2-14-2007

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Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2007;20:91-98

Accepted: February 14, 2007

Regulation of Human Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) by Serum- and Glucocorticoid-Inducible Kinase (SGK1)

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Key Words
Serine/threonine kinase • Ion channel • Electrophysiology

Abstract
Background: Serum- and glucocorticoid-inducible kinase-1 (SGK1) increases CFTR Cl currents in *Xenopus* oocytes by an unknown mechanism. Because SGK increases the plasma membrane expression of other ion channels, the goal of this paper was to test the hypothesis that SGK1 stimulates CFTR Cl currents by increasing the number of CFTR Cl channels in the plasma membrane. Methods: CFTR Cl currents were measured in *Xenopus* oocytes by the two-electrode voltage clamp technique, and CFTR in the plasma membrane was determined by laser scanning confocal microscopy. Results: wt-SGK1 stimulated CFTR Cl currents by 42% and increased the amount of CFTR in the plasma membrane by 35%. A kinase-dead SGK mutant (K127N) had a dominant-negative effect on CFTR, reducing CFTR Cl currents by 38%. In addition, deletion of the C-terminal PDZ-interacting motif (SGK1-ΔSFL) increased CFTR Cl currents by 108%. Thus, SGK1-ΔSFL was more effective than wt-SGK1 in stimulating CFTR Cl currents. Neither wt-SGK nor the K127N mutant had any effect on Cl currents in oocytes when expressed alone in the absence of CFTR. Conclusion: SGK1 stimulates CFTR Cl currents in *Xenopus* oocytes by increasing the number of channels in the plasma membrane. Moreover, the effect of SGK may be mediated by protein-protein interactions involving the PDZ interacting motif.

Introduction

Serum and glucocorticoid-inducible serine/threonine kinase, SGK, was discovered in rat mammary tumor cells [1] and has been shown to regulate epithelial sodium channels (ENaC) in frog [2, 3] and mammalian cells [4, 5]. SGK shares a high degree of similarity in its catalytic region with the kinases protein kinase A (PKA), protein kinase C (PKC), protein kinase B (PKB)/Akt, phosphatidylinositol-dependent kinase-1 (PDK1), and p70 S6 kinase [1, 6]. Two additional isoforms of human SGK, SGK2 and SGK3, have been described [7]. In mammalian cells SGK1 activity is regulated by phosphatidylinositol(PI) 3-kinase through PDK1 and PDK2, and activation of

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SGK1 is abolished by specific inhibitors of PI3-kinase [6, 8]. SGK1 gene transcription is regulated by the p38 MAP kinase pathway [9], and SGK1 transcript levels are up regulated by serum, dexamethasone and environmental stresses that induce p38 MAPK expression such as hypertonicity [10, 11].

SGK regulates an increasingly large number of transport proteins. SGK integrates the regulation of ENaC activity by insulin and mineralocorticoids in *Xenopus* A6 renal epithelial cells [12, 13], it is involved in the regulation of K^+ channels [14] and the NKCC2/BSC1 Na-K-2Cl cotransporter [15], and SGK1 mediates the stimulation of Na^+/H^+ exchanger isoform 3 (NHE3) activity in intestinal cells by glucocorticoids [16]. A common theme that emerges from these studies is that SGK binds to cytoskeleton-bound scaffold/regulatory proteins that directly interact with ion channels or transporters, and it regulates ion transport by phosphorylating either the scaffold/regulatory protein or the channel or transporter proteins. For example, NHE regulatory factor 2 (NHERF2)-bound SGK1 directly phosphorylates NHE3 [16] while SGK indirectly regulates ENaC by phosphorylating and inhibiting the ubiquitin ligase Nedd4-2, which inhibits the degradation of internalized ENaC [17].

