The Role of SGK and CFTR in Acute Adaptation to Seawater in *Fundulus Heteroclitus*

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Key Words

CFTR • SGK • Chloride channel • *Fundulus* • Ion transport • Cystic fibrosis • Cl⁻ secretion • Glucocorticoid receptor • RU486

Abstract

Killifish are euryhaline teleosts that adapt to increased salinity by up regulating CFTR mediated Cl⁻ secretion in the gill and opercular membrane. Although many studies have examined the mechanisms responsible for long term (days) adaptation to increased salinity, little is known about the mechanisms responsible for acute (hours) adaptation. Thus, studies were conducted to test the hypotheses that the acute homeostatic regulation of NaCl balance in killifish involves a translocation of CFTR to the plasma membrane and that this effect is mediated by serum- and glucocorticoid-inducible kinase (SGK1). Cell surface biotinylation and Ussing chamber studies revealed that freshwater to seawater transfer rapidly (1 hour) increased CFTR Cl⁻ secretion and the abundance of CFTR in the plasma membrane of opercular membranes. Q-RT-PCR and Western blot studies demonstrated that the increase in plasma membrane CFTR was preceded by an increase in SGK1 mRNA and protein levels. Seawater rapidly

(1 hr) increases cortisol and plasma tonicity, potent stimuli of SGK1 expression, yet RU486, a glucocorticoid receptor antagonist, did not block the increase in SGK1 expression. Thus, in killifish SGK1 does not appear to be regulated by the glucocorticoid receptor. Since SGK1 has been shown to increase the plasma membrane abundance of CFTR in *Xenopus* oocytes, these observations suggest that acute adaptation (hours) to increased salinity in killifish involves translocation of CFTR from an intracellular pool to the plasma membrane, and that this effect may be mediated by SGK1.

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Introduction

CFTR is a cAMP-activated Cl⁻ channel that is expressed in functionally diverse tissues including the intestine, kidney, lung, heart, and the teleost gill and operculum [1]. CFTR mediates transepithelial salt and water secretion into the airway of the lung and the lumen of kidney tubules and pancreatic ducts. In the intestine, CFTR Cl⁻ secretion is responsible for secretory diarrhea that is induced by *Vibrio cholera* and *Clostridium difficile*, whereas, in the heart, CFTR mediates PKA-

stimulated Cl⁻ currents that may be protective against focal arrhythmias and cardiac ischemia [1-3]. Mutations in the CFTR gene cause cystic fibrosis (CF), one of the most common lethal genetic diseases in Caucasians [3]. CFTR also plays a vital role in regulating NaCl homeostasis in euryhaline teleosts [4-10]. Marine teleosts, including *Fundulus heteroclitus* (killifish) are hypoosmotic to seawater [5] and must excrete the NaCl that diffuses inward across the gill, as well as the NaCl absorbed by the gastrointestinal tract, to maintain plasma concentrations of Na⁺ and Cl⁻ (i.e., plasma osmolality). Although the gill is responsible for the bulk of NaCl secretion into the external environment (i.e., water), the operculum also secretes NaCl.

Transepithelial Cl⁻ secretion by mitochondrial rich cells in the killifish gill and opercular membrane is a two-step process [4-10]. The Na⁺-K⁺-2Cl⁻ cotransporter (i.e., NKCC1) mediates Cl⁻ uptake across the basolateral membrane. The Cl⁻ that enters the cell across the basolateral membrane is secreted into seawater via the CFTR Cl⁻ channel. The Na⁺ that enters the cell across the basolateral membrane with Cl⁻ via the NKCC1 is recycled back into blood via the Na⁺-K⁺-ATPase. The K⁺ that enters the cell with Cl⁻ across the basolateral membrane recycles across the basolateral membrane via a K⁺ channel. Cl⁻ secretion generates an apical-negative, transepithelial voltage, which provides the driving force for the paracellular secretion of Na⁺ across the tight junctions.

CFTR Cl⁻ secretion across epithelial cells is regulated by modulating single channel activity and by adjusting the abundance of CFTR channels in the plasma membrane [1, 11-16]. For example, in airway and intestinal cells cAMP activation of protein kinase A (PKA) enhances Cl⁻ secretion by: 1) increasing the single channel activity, and 2) increasing the plasma membrane abundance of CFTR by inhibiting the endocytic removal of CFTR from the plasma membrane as well as stimulating the exocytic insertion of CFTR from intracellular vesicles into the plasma membrane [1, 11-16].

