Aleongs International, Mississauga, ON, Canada) and held in 200-liter opaque polyethylene tanks supplied with flow-through dechlorinated freshwater (FW, 20°C; pH 7.9) and placed over a stacked three-stage discontinuous Percoll (Sigma-Aldrich Canada) gradient consisting of a 1.03 g/ml top layer, a 1.06 g/ml middle layer, and a 1.13 g/ml the bottom layer. The stacked cell suspension was then centrifuged (2,000 g for 45 min at 4°C), and two interphase fractions were collected for analysis. Interphase fractions were frozen in liquid nitrogen and stored at −80°C for RNA extraction (see RNA extraction and cDNA synthesis), or an aliquot was removed for the examination of mitochondria fluorescence.

To examine whether interphase fractions were composed of pavement cells (PVCs) or mitochondria-rich cells (MRCs), aliquots of each fraction were stained with the mitochondria-specific dye Mitotracker Deep Red FM (100 nM for 20 min at RT; Invitrogen Canada, Burlington, ON, Canada). After incubation with Mitotracker, cells were centrifuged (500 g, 10 min at RT) and then resuspended and fixed in a 3% paraformaldehyde solution (20 min at RT). Stained and fixed cell fractions were centrifuged once more and resuspended in PBS (pH 7.7). For cell counting, a hemocytometer was used. Differential interference contrast and fluorescent images of Mitotracker-stained cells were captured by laser-scanning confocal microscopy (Olympus BX-51, Olympus Canada, ON, Canada).

Electron Microscopy, Gill Morphometrics, and Immunohistochemistry

TEM and SEM. Goldfish gill tissues collected from FW- and IPW-acclimated goldfish were fixed in 2.5% glutaraldehyde (4 h at RT) for electron microscopy. Subsequent tissue preparation and procedures for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) followed those described by Chasiotis and Kelly (11). TEM samples were examined using a Philips EM 201 TEM (Philips, Eindhoven, The Netherlands). SEM images were captured using a Hitachi S-520 SEM (Hitachi High-Technologies Canada, Toronto, Canada) attached to a Quartz PCI Version 6 image capture system (Quartz Imaging, Vancouver, Canada).

Gill cell morphometrics. The depth of TJ complexes formed by adjacent PVCs (PVC-PVC) or MRCs adjacent to PVCs (MRC-PVC) were measured using TEM images captured from the gills of five fish acclimated to FW and five fish acclimated to IPW. For each fish, TEM images of TJ depth were obtained from five different gill filaments. TJ depth was defined as the distance between the apical-most point of the cell-to-cell contact and the beginning of the adherens junction. For MRC surface area morphometric analysis, afferent edge SEM images from five primary gill filaments from each FW- and IPW-acclimated fish sample (n = 5) were used. MRC apical area was measured by digitally tracing around the outer edge of an MRC apical opening (i.e., that were fully visible within a captured SEM image) and calculating the area within the traced edge. MRC apical openings that were only partially visible in an SEM image were not used to calculate MRC apical area. MRC fractional surface area was determined by dividing the total surface area of all MRC apical openings (i.e., those that were whole and partially visible) by the area of the SEM image (MRC fractional surface area = total area of whole and partially visible MRC apical openings/image area). The number of MRCs exposed at the surface of the gill epithelium was determined by dividing the total MRC surface area (i.e., total area of whole and partially visible MRCs) by mean MRC apical area (MRC no. exposed = total area of whole and partially visible MRCs/mean MRC apical area). All morphometric analysis was carried out using ImageJ software (National Institutes of Health, Bethesda, MD, http://imagej.nih.gov/ij/, 1997–2011).

Immunohistochemistry. FW- and IPW-acclimated goldfish gill tissues were fixed in Bouin’s solution for 4 h at RT. Fixed gills were then washed and stored in 70% ethanol at 4°C until processing for the immunohistochemical detection of Na+/K+-ATPase (NKA). Procedures were conducted according to previously outlined methods (10).
NKA α-subunit antibody (α5; Developmental Studies Hybridoma Bank, Iowa City, IA) was used to examine NKA immunoreactivity. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole, and immunohistochemical images were captured using an Olympus DP70 camera (Olympus Canada) coupled with a Reichert Polvvar microscope (Reichert Microscope services).

**Cultured Gill Epithelia and Media Supplementation With Homologous Serum**

Preparation of primary cultured goldfish gill epithelia. Procedures for the preparation and culture of primary cultured goldfish gill epithelia were conducted according to the methods described by Chasiotis and Kelly (11), using fish held in FW at 25 ± 1°C. All other culture conditions were identical to those previously described (see Experimental animals). Briefly, goldfish gill cells were isolated by trypsination and initially cultured in flasks with Leibovitz’s L-15 culture medium supplemented with 2 mM L-glutamine (L15) and 6% fetal bovine serum (FBS). Flasks were held in an air atmosphere at 27°C. At confluence (~2 days in culture), cells were harvested from flasks by trypsinization and seeded into cell culture inserts (polylethylene terephthalate filters, 0.9 μm pore size, 0.4 μm pore density, 1.6 x 10^5 pore/cm^2 pore density; BD Falcon, BD Biosciences, Mississauga, ON, Canada).

