The Serum- and Glucocorticoid-inducible Kinases SGK1 and SGK3 Regulate hERG Channel Expression via Ubiquitin Ligase Nedd4-2 and GTPase Rab11*

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Background: The cardiac hERG (I_{Kr}) potassium channel is important for cardiac repolarization.
Results: Activation of SGK1 and SGK3 increases hERG expression by inhibiting Nedd4-2 activity and promoting Rab11-mediated hERG recycling.
Conclusion: SGK1 and SGK3 regulate hERG through Nedd4-2 and Rab11 pathways.
Significance: Identification of SGK effects on hERG extends our understanding of ion channel regulation and cardiac electrophysiology.

The hERG (human ether-a-go-go-related gene) encodes the α subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}). Dysfunction of hERG channels due to mutations or certain medications causes long QT syndrome, which can lead to fatal ventricular arrhythmias or sudden death. Although the abundance of hERG in the plasma membrane is a key determinant of hERG functionality, the mechanisms underlying its regulation are not well understood. In the present study, we demonstrated that overexpression of the stress-responsive serum- and glucocorticoid-inducible kinase (SGK) isoforms SGK1 and SGK3 increased the current and expression level of the membrane-localized mature proteins of hERG channels stably expressed in HEK 293 (hERG-HEK) cells. Furthermore, the synthetic glucocorticoid, dexamethasone, increased the current and abundance of mature ERG proteins in both hERG-HEK cells and neonatal cardiac myocytes through the enhancement of SGK1 but not SGK3 expression. We have previously shown that mature hERG channels are degraded by ubiquitin ligase Nedd4-2 via enhanced channel ubiquitination. Here, we showed that SGK1 or SGK3 overexpression increased Nedd4-2 phosphorylation, which is known to inhibit Nedd4-2 activity. Nonetheless, disruption of the Nedd4-2 binding site in hERG channels did not eliminate the SGK-induced increase in hERG expression. Additional disruption of Rab11 proteins led to a complete elimination of SGK-mediated increase in hERG expression. These results show that SGK enhances the expression level of mature hERG channels by inhibiting Nedd4-2 as well as by promoting Rab11-mediated hERG recycling.

The human ether-a-go-go related gene (hERG,[2] also known as KCNH2) encodes the α (pore-forming) subunit of the rapidly activating delayed rectifier potassium channel (I_{Kr}) (1). A decrease in the hERG current (I_{hERG}) due to mutations in hERG causes long QT syndrome (LQTS) type 2 (2, 3). LQTS can lead to syncope, ventricular arrhythmias, and sudden death (4, 5). LQTS can also be caused by blockage of hERG channels by certain drugs (6). Although the effects of drugs and mutations on the hERG function have been studied extensively, less is known about the regulation of hERG density at the plasma membrane. Recent studies have revealed some new insights into the internalization and degradation of mature hERG channels. For instance, a decrease in extracellular potassium concentration clinically known as hypokalemia has been found to induce hERG internalization from the plasma membrane (7). Internalization of cell surface hERG channels occurs through a caveolin-dependent pathway (8). Caveolae are plasma membrane pits that form stable membrane domains and function as carriers in the endocytic pathway (9). The principle components of caveolae are caveolins, which compartmentalize and recruit signaling molecules to the caveolae. We have demonstrated that the muscle-specific caveolin isoform caveolin 3 recruits and enhances Nedd4-2 interaction with hERG channels at the cell surface, leading to a decreased expression of hERG channels at the plasma membrane (10). Nedd4-2 is an ubiquitin-protein ligase that is responsible for substrate recognition and ubiquitin transfer to target proteins (11). Covalent attachment of ubiquitin to proteins such as ion channels tags the proteins for proteasomal or lysosomal degradation (11, 12). Presently, the upstream mechanisms regulating Nedd4-2 and the subsequent internalization of hERG are unknown.

Physical and emotional stresses are well known to affect cardiac electrophysiology. They trigger the release of stress hormones such as glucocorticoids (13). The serum- and glucocor-

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[2] The abbreviations used are: hERG, the human ether-a-go-go-related gene; I_{Kr}, the rapidly activating delayed rectifier potassium channel; SGK, serum- and glucocorticoid-inducible kinase; Nedd4-2, neural precursor cell expressed developmentally down-regulated protein 4 subtype 2; LQTS, long QT syndrome.

ticoid-inducible kinase (SGK) phosphorylates and inactivates Nedd4-2 (14). Of the three isoforms of SGK (SGK1, SGK2, and SGK3), SGK1 and SGK3 are expressed in every tissue, including the heart, whereas SGK2 seems to be present primarily in the liver, kidney, pancreas, and brain (15). The effect of SGK on the surface expression of several ion channels has been studied. In particular, SGK1 regulates the cell surface expression of the epithelial Na^+ channel by phosphorylating Nedd4-2 (14). Although SGK-mediated Nedd4-2 phosphorylation represents a mechanism for SGK to regulate ion channel expression levels, other mechanisms also exist. SGK increases the expression of the slowly activating delayed potassium current (I_{Ks}) through a Rab11-dependent pathway distinct from the Nedd4-2-mediated interaction (16). The study of effects of SGK1 and SGK3 on hERG has also been reported (17). It was shown that SGK3 but not SGK1 enhances hERG expression via unknown mechanisms not involving Nedd4-2 (17). The present study demonstrates that SGK1 and SGK3 increase the expression level and the current of hERG channels expressed in HEK cells and native I_{Kr} in cardiomyocytes. Furthermore, the SGK activator dexamethasone enhances the mature ERG protein expression in hERG-HEK cells and neonatal rat cardiac myocytes through increased SGK1 but not SGK3 expression. Mechanistically, SGK achieves its effects through two independent mechanisms: inhibition of Nedd4-2 activity through phosphorylation and activation of a Rab11-mediated pathway.