Killifish and other euryhaline teleosts can adapt to changes in the salinity of their external environment by adjusting the rate of CFTR-mediated Cl⁻ (and Na⁺) secretion by the gill (and operculum) to match NaCl intake. Upon transition from freshwater to seawater, increased NaCl secretion by the gill and operculum can be divided into an acute and a chronic phase [4-10, 17-19]. The chronic phase (days) has been investigated intensively and is mediated by an increase in plasma cortisol, which by binding to the glucocorticoid receptor

increases mRNA and protein abundance of CFTR, Na⁺-K⁺-ATPase and NKCC1 in the mitochondrial rich cells with a delay of > 8-24-hours. Underscoring the importance of cortisol, adaptation can be blocked by RU486, a glucocorticoid receptor antagonist [10, 20]. By contrast, considerably less is known about the acute (hours) phase of adaptation to increased salinity. During this period NaCl intake increases dramatically, yet NaCl balance changes only modestly because of increased NaCl efflux [19, 21]. During the acute phase of adaptation neither CFTR mRNA nor protein levels change, thus, the mechanism underlying the early adaptive response to seawater is unknown. Several reports have suggested that the early increase in NaCl secretion by the gill and opercular membrane is stimulated by a rise in plasma osmolality, which enhances CFTR Cl⁻ secretion, possibly by a mechanism involving the translocation of CFTR from an intracellular compartment into the apical plasma membrane [5, 18, 22]. However, the effect of an acute (hours) increase in salinity on plasma membrane CFTR has not been examined. Accordingly, one of the goals of this study was to test the hypothesis that the early (1-8 hours) adaptive response to elevated salinity is mediated by a translocation of CFTR from an intracellular pool to the plasma membrane.

SGK1, a 50 kDa, serine/threonine protein kinase, is ubiquitously expressed and is transcriptionally activated by a variety of environmental and cytotoxic stressors including plasma hypertonicity and steroids (e, g, glucocorticoids and mineralocorticoids) [23-32]. SGK1 regulates the activity and the subcellular location of several ion channels and transporters. For example, SGK1 increases K⁺ and Ca⁺⁺ transport in epithelial cells by phosphorylating, and enhancing the plasma membrane abundance of ROMK1 and TRPV5 [29, 31]. SGK1 stimulates Na⁺ reabsorption in the kidney by a mechanism involving aldosterone and glucocorticoids, which bind to the mineralocorticoid (MR) and glucocorticoid receptor (GR), respectively, thereby promoting the transcription and subsequent phosphorylation of SGK1 (ppSGK). ppSGK1 in turn phosphorylates, and inhibits Nedd4-2, a E3 ubiquitin ligase, which decreases the ubiquitination of ENaC channels. Because ubiquitinated ENaC is removed from the membrane by the process of endocytosis, reduced ubiquitination of ENaC is thought to lead to accumulation of ENaC in the membrane resulting in enhanced Na⁺ transport [23, 26-29, 33].

Others and we have shown that SGK1 enhances CFTR Cl⁻ currents in *Xenopus* oocytes by increasing the abundance of CFTR Cl⁻ channels in the plasma

membrane [34, 35]. However, the role of SGK1 in regulating the plasma membrane abundance of CFTR *in situ* has not been examined. Accordingly, another goal of this study was to determine if acute (1–8 hr) adaptation to seawater in killifish is associated with increased SGK1 expression.

The data in this paper demonstrate for the first time that freshwater to seawater transfer rapidly (1 hr) increases the abundance of CFTR in the plasma membrane of the operculum. In addition, the rapid increase in plasma membrane CFTR was preceded by an increase in SGK1 mRNA and protein levels. Because SGK1 increases plasma membrane abundance in *Xenopus* oocytes, the most parsimonious interpretation of our data is that the translocation of CFTR to the plasma membrane is mediated by an increase in SGK1, which is stimulated by plasma hypertonicity, and not by the cortisol and the glucocorticoid receptor, since the effect was not blocked by RU486. Thus, in killifish SGK1 does not appear to be regulated by the glucocorticoid receptor.