Transepithelial resistance and [^3H]PEG-4000 permeability. Measurements of transepithelial resistance (TER) were conducted using chopstick electrodes (STX-2) connected to a custom-modified EVOM epithelial voltohmeter (World Precision Instruments, Sarasota, FL). All TER measurements are expressed as kΩ·cm^2 after correcting for TER measured across “blank” culture inserts bathed with appropriate media. Paracellular permeability across cultured epithelia was determined using the paracellular marker [^3H]polyethylene glycol (molecular mass 4,000 Da; PEG-4000; PerkinElmer, Woodbridge, Canada) according to methods and calculations previously described by Wood et al. (43).

Preparation of homologous serum. Goldfish serum (GFS) was obtained from a separate stock of fish (80–200 g) that was acclimated to FW or IPW conditions as detailed in Experimental animals. Goldfish serum (GFS) was goldfish serum (5’-AGGGAAGGGAAUCACUG-GTT-3′) were designed to target a specific sequence starting at nucleotide 1,187 within the 1,500-nucleotide goldfish occludin gene. The scrambled siRNA (5’-GCACACCCUCUGUACAUUUGAUGGAACGAAAGGGUGGCTT-3′) were used as a negative control. Epithelia were collected for RNA and protein extraction (see RNA Extraction and cDNA Synthesis and Western Blotting, respectively) at ~86 h posttransfection. TER measurements were recorded periodically throughout the experiment, and paracellular permeability across cultured epithelia was determined by measuring[^3H]PEG-4000 flux over a 12-h period before sample collection. Alterations in occludin transcript and protein abundance were determined by quantitative real-time PCR (see Quantitative Real-Time PCR Analysis) and Western blotting (see Western Blotting, respectively). Procedures and/or calculations for TER and[^3H]PEG-4000 flux measurements are described in Transepithelial resistance and[^3H]PEG-4000 permeability.

**RNA Extraction and cDNA Synthesis**

Total RNA was isolated from goldfish gill tissue, PVC and MRC fractions, and cultured goldfish gill epithelia using TRIzol Reagent (Invitrogen Canada) according to manufacturer’s instructions. Total RNA yield from each sample was determined using a Multiskan Spectrum UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Nepean, ON, Canada). A fixed quantity of total RNA (2 μg) was treated with DNase I (Amplification Grade, Invitrogen) and used for cDNA synthesis. First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase and Oligo(dT)12–18 primers (Invitrogen).

**Quantitative Real-Time PCR Analysis**

Transcript abundance of the following TJ genes were analyzed in intact goldfish gill tissue, separated PVC and MRC fractions, and cultured goldfish gill epithelia using quantitative real-time PCR analysis (qRT-PCR): occludin; claudin-b, -c, -d, -e, -h, -7, -8d, and -12; and ZO-1. qRT-PCR was performed using SYBR Green I Supermix (Bio-Rad Laboratories Canada, Mississauga, ON, Canada), primer sets (described in Refs. 11 and 12), and a Chromo4 Detection System (CFB-3240; Bio-Rad Laboratories). The following reaction conditions were used: 1 cycle denaturation (95°C, 4 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (51–61°C, 30 s), and extension (72°C, 30 s), respectively. To ensure that a single PCR product was synthesized during reactions, a melting curve was carried out after each qRT-PCR run. For all qRT-PCR analyses, TJ protein...
mRNA abundance was normalized to either β-actin or elongation factor 1-α (EF1-α) transcript abundance. Goldfish β-actin and EF1-α mRNA were amplified using primers previously described by Chasiotis and Kelly (10, 11). As a fixed quantity of total RNA was used for cDNA synthesis (see RNA Extraction and cDNA Synthesis), the veracity of β-actin or EF1-α as reference genes in qRT-PCR experiments was examined by statistically comparing (see Statistical Analysis) β-actin or EF1-α threshold cycle values between experimental groups.

Western Blotting

Occludin protein abundance was examined in cultured gill epithelia by Western blot analysis according to previously validated procedures and antibodies outlined by Chasiotis and Kelly (10, 11). Occludin protein abundance was expressed as a normalized value relative to β-actin protein abundance. Occludin and β-actin protein abundance were quantified using a Molecular Imager Gel Doc XR+ System and Quantity One 1D analysis software (Bio-Rad Laboratories).

Statistical Analysis

All data are expressed as mean values ± SE (n), where n represents the number of fish, or when cultured gill epithelia were used, the number of cell culture inserts. Either a Student’s t-test or one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls test was used to determine significant differences (P < 0.05) between groups as appropriate. All statistical analyses were conducted using SigmaStat 3.5 software (Systat Software, San Jose, CA).