Wagner, et al. [25] reported that wt-SGK1, but not a kinase-dead SGK mutant (K127R), stimulated CFTR-mediated Cl currents in *Xenopus* oocytes. However, the mechanism whereby SGK increased CFTR Cl currents was not examined. Because SGK increases the plasma membrane expression of other ion channels, the goal of this paper was to test the hypothesis that SGK1 stimulates CFTR Cl currents by increasing the number of CFTR Cl channels in the plasma membrane. We report that wt-SGK1 apparently stimulated CFTR Cl currents by increasing the amount of CFTR in the plasma membrane. In addition, deletion of the C-terminal PDZ-interacting motif (SGK1-ΔSFL) increased CFTR Cl currents. Thus, SGK1 stimulates CFTR Cl currents in *Xenopus* oocytes by increasing the number of channels in the plasma membrane, and the effect of SGK may be mediated by protein-protein interactions involving the PDZ interacting motif.

Materials and Methods

Human and Killifish SGK cDNA constructs

Wild-type human SGK cDNA in the pGEM-He Juel cloning vector was generously provided by Dr. Florian Lang (University of Tubingen, Tubingen, Germany) (10; GenBank accession number Y10032). Wild-type killifish SGK cDNA was amplified by RT-PCR and RACE [26] from total liver RNA [27] and cloned in the pCR 2.1 topo-TA cloning vector (Invitrogen, Carlsbad, CA). Initial synthetic oligonucleotide primer sequences for RT-PCR were obtained from kinase subdomains VI and VIII of *D. rerio* SGK cDNA. The complete coding region of killifish SGK cDNA was deposited in GenBank (accession number AY800243). The killifish SGK-K127N kinase-dead mutant was constructed as described [6, 28]. A premature stop codon (TGA) was introduced in the human SGK1-ΔSFL mutant in place of serine-428 (TCT), which deleted the C-terminal PDZ-interacting motif in this protein. The mutations were made with the PCR-based Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), and they were verified by dideoxynucleotide sequencing of purified plasmids in the Marine DNA Sequencing Center at the Mount Desert Island Biological Laboratory.

Synthesis and characterization of cRNAs

Complementary cRNAs were synthesized on cDNA templates using T7 DNA polymerase (Message Machine T7 Ultra kit; Ambion, Inc., Austin, TX). All cRNA preparations were analyzed and quantitated with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) prior to being injected into oocytes.

Expression of cRNAs in Xenopus oocytes

Stage V/VI *Xenopus* oocytes were surgically removed and digested with 2 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) in Ca^{2+}-free ND96 buffer (5 mM Hepes pH7.5, 96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$) for 1-2 hours. Membranes were stripped in hypotonic K_2HPO_4 solution (0.1 M K_2HPO_4 with 0.1% BSA). Each oocyte was injected 1 day after surgery with 100 pg of human CFTR cRNA with or without 1 ng killifish or human SGK cRNAs. In other experiments the amounts of cRNAs co-injected were reduced to 50 pg human CFTR cRNA and 500 pg SGK cRNA (see figure legends). The SGK cRNAs encoded either wild-type or mutant SGK proteins. CFTR-mediated Cl currents were measured by the two-electrode voltage clamp technique (TEVC) one to three days post injection. Oocytes were placed in a chamber containing ND96 buffer and impaled with glass electrodes containing 3 M KCl (resistance 0.3-3 megaohms). The holding potential for each oocyte was the resting transmembrane potential. Voltages were increased stepwise in 20 mV increments from -140 mV