Materials and Methods

Animals

All studies were performed in compliance with Institutional animal care and use guidelines approved by MDIBL (#A3562-01) and Dartmouth Medical School (#A3259-01). Killifish were collected from Northeast Creek (Bar Harbor, ME, USA) and held in aquaria containing running seawater (15°C) at the MDIBL (Salisbury Cove, Maine) for at least two weeks to ensure acclimation to seawater (i.e. seawater-acclimated). Acclimation to fresh water was achieved by gradually (~1 hr) reducing the aquaria salinity to 10% and maintaining the fish in 10% seawater for two weeks. Subsequently, the water was changed gradually (~1 hr) to reconstituted very “soft” fresh water (ASTM, 1985. Annual Book of ASTM Standards. Vol. 11.01, Section 11, Water (I). Philadelphia, PA). Fish were maintained in fresh water for at least two weeks (i.e., fresh water-adapted). Studies investigating seawater acclimation involved direct transfer of fresh water acclimated fish to 100% seawater (15°C).

Using chamber analysis of CFTR-mediated Cl⁻ currents

To isolate opercular membranes for Ussing chamber experiments fish were anesthetized in tricaine (0.2 g/l) and then euthanized by double pithing. Opercular membranes were isolated and mounted in Ussing chambers, and the CFTR-mediated Cl⁻ current (I_{sc}) was measured using a Physiological Instruments Voltage Clamp (Model VCC MC6, San Diego, CA), as previously described in detail [9, 10, 36, 37]. Previous studies have shown that the isoproterenol-stimulated I_{sc} by opercular membranes is equivalent to CFTR-mediated Cl⁻ secretion. A modified Cortland’s saline solution with NaCl added to increase

the osmolality to 350 mOsm/kg H₂O, the osmolality of the blood in animals transferred to seawater [5], bathed the apical and basolateral sides of the opercular membranes, and the solutions were continuously stirred by bubbling with 1% CO₂-air. Isoproterenol (10 μM) was added to the basolateral solution to elevate cAMP levels and stimulate CFTR Cl⁻ currents. Current output from the voltage clamp was digitized by an analog-to-digital converter (iWORX, Dover, NH). Data collection and analysis were conducted with Labscribe software (iWORX).

Drug treatment

Fresh water-acclimated fish were either untreated or injected intraperitoneally (IP) with vehicle (ethanol) or the glucocorticoid receptor antagonist RU486 (40 μg/ml body wt.). This concentration of RU 486 has been used by others and us to induce steroid receptor blockade in killifish [7, 10, 20, 38] and was shown to block the ability of killifish to adapt to seawater [10]. Subsequent to IP injection, fish were either returned to fresh water or placed in 100% seawater. Results in the untreated and vehicle injected fish were similar (see below). Thus, IP injections, which have the potential to induce stress and increase cortisol levels, had no overt effect on the parameters measured in this study.

Cell surface biotinylation and Western blot analysis

To measure the amount of CFTR in the plasma membrane of the operculum, fish were anesthetized and euthanized, as described above, after exposure to seawater for the times described in the Results. Subsequently, opercular membranes attached to the bony operculum were rapidly isolated (6 fish/observation) and, at 4°C, the biochemical determination of plasma membrane CFTR at steady state was performed by cell surface biotinylation using EZ-Link™ Sulfo-NHS-LC-Biotin, as described previously in detail [39, 40]. Preliminary studies were also conducted to measure plasma membrane CFTR expression in the gill by cell surface biotinylation. Although we were able to detect CFTR in gill by Western blot analysis, we were unable to determine the amount of CFTR in the plasma membrane of the gill by cell surface biotinylation. Because the gill is covered by mucus, which may prevent biotinylation of cell surface proteins, studies were conducted with N-acetylcysteine to strip the mucus from the cell surface; however, even after N-acetylcysteine we were unable to measure CFTR in the apical plasma membrane of the gill. We speculate that the mucus prevented access of EZ-Link™ Sulfo-NHS-LC-Biotin to cell surface proteins, and that the methods we used to strip the mucus from the cell surface were not sufficient.

CFTR was detected in the biotinylated samples and in cell lysates of gill and opercular membranes using an anti-CFTR antibody (clone 24-1; R&D Systems) as described in detail previously [40]. Similar results for CFTR in cell lysates were observed in gill and opercular membranes. SGK was detected in gill using anti-rabbit antibodies from Sigma (anti-SGK, #S5188) and Upstate (anti-phospho-SGK, #36-002). Similar results were obtained using both antibodies. Actin was detected using an anti-actin antibody (MP Biomedicals, clone C4, #69100). Because SGK1 expression is low in all tissues studied, even after stimulation with glucocorticoids, it was not possible

to detect SGK1 in opercular membranes since there is very little opercular tissue. Thus, all Western blot and PCR (see below) studies on SGK1 were performed on gill, which is functionally equivalent to the operculum [19].