EXPERIMENTAL PROCEDURES

Molecular Biology—A HEK 293 cell line stably expressing hERG channels (hERG-HEK cells) was provided by Dr. Craig January (University of Wisconsin-Madison); *hERG* cDNA was provided by Dr. Gail Robertson (University of Wisconsin-Madison). *Kv1.5* cDNA (encoding the cardiac ultra rapidly activating delayed rectifier potassium channel) was provided by Dr. Michael Tamkun (Colorado State University, Fort Collins, Colorado); *EAG* (human ether-a-go-go) cDNA was provided by Dr. Luis Pardo (Max-Planck Institute of Experimental Medicine, Göttingen, Germany). The human Nedd4-2 plasmid in pBluescript II was purchased from Kazusa DNA Research Institute (Chiba, Japan). The open reading frame was amplified using PCR and cloned into HA-pcDNA3 (Invitrogen) to generate HA-tagged Nedd4-2. The *SGK1* Myc-DDK plasmid was purchased from Origene Technologies, Inc. (Rockville, Maryland). The *SGK3* and GFP-*Rab11* and Rab11 dominant-negative mutant *Rab11* S25N plasmids were obtained from Addgene (Cambridge, Massachusetts). The hERG C-terminal truncation mutation Δ1073 and hERG Y1078A point mutation were constructed using *pfuUltra* Hotstart PCR Master Mix (Agilent Technologies, Santa Clara, CA) and confirmed by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). The plasmid of the catalytically inactive form of Nedd4-2, Nedd4-2-C801S mutant was obtained from Dr. Hugues Abriel (University of Bern). HEK 293 cells were incubated in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for transfecting plasmids and siRNAs into HEK 293 cells. Kv1.5, EAG, and Δ1073 hERG, and Y1078A hERG stable cell lines were created in HEK using G418 for selection (1

mg/ml) and maintenance (0.4 mg/ml). For electrophysiological experiments on transiently expressed channels, GFP cDNA (*pIRES2-EGFP*, Clontech) was co-transfected for selection of transfected cells. Cardiac myocytes were isolated from neonatal Sprague-Dawley rats using enzymatic dissociation as described previously (18).

Patch Clamp Recording Method—Patch clamp experiments were conducted at room temperature (22 ± 1 °C). The hERG (I_{hERG}), Kv1.5 ($I_{Kv1.5}$), and EAG (I_{EAG}) currents were recorded using whole-cell patch clamp method. The bath solution consisted of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ (pH 7.4 with NaOH). The internal pipette solution consisted of 135 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.2, with KOH). I_{hERG}, $I_{Kv1.5}$, and I_{EAG} were evoked by depolarizing steps to voltages between −70 and 70 mV in 10-mV increments from a holding potential of −80 mV. A repolarizing step to −50 mV was used to record the tail currents. I_{Kr} in cultured neonatal rat cardiomyocytes was recorded using symmetrical Cs^+ solutions by 10-mV incremental depolarization pulses from −80 mV to voltages between −70 mV to 70 mV (19). The current amplitude upon repolarization to the −80 mV holding potential after the depolarizing step of 50 mV was used to measure the amplitude of native I_{Kr}.

Western Blot Analysis and Co-immunoprecipitation—Whole cell protein lysates from stable cell lines as well as neonatal rat ventricular myocytes were used. For the detection of phosphorylated Nedd4-2, a phosphatase inhibitor was added to the lysate to stabilize the phosphorylated proteins. Proteins were separated on 8.0 or 15.0% polyacrylamide gels and electroblotted overnight at 4 °C onto a PVDF membrane. Membranes were then blocked for 1 h using 5% skim milk and 0.1% Tween 20 in TBS. Immunoblotting was then performed for 1 h using appropriate primary antibodies. Protein signals were detected using the corresponding horseradish peroxidase-conjugated secondary antibody and ECL detection kit. The Precision Plus Protein Dual Color Standard was used as the protein ladder (Bio-Rad). To quantify the Western blot data, the intensities of proteins of interest in each gel are first normalized to their respective actin intensities; the normalized intensities are then compared with the intensity of control cells and expressed as relative values to their controls. For co-immunoprecipitation, proteins (0.5 mg) were incubated with the appropriate primary antibody at 4 °C overnight and then precipitated with protein A/G Plus-agarose beads at 4 °C for 4 h. The immunoprecipitates were washed three times with radioimmune precipitation assay lysis buffer. 2× Laemmli sample loading buffer was added to the pelleted immunoprecipitates and boiled for 5 min. The samples were centrifuged at 20,000 × g for 5 min, and the supernatants were loaded into polyacrylamide gels for Western blot analysis.

Immunofluorescence Microscopy—hERG-HEK cells grown on glass coverslips were transiently transfected with SGK1 or SGK3 plasmid. Twenty four hours after transfection, cells were fixed and permeabilized. hERG channels were stained with goat C-20 anti-hERG primary and Alexa Fluor 488-conjugated donkey anti-goat antibodies. Myc-tagged SGK1 or untagged SGK3 was detected with mouse anti-Myc or mouse anti-SGK3 primary antibody, respectively, and visualized using Alexa Fluor
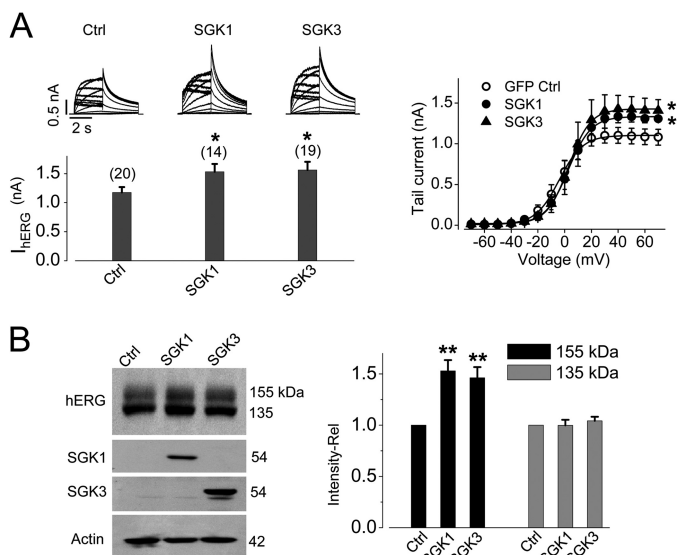
FIGURE 1. **Overexpression of SGK1 or SGK3 increases the hERG expression at the plasma membrane.** *A*, *left panel*: effects of SGK1 and SGK3 on I_{hERG}. Representative currents in pcDNA3- (control, *Ctrl*), SGK1-, or SGK3-transfected cells along with the summarized tail current amplitudes are shown. The peak tail current at -50 mV after the 50-mV depolarizing step was used to analyze I_{hERG} amplitude. The *numbers* in *parentheses above* each bar indicate the number of cells tested. *Right panel*, the activation curves of I_{hERG} recorded from control, SGK1-, or SGK3-transfected cells ($n = 4$, respectively). *B*, effects of SGK1 or SGK3 on the expression level of hERG channel proteins. The relative band intensities (*Intensity-Rel*) of hERG channel proteins in the presence of SGK1 or SGK3 compared with those from pcDNA3-transfected (control) cells are summarized beside the representative Western blot image ($n = 5$). *, $p < 0.05$ and **, $p < 0.01$ *versus* control.

350-conjugated goat anti-mouse antibodies. Endogenous Nedd4-2 protein was detected with rabbit anti-Nedd4-2 primary and Alexa Fluor 546-conjugated donkey anti-rabbit antibodies. Endogenous phosphorylated Nedd4-2 (p-Nedd4-2) protein was detected with rabbit anti-p-Nedd4-2 primary and Alexa Fluor 546-conjugated donkey anti-rabbit antibodies. Images were acquired using a Leica RCS SP2 Multiphoton confocal microscope.