RESULTS

Effects of IPW Acclimation on TJ mRNA Abundance and TJ Depth in Goldfish Gills

Of the 10 TJ protein genes examined in goldfish gill tissue, nine exhibited altered transcript abundance in response to IPW acclimation compared with the gills of FW-acclimated fish. Transcription abundance of occludin and claudin-b, -d, -e, -h, -7, and -8d mRNA were significantly elevated, and claudin-12 and ZO-1 were significantly reduced in the gills of IPW-acclimated goldfish (Fig. 1). The mRNA abundance of claudin-c was unchanged by IPW acclimation (Fig. 1). β-Actin mRNA was used to normalize TJ protein transcript data in this set of tissues. β-Actin mRNA abundance did not significantly change in response to IPW acclimation (P = 0.12).

TEM images were used to measure PVC-PVC and MRC-PVC TJ depth in response to IPW acclimation. PVC-PVC TJ depth significantly increased (approximately twofold) in the gills of goldfish acclimated from FW to IPW (Fig. 2, A, B, and E). MRC-PVC TJ depth also significantly increased in response to IPW acclimation (Fig. 2, C, D, and E). Within the gills of FW-acclimated fish, PVC-PVC and MRC-PVC TJ depth did not differ significantly; however, PVC-PVC TJs were significantly deeper than MRC-PVC within the gills of fish acclimated to IPW (Fig. 2 E).

Effects of IPW Acclimation on Ionocyte Morphometrics of the Goldfish Gill

Primary gill filaments of FW-acclimated goldfish were covered with polygonal PVCs bearing discrete and disordered
microridges (Fig. 3A). In IPW-acclimated goldfish gills, PVCs exhibited prominent microridges on their apical surfaces (Fig. 3B). The surface morphology of MRCs on the afferent gill filament region in fish acclimated to FW and IPW was variable (Fig. 3). In FW and IPW, a subset of MRCs exhibited an apical surface that was large and irregularly shaped, and in IPW these cells appeared to exhibit a greater surface area than in FW (Fig. 3, A and B, closed arrowhead). However, a second type of MRC surface morphology was present in IPW-acclimated fish. These MRCs were recessed in a circular/oval apical opening, and within the recess they exhibited a convex surface with extensive microvillar protrusions (Fig. 3B, open arrowhead). A morphometric comparison of all afferent gill surface MRCs in fish acclimated to FW versus those acclimated to IPW revealed significantly increased MRC apical area (Fig. 3C), fractional surface area (Fig. 3D), and MRC exposure number (Fig. 3E) in IPW-acclimated fish. SEM analysis also revealed the presence of MRC apical openings on the secondary (respiratory) lamellae (SL) of fish acclimated to IPW (data not shown).

Immunohistochemical analysis of FW-acclimated goldfish gills revealed NKA-immunoreactive (NKA-ir) cells at the base of the SL and the surface of the interlamellar region (ILR) (Fig. 3F). The occasional NKA-ir cell was observed in the SL region of the FW fish gill (Fig. 3F). In the gills of fish acclimated to IPW, NKA-ir cells were also found at the base of the SL and in the ILR (Fig. 3G). But in these fish, the SL was also found to possess numerous NKA-ir cells (Fig. 3G).

**Isolation and Separation of Goldfish Gill PVCs and MRCs**

Collagenase digestion of goldfish gill tissue was found to result in gill cell yields in the region of 140–150 million cells/fish. Because of gill perfusion, these mixed cell populations contained negligible numbers of red blood cells. When the mixed cell population was separated into two interphase fractions, 99.4 ± 0.6% (n = 4) of the upper phase fraction did not fluoresce following incubation in Mitotracker Deep Red FM (Fig. 4, A–D). This upper phase was designated the PVC fraction. In contrast, 94.7 ± 3.1% (n = 4) of cells in the lower phase fraction exhibited strong fluorescence following incubation with Mitotracker Deep Red FM (Fig. 4, E and F). This fraction was designated the MRC fraction.

**TJ Protein Transcript Abundance in Percoll-Separated Gill PVC and MRC Fractions**

Transcript encoding for all TJ proteins examined in this study (occludin; claudin-b, -c, -d, -e, -h, -7, -8d, and 12; and ZO-1) were found to be present in PVC and MRC gill fractions, and transcript abundance of occludin and claudin-c, -d, -e, -7, and -12 did not differ between PVCs and MRCs (Fig. 5).

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**Fig. 3.** Scanning electron microscope, morphometric and Na⁺-K⁺-ATPase (NKA) immunohistochemical analysis of gill epithelia from goldfish acclimated to FW or IPW. Representative surface ultrastructure images of FW- (A) and IPW-acclimated (B) goldfish gill epithelia are shown along with a comparison of FW and IPW fish MRC apical area (C), fractional surface area (D), and exposure number (E). NKA-immunoreactivity (NKA-ir; green) in gills is shown for goldfish acclimated to FW (F) and IPW (G). Scale bars = 10 μm. An asterisk in A and B indicates a PVC, whereas the closed arrowhead indicates an MRC surface morphology found in both FW- and IPW-acclimated fish. An open arrowhead in B indicates an MRC surface morphology only found in fish acclimated to IPW. Data in C–E are expressed as mean values ± SE (n = 5). * in C–E, significant difference (P ≤ 0.05) between FW and IPW. SL, secondary lamellae; ILS, interlamellar space; nuclei in F and G are stained with DAPI (blue).
In contrast, mRNA abundance of claudin-h and -8d was significantly greater in gill MRCs, and claudin-b and ZO-1 mRNA abundance were greater in PVCs (Fig. 5). EF1-α mRNA was used to normalize TJ protein transcript data in this set of tissues. EF1-α mRNA abundance was not significantly different between PVC and MRC populations (P = 0.99).