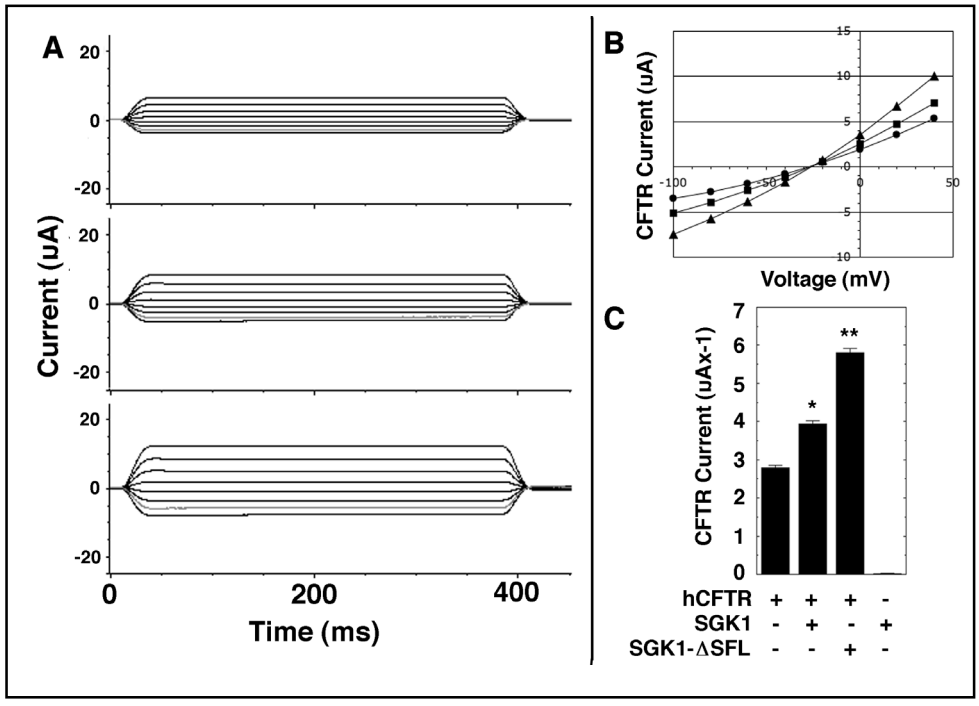

Fig. 1. Effects of wild-type and human SGK1 on IBMX-stimulated CFTR currents in oocytes. Oocytes were injected with 50 pg human CFTR cRNA only (n=30), human CFTR cRNA and 500 pg human SGK1 cRNA (n=35), or human CFTR cRNA and 500 pg human SGK1-ΔSFL cRNA (n=32). IBMX-stimulated CFTR currents were measured by the TEVC technique. Panel A, Current traces recorded at clamp voltages between -140 mV and +60 mV in 20 mV increments for representative oocytes expressing CFTR (top), CFTR and wt-SGK1 (middle) or CFTR and SGK1-ΔSFL (bottom). Currents were filtered at 20 Hz. Panel B, I-V plots displaying mean current values at each voltage for oocytes expressing CFTR (closed circles), CFTR and SGK1 (closed squares), or CFTR and SGK1-ΔSFL (closed triangles). Panel C, Summary of currents measured at -80 mV. *P<0.05 versus CFTR alone and **P<0.05 versus CFTR+wt-SGK. The currents before IBMX treatment were similar in oocytes injected with vehicle and CFTR cRNA.

to +60 mV adjusting for resting transmembrane potential. Currents were recorded at each voltage step with a Gene Clamp 500B voltage amplifier (Molecular Devices, Mountain View, CA) both prior to and following 15 minutes of stimulation by 1 mM IBMX in ND96 buffer. In data analyses Cl currents in the absence of IBMX were subtracted from IBMX-stimulated currents. The CFTR inhibitor CFTR 172 was used in some experiments to demonstrate that IBMX-stimulated currents were mediated by CFTR. SGK-injected oocytes were used as negative controls for Cl currents. Data were collected with Clampex/Clampfit software (Molecular Devices). The statistical significance of the various treatments on CFTR currents was evaluated by comparing average currents at -80 mV with the Student's t-test.

Localization and quantitation of CFTR in oocytes by confocal microscopy

Oocytes were fixed and prepared for immunofluorescence microscopy. Oocytes were fixed for 20 min. at room temperature in 2% (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in ND96 buffer. The oocytes were washed three times in ND96 and permeabilized in 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBAG (PBS with 0.5% BSA and 0.15% glycine, w/v) for 30 min. The oocytes were washed three times with PBAG and incubated for 3 hours with mouse anti-human CFTR (R and D Systems, Minneapolis, MN) diluted 1:20 in PBAG solution. After the oocytes were washed in PBAG solution they were co-incubated with Cy5-labeled goat anti-mouse antibodies (Invitrogen; 1:200 dilution) and rhodamine-labeled phalloidin to label the subplasma membrane actin pool (Invitrogen; 1:100 dilution) in PBAG solution for 1 hour, and subsequently washed three times in PBAG. All oocytes were imaged using identical settings with a FluoView 1000 scanning laser confocal microscope (Olympus America, Inc., Center Valley, PA) with a PlanApo 60x oil emersion objective. The intensity of CFTR fluorescence in TIFF images was measured with the NIH Image application. The results were expressed as mean relative fluorescence units (RFU) ±SEM.

