Regulation of Endocytic Recycling of KCNQ1/KCNE1 Potassium Channels


Abstract—Stress-dependent regulation of cardiac action potential duration is mediated by the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. It is accompanied by an increased magnitude of the slow outward potassium current, \( I_{Ks} \). KCNQ1 and KCNE1 subunits coassemble to form the \( I_{Ks} \) channel. Mutations in either subunit cause long QT syndrome, an inherited cardiac arrhythmia associated with an increased risk of sudden cardiac death. Here we demonstrate that exocytosis of KCNQ1 proteins to the plasma membrane requires the small GTPase RAB11, whereas endocytosis is dependent on RAB5. We further demonstrate that RAB-dependent KCNQ1/KCNE1 exocytosis is enhanced by the serum- and glucocorticoid-inducible kinase 1, and requires phosphorylation and activation of phosphoinositide 3-phosphate 5-kinase and the generation of \( \text{PI}(3,5)P_2 \). Identification of KCNQ1/KCNE1 recycling and its modulation by serum- and glucocorticoid-inducible kinase 1-phosphoinositide 3-phosphate 5-kinase -\( 
\text{PI}(3,5)P_2 \) provides a mechanistic insight into stress-induced acceleration of cardiac repolarization. (Circ Res. 2007;100:0-0.)

Key Words: kinase ■ PIP2 ■ RAB ■ trafficking ■ PIKfyve

Emotional stress activates the sympathetic nervous system and the release of stress hormones such as cortisol via the hypothalamic-pituitary-adrenal (HPA) axis and is a common trigger of sudden cardiac death.1,2 One of the many genes regulated by cortisol is the serum- and glucocorticoid-inducible kinase 1 (SGK1).3,6 In vitro experiments have shown that SGK1 stimulates \( I_{Ks} \), a repolarizing potassium current conducted by channels composed of KCNQ1 \( \alpha \)-subunits and KCNE1 \( \beta \)-subunits.8,9 Moreover, a gain-of-function variant of SGK1 is associated with shortening of the QT interval.10 SGK1-mediated regulation of \( I_{Ks} \) might be particularly important in patients with KCNQ1 (Kv7.1, KvLQT1) or KCNE1 (minK) mutations that are prone to fatal cardiac arrhythmias triggered by physical and psychological stress.2

The mechanism responsible for regulation of \( I_{Ks} \) channels by SGK1 have remained elusive. SGK1 enhances the abundance of other types of potassium channel in the plasma membrane by inhibiting the ubiquitin ligase Nedd4–211 in addition to other mechanisms (summarized by Lang et al 200612).

Other candidate signaling molecules that may affect channel trafficking include RAB family proteins, GTPases involved in vesicle cycling.13–18 RAB5, a monomeric GTPase of the Ras superfamily, has been implicated in the regulation of early steps in the endocytic pathway, whereas the RAB11 GTPase is localized at the trans-Golgi network, post-Golgi vesicles and the recycling endosome.19 Both RAB5 and RAB11 are expressed in cardiac tissue.17 Mammalian cells and Xenopus laevis oocytes have been shown to possess and use highly conserved RAB-dependent trafficking pathways.20,21 Endocytosis by RAB5 and exocytosis by RAB11 have been reported to participate in the regulation of CFTR chloride channels22 and the glucose transporter GluT4.15,23 The RAB dependent regulation of GLUT4 also involves the phosphoinositide 3-phosphate 5-kinase (PIKfyve)23 that generates the phosphatidylinositol \( \text{PI}(3,5)P_2 \). The closely related phosphatidylinositol \( \text{PI}(4,5)P_2 \) has been implicated in the...
regulation of \( I_{Ks} \) channel activity.\textsuperscript{25} PIKfyve is stimulated by protein kinase B\textsuperscript{23}, a close relative of SGK1, phosphorylating serine and threonine residues within a similar core consensus sequence (RXRXX[S/T]).\textsuperscript{6}

Here we determine the mechanisms involved in the stimulation of KCNQ1/KCNE1 channel activity by SGK1. We identify a novel mechanism involving SGK1 dependent stimulation of PIKfyve with subsequent formation of PI(3,5)P\textsubscript{2}, which modulates RAB11A guided vesicle exocytosis, leading to an increased membrane abundance of KCNQ1/KCNE1 channels in the plasma membrane.