Reagents and Antibodies—Rabbit anti-Kv11.1 (hERG) and anti-Kv10.1 (EAG-1), mouse anti-Myc, anti-SGK1, anti-HA, and anti-actin antibodies, and G418, insulin, and dexamethasone were purchased from Sigma. Goat anti-hERG (C-20), anti-actin, anti-GAPDH, rabbit anti-Kv1.5, anti-Rab11, anti-GAPDH, mouse anti-SGK3, and anti-GAPDH antibodies; siRNAs for SGK1, SGK3, and scrambled control; and A/G plus-agarose for immunoprecipitation assay were purchased from Santa Cruz Biotechnology. Rabbit anti-Nedd4-2 and anti-phosphorylated Nedd4-2 antibodies were purchased from Cell Signaling. PhosSTOP phosphatase inhibitor mixture was purchased from Roche Applied Science.

All data are expressed as the mean ± S.E. A one-way analysis of variance or two-tailed Student's *t* test was used to determine statistical significance between the control and test groups. A *p* value of 0.05 or less was considered significant.

RESULTS

SGK1 and SGK3 Increase hERG Expression in the Plasma Membrane—Fig. 1 illustrates the effects of SGK1 or SGK3 overexpression on the function and expression of hERG channels

stably expressed in HEK 293 (hERG-HEK) cells. SGK1, SGK3, or empty pcDNA3 plasmid (control) was transiently transfected into the hERG-HEK cells. Twenty four hours after transfection, whole-cell patch clamp recording and Western blot analysis were performed to determine the function and expression level of the hERG channel. SGK1 or SGK3 overexpression significantly increased I_{hERG} (Fig. 1*A*) without affecting the biophysical properties of I_{hERG}. The half-activation voltage and the slope factor of I_{hERG} were -4.1 ± 0.7 mV and 8.2 ± 0.5 mV ($n = 4$) for control cells, 2.6 ± 0.8 mV and 8.4 ± 0.6 mV ($n = 4$) for SGK1-overexpressed cells, and 3.0 ± 0.5 mV and 8.0 ± 0.5 mV ($n = 4$) for SGK3-overexpressed cells ($p > 0.05$ compared with control).

hERG proteins display two distinct bands in the Western blot analysis: a mature fully glycosylated form in the plasma membrane with a molecular mass of 155 kDa and an immature coreglycosylated form with a molecular mass of 135 kDa (18, 20). SGK1 or SGK3 overexpression significantly increased the expression of the mature hERG protein (155 kDa) but had no significant effect on the expression of the immature hERG protein (135 kDa) (Fig. 1*B*).

SGK1 and SGK3 Phosphorylate Nedd4-2—Nedd4-2 is an E3 ubiquitin-protein ligase and is responsible for recognition and ubiquitin transfer to substrate proteins such as ion channels, leading to the internalization of substrates from the membrane (11, 21, 22). Nedd4-2 possesses WW domains that bind to the PP*x*Y (PY motif) sequence of various target proteins (23). The PY motif exists in the hERG channel but not in Kv1.5 or EAG (10). Recent works by us and Albesa *et al.* have demonstrated that Nedd4-2 ubiquitinates and subsequently internalizes mature hERG channels from the plasma membrane (10, 24, 25). SGK is a serine/threonine kinase (26). It has been shown that SGK inhibits Nedd4-2 activity by phosphorylating Nedd4-2 at Ser-444 (and to a lesser extent Ser-338) residues present in the WW domains (14); this consequently enhances the cell surface expression of the epithelial Na$^+$ channel, which is a substrate of Nedd4-2 (14).

As a first step to investigate whether Nedd4-2 is involved in the SGK-mediated increase in hERG expression, we examined the effects of SGK overexpression on Kv1.5 and EAG channels, neither of which possesses the Nedd4-2 binding motif PP*x*Y (10). Our data show neither SGK1 nor SGK3 overexpression significantly affected $I_{Kv1.5}$ or I_{EAG} (Fig. 2*A*). In addition, the expression of Kv1.5 or EAG channel was not affected by SGK1 or SGK3 overexpression (Fig. 2*B*). These data suggest that SGK may affect hERG expression through Nedd4-2.