Results

SGK1 stimulates CFTR Cl currents

To confirm the observation by Wagner [25] that human SGK1 stimulates CFTR Cl currents, CFTR Cl currents were measured in *Xenopus* oocytes by the TEVC technique. In the presence of IBMX wt-SGK1 stimulated CFTR Cl currents by 42% from -2.784± 0.066

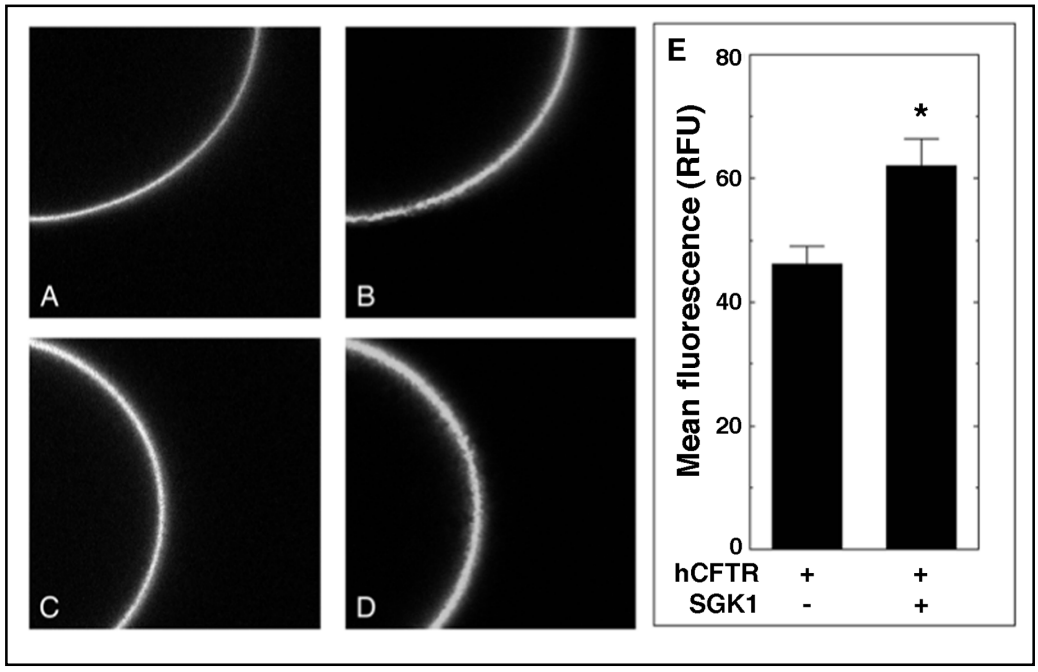

Fig. 2. SGK increases plasma membrane CFTR. Panel B, image of CFTR in an oocyte expressing wt-CFTR. Panel D, image of CFTR in an oocyte expressing wt-CFTR+wt-SGK. Panel A, image of the submembrane actin cytoskeleton in the same oocyte as imaged in Panel B. Panel C, image of the submembrane actin cytoskeleton in the same oocyte as imaged in Panel D. Panel E, summary of data. After being assayed for CFTR currents, oocytes expressing human CFTR alone or CFTR+wt-SGK were fixed and examined by scanning laser confocal microscopy. CFTR was detected with Cy5 (panels B and D), and the submembrane actin cytoskeleton was detected with rhodamine-labeled phalloidin (panels A and C). * indicates $P<0.05$ versus hCFTR. Immunofluorescence in the plasma membranes was quantitated from confocal images with NIH Image. In vehicle and SGK injected oocytes the fluorescence in the Cy5 channel was not different from background. N = 6 oocytes expressing wt-CFTR, and n = 7 oocytes expressing CFTR and SGK.

