Comparative Biochemistry and Physiology, Part A 182 (2015) 45–52

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Transepithelial resistance and claudin expression in trout RTgill-W1 cell line: Effects of osmoregulatory hormones

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ARTICLE INFO

Article history:
Received 18 July 2014
Received in revised form 27 November 2014
Accepted 1 December 2014
Available online 6 December 2014

Keywords:
Cortisol
Claudin-10e
Claudin-30
Growth hormone
Na^+,K^+-ATPase
Osmoregulation
Prolactin
Rainbow trout
Tight junctions
Zonula occludens-1

ABSTRACT

In the present study, we examined the trout gill cell line RTgill-W1 as a possible tool for *in vitro* investigation of epithelial gill function in fish. After seeding in transwells, transepithelial resistance (TER) increased until reaching a plateau after 1–2 days (20–80 $\Omega \cdot cm^2$), which was then maintained for more than 6 days. Tetrabromocinnamic acid, a known stimulator of TER via casein kinase II inhibition, elevated TER in the cell line to 125% of control values after 2 and 6 h. Treatment with ethylenediaminetetraacetic acid induced a decrease in TER to <15% of pre-treatment level. Cortisol elevated TER after 12–72 h in a concentration-dependent manner, and this increase was antagonized by growth hormone (Gh). The effects of three osmoregulatory hormones, Gh, prolactin, and cortisol, on the mRNA expression of three tight junction proteins were examined: claudin-10e (Cldn-10e), Cldn-30, and zonula occludens-1 (Zo-1). The expression of *cldn-10e* was stimulated by all three hormones but with the strongest effect of Gh (50-fold). *cldn-30* expression was stimulated especially by cortisol (20-fold) and also by Gh (4-fold). Finally, *zo-1* was unresponsive to hormone treatment. Western blot analysis detected Cldn-10e and Cldn-30 immunoreactive proteins of expected molecular weight in samples from rainbow trout gills but not from RTgill-W1 cultures, possibly due to low expression levels. Collectively, these results show that the RTgill-W1 cell layers have tight junctions between cells, are sensitive to hormone treatments, and may provide a useful model for *in vitro* study of some *in vivo* gill phenomena.

1. Introduction

In gills of euryhaline fish, such as rainbow trout (*Oncorhynchus mykiss*), extensive morphological and molecular remodeling occurs during acclimation between freshwater (FW) and seawater (SW), and *vice versa*. Therefore, studies of such fish species can give important insights into general gill function, as well as regulatory mechanisms controlling changes in response to the environment. The signals that direct physiological changes associated with osmoregulation are very complex. However, current models include a series of endocrine factors, including cortisol, growth hormone (Gh), and prolactin (Prl; McCormick, 2001; Sakamoto and McCormick, 2006; Tipsmark et al., 2011). It has thus been known for a while that Gh and cortisol interact to improve SW tolerance in salmonids (Madsen, 1990; McCormick, 1996), in part, due to synergistic stimulation of a specific gill Na^+, K^+-ATPase isoform (α-1b; Tipsmark and Madsen, 2009).

The gill epithelium of teleosts acclimated to SW is usually considered "leaky" when compared with fishes acclimated to FW. It has been suggested that the "leakiness" of SW-gills arises from altered composition in gill claudin isoforms, which create shallow tight junctions (TJs) between aggregated mitochondrion-rich cells (MRCs) and accessory cells (Sardet et al., 1979). Claudins comprise a large family of TJ proteins, with 54–56 isoforms in zebrafish (Baltzegar et al., 2013) and Japanese pufferfish (Loh et al., 2004). They are four-transmembrane domain proteins that make homo- or hetero-dimeric interactions between adjacent cells, thereby forming apicolateral TJs (Angelow et al., 2008). While other proteins like occludin, junctional adhesion molecules, and zonula occludens (Zo) proteins also participate in physical aspects of TJ formation, claudins are the critical determinants of the electrophysiological permeability characteristics (Saitou et al., 2000), through their specific charged distribution of amino acids in the first extracellular loop (Hou et al., 2013). Accordingly, the expression of claudin isoforms depends on tissue, stage of development, and salinity to which fish are exposed (Bagherie-Lachidan et al., 2008; C.K. Tipsmark et al., 2008a; C.K. Tipsmark et al., 2008b).