Effect of IPW Acclimation on TJ Protein Transcript Abundance in Percoll-Separated Gill PVC and MRC Fractions

Occludin, claudin-b, -e, and -8d, and ZO-1 mRNA abundance was significantly greater in gill MRCs, and claudin-b and ZO-1 mRNA abundance were greater in PVCs (Fig. 5). EF1-α mRNA was used to normalize TJ protein transcript data in this set of tissues. EF1-α mRNA abundance was not significantly different between PVC and MRC populations (P = 0.99).

Supplementation of cultured goldfish gill epithelia with homologous serum (i.e., GFS harvested from either FW- or IPW-acclimated goldfish gills) significantly increased claudin-b and -e mRNA abundance in MRCs and decreased claudin-h mRNA abundance in MRCs (Fig. 6A). OCCL, claudin-c, -d, -h, -7, -8d, and -12 mRNA abundance was unaltered in MRCs of fish acclimated to IPW (Fig. 6B). OCCL, claudin-c, -d, -7, -8d, and -12 mRNA abundance was unaltered in the MRC fraction of IPW-acclimated goldfish gills (Fig. 6B). EF1-α mRNA abundance was used to normalize TJ protein transcript data in this set of tissues. EF1-α mRNA abundance did not change significantly in response to IPW acclimation in either PVCs (P = 0.38) or MRCs (P = 0.20).

Permeability Properties and TJ Protein Transcript Abundance in Primary Cultured Gill Epithelia Supplemented with GFS

Supplementation of cultured goldfish gill epithelia with homologous serum (i.e., GFS harvested from either FW- or IPW-acclimated goldfish gills) significantly increased claudin-b and -e mRNA abundance in MRCs and decreased claudin-h mRNA abundance in MRCs (Fig. 6A). OCCL, claudin-c, -d, -h, -7, -8d, and -12 mRNA abundance was unaltered in MRCs of fish acclimated to IPW (Fig. 6B). OCCL, claudin-c, -d, -7, -8d, and -12 mRNA abundance was unaltered in the MRC fraction of IPW-acclimated goldfish gills (Fig. 6B). EF1-α mRNA abundance was used to normalize TJ protein transcript data in this set of tissues. EF1-α mRNA abundance did not change significantly in response to IPW acclimation in either PVCs (P = 0.38) or MRCs (P = 0.20).

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IPW-acclimated fish) significantly elevated TER and reduced $[^{3}H]PEG-4000$ flux compared with gill preparations supplemented with FBS (Fig. 7, A and B). Furthermore, TER was significantly increased and $[^{3}H]PEG-4000$ permeability was significantly decreased in the IPW serum group relative to the FW serum group (Fig. 7, A and B).

Occludin and claudin-e, -h, and -8d mRNA abundance was significantly elevated in GFS-supplemented cultured gill epithelia compared with those supplemented with FBS (Fig. 7C). In addition, occludin and claudin-e and -h transcript abundance was significantly greater in epithelia treated with serum derived from fish acclimated to IPW compared with epithelia treated with serum derived from FW fish (Fig. 7C). In contrast, claudin-d and ZO-1 mRNA abundance significantly decreased following GFS treatment (Fig. 7C). Claudin-b, -c, -7, and -12 transcript abundance was unaffected by the presence of GFS (Fig. 7C). EF1-α mRNA was used to normalize TJ protein transcript data. EF1-α mRNA abundance was not significantly altered by GFS supplementation ($P = 0.33$).

Effects of Apical FW and IPW Exposure on Permeability Properties of Primary Cultured Gill Epithelia Supplemented with GFS

Within the FBS group, TER was significantly elevated by apical IPW exposure compared with apical FW exposure (Fig. 8A); however, $[^{3}H]PEG-4000$ permeability was unchanged (Fig. 8B). Within the FW GFS group, apical IPW exposure significantly increased TER and decreased $[^{3}H]PEG-4000$ flux relative to apical FW exposure (Fig. 8). With respect to apical FW exposure, the FW GFS group exhibited significantly elevated TER but no change in $[^{3}H]PEG-4000$ permeability compared with the FBS group (Fig. 8). On the other hand, the FW GFS group exhibited a significantly increased TER and reduced $[^{3}H]PEG-4000$ flux relative to the FBS group during apical IPW exposure (Fig. 8). When exposed to apical IPW, the IPW GFS group exhibited significantly elevated TER and reduced $[^{3}H]PEG-4000$ permeability compared with all other treatment groups (Fig. 8).

siRNA-Mediated Reductions in Occludin Expression and the Effect on Cultured Goldfish Gill Epithelium Permeability

Transfection of cultured goldfish gill epithelia with occludin siRNA resulted in an ~60% reduction in goldfish occludin transcript abundance (Fig. 9A) and an ~50% decrease in occludin protein abundance (Fig. 9B). These changes were not associated with altered TER (Fig. 9C) but did result in a significant increase in paracellular $[^{3}H]PEG-4000$ flux (Fig. 9D). EF1-α mRNA was used to normalize occludin transcript abundance in these experiments, and β-actin was used to normalize protein abundance. EF1-α mRNA abundance ($P = 0.63$) or β-actin protein abundance ($P = 0.88$) was not found to be significantly different between treatments.

