

Regulation of Endocytic Recycling of KCNQ1/KCNE1 Potassium Channels

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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 ion 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 PI(3,5)P₂. Identification of KCNQ1/KCNE1 recycling and its modulation by serum- and glucocorticoid-inducible kinase 1-phosphoinositide 3-phosphate 5-kinase -PI(3,5)P₂ provides a mechanistic insight into stress-induced acceleration of cardiac repolarization. (*Circ Res.* 2007;100:686-692.)

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

Emotional stress activates the sympathetic nervous system¹ and the release of stress hormones such as cortisol via the hypothalamic-pituitary-adrenal (HPA) axis² and is a common trigger of sudden cardiac death.^{3,4} One of the many genes regulated by cortisol is the serum- and glucocorticoid-inducible kinase 1 (SGK1).^{5,6} In vitro experiments have shown that SGK1 stimulates I_{Ks} ,⁷ a repolarizing potassium current conducted by channels composed of KCNQ1 α -subunits and KCNE1 β -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.⁴

The mechanism responsible for regulation of I_{Ks} channels by SGK1 have remained elusive. SGK1 enhances the abundance of other types of channel protein 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.^{15,23,24} The RAB dependent regulation of GLUT4 also involves the phosphoinositide 3-phosphate 5-kinase (PIKfyve)²³ that generates the phosphatidylinositol PI(3,5)P₂. The closely related phosphatidylinositol PI(4,5)P₂ has been implicated in the

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regulation of I_{Ks} channel activity.²⁵ PIKfyve is stimulated by protein kinase B²³, a close relative of SGK1, phosphorylating serine and threonine residues within a similar core consensus sequence (RXXRX[S/T]).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)P₂, 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 μ L H-buffer (in mM: 100 NaCl, 20 Tris-HCl, pH 7.4, 1% Triton X-100, plus a mixture of proteinase 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 μ L washed immobilized NeutrAvidin 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 μ L SDS-PAGE loading buffer (sodium dodecylsulphate-polyacrylamide gel electrophoresis, 0.8 mol/L β -mercaptoethanol, 6% SDS, 20% glycerol, 25 mmol/L Tris-HCl, 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.²⁶ 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.^{19,20,27,28} RAB11(T77A) was introduced into RAB11-HA in pCDNA3.1. Human KCNE1 was subcloned into pCDNA3.1 for transfection into COS-7 cells. The FLAG-tagged and MYC-tagged KCNQ1 (kindly provided by DM Roden) were described before.²⁹ SGK1(S422D) and SGK1(K127N) were subcloned into pIRES-EGFP. All constructs were confirmed by automated DNA sequencing. In vitro synthesis of poly-A capped cRNA was performed with SP6 and T7 mMessage mMachine kits (Ambion).

Electrophysiology-Xenopus laevis oocytes were harvested as described previously³⁰ (using procedures approved by local animal use authorities). Stage IV-V oocytes were collected and injected with 30 to 50 nL of RNA. Noninjected oocytes express some endogenous SGK activity¹² but do not express significant slowly activating K^+ current. Oocytes were injected with 1 ng or 5 ng of KCNQ1 cRNA with or without additional injection of 1 ng KCNE1 cRNA (or as indicated in the figure legends), 5 ng SGK1 RNA and/or 5 ng RAB5/11 RNA. After cRNA injections, oocytes were incubated at 17 to 18°C in ND96 solution containing (in mM: 96 NaCl, 4 KCl, 1.8 MgCl₂, 0.1 CaCl₂, 5 HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid], gentamycin (50 μ g/L); pH 7.6). A TurboTEC10CX amplifier (NPI, Germany) was used to record KCNQ1/KCNE1 currents at room temperature (22 to 23°C) in oocytes 3 to 4 days after injection with cRNAs using standard 2-electrode voltage clamp techniques as described before.³⁰ Glass pipettes were filled with 3 mol/L KCl and had resistances of 0.4 to 1.3 mol/L Ω . For all experiments the amplifiers integrator (set to 5 ms) was used to enable robust clamp-performance of currents. The clamp was always controlled via the PI-controller. Data acquisition was performed