Q-RT-PCR

In a previous study we reported that killifish gill and operculum express SGK1. Overall killifish and human SGK1 proteins are 87.9% identical and 95.4% similar in sequence [35]. No orthologs of human SGK2 or SGK3 were identified in killifish [35]. Thus, quantitative-RT-PCR (Q-RT-PCR) experiments were conducted to examine the effects of increased salinity on SGK1 mRNA expression by the gill. To isolate total RNA from gill, tissue was obtained as described above and immediately stored in RNeasy lysis buffer (Qiagen, Valencia, CA). Total RNA was isolated from 30 mg of tissue (from three animals to reduce animal to animal variation [41]) per observation using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was treated with DNase (DNA-Free, Ambion, Austin, TX) to remove contaminating DNA. Total RNA was quantified using spectrophotometric OD_{260/280} measurements (NanoDrop, NanoDrop Technologies, Rockland, DE) and RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE). Two-Step RT-PCR was performed with 1 µg of total RNA (Retroscript Reverse Transcriptase, Ambion, Austin, TX) and random decamers. Primers and probe for real-time PCR were synthesized using the Assays-by-Design service (Applied Biosystems, Foster City, CA). The sequence for killifish SGK1 [35] was submitted for primer/probe design, and probe target was designed to span a predicted exon-exon splice junction. Sequence of the primers and probe used (5' to 3') were: SGK Exon 24 Forward: TCC TCA AAG TGA TCG GCA AGG SGK Exon 24 Reverse: TGC GTA AAA CTG GTC GTC TGT SGK Exon 24 Internal probe: CTT CGG CAA GGT GCT GC.

The probe was 6-FAM dye-labeled with a minor groove binding modification and non-fluorescent quencher on the 3' end. The Assays-by-Design primers and probe premixed to a concentration of 18 µM for each primer and 5 µM for each probe (equivalent to a 20X mix) were combined with TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) and cDNA diluted in RNase-free H₂O in a 20 µl reaction and placed in a 96-well format spectrofluorometric thermal cycler (ABI Prism 7500 Sequence Detection System, Foster City, CA). Duplicate and/or triplicate reactions of each sample were incubated at 95° for 10 min, followed by 40 cycles of 15 sec at 95° and 1 min at 60°. In preliminary studies, Q-RT-PCR products were run on an LMP agarose gel to confirm product size, subcloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) and submitted for sequence analysis to confirm identity of the products. Dilutions of SGK plasmid DNA prepared from the killifish Q-RT-PCR products were used to construct a standard curve. The standard curves showed a correlation coefficient close to 1 ($R^2 = >0.99$) and were linear over a 6-log range. Equivalent amplification efficiencies of standard and target molecules were observed and SYBR green melting curve dissociation analysis and sequencing revealed a single PCR product for each target. Raw data were analyzed; baseline and threshold values set and gene expression interpolated using

the external standard curves. The cDNA generated during reverse transcription and used as template was quantified (NanoDrop) and data were calculated as gene expression (fg/ng cDNA).

Analysis of data

Data are presented as mean ± SE. Statistical significance of experimental treatments was determined by the paired or unpaired Student's *t*-test, or ANOVA and the Tukey-Kramer Multiple Comparison Test, as appropriate, using GraphPad Instat version 3.0a for Macintosh (GraphPad Software, San Diego, California USA). A *P* value <0.05 was considered significant.

Results

CFTR Cl⁻ secretion

Ussing chamber studies were conducted at early time points (1 hr, 3 hr, and 8 hr) after fish were transferred from freshwater to seawater to determine when CFTR-mediated Cl⁻ current (I_{sc}) increased in response to seawater. Fish were transferred to seawater for the indicated times and opercular membranes were subsequently isolated and mounted in an Ussing chamber. A modified Cortland's solution (350 mOsm/kg/H₂O) was added to the basolateral side to reflect the plasma osmolality of fish transferred to seawater. The same solution bathed the apical solution to eliminate any concentration gradients across the opercular membranes, a requirement for Ussing chamber measurements of I_{sc}. The isoproterenol (10 µM) stimulated I_{sc} was increased 1 hour after transfer to seawater, compared to fresh water control (Figure 1). I_{sc} increased further after 3 hrs. However, longer exposure to seawater (8 hrs) did not increase I_{sc} compared to 3 hrs (Figure 1).