To investigate the involvement of Nedd4-2 in the SGK1 or SGK3-induced hERG increase, we analyzed the interaction between SGK and Nedd4-2 by performing co-immunoprecipitation experiments. Whole cell lysates were extracted from HEK cells 24 h after SGK1 transfection. An anti-SGK1 antibody was used to immunoprecipitate SGK1 and its associated proteins. The precipitated proteins were immunoblotted to detect endogenous Nedd4-2. As shown in Fig. 3*A*, Nedd4-2 bands were detected in the SGK1-precipitated proteins. In our study, with a Nedd4-2 antibody from Cell Signaling, endogenous Nedd4-2 extracted from HEK cells displayed two bands with molecular masses of 110 and 120 kDa,
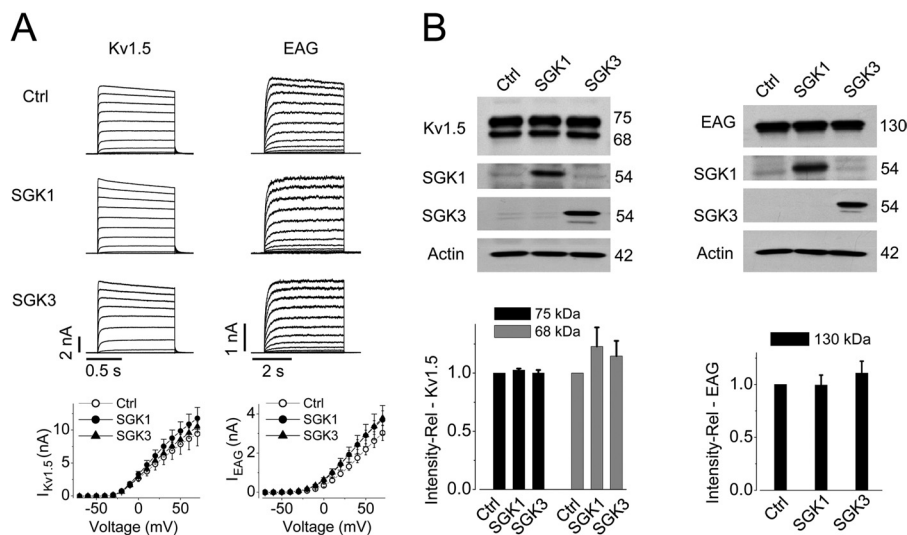
FIGURE 2. **Overexpression of SGK1 or SGK3 does not affect the Kv1.5 or EAG channels.** *A*, effects of SGK1 or SGK3 on $I_{Kv1.5}$ and I_{EAG}. Representative currents in pcDNA3- (control, *Ctrl*), SGK1, or SGK3-transfected cells along with their summarized current-voltage relationships of $I_{Kv1.5}$ and I_{EAG} are shown ($n = 4$–15). *B*, effects of SGK1 or SGK3 on the expression levels of Kv1.5 and EAG channel proteins. The relative band intensities (*Intensity-Rel*) from cells transfected with SGK1 or SGK3 are normalized to those from cells transfected with pcDNA3 (control) and summarized below the representative Western blot images ($n = 4$ for Kv1.5 and EAG, respectively).
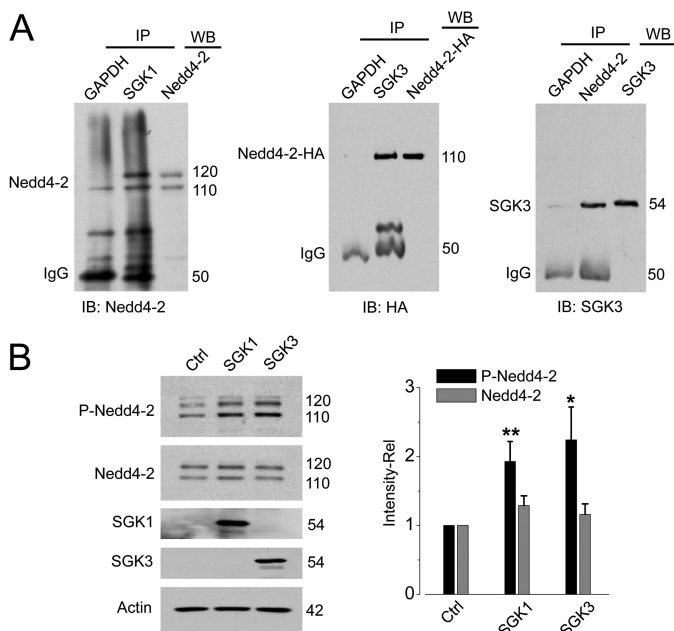
FIGURE 3. **SGK1 and SGK3 interact with Nedd4-2 and enhance its phosphorylation.** *A*, SGK1 and SGK3 interact with Nedd4-2. Nedd4-2 was detected in proteins precipitated with an anti-SGK1 or an anti-SGK3 antibody. Additionally, SGK3 was detected in proteins precipitated with an anti-Nedd4-2 antibody. In each case, a fraction of proteins used for the immuno-precipitation assay was included for the immunoblotting assay to show the protein bands of interest. *B*, effects of SGK1 or SGK3 overexpression on Nedd4-2 and phosphorylated Nedd4-2 (P-Nedd4-2). The relative intensities (*Intensity-Rel*) of Nedd4-2 and P-Nedd4-2 bands from cells transfected with SGK1 or SGK3 compared with those from pcDNA3-transfected (control, *Ctrl*) cells are summarized next to the representative Western blot (*WB*) images ($n = 5$–8). *, $p < 0.05$ and **, $p < 0.01$ *versus* control.

whereas the transfected Nedd4-2 displayed primarily the 110-kDa band. As shown in Fig. 3*A*, an interaction between SGK3 and Nedd4-2-HA was also detected in cells co-transfected with SGK3 and Nedd4-2-HA.

To determine whether SGK1 or SGK3 can lead to increased phosphorylation of Nedd4-2, levels of basal as well as phosphor-

ylated endogenous Nedd4-2 were detected using Western blot analysis. Whole cell protein was extracted from hERG-HEK cells after transfection with SGK1, SGK3, or an empty pcDNA3 plasmid (control) for 24 h. Overexpression of SGK1 or SGK3 did not affect the total level of endogenous Nedd4-2 but significantly increased the level of phosphorylated Nedd4-2 (Fig. 3*B*).

The effects of SGK transfection on Nedd4-2 phosphorylation and hERG expression were further studied using immunocytochemistry. Myc-tagged SGK1 plasmid was transiently transfected into hERG-HEK cells for 24 h. Cells were fixed, and various protein expressions were detected. Compared with non-transfected cells, SGK1 (*blue*) transfected cells portrayed an increased hERG expression (*green*) with a concomitant increase in the phosphorylated Nedd4-2 (*red*) (Fig. 4). However, the level of total Nedd4-2 was not affected by SGK1 overexpression (Fig. 4). The SGK3 overexpression displayed effects on Nedd4-2 and hERG similar to SGK1 overexpression (data not shown).

SGK Affects hERG via Nedd4-2 and Rab11-mediated Pathways—Our data so far have demonstrated that SGK overexpression enhances Nedd4-2 phosphorylation, which is known to inhibit Nedd4-2 activity. To determine the role of Nedd4-2 in SGK-mediated effects on hERG channels, two hERG mutations, the point mutation Y1078A and C-terminal truncation mutation Δ1073, were used. As shown in Fig. 5*A*, both of the mutations abolished the Nedd4-2 overexpression-mediated decrease in the mature hERG expression. However, these mutations failed to abolish the SGK-mediated increase in the mature hERG expression. As shown in Fig. 5*B*, the expression level of the mature form (upper band) of Y1078A and Δ1073 mutant channels was still increased by SGK1 or SGK3 transfection. Thus, SGK-mediated increase in hERG is not entirely dependent on the Nedd4-2 activity, and other pathways must be involved.