### Materials and Methods

#### Western Blot of Plasma Membrane Proteins

Intact healthy oocytes were incubated in Sulfo-NHS-LC-Biotin (1 mg/mL, Pierce) for 30 minutes at room temperature and washed 5 times for 10 minutes in ND96. 20 intact oocytes were homogenized with a Teflon pestle in 400 \( \mu \)L H-buffer (in mM: 100 NaCl, 20 Tris-Cl, pH 7.4, 1\% Triton X-100, plus a mixture of protease inhibitors [Complete tablets, Boehringer]). The samples were kept at 4°C for 1 hour on a rotator, then centrifuged for 60 s at 13,000 rpm. The supernatants were supplemented with 25 \( \mu \)L washed immobilized NeutraAvidin Biotin-Binding Protein (Pierce) and incubated at 4°C for 3 hours on a rotator. The beads were then pelleted by a 120 s spin at 13,000 rpm, and washed in H-buffer (3 times). The pellets were boiled in 40 \( \mu \)L SDS-PAGE loading buffer (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, 0.8 mol/L \( \beta \)-mercaptoethanol, 6\% SDS, 20\% glycerol, 25 mmol/L Tris-Cl, pH 6.8, 0.1\% bromophenol blue). The samples were Western-blotted and probed with primary KCNQ1 antibody (KCNQ1, Santa Cruz: sc-10646). All Western blots were repeated at least 3 times.

#### Molecular Biology

Most of the procedures have been previously described.\textsuperscript{32} Clones used were subcloned into oocyte expression vectors, a modified pcDNA3 vector or pSP64 or pSGEM. The clones were mutated at the positions mentioned in the text by site-directed mutagenesis using PCR with cloned Pfu-polymerase. RAB5 and RAB11 clones have been described previously.\textsuperscript{19,20,33,34} RAB11(T77A) was introduced into RAB1-1HA in pcDNA3.1. Human KCNE1 was subcloned into pcDNA3.1 for transfection into COS-7 cells. The FLAG-tagged KCNQ1 in pCI using FuGene6 (Roche) according to the manufacturers instructions. COS-7 cells were fixed 3 days after transfection in 4\% paraformaldehyde. Cells were subsequently stained with an Anti-FLAG antibody (Sigma) to detect FLAG-tagged KCNQ1. Fluorescence was detected using a confocal microscope (Zeiss LSM 510, Germany) with appropriate filter sets.

#### Chemiluminescence Assay for Detection of Surface Expressed HA-KCNQ1 in Oocytes

Experiments were performed as recently described.\textsuperscript{37} Oocytes expressing KCNQ1 with an external Myc-tag attached between S1-S2\textsuperscript{33} were incubated for 30 minutes in ND96 with 1\% bovine serum albumin (BSA) at 4°C to block nonspecific antibody binding. Oocytes were subsequently incubated for 60 minutes at 4°C with rat monoclonal anti-MYC antibody (Roche, 100 \( \mu \)g/mL, 1:100 in 1\% BSA/ND96), washed 5 times at 4°C with 1\% BSA/ND96 and incubated with 2 \( \mu \)g/mL peroxidase-conjugated affinity purified F(ab\textsuperscript{2}) fragment goat anti-rat IgG antibody (Jackson ImmunoResearch) in 1\% BSA/ND96 for 60 minutes. Oocytes were washed thoroughly 10 times, initially in 1\% BSA/ND96 (at 4°C, 5 minutes), and then 5 times in ND96 without BSA (at 4°C, 5 minutes). Individual oocytes were transferred in 100 \( \mu \)L Power Signal Elisa solution (Pierce) and chemiluminescence was measured with a multilabel counter (Wallac Victor, Perkin Elmer). The results from 20 oocytes were averaged and the results presented in relative light units (RLU).