The fish gill has a complex three-dimensional structure at both the macro and microscopic levels (Wilson and Laurent, 2002), making especially electrophysiological studies difficult. Therefore, gill surrogate models in two dimensions have been approached for decades for this purpose (see Marshall and Bellamy, 2010). For SW fish, the

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http://dx.doi.org/10.1016/j.cbpa.2014.12.005
1095-6433/

predominant gill model is the opercular membrane from species like killifish and tilapia containing high densities of MRCs (Degnan et al., 1977; Marshall et al., 1998). However, for studies of NaCl transport in the FW gill, opercular membranes appear less useful (Marshall et al., 1997). As an alternative model, primary gill cell culture was pioneered by Pärt et al. (1993). Their initial preparation of single-seeded insert (SSI) monolayers contained only pavement cells (PVCs), and a double-seeded insert (DSI) approach has since been used to improve the system by including MRCs (Fletcher et al., 2000). These primary gill cell monolayers generally have a relative high transepithelial resistance (TER: 1–20 $k\Omega \cdot cm^2$) and have been used in a series of studies of hormonal and direct osmotic effects (Gilmour et al., 1998; Zhou et al., 2003; Leguen et al., 2007; Kelly and Chasiotis, 2011; Sandbichler et al., 2011). Unfortunately, such primary cell cultures have a number of limitations, including short life span, need for live fish, time for isolation and culture, and issues of experimental consistency. Following its development, the rainbow trout gill cell line RTgill-W1 (Bols et al., 1994) has been used in studies of toxicology and fish health (Lee et al., 2009), and except for one study of intracellular Map-kinase signaling (Ebner et al., 2007), no studies have to our knowledge examined its potential use as a physiological model.

The aim of the present studies was to examine aspects of the RTgill-W1 cell line as an alternative model to study osmoregulatory mechanisms. This included an initial examination of TER, TJ modulators, and sensitivity to osmoregulatory hormones. We examined the time course development of TER across cell layer after seeding on transwells with or without cortisol, a hormone known to elevate TER in primary gill cultures from rainbow trout (Zhou et al., 2003; Leguen et al., 2007). Next, we examined the cell layers' sensitivity to known pharmacological modulators of paracellular permeability: tetrabromocinnamic acid (TBCA) known to elevate TER by reducing paracellular cation flux (Raleigh et al., 2011) and ethylenediaminetetraacetic acid (EDTA) to disrupt TJs and thereby remove this epithelial barrier (Okamoto et al., 1995). Hormonal effects and interactions on TER and mRNA expression of selected TJ proteins (zonula occludens, Zo-1; claudin-10e, Cldn-10e; and claudin-30, Cldn-30) were also assayed using cortisol, carp Gh (cGh), and ovine Prl (oPrl). Cldn-10e and Cldn-30 are known to be highly expressed in salmonid gill and their expressions have some degree of regulation by salinity, smoltification, and cortisol (C. Tipsmark et al., 2008; Tipsmark and Madsen, 2009; Kelly and Chasiotis, 2011; Engelund et al., 2012; Kolosov et al., 2014). Finally, we examined the protein expression of Cldn-10e and Cldn-30 in RTgill-W1 cells compared with gill samples from intact rainbow trout. These investigations also includes the MRCs markers Na^+,K^+-ATPase and Na^+,K^+,$2Cl^-$-cotransporter.

2. Materials and methods

2.1. Experimental animals

Rainbow trout were obtained from Norfork National Fish Hatchery in Arkansas and kept in recirculated dechlorinated tap water at 20 °C for 1 month prior to experiment. They were fed daily. All handling and experimental procedures involving animals were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC protocol number 11005).