μA to -3.943 ± 0.078 μA. IBMX did not increase Cl currents in oocytes expressing wt-SGK alone, thus SGK did not activate endogenous Cl channels (Fig. 1).

A PDZ-interacting motif in SGK regulates SGK activity

Inspection of the C-terminal amino acid sequence in human SGK1 identified a putative PDZ-interacting motif (SFL). Because PDZ motifs are known to regulate protein-protein interactions and regulate CFTR activity [18] we tested the hypothesis that this putative PDZ-interacting motif may regulate the ability of SGK to activate CFTR Cl currents. Compared to wt-SGK1, which increased the IBMX stimulated Cl current by 42%, SGK1-ΔSFL stimulated CFTR Cl currents by 108% (Fig. 1). Thus, SGK1-ΔSFL was 2.7-times more active than wt-SGK in enhancing CFTR Cl currents.

Plasma membrane CFTR is increased by SGK

To determine if SGK stimulated CFTR Cl currents by increasing the amount of CFTR in the plasma membrane we used immunofluorescent confocal microscopy to quantify the amount of human CFTR in the plasma membranes of oocytes co-expressing CFTR and wt-SGK relative to oocytes expressing CFTR alone. Plasma membrane CFTR fluorescence in oocytes expressing human CFTR only was 46.16 ± 2.9 relative fluorescence units (RFU) while CFTR fluorescence in oocytes expressing CFTR and wild-type SGK was 62.10 ± 4.34 RFU (Fig. 2). This 35% increase in CFTR abundance in the plasma membrane ($P<0.05$) was similar in magnitude to the SGK1-stimulated 42% increase in CFTR Cl currents. These results are consistent with the hypothesis that SGK1 regulates CFTR activity by increasing CFTR expression in the plasma membrane.

The ortholog of SGK1 cloned from the euryhaline teleost, Fundulus heteroclitus, enhances CFTR Cl currents

We and others have shown that increased salinity stimulates CFTR Cl secretion by the gill and operculum of *Fundulus heteroclitus* (killifish) [29-31]. Because transfer from freshwater to saltwater is associated with an increase in the blood osmolality and cortisol, both factors that activate SGK, we speculated that either or both of these facts may activate SGK, thereby enhancing CFTR Cl secretion. The effects of fresh to saltwater transfer on SGK activity in killifish gill will be described elsewhere. In the present study our goal was to first clone killifish SGK (kfSGK) and then determine whether kfSGK,