In addition to Nedd4-2, Rab family proteins (GTPases) regulate channel density at the plasma membrane by affecting
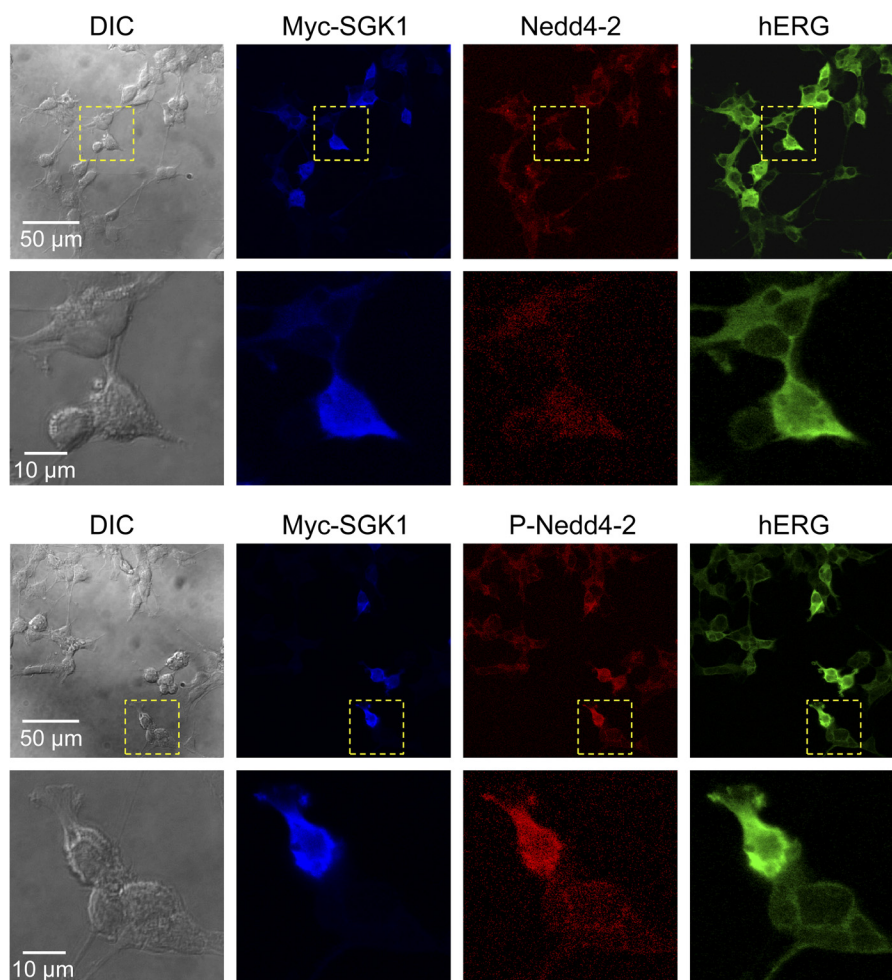
FIGURE 4. **SGK1 enhances phosphorylated Nedd4-2 and hERG expression.** Confocal images show that SGK1 overexpression does not affect the total level of Nedd4-2 expression but does increase phosphorylated Nedd4-2 and hERG expression. Myc-tagged-SGK1 was stained in *blue*. Nedd4-2 or phosphorylated Nedd4-2 (p-Nedd4-2) was stained in *red*. The hERG protein was stained in *green*. *Insets* of individual cells are magnified to enhance the visualization. Differential interference contrast (*DIC*) images of cells are shown on the *left panels*.

channel trafficking (27). Of interest is Rab11, which is highly expressed in cardiac myocytes (28) and is involved in the recycling/trafficking of various ion channels such as cystic fibrosis transmembrane conductance regulator chloride channels, Cav1.2 Ca^{2+} channels, and the glucose transporter GluT4 (28 – 30). In particular, SGK1 has been shown to increase the expression of the KCNQ1/KCNE1 (I_{Ks}) channel in the plasma membrane, and this increase is via the activation of a Rab11-mediated pathway (16).

To study whether a Rab11-recycling pathway is involved in the SGK-mediated hERG increase, we performed a co-immunoprecipitation analysis to determine whether Rab11 interacts with hERG channels. Whole-cell lysates were extracted from hERG-HEK cells transfected with GFP-tagged Rab11 plasmid. An anti-GFP antibody was used to precipitate Rab11 and associated proteins. Precipitated proteins were analyzed using Western blots to detect for hERG. An interaction between Rab11 and the mature hERG was evident as the 155-kDa hERG band was detected in the Rab11-precipitated proteins (Fig. 6*A*, *upper panel*). Conversely, GFP-tagged Rab11 was detected in the proteins precipitated by an anti-hERG antibody (Fig. 6*A*, *lower panel*).

To determine the role of Rab11 in SGK-mediated increase in mature hERG, a dominant negative Rab11 mutant, Rab11SN (Rab11 S25N) plasmid was transfected into Y1078A or Δ1073 hERG-HEK cells to suppress endogenous Rab11 function, and SGK effects were analyzed. As shown in Fig. 6*B*, dominant negative Rab11SN expression completely eliminated the SGK3-induced increase in expression of mature channels of these two Nedd4-2-insensitive hERG mutants. However, Rab11SN expression failed to eliminate the SGK3-mediated increase in mature channels of the WT hERG (Fig. 6*C*), indicating that altered Nedd4-2 activity also plays a role in the SGK-mediated hERG increase. To confirm this, we disrupted the function of either Rab11 alone or Rab11 and Nedd4-2 together by overexpressing dominant-negative Rab11SN and catalytically inactive Nedd4-2, Nedd4-2CS (Nedd4-2-C801S) into hERG-HEK cells and examined SGK3 effects. As shown in Fig. 6*C*, although SGK3 increased the mature hERG expression in Rab11SN-transfected cells, SGK3 did not affect the hERG expression in cells co-transfected with Rab11SN and Nedd4-2CS.

