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Cold acclimation allows regulation of chloride secretion in a eurythermic teleost fish *Fundulus heteroclitus*

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ABSTRACT

Fundulus heteroclitus (mummichog or common killifish) is an ideal model for ion transport regulation in chloride cells of the opercular epithelium (OE) and the response to thermal challenge. Mummichogs were acclimated to warm (20 °C) and cold (5 °C) seawater and opercular epithelia dissected and mounted in isolated Ussing-style epithelia chambers. The α_2 adrenergic agonist clonidine inhibited the Cl^- secretion (measured as short-circuit current, I_{sc}), while the β-adrenergic agonist isoproterenol and 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP) plus 0.1 mM isobutyl methylxanthine (IBMX) stimulated I_{sc} in OE from warm and cold acclimated fish, measured at 20 °C. In contrast, rapid cooling partially inhibited I_{sc}, but totally blocked the inhibition by clonidine and stimulation by isoproterenol and db-cAMP + IBMX in OE from warm-acclimated fish, while OE from cold-acclimated animals responded normally at 5 °C. Warming epithelia from 5 °C to 20 °C restored I_{sc} and stimulation by db-cAMP + IBMX markedly increased I_{sc} to levels similar to warm acclimated epithelia, while isoproterenol was much less effective. The isoproterenol insensitivity suggests a downregulation of β-adrenergic receptors in the cold. We infer from present results and previous work (Buhariwalla et al. 2012) that cold shock of plasma membranes induces a phase shift from liquid to gel state that impaired plasma membrane protein mobility of necessary hormone regulatory functions, while cold acclimation preserved ion transport regulation via homeoviscous adaptation of plasma membrane lipids.

1. Introduction

Fundulus heteroclitus, (mummichog) are euryhaline and eurythermic teleost fish able to acclimate to extreme salinity, fresh water to over 2× seawater habitats (Griffith, 1974; McCormick 1994) and temperatures ranging from −1.5 °C to 38 °C (Fangue et al., 2009). Previously, we determined that acclimation of mummichogs to the cold, 5 °C, slows down but does not stop NaCl secretion by the mitochondrion-rich cells of the opercular epithelium and gill of seawater animals (Buhariwalla et al., 2012; Barnes et al., 2013). Importantly, acclimation to the cold for >30 days causes significant changes in fatty acid saturation, specifically a reduction in C18:0 and an increase in monounsaturated n − 9 fatty acids in membranes from the liver (Buhariwalla et al., 2012) and gill epithelium (Barnes et al., 2013). This demonstration of phenotypic plasticity is also seen in Antarctic fish as a mechanism to regulate metabolic and cardiovascular function in correlation to temperature fluctuations (Guderley et al., 2004). These changes also help maintain sensitivity of the osmotic control pathway that controls NaCl secretion, a pathway that involves Integrin α/β mechanosensing of changes in plasma membrane, transduction to a kinase cascade that includes proto oncogene c-SRC kinase (c-SRC), Focal Adhesion Kinase (FAK), ste20-related kinase (SPAK) and Oxidative Response Kinase (OSR1) that ultimately activates Sodium, Potassium, 2 Chloride cotransporter (NKCC) in the basolateral membrane of mitochondrion-rich cells and increases NaCl secretion (Marshall et al., 2000, 2005). We also determined that a similar pathway involving FAK may also activate the anion channel Cystic Fibrosis Transmembrane conductance Regulator (CFTR) in the apical membrane, thus the Cl^- transcellular secretion pathway can be rapidly activated by the simultaneous actions at basolateral and apical membranes (Marshall et al., 2009).

The plasma membrane is a temperature sensitive structure (Cossins and MacDonald, 1989; Hazel, 1995) that houses many molecular components that interact to mediate ion transport, diffusion, and cell maintenance (Cossins et al., 1995). Temperature-induced changes in plasma membranes affect cellular processes including protein mediated ionic solute transport and signal transduction pathways important for organism survival (McKinley and Hazel, 2000). Plasma membranes are able to acclimate to temperature fluctuations by altering lipid composition that allows for retention of membrane fluidity at low temperatures and thus maintenance of crucial cellular mechanisms (Williams and Hazel, 1995), but little is known specifically about transepithelial ion transport and its regulation in extreme cold.