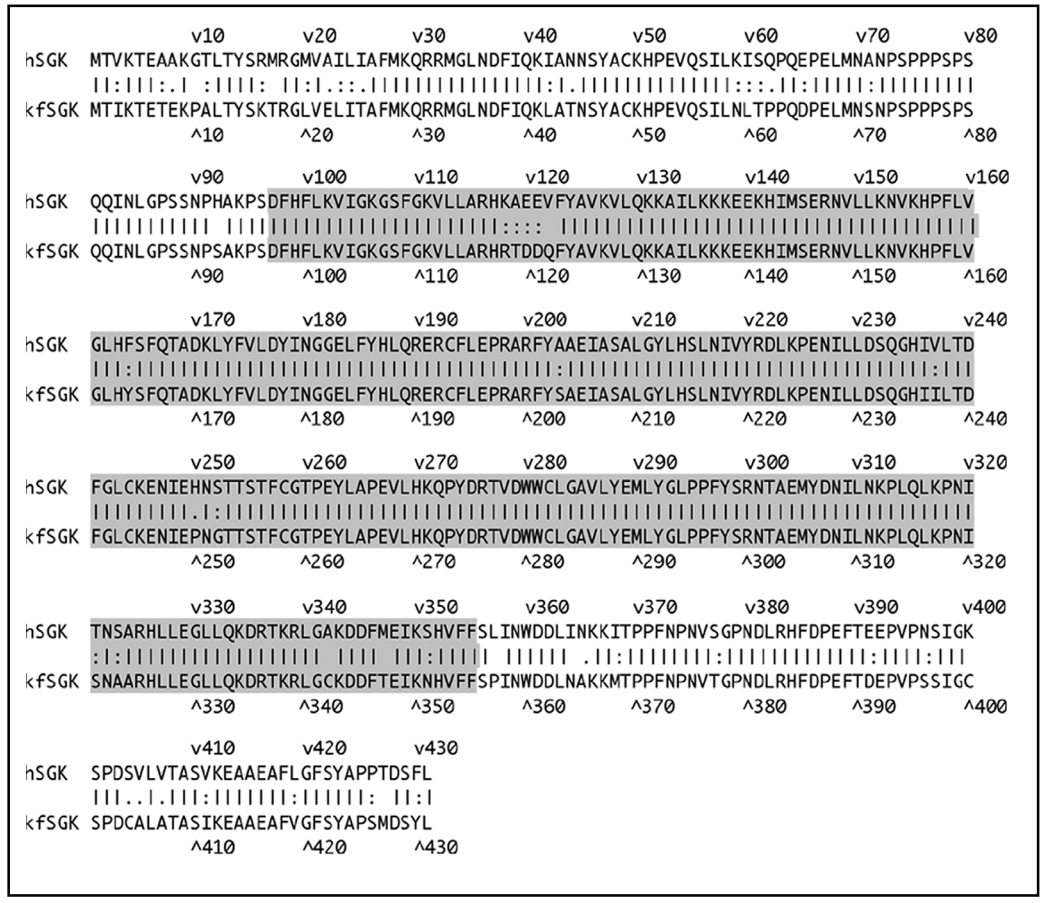

Fig. 3. Alignment of the amino acid sequences of human SGK1 and killifish SGK. Overall the proteins are 87.9% identical and 95.4% similar in sequence. The kinase domains of the proteins (shaded residues 97 to 355) are 98.5% similar in sequence. No orthologs of human SGK2 or SGK3 have been found in killifish.

like human SGK1, stimulates CFTR Cl currents in *Xenopus* oocytes.

Using RT-PCR we cloned the cDNA for the SGK1 ortholog from the killifish [27, 28]. The deduced amino acid sequence of killifish kfSGK was 87.9% identical to and 95.4% similar to human SGK1 while their kinase domains were 98.5% similar in sequence (Fig. 3). We did not find any evidence for orthologs of human SGK2 or SGK3 in killifish, and no orthologs of these SGK isoforms have been identified in the zebrafish (*Danio rerio*) genome. Like human SGK1 [25] kfSGK stimulated CFTR Cl currents when co-expressed in oocytes (Fig. 4.). In the presence of IBMX, kfSGK increased CFTR Cl currents by 66% from -3.845 ± 0.083 μA to -6.370 ± 0.151 μA. The kinase-dead kfSGK-K127N mutant had a dominant-negative effect on CFTR-mediated currents in oocytes (Fig. 4). Co-expression of SGK-K127N decreased CFTR Cl currents by 38% from -4.054 ± 0.174 μA to -2.517 ± 0.085 μA. Neither wild-type kfSGK nor the K127N mutant had any effect on currents when expressed alone.

Discusion

The major new findings in this report are that SGK1 stimulates CFTR Cl currents in *Xenopus* oocytes at least in part by increasing the number of channels in the plasma membrane and that the effect of SGK may be mediated by protein-protein interactions involving the PDZ-interacting motif. SGK1 regulates the activities of several other ion channels and transporters including the ENaC sodium channel, the ROMK1 and Kv1.3 potassium channels, and the Na^+/H^+ exchanger NHE3 [reviewed in 32]. In this study we confirmed and extended the observation by Wagner, et al. [25] that human SGK1 stimulated CFTR-mediated Cl currents in *Xenopus* oocytes. We found that both human SGK1 and the wild-type SGK ortholog in the euryhaline telost *Fundulus heteroclitus* stimulated human CFTR currents when co-expressed in *Xenopus* oocytes. In addition we found that the killifish SGK-K127N kinase-dead mutant had a dominant-negative effect on CFTR activity. Although it is not clear why our results differ from that of Wagner et