using an IBM compatible computer with Pentium 3 processor, a Digidata 1322 A/D interface and pClamp 8 software (Axon Instruments). Specific pulse protocols used to voltage clamp oocytes are described in the Results and figure legends. Taxol (Tocris, USA) and colchicine (Tocris, USA) were prepared as 1 mmol/L and 10 mmol/L DMSO stock solutions, respectively. Active and inactive SGK1 proteins were purchased from United States Biological, USA. Where indicated, oocytes were incubated at 4°C after injection of active SGK1 protein, or have been treated with 1 μ mol/L Taxol (Tocris, USA) or 10 μ mol/L Colchicine (Tocris, USA) for 4 hours before injection of active SGK1 protein and stored at 22°C.

Immunocytochemistry

COS-7 cells were grown on glass coverslips and transfected with 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 Myc-KCNQ1 in Oocytes

Experiments were performed as recently described.³¹ Oocytes expressing KCNQ1 with an external Myc-tag attached between S1-S2²⁹ 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 μ g/mL, 1:100 in 1% BSA/ND96), washed 5 times at 4°C with 1% BSA/ND96, and incubated with 2 μ g/mL peroxidase-conjugated affinity purified F(ab')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 μ 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.³² The beads (10 to 20 mL) were washed in 50 to 100 μ L Kinase Assay Buffer (KAB; 20 mmol/L HEPES, pH 7.5, 20 mmol/L β -glycerophosphate, 1 mmol/L EDTA, 0.1% β -mercaptoethanol and 5 mmol/L MgCl₂). A volume of 20 μ L of these beads were then incubated in a further 50 μ L KAB containing 1 to 2 U of recombinant PKB or SGK1 (Upstate, Dundee, UK) and 50 μ mol/L [γ -³²P]ATP (40 kBq/mol). 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 (α SP318) or 1:500 dilution of rabbit anti-PIKfyve antibody, as previously described.³² Blots were developed using ECL reagent (GE Healthcare, Amersham, UK) for 2 minutes before being exposed to photographic film. For tryptic 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 iodoacetamide 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

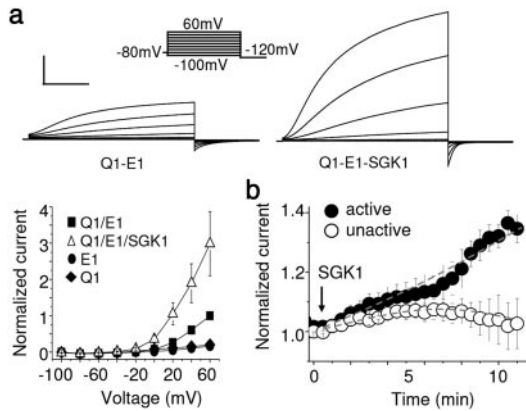


Figure 1. SGK1 increases KCNQ1/KCNE1 current density. a, KCNQ1/KCNE1 (Q1/E1) coexpressed with SGK1 yielded larger currents than KCNQ1/KCNE1 expressed alone in *Xenopus* oocytes. Horizontal scale bar=2 s, vertical scale bar=3 μ A, KCNQ1/KCNE1 currents in the presence of SGK1 were normalized relative to WT KCNQ1/KCNE1 currents at +60 mV (n=19 to 24). b, Active (in vitro phosphorylated) and inactive (not in vitro phosphorylated) SGK1 protein (\approx 0.17 μ g) were injected into oocytes via a third pipette resulting in an increase of currents following injection of active but not inactive SGK1 protein (n=5 each). Data are shown as mean \pm SEM and statistical differences ($P<0.05$) are marked by *.