Activation of Endogenous SGK Increases the Expression of hERG Channels—SGK1 is regulated by a number of stimulatory agents such as serum, glucocorticoids, mineralocorticoids,
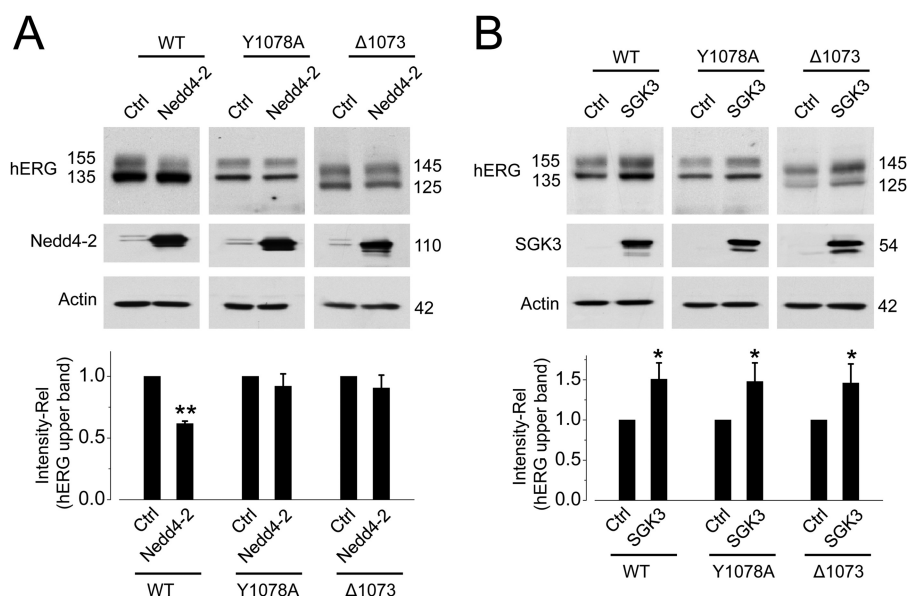
FIGURE 5. Mutations disrupting Nedd4-2 targeting site in hERG do not eliminate SGK3-mediated increase in hERG expression. *A*, effects of Nedd4-2 transfection on the expressions of WT, Y1078A, or Δ1073 hERG channels. *B*, effects of SGK3 transfection on the expressions of WT, Y1078A, or Δ1073 hERG channels. Stable HEK cell lines expressing WT, Y1078A, or Δ1073 mutant hERG channels were transfected with pcDNA3 (control, *Ctrl*), Nedd4-2, or SGK3 plasmid. Experiments were performed 24 h after transfection. The relative upper band intensities (*Intensity-Rel*) of WT, Y1078A, and Δ1073 hERG channel proteins from cells transfected with Nedd4-2 or SGK3 compared with those in pcDNA3-transfected (control, *Ctrl*) cells are summarized below the respective Western blot images ($n = 4$–7). *, $p < 0.05$ and **, $p < 0.01$ *versus* control.
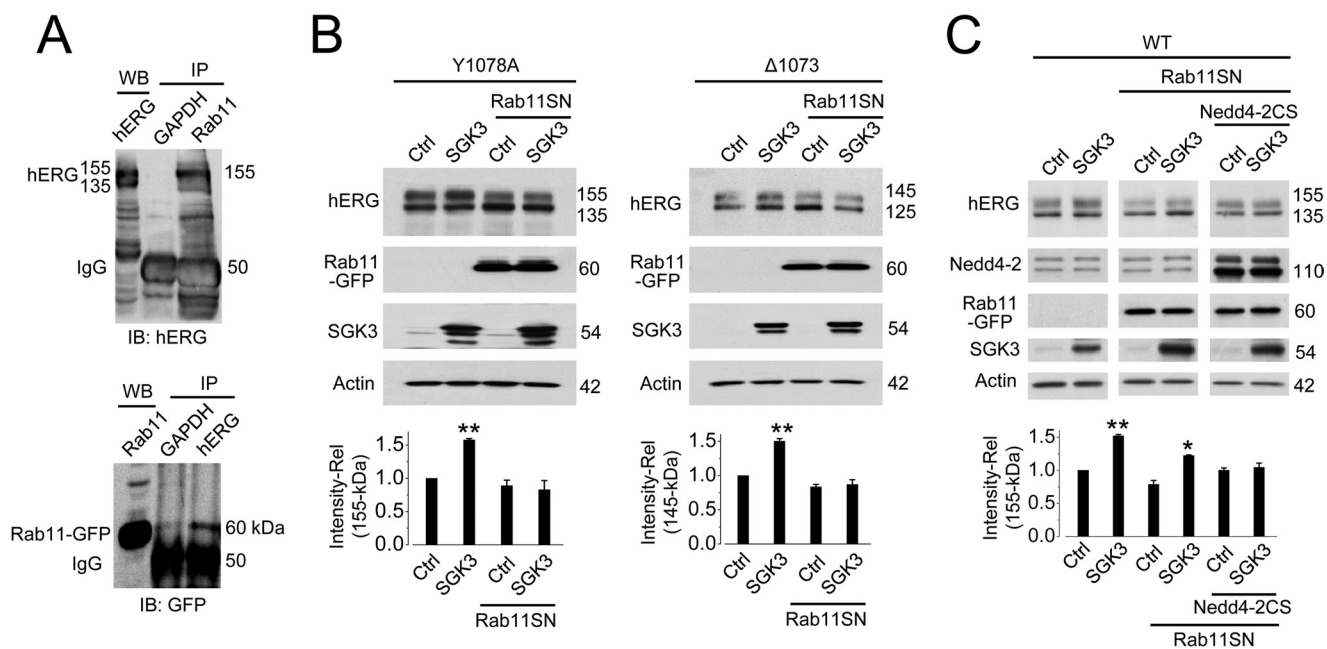
FIGURE 6. Disruption of Rab11 and Nedd4-2 eliminates SGK3-mediated increase in hERG expression. *A*, Rab11 interacts with mature hERG channels. *Upper panel*: detection of hERG in proteins precipitated with anti-GFP antibody from whole-cell lysate extracted from hERG-HEK cells transfected with GFP-tagged Rab11 (Rab11-GFP) plasmid. A fraction of proteins used for pulldown assay was also immunoblotted to show hERG. *Lower panel*: detection of Rab11-GFP in the anti-hERG antibody precipitated proteins. A fraction of proteins used for pulldown assay was also immunoblotted to show Rab11-GFP. *B*, stable HEK cell lines expressing Y1078A or Δ1073 mutant hERG channels were transfected with either SGK3 or empty pcDNA3 (control, *Ctrl*) plasmids. An additional group of cells co-transfected with a dominant negative Rab11SN-GFP plasmid. *C*, WT hERG-HEK cells were transfected with either SGK3 or empty pcDNA3 (control) plasmids. Additional groups of cells co-transfected with either a Rab11SN-GFP plasmid or Rab11SN-GFP plus Nedd4-2CS plasmids were used to inhibit endogenous Rab11 and Nedd4-2. In *B* and *C*, the relative upper band intensities (*Intensity-Rel*) of hERG compared with their controls are summarized *below* the representative Western blot images ($n = 4$–6). *, $p < 0.05$ and **, $p < 0.01$ *versus* control.

cytokines, follicle-stimulating hormone, luteinizing hormone, insulin, and insulin growth factor (31). In particular, glucocorticoids and/or mineralocorticoids that are widely used for various purposes, including immunosuppression and anti-inflammation, are known to enhance SGK1 but not SGK3 activity (31).