DISCUSSION

Overview

The current work strongly supports the view that altered paracellular permeability in the gill of fishes plays a crucial role in the maintenance of salt and water balance under conditions where 1) the acquisition of ions is severely limited and 2) the internal hyperosmotic/external hypotonic gradient is greater than in regular FW. In this
particular suite of studies, goldfish were acclimated to ion-poor surroundings to introduce the aforementioned conditions. Based on the observed results, it would seem that the response of the paracellular pathway to these environmental circumstances can also be mimicked in vitro by supplementing a primary cultured "reconstructed" gill epithelium with homologous serum. Although the primary cultured gill preparation is composed of PVCs only, density gradient separation of gill cells into populations of PVCs or MRCs revealed that these two different gill cell types respond to ion-poor conditions in a similar manner; i.e., with generally the same alterations in molecular TJ components as well as increased TJ depth. In the case of PVCs, the observed biochemical, morphological, and physiological changes are typically indicative of reduced epithelium permeability. This may not be unexpected and would, in fact, be advantageous for the animal. This is because the gill represents a very large surface area of direct exposure to the external environment, and gill PVCs are estimated to comprise over 90% of this epithelial interface (18). Therefore, a

Fig. 7. Effect of fetal bovine serum (FBS) or goldfish serum (GFS) derived from fish acclimated to FW or IPW on transepithelial resistance (TER) (A), [³H]PEG-4000 permeability (B), and TJ (C) protein mRNA abundance in primary cultured goldfish gill epithelium. Gill epithelia were maintained in symmetrical culture conditions (L15 apical/L15 basolateral) and exposed to homologous serum supplements for ~24 h. TJ protein mRNA was normalized with EF1-α, and FW- and IPW GFS-treated epithelia transcript abundance were expressed relative to FBS-treated epithelia transcript abundance assigned a value of 1.0. Data are expressed as mean values ± SE (n = 9–12). *Significant difference (P ≤ 0.05) from FBS-treated epithelia. †Significant difference between FW- and IPW GFS-treated epithelia.

Fig. 8. Effect of FBS or GFS derived from fish acclimated to FW or IPW on TER (A) and [³H]PEG-4000 permeability (B) across primary cultured goldfish gill epithelia after exposure to asymmetrical culture conditions (FW apical/L15 basolateral or IPW apical/L15 basolateral). Data are expressed as mean values ± SE (n = 6–9). Different letters denote significant differences (P ≤ 0.05) between treatment groups.
reduction in PVC-PVC paracellular permeability would contribute greatly to a decline in passive ion loss. In contrast, and in terms of surface area, MRCs present a smaller water-to-blood interface through which high rates of active transcellular ion flux occur (33). Nevertheless, data from these studies suggest that the increase in MRC number and MRC surface area exposure that may occur to facilitate ion acquisition in IPW (e.g., see Ref. 36) likely does so in conjunction with a decrease in MRC-PVC TJ permeability. This represents an added contribution to the management of passive ion movement across the gill epithelium in IPW and provides further insight into strategies of maintaining salt and water balance under conditions where the capacity for ion uptake is narrowed due to extrinsic factors.

**TJ Protein mRNA Abundance in Gill Tissue Following Acclimation to IPW**

A significant increase in the transcript abundance of gill TJ proteins occludin and claudin-b, -d, -e, -h, -7, and -8d was found following acclimation of goldfish to IPW. These observations are consistent with previous studies that reported an increase in the protein abundance of gill occludin in goldfish (14) and mRNA abundance of several claudin isoforms in gill tissue of puffer fish (17) following acclimation to IPW. Furthermore, occludin and claudin-b, -e, -h, -7, and -8d have previously been characterized as barrier-forming TJ elements in vertebrate epithelia or have been associated with reduced paracellular permeability across gill epithelia of fishes (2, 4, 5, 12, 15, 19, 25, 34, 44). Therefore, an increase in the abundance of these proteins in goldfish gills following acclimation to IPW seems very likely to be involved in reducing paracellular permeability across gill tissue. Accordingly, TJ depth between adjacent PVCs and MRCs and PVCs in goldfish gill tissue was also significantly increased following IPW acclimation (Fig. 2). Taken together, these changes would result in a beneficial reduction in passive ion loss in an ion-poor environment. Indeed, reduced ion efflux has previously been reported to occur across the gills of goldfish under ion-poor conditions (16), thus supporting the idea of TJ involvement.