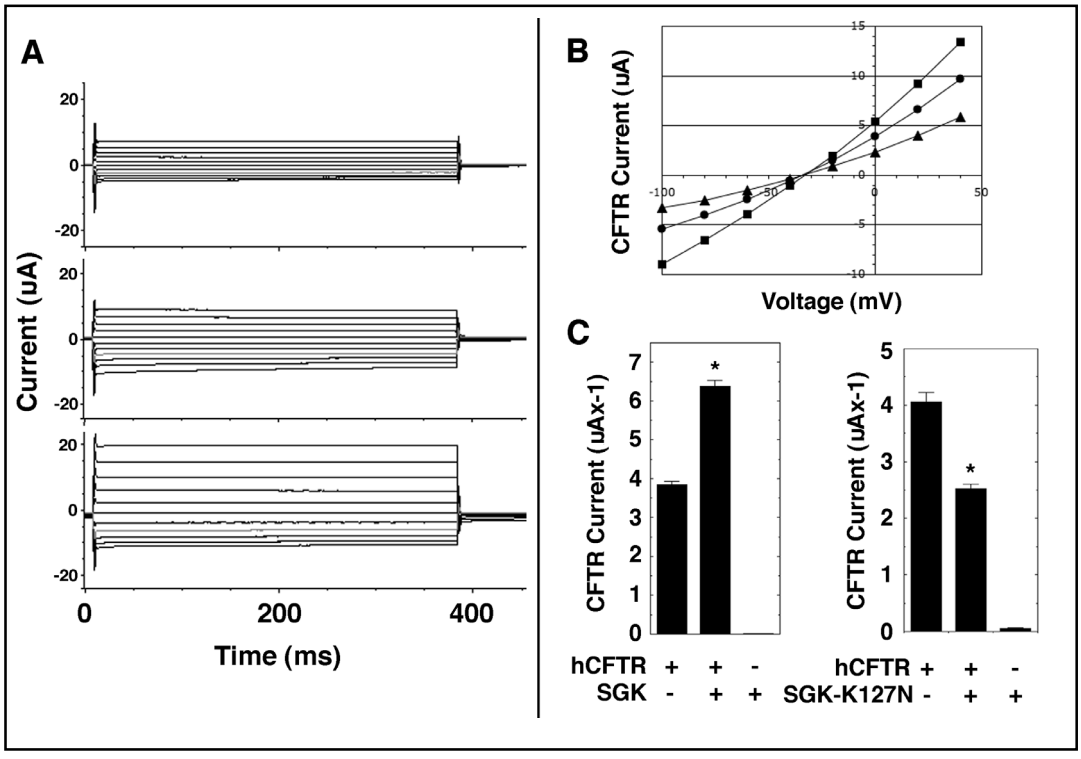

Fig. 4. Effects of wild-type and mutant killifish SGK on IBMX-stimulated CFTR currents in oocytes. Oocytes were injected with 100 pg human CFTR cRNA only or with CFTR cRNA and 1 ng wild-type kfSGK cRNA or kinase-dead kfSGK-K127N cRNA. IBMX-stimulated CFTR currents were measured by the TEVC technique. Panel A, Current traces recorded at clamp voltages between -140 mV and +60 mV in 20 mV increments for representative oocytes expressing kfSGK-K127N (top), CFTR only (middle) or CFTR and kfSGK (bottom). Currents were filtered at 2000 Hz. Panel B, I-V plots displaying mean current values at each voltage step for oocytes expressing CFTR only (closed circles), CFTR and kfSGK (closed squares), or CFTR and kfSGK-K127N (closed triangles). Panel C, Summary of short circuit currents measured at -80 mV. *P<0.05 versus CFTR alone. The currents before IBMX treatment were similar in oocytes injected with vehicle and CFTR cRNA.