#### Phosphorylation of purified PIKfyve by recombinant PKB and SGK1

Wild-type and mutant (S318A) GST-PIKfyve were heterologously expressed in yeast and purified using glutathione-Sepharose beads as previously described.\textsuperscript{29} The beads (10 to 20 mL) were washed in 50 to 100 \( \mu \)L Kinase Assay Buffer (KAB; 20 mmol/L HEPES, pH 7.5, 20 mmol/L \( \beta \)-glycerophosphate, 1 mmol/L EDTA, 0.1\% \( \beta \)-mercaptoethanol and 5 mmol/L MgCl\textsubscript{2}). A volume of 20 \( \mu \)L of these beads were then incubated in a further 50 \( \mu \)L KAB containing 1 to 2 U of recombinant PKB or SGK1 (Upstate, Dundee, UK) and 50 \( \mu \)mol/L [\( ^{32}P \)]ATP (40 kBq/mmol). Incubations were performed at 30°C for 1 hour. Reactions were stopped by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, and transferred to PVDF membrane by wet transfer. The membrane was first subjected to autoradiography, and then to Western blotting after first blocking in 10\% bovine serum albumin in TBS-T buffer (20 mmol/L Tris, pH 7.6, 137 mmol/L NaCl, 0.1\% Tween). For Western blotting, the membranes were incubated with 1:500 dilution of rabbit anti-PIKfyve-phosphoserine-318 antibody (rpS318) or 1:500 dilution of rabbit anti-PIKfyve antibody, as previously described.\textsuperscript{28} Blots were developed using ECL reagent (GE Healthcare, Amersham, UK) for 2 minutes before being exposed to photographic film. For
trypsinic phosphopeptide mapping, 32P-labeled protein was isolated by SDS-PAGE, excised and the gel chip subjected to reduction and alkylation for 30 minutes by incubation first in 10 mmol/L DTT and 50 mmol/L NH4HCO3 (pH 8.2 at 56°C), followed by a further 20 minutes in 55 mmol/L iodoaceticamide and 50 mmol/L NH4HCO3 (pH 8.2 at 20°C). The gel chips were washed 3 times with 50 mmol/L NH4HCO3 pH 8.2, and then incubated overnight in 50 mL of 50 mmol/L NH4HCO3 pH 8.2 containing 0.002 μg trypsin (Promega) at 37°C. Tryptic peptides were lyophilized and subjected to reverse-phase chromatography on a μRPC C2/C18 ST 1.0/150 column using an Ettan LC chromatography system (GE Healthcare, Amersham, UK) which had been pre-equilibrated in 100% Buffer A (0.065% TFA). Peptides were eluted from the column using a gradient from 0% to 60% Buffer B (0.05% TFA, 84% acetonitrile). Fractions (50 mL) were collected in a 96-well plate and counted using a Wallac Microbeta counter.

Results and Discussion

As assayed by 2-electrode voltage-clamp of Xenopus oocytes, the constitutive expression of SGK1 caused an increase of KCNQ1/KCNE1 channel currents (Figure 1a). Similar to previous observations,10 the effect was not associated with marked alterations of current kinetics (supplemental Figure I in the online data supplement available at http://circres.ahajournals.org). To further analyze the effects of SGK1, purified activated/phosphorylated SGK1 protein was injected into Xenopus oocytes which were previously injected with cRNA encoding KCNQ1/KCNE1. Injection of purified active SGK1 protein during continuous recording was followed 11 minutes later by a ~40% increase in KCNQ1/KCNE1 currents (Figure 1b), indicating that the kinase acted directly and not at a transcriptional level. Injection of SGK1 caused an increase in cell volume by ~0.3%. This method of application is less perturbing than intracellular application of proteins via a perfused pipette in patch clamp experiments, which is associated with serious dilution of potentially important intracellular components. Injection of purified inactive (not in vitro phosphorylated by PDK) SGK1 protein failed to stimulate KCNQ1/KCNE1 currents, thus confirming that the effect required active SGK1.

Western blotting and a chemiluminescence assay indicated that the SGK1-induced enhancement of I\textsubscript{Ks} resulted from an increased insertion of KCNQ1/KCNE1 channel proteins into the plasma membrane (Figure 2a and b). This effect was confirmed in COS-7 cells by enhanced immunofluorescent staining of KCNQ1/KCNE1 coexpressed with a constitutively active mutant form of SGK1 (S422D), but not an inactive mutant (K127N) (Figure 2c, supplemental Figure II in the online data supplement available at http://circres.ahajournals.org). Thus, active SGK1 increases the plasma membrane abundance of KCNE1/KCNQ1 channel protein in both Xenopus oocytes and COS-7 cells.