We treated hERG-HEK cells with serum, insulin, or dexamethasone, a potent synthetic glucocorticoid steroid drug. Control hERG-HEK cells were cultured in serum-free medium for 24 h. Serum-treated hERG-HEK cells were cultured in normal minimum essential medium supplemented with 10% fetal bovine
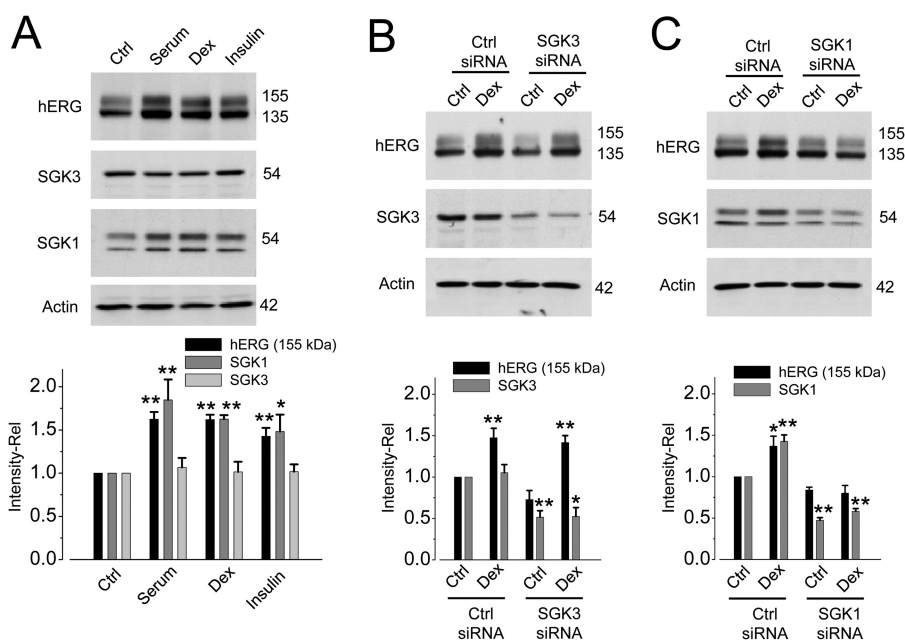
FIGURE 7. **Serum, dexamethasone, and insulin increase mature hERG protein by enhancing SGK1 expression.** *A*, effects of serum, dexamethasone (*Dex*), or insulin treatment on the expression of hERG, SGK1, and SGK3 in hERG-HEK stable cell lines. Cells were collected 6–12 h after treatment with 10% fetal bovine serum medium, 1.0 μM dexamethasone, or 0.1 μM insulin, respectively. Serum-free minimum essential medium-treated cells (24 h) were used as control (*Ctrl*). *B* and *C*, effects of hERG-HEK cells transfected with scrambled control siRNA, SGK1 siRNA, or SGK3 siRNA and treated with or without dexamethasone for 24 h. In *A–C*, the relative band intensities (*Intensity-Rel.*) of hERG, SGK1, or SGK3 in cells with various treatments compared with their respective controls are summarized below the Western blot images ($n = 3$–7). *, $p < 0.05$ and **, $p < 0.01$ *versus* control.

serum. For dexamethasone or insulin treatment, hERG-HEK cells were incubated for 24 h with serum-free medium containing 1.0 μM dexamethasone or 0.1 μM insulin. As depicted in Fig. 7*A*, serum, insulin, and dexamethasone significantly increased mature (155 kDa) hERG expression compared with serum-free (control) hERG-HEK cells. These treatments concomitantly increased endogenous SGK1 expression levels but not SGK3 expression levels (Fig. 7*A*). Consistent with the enhanced hERG expression, dexamethasone (1.0 μM) treatment also significantly increased I_{hERG} as revealed by whole cell patch clamp experiments (data not shown).

To confirm that dexamethasone increases hERG expression by elevating SGK1 expression, SGK1 or SGK3 siRNA was used to knockdown endogenous SGK1 or SGK3, respectively. hERG-HEK cells were transfected with either control (scrambled), SGK1, or SGK3 siRNA. Twenty four hours after siRNA transfection, cells were treated with either serum-free medium (control) or 1.0 μM dexamethasone for 12 h for Western blot analysis. In control or SGK3 siRNA-treated cells, dexamethasone significantly increased mature hERG expression (Fig. 7, *B* and *C*) as well as SGK1 expression (Fig. 7*C*). However, in SGK1 siRNA-treated cells, dexamethasone failed to increase the expression of mature hERG (155 kDa) proteins (Fig. 7*C*). Thus, dexamethasone enhances the mature hERG expression through SGK1 activation.

Dexamethasone Enhances I_{Kr} in Neonatal Ventricular Myocytes—To test whether dexamethasone regulates native I_{Kr} (ERG proteins), cultured neonatal rat cardiomyocytes were treated with serum-free (control) medium with or without 1.0 μM dexamethasone for 6 h. After treatment, I_{Kr} was recorded using whole cell patch clamp with symmetrical Cs^+ solutions (19). Dexamethasone treatment significantly increased I_{Kr} in neonatal cardiomyocytes (Fig. 8*A*). Additionally, dexamethasone treatment increased the ERG protein expression (Fig. 8*B*). Consistent with data obtained from the cell line, dexamethasone treatment also increased the expression level of SGK1 but not SGK3 in neonatal rat cardiomyocytes.

DISCUSSION

hERG (I_{Kr}) is critical for repolarization of the cardiac action potential (6). Disruptions of the hERG functionality caused by mutations or drugs can induce LQTS, leading to life-threatening cardiac arrhythmias (32). The density of hERG channels in the plasma membrane is one of the primary determinants of the hERG current amplitude and is controlled by a delicate balance between anterograde trafficking to and retrograde trafficking from the cell surface. Mutations altering forward trafficking of hERG channels to the plasma membrane have been reported previously (33). We recently demonstrated that Nedd4-2 plays an important role in the degradation pathway of hERG retrograde trafficking (10). In the present study, we extend our study on hERG retrograde trafficking by focusing on the role of SGK in this process.
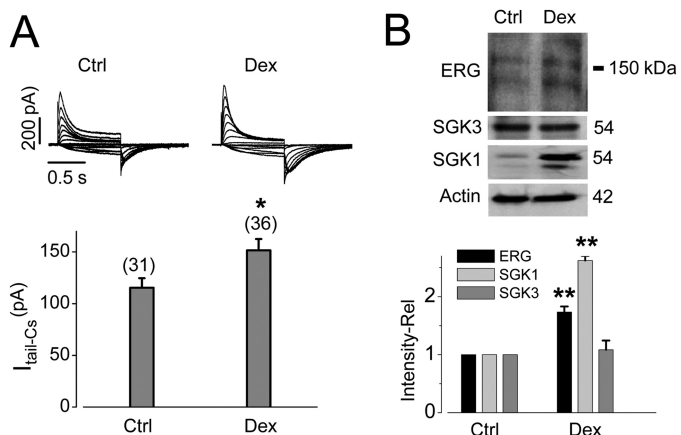
The effect of SGK on hERG channels has previously been reported (17). However, the mechanisms underlying the SGK-induced increase in hERG expression were unknown (17). Furthermore, although this previous study showed that expression of SGK3 but not of SGK1 in hERG-expressing *Xenopus* oocytes increases steady state hERG current and cell membrane protein abundance, our data show that both SGK1 and SGK3 increase the expression level of mature hERG channels. The reasons for this discrepancy are unknown and may be related to the expression systems used in the previous (*Xenopus* oocytes) and the present studies (HEK 293 cells). Our data further show that overexpression of SGK1 and SGK3 enhances Nedd4-2 phos-
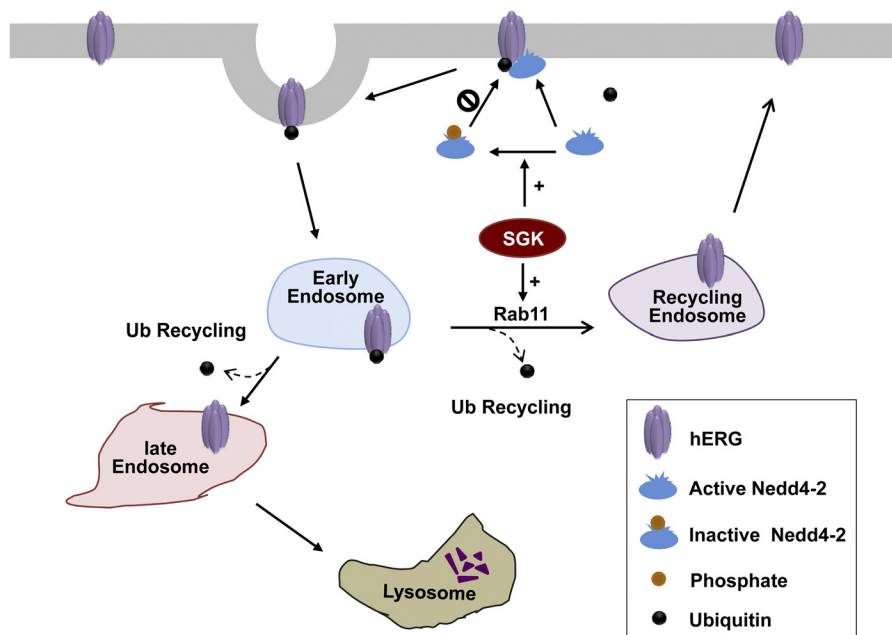
phorylation (Figs. 3 and 4), which is known to inhibit Nedd4-2 activity. These data suggest that SGK1 and SGK3 increase hERG expression through inhibiting Nedd4-2 activity, thereby decreasing ubiquitination of hERG channels by Nedd4-2. Interestingly, although disruption of Nedd4-2 binding site eradicated Nedd4-2-induced hERG reduction, it did not eliminate SGK-induced hERG increase (Fig. 5). Thus, our data provide evidence that SGK affects hERG expression through a pathway besides Nedd4-2. Rab11 has been shown to recycle membrane proteins to the cell surface via intracompartmental vesicles (27,