50 mmol/L NH_4HCO_3 , pH 8.2 containing 0.002 μ g trypsin (Promega) at 37°C. Tryptic peptides were lyophilized and subjected to reverse-phase chromatography on a μ RPC 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 \approx 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 \approx 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_{Ks} resulted from an increased insertion of KCNQ1/KCNE1 channel proteins into the plasma membrane (Figure 2a and 2b). 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,³⁴ taxol³³ and colchicin.³⁵ 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 μ mol/L Taxol or 10 μ mol/L colchicine 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).²² 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_{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, overexpression of a mutant form of RAB11 (T77A) disrupted the ability of activated SGK1 to increase KCNQ1/KCNE1 current magnitude (Figure 3c). 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).³² These findings suggest

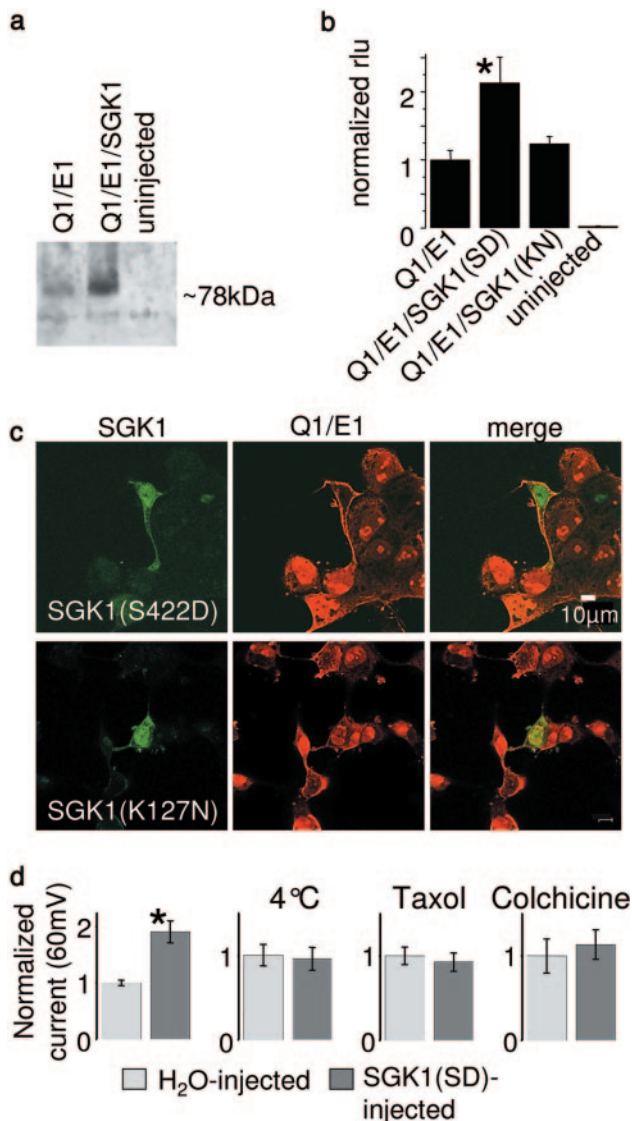


Figure 2. SGK1 enhances plasma membrane insertion of KCNQ1 channel. **a**, Western blot of oocyte plasma membrane preparation (30) performed by a biotinylation-based assay probed with anti-KCNQ1 antibody. **b**, KCNQ1 tagged with an extracellular Myc-epitope²⁹ was coexpressed with WT KCNE1 subunits in oocytes. Myc-tagged KCNQ1 was detected by a standard chemiluminescence assay. Channels were coexpressed with constitutively active SGK1(SD) or inactive SGK1(KN). SGK1(SD) increased KCNQ1 cell surface expression. **c**, KCNQ1(Flag) and KCNE1 was coexpressed with a GFP-tagged constitutively active mutant SGK1(S422D) or with a GFP-tagged inactive mutant SGK1(K127N) in COS-7 cells. KCNQ1(Flag) was probed by immunostaining with an anti-Flag antibody. The constitutively active mutant increased, whereas the inactive mutant decreased, plasma membrane expression of channels. **d**, Oocytes expressing KCNQ1/KCNE1 were injected with 0.17 pg active SGK1 protein and recorded 30 to 60 minutes after injection and subsequent storage at either 22°C (n=11/13) or 4°C (n=10). In the second set of experiments the KCNQ1/KCNE1 expressing oocytes were incubated in either 1 μ mol/L Taxol or 10 μ mol/L colchicine for 4 hour before injection of active SGK1 protein and storage at 22°C (n=11 to 15). Data are shown with the corresponding mean \pm SEM and statistical differences ($P<0.05$) are marked by *.