The increased membrane insertion of KCNQ1/KCNE1 channels could result from SGK1-mediated alterations in KCNQ1/KCNE1-trafficking from intracellular compartments to the cell surface. Vesicle trafficking and exocytosis are known to be impaired by low temperature,27 taxol26 and colchicin.28 As illustrated in Figure 2d, the stimulating effect of SGK1 on KCNE1/KCNQ1 channel current was indeed abrogated by lowering the temperature to 4°C, or application of 1 μmol/L Taxol or 10 μmol/L colchicin for 4 hours before injection of active SGK1 protein. We hypothesized that if SGK1 increased recycling of KCNQ1/KCNE1 enriched vesicles from intracellular stores to the plasma membrane, then excessive overexpression of KCNQ1/KCNE1 should lead to maximal channel insertion independent from further stimulation by SGK1. As predicted, the SGK1-mediated stimulation saturated at high levels of KCNQ1/KCNE1 expression (supplemental Figure III).

Trafficking of membrane vesicles containing channels can be stimulated by injection of GTP, the physiological activator of RAB-GTPase-dependent vesicle trafficking.13–17 Injection of GTP increased channel current in the presence of RAB5(N133I), a mutant with reduced GTP binding affinity. This effect was completely blocked on introduction of a dominant-negative RAB11(S25N) (Figure 3a). Using GFP-tagged KCNQ1 and dsRED-RAB11 constructs we observed colocalization of RAB11 with KCNQ1/KCNE1 channels in COS-7 cells (Figure 3b). Taken together, these results suggest that KCNQ1/KCNE1 channels are endocytosed via a RAB5-dependent pathway and reinserted into the plasma membrane under the influence of RAB11. Thus, I\textsubscript{Ks} channels are contained in storage vesicles that are positionally poised for rapid insertion into the plasma membrane in response to specific physiological triggers.

In theory, phosphorylation could stabilize the GTP-bound conformation of RAB11. However, RAB11 is not a direct SGK1 substrate because we could not detect phosphorylation of purified RAB11 by a radioactive assay or by mass spectrometry (data not shown). Interestingly, over-expression of a mutant form of RAB11 (T77A) disrupted the ability of activated SGK1 to increase KCNQ1/KCNE1 current magni-
Thr77 is located in a domain of RAB11 called switch 2 (Figure 3d) that undergoes conformational changes on GTP-binding and is the docking interface for GTPase-activating proteins (GAPs).29 These findings suggest that T77A disrupts interaction of SGK1-dependent factors with RAB11.

Further experiments were performed to determine whether SGK1, similar to protein kinase B23, phosphorylates PIKfyve. As shown in Figure 4, recombinant active SGK1 phosphorylates purified PIKfyve in the presence of [γ-32P]ATP. This phosphorylation occurred on Ser318 in the RNRSA motif (Figure 4a,b), as demonstrated by immunoreactivity of WT PIKfyve with an antibody (anti-pS318) that is selective for (Ser318)-phosphorylated PIKfyve.23 Immunoreactivity of PIKfyve with the anti-pS318 antibody and 32P incorporation into PIKfyve after SGK1 incubation was almost completely eliminated when Ser318 was substituted with an Ala, again suggesting phosphorylation of Ser318 (Figure 4a, middle panel and Figure 4b). Interestingly, the phosphorylation of PIKfyve by PKB, in the presence of 32P]ATP was not eliminated when Ser318 was mutated to Ala. This finding suggests that the major SGK1 phosphorylation site in vitro is Ser318 and that PKB phosphorylates PIKfyve at an additional site, as supported by tryptic phosphopeptide mapping of PIKfyve after phosphorylation by PKB and SGK1 (Figure 4c).

Phosphorylation of Ser318 PIKfyve leads to activation and increased PI(3,5)P2 production.23 The SGK1 effects on KCNQ1/KCNE1 channel currents were mimicked by overexpression of PIKfyve and abrogated by site directed mutagenesis that replaced Ser318 by Ala (Figure 5a). Further, KCNQ1/KCNE1 channel currents were enhanced to the same level in response to injection of PI(3,5)P2 in the absence of SGK1, PIKfyve or PIKfyve(S318A) (Figure 5a). The effect of SGK1 was not additive to that of PIKfyve or PI(3,5)P2. The stimulating effect on KCNQ1/KCNE1 current was not specific for PI(3,5)P2 as PI(4,5)P2 similarly stimulated KCNQ1/KCNE1 current. To explore whether PI(3,5)P2 and PI(4,5)P2 both stimulated KCNQ1/KCNE1 currents, channels coexpressed together with RAB11(S25N) were insensitive to the subsequent injection of PI(3,5)P2, suggesting that PI(3,5)P2 acts as a positive regulator of RAB11-mediated exocytosis of vesicles containing KCNQ1/KCNE1 channels. Interestingly, PI(4,5)P2 stimulation did not depend on intact RAB11 and therefore PI(3,5)P2 and PI(4,5)P2 apparently stimulate KCNQ1/KCNE1 channels by different mechanisms (Figure 5b). Taken together, these findings strongly suggest that SGK1-mediated phosphorylation of PIKfyve and subsequent PI(3,5)P2 production act to regulate channel activity via RAB11 dependent vesicle exocytosis (Figure 5).