In contrast to the above, claudin-12 and ZO-1 mRNA abundance decreased in intact gill tissue of goldfish following acclimation to IPW. However, it seems likely that these changes may have been driven by alterations in the endothelial TJ components of the intact goldfish gill, since claudin-12 was unaltered in isolated PVCs and MRCs, and ZO-1 mRNA was significantly elevated in both of these cell populations (see Fig. 6). In this regard, ZO-1 has been found to localize to the capillary endothelium in goldfish gills (Chasiotis H and Kelly SP, unpublished observations) as well as the capillary endothelium of puffer fish gills (24). In addition, claudin-12 is a component of capillary endothelial cells in mammals (6), although the presence of claudin-12 in the capillary endothelium of fishes has yet to be confirmed. Nevertheless, the observations made in the present study, along with literature reports, highlight the importance of considering the heterogeneous nature of the fish gill and the potential contribution of the capillary endothelium to alterations in whole tissue TJ protein or transcript abundance.
TJ Protein mRNA Abundance in Separated Gill PVCs and MRCs

To the best of our knowledge, this is the first study to examine the presence of TJ proteins in separated cells from the fish gill epithelium. Although it has previously been demonstrated that transcripts encoding for select TJ proteins are not present in primary cultured gill PVCs (i.e., claudin-6, -10d, and -10e in the euryhaline puffer fish, *Tetraodon nigroviridis*; see Refs. 7 and 8), the current study demonstrates that occludin, claudin-b, -c, -d, -e, -h, -7, -8d, and -12, and ZO-1 mRNA are all present in goldfish PVCs and MRCs. Nevertheless, mRNA encoding for several of these TJ proteins (e.g., claudin-b, -h, -8d, and ZO-1) appears to vary in abundance when gill PVCs and MRCs are compared (Fig. 5). Therefore, even when TJ proteins are present in both of these cell types, differences in abundance would suggest that some measure of heterogeneity exists in the paracellular pathway properties of FW fish gill PVCs and MRCs. This is unexpected because there is currently no physiological evidence to suggest TJ heterogeneity in the gills of a FW fish, even though TJ heterogeneity is a generally accepted feature of SW fish gill epithelia. However, the possibility that TJ heterogeneity is present in the gill cells of FW fishes is further supported by the differential response of claudin-8d and -h in gill PVCs and MRCs following acclimation of goldfish to IPW (see Fig. 6). More specifically, claudin-8d mRNA was found to increase in gill PVCs following IPW acclimation, which is in line with the proposed barrier-forming properties of claudin-8 protein (and claudin-8 isoforms) in vertebrate epithelia (2, 5, 12, 13, 17, 25, 44). In MRCs, however, claudin-8d did not alter in response to IPW acclimation. In contrast, claudin-h was unchanged in gill PVCs following IPW acclimation but decreased in MRCs. Taken together, the observations generated by the current suite of studies introduce the idea that TJ heterogeneity is a distinct possibility in the gills of FW fishes. The potential mechanistic reasons for this will be an intriguing avenue for further study.

Cultured Gill Epithelia and Homologous Serum Supplements

Permeability properties under symmetrical culture conditions. Under symmetrical culture conditions (i.e., L15 apical/L15 basolateral), an increase in TER and reduction in \( [\text{H}] \)PEG-4000 permeability was observed in cultured gill epithelia supplemented with GFS instead of FBS. These changes suggest that homologous serum supplements derived from a FW fish may contain factors that promote a reduction in cultured gill epithelium permeability. This may not be unexpected since FW fishes would need to maintain adequate levels of circulating signaling molecules that communicate the need to maintain low paracellular permeability across tissues that interface with the surrounding environment (e.g., the gill). Under natural conditions, this would help to minimize passive ion loss to hypo-osmotic surroundings. In addition, the idea that serum from goldfish reduces cultured gill permeability due to inherent properties that, under natural circumstances, contribute to the maintenance of salt and water balance in vivo are further strengthened by the observation that serum taken from fish held in IPW reduced epithelium permeability to a greater extent than serum from fish held in regular FW. This is consistent with previous studies that have reported reduced ion loss (efflux) across the gill epithelium of goldfish (16) and other FW species (36) acclimated to ion-poor conditions. In contrast, the current results differ from a previous study that reported a reduction in TER of primary cultured sea bass gill epithelia when homologous serum was used instead of FBS (3). However, a key difference is that the cultured sea bass gill preparation was generated using marine fish, and the serum used for supplementation was derived from sea bass residing in SW (3). It is broadly accepted that the gill epithelium of marine fishes is considerably leakier than the gill epithelium of FW fishes (see Ref. 33). Furthermore, in surrogate models of the SW fish gill epithelium, TER is substantially lower than TER across equivalent preparations isolated from FW fish (e.g., Ref. 20). Therefore, it seems plausible that in contrast to the tightening influence of FW fish serum, SW fish serum may contain factors that promote an increase in permeability. It would be interesting to develop this idea further given that the aforementioned cultured sea bass gill preparation was composed exclusively of PVCs, whereas SW fish gill “leakiness” is attributed to the presence of shallow “leaky” TJs between gill MRCs and accessory cells (33).