Plasma membrane CFTR

To determine if the acute (1-8 hrs) increase in CFTR Cl⁻ secretion is mediated, at least in part, by an increase in the plasma membrane abundance of CFTR, plasma membrane CFTR was measured by cell surface biotinylation, after fish were transferred to salt water for 1, 3 and 8 hrs. Plasma membrane CFTR was increased in all groups of fish transferred to salt water compared to fresh water-adapted fish (Figure 2). Moreover, during the 8 hr period after transfer to seawater, total CFTR in cells did not change, as determined by Western blotting (Figure 2). Comparison of Figures 1 and 2 reveals that there is a good correlation between CFTR Cl⁻ secretion and plasma membrane abundance of CFTR. Taken together, these data suggest that the acute (1-8 hrs)

Fig. 1. Opercular membranes were isolated from killifish adapted to freshwater (FW) and from fish adapted to freshwater and then transferred to seawater (SW) for 1, hr, 3 hr, or 8 hr. Membranes were isolated and bathed in hypertonic Cortland's solution, which mimics plasma osmolality of killifish transferred to SW. CFTR Cl^- currents were measured as described in Methods. $N=6-8$ membranes/time point. * $P<0.05$ versus FW. ** $P<0.05$ versus SW 1 hr and FW.

adaptation to seawater is mediated, at least in part, by an increase in the abundance of CFTR in the plasma membrane, which is most likely due to a translocation of CFTR from an intracellular pool to the plasma membrane.

SGK1

As noted in the Introduction, SGK1 stimulates CFTR Cl^- currents in *Xenopus* oocytes by increasing the plasma membrane abundance of CFTR [34, 35]. However, the role of SGK1 in the adaptation to seawater in killifish is unknown. Because cortisol and plasma osmolality are elevated in killifish transferred to seawater, and because cortisol and hypertonicity stimulate SGK1 in mammalian cells, studies were conducted to test the hypothesis that SGK1 expression is stimulated in killifish exposed to seawater. Q-RT-PCR and Western blot studies were performed to measure SGK1 mRNA levels and protein abundance. Seawater increased SGK1 mRNA levels at the earliest time point examined (15 minutes), and SGK1 mRNA remained elevated for 180 minutes (Figure 3). SGK1 protein abundance was increased 1 hr after transfer to seawater, but returned to freshwater control levels after 3 hrs (Figure 4). This rapid increase in SGK1 mRNA and protein abundance is similar to what is observed in mammalian cells, and, importantly, preceded the increase in the CFTR Cl^- secretion and the increase in the plasma membrane abundance of CFTR (See Figure 2 and the Discussion). Unfortunately, because there are no drugs or compounds that directly inhibit SGK1 activity, because transgenic killifish are not available, and because siRNA and other genetic approaches are not feasible in

Fig. 2. Transfer from freshwater (FW) to seawater increased plasma membrane CFTR without changing total CFTR (1-8 hr). A. Summary of data. Fish were adapted to fresh water (FW) for two weeks and placed in seawater (SW) for 1 hr, 3 hr, or 8 hr. After the indicated times, opercula were isolated and the amount of CFTR in cell lysates (solid bars) and the plasma membrane (hatched bars) were measured as described in Methods. Data presented as percent of FW. $N=7/\text{group}$. * $P<0.05$ versus FW. B. Representative Western blot of biotinylated CFTR in the plasma membrane. C. Representative Western blot of CFTR in cell lysates, which includes plasma membrane and intracellular CFTR.

Fig. 3. Fish were adapted to freshwater (FW) for two weeks and transferred from freshwater to seawater (SW) for 15 min, 30 min, 60 min, or 180 min. SGK mRNA expression was measured in gill by Q-RT-PCR as described in Methods. $n=6/\text{group}$. * $P<0.05$ versus FW.

Fig. 4. A. Fish were adapted to freshwater (FW) for two weeks and transferred from freshwater to seawater (SW) for 1 hr, 3 hr, and 8 hr. SGK protein expression was measured in gill by Western blot as described in Methods. $n=4/\text{group}$. Each group equals 6 fish. $*P<0.05$ versus FW. B. Representative Western blot of SGK1 in cell lysates. C. Representative Western blot of actin in cell lysates.