Various stress-induced stimuli are known to activate SGK1. Here we demonstrate that SGK1 phosphorylates PIKfyve, a modification previously reported to activate the enzyme.23 We propose that an SGK1-PIKfyve-mediated in-
crease of PI(3,5)P2 promotes exocytosis of KCNQ1/KCNE1 to the plasma membrane via a Rab11-dependent pathway (Figure 6). Apparently PIKfyve has a similar role in GLUT4 exocytosis.23 Re-internalization of channels on removal of the stimulus occurs via a Rab5-dependent pathway.

SGK1-dependent phosphorylation and regulation of PIKfyve can also modulate RAB11 controlled recycling of some other vesicle-bound channels. For example, SGK1 stimulates recycling of Kv1.530 and Kv4.3,31 but not Kir2.1 (data not shown). Simulations with the LabHeart action potential model (http://www.labheart.org/) suggest that the effects of SGK1 on Kv4.3 and Kv1.5 could not explain the shortened QT interval found in twins carrying a gain-of-function SGK1 mutation.10 In contrast, the strong functional upregulation of I\textsubscript{Ks} channels is sufficient to explain the shortened QT interval.
In summary, our studies demonstrate a novel link between trafficking of a membrane-bound K⁺ channel protein to PIP₂-dependent vesicle recycling that is regulated by the stress-dependent kinase SGK1. The exocytosis of K⁺ channels from the intracellular pool to the plasma membrane could underlie fast adaptations of cells to altered physiological conditions. Stress-induced increase of PI(3,5)P₂/PI(4,5)P₂ 30 to 60 minutes before recording and data analysis were described above (n = 12 to 13). Injection of oocytes with P(3,5)P₂ (n = 12 to 13) or P(4,5)P₂ (n = 11 to 13) 30 to 60 minutes before recording abated the differences in current amplitudes. For better comparison we include the results for KCNQ1/KCNE1 without PIP₂ (curve) in all figures. The filled circle curves with the star in panels b and c represent Q1/E1 without PIP₂ (n = 11 to 13) 30 to 60 minutes before recording and data are analyzed as described above (n = 12 to 13).

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Disclosures
None.

References


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Figure a: SGK1 does not markedly modulate KCNQ1/KCNE1 kinetics.

Kinetic analysis of KCNQ1/KCNQ1 alone or coexpressed with SGK1 or injected with PI(3,5)P2 or PI(4,5)P2. The activation and deactivation phases (at -100mV) were fitted to single-exponential functions. The initial cursor used to define the region of fit were positioned after the delay-of-activation of KCNQ1/KCNE1 currents (n=8-13). The isochronal (7 s) activation curves were calculated from initial tail currents. The V1/2 was derived by fit of the curves to a Boltzmann function and were 24.1 ± 2.2 mV (n=13) for KCNQ1/KCNQ1 alone, 18.6 ± 2.9 mV (n=11) when coexpressed with SGK1, 21.1 ± 1.9 mV (n=12) after injection with PI(3,5)P2 , and 20.4 ± 2.1 mV (n=12) after injection with PI(4,5)P2.
Supplementary figure b: Functional SGK1 may increase KCNQ1/KCNE1 density at the plasma membrane.
2D-pixel intensity scan (ImageJ software) of transfected COS-7 cells reveals an enhanced localization of KCNQ1/KCNE1-channels close to the plasma membrane in the cell co-transfected with the constitutive active SGK1SD as compared to cells transfected with KCNQ1/KCNE1 alone or cells co-transfected with the constitutive inactive SGK1KN.
Supplementary figure c: SGK1 effects depend on moderate overexpression.
KCNQ1 and KCNE1 cRNA were injected in different amounts into oocytes. The oocytes were also coinjected with 5ng SGK1 cRNA or with water (n=5-8). The stimulation by SGK1 was calculated by normalizing to the respective current amplitude without SGK1.