TJ Protein mRNA Abundance in Cultured Gill Epithelia

For the most part, alterations in TJ components match up well when comparing the in vivo and in vitro response of PVCs to “ion-poor conditions.” For example, occludin and claudin-e and -8d mRNA increased in gill tissue and separated PVCs from IPW-acclimated goldfish and in cultured gill (PVC) epithelia following treatment with IPW GFS in vitro. Furthermore, in isolated gill PVCs, transcript encoding for claudin-c, -7, and -12 was unaltered following IPW acclimation, and mRNA encoding for these same TJ components did not change in cultured PVC epithelia following treatment with either FW or IPW GFS. The alterations observed in occludin and claudin-e and -8d in association with increased TER and decreased \( [\text{H}] \)PEG-4000 flux further strengthen the suggestions that these TJ elements are involved in reducing paracellular permeability of gill tissue in fish at least. These results are consistent with previous reports of increased occludin, claudin-e (= claudin-28b), and claudin-8d protein and/or mRNA abundance in association with corticosteroid-induced reductions in the paracellular permeability of primary cultured gill epithelia (12, 15, 25). In addition, when occludin abundance is reduced by siRNA in cultured gill epithelia, \( [\text{H}] \)PEG-4000 flux can be observed to increase (see *siRNA-Mediated Reductions in Occludin Abundance and Gill Epithelium Permeability*). Not all alterations in PVC TJ components corresponded when in vivo and in vitro results were compared. Notably, claudin-d and ZO-1 mRNA abundance decreased following GFS treatment of cultured gill epithelia, whereas PVCs isolated from fish acclimated to IPW showed unaltered and marginally (but significantly) increased claudin-d and ZO-1 mRNA, respectively (see Fig. 6). The specific reasons for this discrepancy are unclear. However, it is noteworthy that the decline in claudin-d and ZO-1 mRNA abundance in vitro following exposure to GFS did not vary between FW GFS and IPW GFS. Therefore, the effect appeared to occur irrespective of the physiological state of the fish from which the serum was derived. This response to GFS differs from TJ protein mRNA changes that match between in vivo and in vitro studies (e.g., occludin and claudin-e), where mRNA abundance was altered.
in response to IPW GFS treatment to a greater extent than FW GFS treatment. Previous studies using mammalian cell lines have reported that serum-derived factors can weaken the TJ complex, and this has been associated with an increase in permeability, reduction in ZO-1 abundance, and delocalization of ZO-1 (9, 35). Under certain circumstances, this appears to occur when cultured epithelia or endothelia express a pathophysiological response to the presence of serum where it is normally absent [e.g., basolateral side of brain capillary endothelial cells; (35)]. In these cases, the deleterious effects of serum supplementation in vitro match the in vivo pathological response of tissue when disease or injury results in serum leakage and contamination of a normally serum-free compartment (9, 35). In the current study, the presence of GFS reduced ZO-1 (and claudin-d) mRNA abundance, but GFS did not have a deleterious effect on the physiological properties of the cultured goldfish gill epithelium (see Figs. 7 and 8). Nevertheless, the fact that the apical surface of the gill epithelium in vivo is serum free under natural conditions (i.e., exposed directly to water) suggests that in future studies it may be interesting to reduce the presence of GFS on the apical side of cultured gill epithelia to see if this curtails the observed reduction in ZO-1 and claudin-d transcript abundance. In this regard, cultured gill epithelia have previously been shown to tolerate reduced media presence on the apical surface during development (45), but this has never been matched with the presence of homologous serum or the effects examined at the molecular level.

A final curious discrepancy between PVC TJ protein mRNA alterations following GFS treatment in vitro versus ion-poor conditions in vivo was the response of claudin-b and -h. More specifically, transcript encoding for claudin b significantly increased in PVCs isolated from the gills of fish acclimated to IPW but did not alter in vitro following GFS treatment. In a reverse situation, claudin-h did not exhibit any change in mRNA abundance in PVCs isolated from the gills of IPW-acclimated fish but did significantly increase in vitro in response to FW GFS treatment and, in a more pronounced manner, IPW GFS treatment. Once again, the specific reasons for this are currently unknown and will require further study; however, a number of factors could contribute. For example, in the current study, cultured epithelia were exposed to 10% GFS, which represents a 90% reduction in any element that, in vivo, may be stimulating (or suppressing) claudin TJ protein abundance in PVCs. Also, temporal factors could play a role since cultured gill epithelia were exposed to GFS for 24 h, whereas gill tissue was taken from fish that have been acclimated to IPW for at least 14 days. Nevertheless, an increase in claudin-h (= claudin-3a) abundance may also have contributed to reduced epithelia permeability in GFS-treated epithelia, since claudin-3 is generally considered to be a barrier-promoting TJ protein (34).