Fig. 6. Fish were adapted to fresh water for two weeks and given RU486 (RU) or vehicle by IP injection and returned to FW. One hour later fish were removed from freshwater and placed in either freshwater (FW) or seawater (SW) for 1 hour. FW: fish injected with vehicle and returned to FW. SW 1 hr: fish injected with vehicle and transferred to SW. SW RU 1 hr: fish injected with RU486 and transferred to SW. $n=4/\text{group}$. A: SGK protein was measured by Western blot as described in Methods. SW 1 hr and SW RU 1 hr are $*P<0.05$ versus FW. However, SW 1 hr is not different from SW RU 1 hr. B. Representative Western blot of SGK1 in cell lysates. The two lanes labeled SW 1 hr represent two different experiments. When normalized for actin (See panel C), the abundance of SGK1 was similar in these two experiments. C. Representative Western blot of actin in cell lysates.

killifish it was not possible to directly examine the role of SGK1 in regulating the plasma membrane abundance of CFTR. Nevertheless, the most parsimonious interpretation of these data is that the increase in SGK1 mRNA and protein is likely to be responsible for the translocation of CFTR to the plasma membrane (see Discussion).

To determine if the increase in SGK1 mRNA and protein expression was mediated by increased cortisol levels, and activation of the glucocorticoid receptor,

Fig. 5. Fish were adapted to fresh water (FW) for two weeks and given RU486 (RU) or vehicle by IP injection and returned to FW. 24 hours later fish were removed from freshwater and then placed in freshwater (FW) or seawater (SW) for 1 hour. FW: fish injected with vehicle and returned to FW. SW 1 hr: fish injected with vehicle and transferred to SW. SW RU 1 hr: fish injected with RU486 and transferred to SW. $n=4/\text{group}$. $*P<0.05$ for SW 1 hr and SW RU 1 hr versus FW. However, SW 1 hr is not different from SW RU 1 hr.

freshwater adapted fish were given RU486, a glucocorticoid antagonist, by IP injection, transferred to seawater, and SGK1 mRNA was measured by Q-RT-PCR, and SGK1 protein was measured by Western blotting. RU486 did not block the seawater-induced increase in SGK1 mRNA (Figure 5). In addition, RU486 had no effect on the seawater-induced increase in SGK1 protein abundance (Figure 6), consistent with the view that plasma hypertonicity and not plasma cortisol increased

SGK1 expression. The lack of effect of RU486 suggests that the promoter of killifish SGK1 gene may not contain the transcription factor binding site for the glucocorticoid receptor in contrast with the promoter of the rat and human SGK1 gene [24, 42] (See the Discussion).

Discussion

There are three major new findings in this paper. First, acute (hours) adaptation to increased salinity in killifish involves the rapid (1 hr) translocation of CFTR from an intracellular pool to the plasma membrane of the operculum, which is associated with increased CFTR Cl⁻ secretion. Second, our data suggests that SGK1 increases the plasma membrane abundance of CFTR in fish operculum. Third, the lack of effect of RU486 suggests that the promoter of the killifish SGK1 gene may not contain the transcription factor binding site for the glucocorticoid receptor in contrast with the promoter of the rat and human SGK1 gene [24, 42].

Acute adaptation to increased salinity is mediated by translocation of CFTR from an intracellular pool to the plasma membrane.

The data presented here demonstrate that acute (1-8 hrs) adaptation to salinity in killifish is mediated in part by a translocation of CFTR from an intracellular pool to the plasma membrane of the operculum. Presumably, the same phenomenon occurs in the gill, although we were unable to monitor plasma membrane CFTR due to technical limitations. Numerous studies have shown that CFTR Cl⁻ secretion across epithelial cells is regulated by modulating the number of CFTR channels in the plasma membrane. For example, in airway and intestinal cells activation of protein kinase A (PKA) by cAMP enhanced Cl⁻ secretion in part by increasing the plasma membrane abundance of CFTR. The mechanism of increased surface expression of CFTR by PKA involved inhibition of the endocytic removal of CFTR from the plasma membrane as well as a stimulation of the exocytic insertion of CFTR from intracellular vesicles into the plasma membrane [1, 11-16]. Similarly, patch clamp studies showed that cAMP/IBMX rapidly increased the number of CFTR channels in the plasma membrane of cultured killifish opercular membranes [43]. In the present study we observed that one hour after killifish were transferred to seawater there was an increase in the amount of CFTR in the plasma membrane, as determined by cell surface biotinylation, and an increase in CFTR Cl⁻ secretion,