NaCl secretion is also under complex regulatory control and is stimulated by β-adrenergic agonists, Urotensin I, vasoactive intestinal polypeptide and glucagon, all via cAMP, and is inhibited by α_2-

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adrenergic agonists, Urotensin II, acetylcholine and mediators that increase intracellular Ca^{2+}, such as ionomycin (Burnett et al, 2007; Marshall, 2013). The opposing roles of α_2- and β-adrenergic pathways on Cl^- secretion are well known (May and Degnan, 1985; Marshall et al, 1993; McCormick, 1994) and both pathways involve multiple protein interactions and at least one kinase or phosphatase reaction. Previous studies in our lab have included dose-responses with several agonists, which allowed us to determine that α_2-receptors were dominant in our system and the sensitivity is comparable to higher animal responses (Marshall et al., 1993). We previously demonstrated that neurally derived activation of α_2-adrenergic receptors using an isolated nerve-epithelium preparation, is the physiologically relevant action in context of the animals moving from seawater to more dilute environments and rapidly downregulating Cl^- secretion (Marshall et al., 1998). Here mummichogs were acclimated to warm (20 °C) or cold (5 °C) seawater, mimicking summer and winter conditions in the estuary, then tested in vitro for the up- and down-regulation of Cl^- secretion by adrenergic pathways at these same temperatures. We hypothesized that cold shock would interrupt the up- and downregulation of Cl^- secretion whereas cold acclimation might preserve the ability of the animal to regulate Cl^- secretion by maintaining plasma membrane fluidity. The present study extends previous findings to test whether hormonal regulatory responses of active Cl^- secretion behave similarly to osmotic perturbation, being maintained by acclimation to the cold and restored by warming.

Some adrenergic agonists such as epinephrine and drugs that mimic this hormone selectively activate the cAMP signal transduction pathway via cAMP dependent Protein Kinase (PKA). Multiple PKA-dependent phosphorylation sites exist in the R (regulatory) domain of CFTR, consistent with the theory that CFTR stimulation is elicited by PKA activation via the cAMP-dependent pathway (Gadsby and Nairn, 1999; Marshall, 2002). Multiprotein complexes along the signal transduction pathway interact to regulate CFTR, influencing ion transport functions of this protein (Li et al., 2007a,b). Activation of PKA by 3′-5′cyclic adenosine monophosphate (cAMP) is necessary for the stimulation of CFTR and subsequent ion transport (Cheng et al, 1991; Kelley et al., 1995). The initial activation of this cAMP-dependent pathway is via adrenergic receptors, which are present in the basolateral plasma membrane of the chloride cell. Adrenergic receptors are coupled with G-proteins and when activated, initiate the attendant signal transduction pathway (O'Dowd et al., 1989). The G-protein can then activate adenylate cyclase (Kelley et al., 1995) to increase the production of intracellular cAMP, which subsequently activates PKA which in turn phosphorylates of CFTR, in the apical membrane, increasing efflux of Cl^- through these anion channels (Singer et al., 1998). The response of ion transport to activation of the G-protein coupled β-receptors was observed using β-adrenergic agonist isoproterenol. Dibutyryl cyclic AMP (db-cAMP) and isobutyl methylxanthine (IBMX) were used to increase intracellular levels of cAMP. This more direct activation bypasses hormone–receptor and amplification steps and requires only PKA and CFTR to produce an increase in Cl^- secretion.

In contrast, when α_2-adrenergic receptors are activated by α_2-adrenergic agonists, adenylate cyclase is unaffected. Activation of these receptors causes inhibition of chloride transport by a mechanism that is not well understood, but is mediated via increased intracellular calcium (Marshall et al., 1993; Lam et al., 2003). There is evidence to suggest that K^+ channels localized in the basolateral membrane are affected by binding of adrenergic agonists to α_2-adrenergic receptors (Lam et al., 2003). In this study, clonidine was used as the α_2-adrenergic agonist that leads to a rapid decrease in short-circuit current (I_{sc}), and in active Cl^- secretion by the OE (May and Degnan, 1985; Marshall et al., 2009).