**Permeability Properties Under Asymmetrical Culture Conditions**

Under asymmetrical culture conditions, epithelia supplemented with GFS exhibited an elevated TER relative to those supplemented with FBS. In addition, epithelia treated with GFS exhibited lower [3H]PEG-4000 permeability flux when exposed to apical IPW relative to those supplemented with FBS. Once again, a significant difference between epithelia supplemented with GFS derived from FW- and IPW-acclimated fish was apparent (i.e., highest TER and lowest [3H]PEG-4000 flux observed in epithelia treated with IPW GFS) (see Fig. 8). This distinction clearly suggests that the presence of IPW GFS allowed epithelia to better respond to in vitro IPW exposure. This, as well as the response of epithelia to IPW GFS supplementation under symmetrical culture conditions (see above), strongly supports the idea that IPW GFS contains factors that lead to the differentiation of an IPW-like epithelial phenotype in vitro. As previously discussed, the serum components responsible for this are currently unknown, but it seems reasonable to anticipate that osmoregulatory endocrine factors most likely play a pivotal role. In this regard, hormones that are linked to vertebrate salt and water balance have previously been demonstrated to significantly impact the permeability of cultured gill epithelia (15, 25–29) as well as molecular components of the TJ complex (8, 12, 15, 25, 39).

For example, elevated circulating levels of the osmoregulatory hormone cortisol in goldfish were reported to significantly increase gill protein and/or mRNA abundance of the same TJ components that have been shown to respond to ion-poor conditions in the current in vivo and in vitro studies (i.e., occludin, claudin-e and -8d; see Ref. 13). Furthermore, the observation that cultured goldfish gill epithelia exhibit a differential response to homologous serum derived from fish in different physiological states is consistent with a previous report where cultured tilapia gill epithelia were shown to respond very differently to homologous serum derived from fish that were unstressed versus serum from fish that had been stressed (i.e., intermittently net chased over a 20-min period: see Ref. 28). In the tilapia study, the stress (and osmoregulatory) hormone cortisol was found to be largely responsible for the differential response of cultured gill preparations (28).

**siRNA-Mediated Reductions in Occludin Abundance and Gill Epithelial Permeability**

After a siRNA-mediated reduction in occludin abundance in cultured goldfish gill epithelia, paracellular [3H]PEG-4000 flux increased, suggesting that the paracellular pathway became “leakier” as a result of occludin loss. This supports the idea that occludin plays a barrier-forming or “tightening” role in the regulation of fish gill permeability as has previously been suggested (14, 15). A barrier-forming role for occludin is further supported in the present study by increased occludin abundance in gill tissue (Fig. 1), gill PVCs, and gill MRCs (Fig. 6) following IPW exposure. In addition, occludin abundance also increased in cultured gill epithelia following treatment with IPW GFS and in association with reduced epithelium permeability (Fig. 7). Despite these observations, TER across cultured gill epithelia did not significantly alter in association with siRNA-mediated reductions in occludin abundance. However, TER represents measurements of resistance to the flow of charged solutes, and the results of the current study could suggest that occludin may play a role in regulating the paracellular movement of uncharged or large molecules. This idea will require further testing but is consistent with recent observations of siRNA-mediated occludin “knockdown” in Caco-2 cells that significantly increased the paracel-
lular flux of various sized macromolecules without altering TER (1).

**Perspectives and Significance**

It is broadly accepted that the presence of a “tight” branchial epithelium in hypo-osmotic surroundings prevents detrimental ion loss across the gill epithelium of fishes and that this plays a key role in the maintenance of salt and water balance in these organisms. In addition, TJ heterogeneity in the gill epithelium also performs an important role in the physiological plasticity of fishes when they are exposed to changing environmental ion levels. However, the aforementioned paradigms are based largely on morphological evidence because the physiological properties and molecular components of the gill epithelium paracellular pathway are not well characterized. This is partly due to obstacles that make the gill epithelium a challenging tissue to study (e.g., architectural complexity, cellular heterogeneity, etc.) but also relates to the fact that large numbers of TJ proteins have only recently been described in fishes. Recent progress clearly indicates that the molecular physiology of TJs in the gill epithelium of fishes is complicated. This is exemplified in the current study that used different approaches to experimentally dissect the response of select gill cell TJ components following exposure to ion-poor conditions and found specific cell-type alterations in TJ factors. In the present study, a distinction is only made between gill cells that exhibit an abundant complement of mitochondria (i.e., MRCs) versus those that do not (i.e., PVCs). This does not take into account a growing body of evidence that supports the idea of gill MRC heterogeneity (23), and this is an additional layer of complexity to be considered in future studies. However, the current study does demonstrate that gill PVCs, which are normally overlooked in transepithelial ion transport studies, are likely to be major players in overall strategies that strive to regulate passive solute movement across this epithelium. Taken together, these factors provide numerous avenues for further study, and when considered with the knowledge that proteins of the TJ complex have not yet been fully enumerated in any fish species, gill epithelia of fishes exhibit spectacular physiological flexibility, and fishes represent the largest vertebrate group within the chordate lineage, the potential for new insights into gill function promises to be exceptionally fruitful.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