measured in Ussing chamber studies. Because the total amount of CFTR (cell surface plus intracellular CFTR) did not change during this time period (Figure 2), these observations are consistent with the conclusion that there was a translocation of CFTR from an intracellular pool to the plasma membrane. Others have reported that more prolonged (12-24 hours) exposure to seawater enhances the number of mitochondrial rich cells in the operculum that express CFTR near the apical membrane. Marshall and his colleagues [4] reported that the number of mitochondrial rich cells that express apical membrane CFTR increased ~2-fold 24 hours after transfer to seawater, the earliest time point reported. Hiro et al [44] also reported that the number of mitochondria rich cells expressing apical membrane CFTR increased in talapia 12 hours after transfer to seawater. Moreover, it has been suggested that adaptation to seawater in striped bass may be mediated in part by an increase in the plasma membrane expression of CFTR [45]. Our finding that increased salinity increases the abundance of CFTR in the apical plasma membrane demonstrates for the first time that the increase in plasma membrane CFTR abundance occurs one hour following the transfer to seawater, and is consistent the observation by us and others that killifish rapidly (1 hr) enhance NaCl excretion after transfer to seawater [19, 21].

SGK1 and plasma membrane expression of CFTR. Transfer to saltwater increased SGK1 mRNA in killifish gill with 15 minutes and enhanced SGK1 protein expression after 1 hour, the earliest time point measured. These changes in SGK1 preceded the rise in plasma membrane CFTR abundance and CFTR Cl⁻ secretion and are consistent with observations in other tissues that corticosteroids and hypertonicity rapidly (~20 min), increase SGK1 mRNA and protein levels [29, 32]. The correlation between SGK1 expression and CFTR Cl⁻ secretion does not *a priori* prove a cause and effect relationship between SGK1 and plasma membrane abundance of CFTR. However, taken together with studies in *Xenopus* oocytes revealing that SGK1 stimulates CFTR Cl⁻ currents by increasing the plasma membrane abundance of CFTR [34, 35], these observations are consistent with the view that SGK1 mediates the increase in plasma membrane CFTR. SGK1 also increases the plasma membrane abundance of ROMK1, TRPV5 and ENaC ion channels [23, 26-29, 31, 33]. Thus, enhancing plasma membrane abundance of ion channels is a common mechanism whereby SGK1 stimulates ion transport in epithelial cells. Unfortunately, because there are no drugs

or compounds that directly inhibit SGK, because transgenic killifish are not available, and because siRNA and other genetic approaches are not feasible in killifish it was not possible to directly examine the role of SGK1 in regulating the plasma membrane abundance of CFTR in killifish operculum. More direct proof that SGK1 is directly responsible for the translocation of CFTR from an intracellular pool to the plasma membrane requires additional studies in a model epithelium in which SGK1 levels can be modulated by siRNA and in which constitutively active and dominant negative SGK1 can be expressed. These studies are currently underway in a mammalian cell line.

Although studies to elucidate the mechanism whereby SGK1 increases the plasma membrane abundance of CFTR are beyond the scope of this report, and such experiments are not technically feasible in killifish, we speculate that SGK1 may increase membrane CFTR by a mechanism that involves the phosphorylation of CFTR and/or by a mechanism that involves phosphorylation, and inactivation, of the E3 ubiquitin ligase Nedd4-2. SGK1 increases the cell surface abundance of several ion channels and transporters, possibly by direct phosphorylation [29, 32]. CFTR has several SGK1 consensus phosphorylation sites (R-X-R-X-X-(S/T)) [32], and as noted above, phosphorylation of CFTR by PKA increases the plasma membrane abundance of CFTR. Thus, it is possible that SGK1 enhances the abundance of CFTR in the plasma membrane by phosphorylating CFTR. Additional studies, beyond the scope of this manuscript, are required to test this hypothesis. We do not favor the hypothesis that SGK1 enhances the cell surface expression of CFTR by phosphorylating and inactivating Nedd4-2, a E3 ubiquitin ligase, because CFTR does not contain a PY motif that is thought to be essential for Nedd4-2 to bind to and ubiquitinate target proteins, which would promote their removal from the plasma membrane and their subsequent degradation [23, 26-29, 33]. Although a recent study has shown that CFTR is ubiquitinated and that ubiquitinated CFTR is redirected from recycling endosomes to lysosomes for degradation [46], the hormones and factors that regulate CFTR ubiquitination is unknown. Thus, additional studies are required to determine the mechanisms whereby SGK1 regulates the plasma membrane abundance of CFTR.

Because SGK1 also increases the abundance of plasma membrane $\text{Na}^+\text{-K}^+\text{-ATPase}$ [47, 48], it must be considered that SGK1 may enhance Cl^- secretion across opercular membranes by facilitating the expression of $\text{Na}^+\text{-K}^+\text{-ATPase}$ in the basolateral membrane.