al [25] who reported that SGK-K127R had no effect on CFTR currents in oocytes, it should be noted that we and others have shown that SGK-K127N had a dominant-negative effect on CFTR and ENaC currents in *Xenopus* A6 renal epithelial cells [13, 33]. It is possible that the nature of the inactivating mutation may alter the function of SGK. Whereas we report that SGK-K127N reduced CFTR Cl currents, Wagner et al [25] reported that SGK-K127R had no effect on CFTR currents. Finally, we found the human SGK1-ΔSFL mutant lacking the C-terminal PDZ-interacting motif was more effective than wild-type SGK1 in stimulating CFTR-mediated Cl currents in oocytes. The deletion of this mediator of protein-protein interactions likely altered the cellular localization of the SGK1-ΔSFL protein and may have increased its access to downstream target proteins. By contrast, a similar SGK1 mutant was indistinguishable from wild-type SGK1 in its stimulation of ENaC activity and cellular localization in mouse M1 renal collecting duct cells [34].

Our results along with those of Wagner, et al. [25] indicate that SGK1 regulates CFTR. Our results further suggest that the kinase activity of SGK1 is required for the observed stimulation of CFTR Cl currents and that the PDZ-binding motif of SGK1 is involved in modulating the effects of the kinase on CFTR activity. The mechanism by which SGK1 regulates CFTR activity is currently unknown. However, the observed SGK-related increase in CFTR levels in oocyte plasma membranes is consistent with the idea that SGK stimulated CFTR currents by increasing the number of CFTR channels in the plasma membrane rather than by increasing the single channel open probability. SGK1 increases the number of ENaC channels in the plasma membrane by phosphorylating and thus inactivating the ubiquitin ligase Nedd4-2 [17, 35]. Thus, SGK1 inhibits the ubiquitination and degradation of ENaC [36], allowing ENaC to accumulate in the plasma membrane [5, 25]. Further studies are necessary to determine whether SGK regulates CFTR in the same manner in mammalian epithelial cells that express endogenous CFTR.

The finding that SGK1 regulates CFTR function in *Xenopus* oocytes may open up new approaches to discover therapies for cystic fibrosis if these results can be replicated in human airway epithelial cell lines or other

relevant experimental models. The most commonly occurring CFTR mutation, the ΔF508 mutation, decreases the stability and increases the turnover rate of CFTR while retaining some protein function [18, 24]. Thus, therapies that result in the accumulation of ΔF508-CFTR in the plasma membranes of airway epithelial cells by stimulating SGK1-mediated signaling may promote lung function and alleviate some of the symptoms of cystic fibrosis. Several research groups have reported that hypertonicity stimulates wt-CFTR activity in nasal epithelium [37] and ΔF508-CFTR in mouse renal collecting duct cells [38] and in human bronchial epithelial cells [39]. Thus, increasing the osmolarity of airway surface liquid may be a simple and effective method of stimulating the function of both wild-type and ΔF508-CFTR in bronchial epithelial cells. Indeed, in two clinical trials testing the effects of inhaled hypertonic saline (7% NaCl solution), cystic fibrosis patients showed an enhanced ability to clear mucus over two weeks [40] and, over a longer treatment period, had improved lung function and fewer pulmonary exacerbations [41]. Since SGK1 is up regulated by hypertonicity [9-11] and hypertonicity stimulates CFTR currents, we speculate that the improved lung function in CF patients with hypertonic saline may be mediated in part by SGK1 activation of CFTR. Finally, our observation that SGK1-ΔSFL was more potent than wild-type SGK1 in stimulating CFTR currents suggests that specific inhibitors of protein-protein interactions mediated by the SGK1 PDZ-interaction motif may further enhance the effects of SGK1 on mutant forms of CFTR that retain some channel activity.

Acknowledgements

This research was supported by grant RO1-DK45881 from NIDDK to BAS, a Cystic Fibrosis Foundation research development program grant to BAS, grant R01-DK68196 from NIDDK to RAF, NIEHS Center grant P30-ES03828, and by INBRE grant P20-RR016463 from NCRR. RT was supported by NSF Research Experience for Undergraduates grant NSF DBI-0453391. The authors thank Pearl Ryder, Ciara Clarke, Sonal Patel, Kristin Gabor and Caitlin Stanton for technical assistance, and Christine Smith of the MDIBL Marine DNA Sequencing Center for DNA sequencing.

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