A few studies have highlighted the use of adrenergic agonists to induce a response in membrane current (Llach et al., 2004; Timmons et al., 2004), but none have addressed the differences in response to drugs between cold acclimated, warm acclimated, and cold shocked mitochondrion rich cells. Killifish have been able to adapt their physiology in response to changes in temperature by adjusting their biological components on a structural and a molecular level (Podrabsky and Somero, 2004). Because previous evidence of osmotic dysregulation in cold-shocked membranes (Cossins et al., 1995; Buhariwalla et al. 2012) and plasma membrane lipid transition in fish acclimated to cold temperatures (Williams and Hazel, 1995), it is suspected that alteration of membrane fluidity will elicit a differential response to drugs between cold acclimated and cold shocked OE in *F. heteroclitus*. Here we found that epithelia from cold acclimated animals can respond to hormonal inhibition and stimulation, whereas epithelia from warm acclimated animals are unresponsive in the cold to stimulation or inhibition.

2. Materials and methods

2.1. Animals

Male and female adult killifish (*F. heteroclitus*), mass 6 to 10 g, were trapped in Jimtown estuary located in Antigonish County, Nova Scotia. The fish were transferred from the estuary in coolers containing brackish water to 400 l aquaria located in the St. Francis Xavier animal care facility. Aquaria contained full strength seawater (30‰) at room temperature (20 °C) and at ambient photoperiod under artificial light (summer, approx. 15L9D). The mummichogs were fed twice daily commercial fish flake food plus freeze dried *Tubifex* worms and live mealworms in alternating afternoon feedings. A sample of fish was transferred into a separate aquarium and acclimated to a temperature of 5 °C by lowering the temperature by 2 °C every other day for a period of two weeks. Experimental procedures were approved by the St. Francis Xavier Animal Care Committee and followed the guidelines administered by the Canadian Council on Animal Care (protocol 12-001-N). All fish were euthanized by pithing; the fish opercula were removed and the opercular epithelia (OEs) were dissected under a stereo-microscope.

2.2. Bathing solutions

Cortland's balanced saline solution was the saline used in all experimental procedures. This isotonic solution (in mmol l^{-1}) is composed of 159.9 NaCl, 2.55 KCl, 1.56 $CaCl_2$, 0.93 $MgSO_4$, 17.85 $NaHCO_3$, 2.97 NaH_2PO_4, and 5.55 glucose and had an osmolality of approximately 310 mOsmol kg^{-1}, pH 7.6–7.8 after being bubbled with a gas mixture composed of 99% O_2/1.0% CO_2 prior to use in experiments.

2.3. Drugs

Clonidine hydrochloride (Sigma-Aldrich, St Louis, MO, USA) was prepared as a 10 mg/ml stock using Cortland's and added to a final concentration of 10 μM, a maximally effective dose (Marshall et al., 1993). Activators of the cAMP pathway include isoproterenol (Sigma-Aldrich, 10 μM final concentration) or 1.0 mM dibutyryl cyclic adenosine monophosphate (db-cAMP) plus 0.1 mM isobutyl methylxanthine (IBMX) (Sigma-Aldrich), dissolved in Cortland's saline.

2.4. Electrophysiology

Opercular epithelia were placed over a 0.125 cm^2 circular aperture in Plexiglas® inserts. The epithelium and insert were placed between two Ussing-style epithelium chambers that were filled with gas mixture equilibrated Cortland's saline. Two Ussing chambers were water jacketed for temperature control. All OEs were initially kept at the acclimation temperature to which that particular fish was acclimated (5.0 °C or 20 °C), Chambers were connected via 4% agar-saline bridges to a current–voltage clamp (D. Lee Co, Sunnyvale, CA). The secretion rate of Cl^- across the OE was measured by short circuit current (I_{sc}, μamp/cm^2), which has been shown previously to be equal to the net flux of Cl^- (e.g. Degnan et al., 1977). The transepithelial potential (V_t, mV) and