However, such an effect is unlikely to participate in acute (1-8 hrs) adaptation since immunofluorescent studies reveal that the plasma membrane expression of $\text{Na}^+\text{-K}^+\text{-ATPase}$ does not increase until at least 8 hrs after transfer to seawater [48]. Furthermore, ouabain-binding studies reveal that the more rapid, seawater-induced increase in $\text{Na}^+\text{-K}^+\text{-ATPase}$ transporter activity is mediated by changes in the catalytic rate rather than the abundance of $\text{Na}^+\text{-K}^+\text{-ATPase}$ [49]. Moreover, SGK1 may also up regulate basolateral K^+ channels [8, 29, 32], which would enhance the turnover of the $\text{Na}^+\text{-K}^+\text{-ATPase}$. However, the identity of the basolateral K^+ channel in the gill and the operculum are unknown, as is the ability of SGK1 to regulate K^+ channel activity.

Cortisol and the glucocorticoid receptor do not regulate SGK1 expression.

RU486, a glucocorticoid receptor antagonist, did not inhibit the seawater-induced increase in SGK1 mRNA or protein expression, a result consistent with the conclusion that in killifish SGK1 expression is not regulated by cortisol and the glucocorticoid receptor. The lack of effect of RU486 suggests that the promoter of the killifish SGK1 gene may not contain the transcription factor binding site for the glucocorticoid receptor in contrast with the promoter of the rat and human SGK1 gene [24, 42]. Additional studies, beyond the scope of this study are required to test this suggestion.

Because RU486 did not inhibit the seawater induced increase in SGK1 expression, it is most likely that SGK1 expression was elevated by plasma hypertonicity, a well known stimulus of SGK1 expression [50]. Previously, others and we showed that RU486 reduced the ability of killifish to adapt to seawater by blocking the cortisol-induced increase in CFTR mRNA [7, 10]. Thus, the lack of effect of RU486 on SGK1 is not related to an inability to antagonize the effect of cortisol in killifish. The most parsimonious interpretation of our data is that in killifish hypertonicity alone is capable of elevating SGK1 mRNA to levels that are sufficient to increase CFTR Cl^- secretion to meet the acute challenge of excreting the excess NaCl ingested in seawater.

Data from several other laboratories, but not all, are consistent with our conclusion that hypertonicity *per se* stimulates CFTR Cl^- secretion across the opercular membrane of killifish. In studies similar to ours where fresh water-adapted killifish were transferred to seawater for one hour and CFTR Cl^- secretion was measured when opercular membranes were bathed in hypertonic bath solutions, currents increased to $\sim 100 \mu\text{A}/\text{cm}^2$, a value

similar to those measured in this report (Figure 1)[5]. By contrast, others have reported that ~1 day is required before CFTR Cl⁻ secretion by the operculum is elevated upon transfer to seawater [18, 19, 22]. However, in these studies Cl⁻ secretion was measured when opercular membranes were bathed in a solution with an osmolality of 300 mOsm/kg/H₂O, rather than the osmolality of 350 mOsm/kg/H₂O used in the present study, which mimics that of plasma in fish acutely transferred to seawater. These observations suggest that acute exposure to hypertonicity is an important determinant of CFTR Cl⁻ secretion in opercular membranes isolated from fish transferred from fresh water to seawater.

Finally, it should also be mentioned that the rapid (hours) increase in CFTR Cl⁻ secretion across the gill and opercular membrane following transfer to seawater is also enhanced by hormones including arginine vasotocin and urotensin I (and their second messengers cAMP and PKA), as well as by the neurotransmitter vasoactive intestinal peptide, and cell shrinkage, which activates NKCC1 [8].

In conclusion, there are three major new findings in

this paper. First, acute adaptation to increased salinity in killifish involves the rapid (1 hr) translocation of CFTR from an intracellular pool to the plasma membrane of the operculum, which is associated with increased CFTR Cl⁻ secretion. Second, our data suggests that SGK1 increases the plasma membrane abundance of CFTR in fish operculum. Third, the lack of effect of RU486 suggests that the promoter of the killifish SGK1 gene may not contain the transcription factor binding site for the glucocorticoid receptor in contrast with the promoter of the rat and human SGK1 gene [24, 42].

Acknowledgements

This study was supported by NIH-NIDDK grant RO1-DK45881 (BAS), NIH-NIEHS Superfund Basic Research Program Project grant P42 ESO7373 (BAS), an MDIBL New Investigator Award to JRS, INBRE grant P20-RR016463 from NCRR, NSF REU grant DBI-0453391 to J.V. and an MDIBL Hancock Scholars Studentship to T.L.

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