FIGURE 8. **Dexamethasone increases I_{Kr} via enhanced SGK1 expression in neonatal rat ventricular myocytes.** *A*, treatment of cultured neonatal rat ventricular myocytes with dexamethasone increases I_{Kr}. Families of Cs^+-mediated I_{Kr} in control (*Ctrl*) and dexamethasone (*Dex*)-treated cells along with the summarized tail current amplitudes are shown. The *numbers* in *parentheses above* the *bars* indicate the number of cells tested. *B*, effects of dexamethasone treatment on the expression of ERG, SGK1, and SGK3 protein in neonatal rat ventricular myocytes. The relative band intensities (*Intensity-Rel.*) of ERG, SGK1, and SGK3 from cardiomyocytes treated with or without dexamethasone are summarized below the Western blot images (*n* = 4). Cells incubated in serum free medium were used as control. *, $p < 0.05$ and $p < 0.01$ *versus* control.

34). Disruption of endogenous Rab11 abolishes the SGK-induced increase in the expression of mutant hERG channels whose Nedd4-2 interacting site is disrupted (Fig. 6). Co-immunoprecipitation results demonstrated that Rab11 interacts with mature hERG proteins. Because both Nedd4-2 and Rab11 interact directly with mature hERG channels, it is likely that the SGK-mediated increase in hERG expression occurs through a Nedd4-2 pathway and a Rab11 recycling pathway (Fig. 9). For the former pathway, our data indicate that SGK inhibits Nedd4-2 through phosphorylation. For the latter pathway, it has been reported that SGK phosphorylates and stimulates PIKfyve, a FYVE finger-containing phosphoinositide kinase that acts on phosphatidylinositol 3-phosphate to generate $PI(3,5)P_2$ (35). SGK-mediated activation of PIKfyve as well as addition of $PI(3,5)P_2$ to the cell have been shown to positively regulate Rab11-mediated insertion of KCNQ1/KCNE1 channels to the membrane (16).

Mutations in hERG cause type 2 LQTS (LQT2) and most LQT2 mutations reduce the hERG current due to defective trafficking, leading to reduced hERG expression in the plasma membrane (33). It would be interesting to investigate whether a stimulated SGK activity can rescue trafficking-deficient mutant hERG channels to correct the prolonged QT intervals. In this regard, agents that function through a glucocorticoid receptor signaling pathway have been shown to rescue LQTS type 2 by shortening the cardiac action potential in a zebrafish model (36). Also, QT prolongation in diabetic patients has drawn particular attention due to increased risk of ventricular arrhythmias and sudden death in this population. It has been demonstrated that insulin treatment restored depressed hERG channel function and consequently corrected the prolonged cardiac action potential (37). Glucocorticoids and insulin are potent activators of SGK (31). Notably, glucocorticoids have

FIGURE 9. **A diagram illustrating that SGK interacts with Nedd4-2 and Rab11 to regulate hERG channels in the plasma membrane.** Nedd4-2 binds to hERG channels to cause hERG ubiquitination (*Ub*) and degradation. SGK phosphorylates and inactivates Nedd4-2, leading to the inhibition of Nedd4-2-mediated hERG degradation. In addition, Rab11 mediates insertion of internalized hERG channels to the plasma membrane via recycling endosomes, which is promoted by SGK activity.

been shown to directly stimulate SGK1 mRNA expression but not SGK3 expression (31). Webster *et al.* (38) reported that treatment of mammary epithelial cells with 1.0 μM dexamethasone elevated SGK1 expression by 2-fold within 30 min. We demonstrated that treatment of hERG-HEK cells with serum, insulin (0.1 μM), and dexamethasone (1.0 μM) increased mature hERG expression with a concomitant increase in the expression level of SGK1 but not SGK3 (Fig. 7). The dexamethasone-induced concomitant increases in I_{Kr}/ERG and SGK1 were also observed in cultured rat ventricular myocytes (Fig. 8). Our data also show that knockdown of endogenous SGK1, but not SGK3, abrogated the dexamethasone effect on hERG channels (Fig. 7). These results indicate that dexamethasone enhances hERG expression levels by stimulating SGK1 expression, which activates Rab11 recycling of hERG and prevents hERG degradation by inhibiting Nedd4-2 activity. The enhanced SGK activity would accelerate cardiac action potential repolarization to adapt to the stressed physiological conditions. However, over-stimulated SGK activity may be detrimental. For example, it has been reported that psychosocial stress plays a role in otherwise unexplained cardiac arrest (39). Furthermore, a gain-of-function SGK1 mutation has been reported in twins who displayed the shortened QT interval, which induces arrhythmias and sudden death (40, 41).

In summary, the present study demonstrated that stress hormones such as dexamethasone can enhance hERG expression on the plasma membrane through stimulating SGK1 activity. These data extend our understanding of the regulation of cardiac ion channels and shed some light onto strategies for the management of altered QT intervals in patients.

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