2.2. Cell culture

The gill derived rainbow trout cell line RTgill-W1 was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and largely cultured in accordance with protocols developed by the research group isolating the cell line (Bols et al., 1994; Lee et al., 2009). Briefly, cells were maintained at 19 °C in 75 cm^2 plastic flasks (Nunc, Roskilde, Denmark). The growth medium was Leibowitz L-15 media (GIBCO, Grand Island, NY, USA) supplemented with 10% of fetal bovine serum (HyClone Laboratories Inc, Logan, UT, USA), 100 μg/ml penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO), 0.25 μg/ml amphotericin B (GIBCO), 20 μg/ml gentamycin (Amresco, Solon, OH, USA), and 2 mM L-glutamine (GIBCO). They were harvested at 95% confluence using TrypLE (GIBCO) and seeded to full confluence in 12-well plates (Greiner Bio-one, Monroe, NC, USA) with Transwell permeable inserts (0.4 μm pore size). For experiments examining mRNA and protein expression, they were grown to around 95% confluence in 6-well plates, treated without or with hormones for 72 h and then harvested for further processing.

2.3. Analytical techniques

2.3.1. TER

Measurements of the TER were performed with a chopstick-type Ag/AgCl electrode (STX-2) connected to an EVOM chopstick voltmeter (World Precision Instruments, Sarasota, FL, USA), as described previously (Engelund et al., 2012). The resistance measured across a culture insert with no seeded cells was used for background correction of all TER measurements.

2.3.2. Total RNA purification and reverse transcriptase reaction

Total cell RNA was extracted using 100 μl of TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's directions. Quantity and purity of RNA were assessed with the NanoDrop® ND-2000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), where a 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. The cDNA was obtained using 1 μg of total RNA for each sample and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol.

2.3.3. Quantitative PCR (QPCR)

The mRNA expression levels of the TJ proteins Zo-1, Cldn-10e, and Cldn-30, were quantified by QPCR using a SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) kit in a C1000 Thermal Cycler, CFX96 Real Time System (BioRad, Hercules, CA, USA). The following QPCR protocol was employed: heating to 95 °C for 2 min; 45 cycles of 95 °C for 30 s and 60 °C for 60 s; followed by dissociation curve analysis to confirm the generation of a single specific amplicon. In all case, only one product with the previously confirmed melting point was obtained. All mRNA expression data were normalized against the geometric mean of two references genes: elongation factor-1a (*ef-1a*) and beta-actin (*act-b*). Primers used in this study have been described previously (Richards et al., 2003; C. Tipsmark et al., 2008; Madsen et al., 2009) or were designed using Primer3 software (Rozen and Skaletsky, 2000; see Table 1).

2.3.4. Western blot analysis

Two days after obtaining full confluence RTgill-W1 cultures were rinsed with PBS and harvested with ice-cold SEID buffer (300 mM sucrose, 10 mM Na_2-EDTA, 50 mM imidazole and 0.1% of sodium deoxycholate) with 1% protease inhibitor cocktail (Sigma-Aldrich; P8340). Cells were detached from the wells using a cell scraper, transferred into a clean tube and homogenized with a VWR Power

Table 1
Primers sequences used for quantitative PCR of rainbow trout transcript targets.

Target name	Forward sequence	Reverse sequence	Acc. No.
ef-1a	agaaccattgagaagttcgagaag	gcacccaggcatacttgaaag	BT046846
act-b	tcctcggtatggagtcttgc	agcactgtgttggcgtacag	AF254414
cldn-10e	atcaaggtggcctggtactg	gaccagagcacagggaagtc	BK006391
cldn-30	tgatcattggaggagggttc	aacatagtccctgggtgctg	BK006405
zo-1	aggctgtgctgttcctccta	tccgacggtaaacatccttc	HQ656020
a1a	cccaggatcactcaatgtcac	ccaaaggcaaatgggtttaat	AY319391
a1b	ctgctacatctcaaccaacaacatt	caccatcacagtgttcattggat	AY319390
a1c	gagagggagacgtactactagaaagca	cagcaagacaaccatgcaaga	AY319389