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 RNRSA³¹⁸ motif (Figure 4a and 4b), 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 [γ 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 current by a RAB dependent mechanism, we studied PIP₂ effects on KCNQ1/KCNE1 channels in the presence of wild-type and a dominant-negative form (S25N) of RAB11 (Figure 5b). KCNQ1/KCNE1 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 increase 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

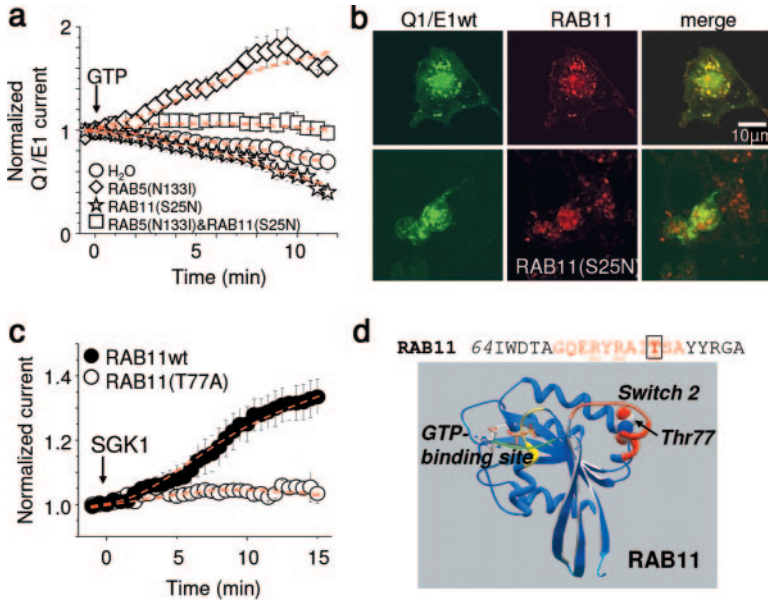


Figure 3. KCNQ1/KCNE1 is endocytosed and recycled by a RAB5-RAB11-dependent SGK1 pathway. a, Oocytes expressing KCNQ1/KCNE1 (Q1/E1) or KCNQ1/KCNE1 without or with RAB5(N133I) or KCNQ1/KCNE1 plus dominant-negative RAB11(S25N) or KCNQ1/KCNE1 with both mutant RABs were recorded. Modulation by GTP was tested by injection of 0.23 nmol GTP into oocytes during voltage clamp recording (n=5 to 9). b, GFP-tagged KCNQ1/KCNE1 was coexpressed in COS-7 cells with dsRED-fused RAB11 or RAB11(S25N) as a negative control. KCNQ1/KCNE1 channels were expressed in the plasma membrane and colocalized with intracellularly expressed RAB11, but not RAB11(S25N), which is no longer specifically localized to the RAB11 positive vesicles. c, Coexpression of RAB11(T77A) mutant, but not WT RAB11, with KCNQ1/KCNE1 in oocytes abrogated the stimulation by constitutively active SGK1 protein (~0.17 pg, n=5/8). d, A homology model of RAB11 was generated and the switch 2 region (red) and the GTP binding site (green/yellow) are highlighted. The residue Thr77 (boxed) is shown in space-fill presentation. Mutation of this residue (T77A) uncouples RAB11 from activating effects, whereas basal GTP hydrolysis is unaltered.

exocytosis.²³ Reinternalization 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 Kv1.5³⁶ and Kv4.3,³⁰ 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 func-

tional upregulation of I_{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 PI(3,5)P₂-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 I_{Ks} would accelerate action potential repolarization and thus limit Ca^{2+} entry trough I_{Ca} and Ca^{2+} -overload of cardiac myocytes.

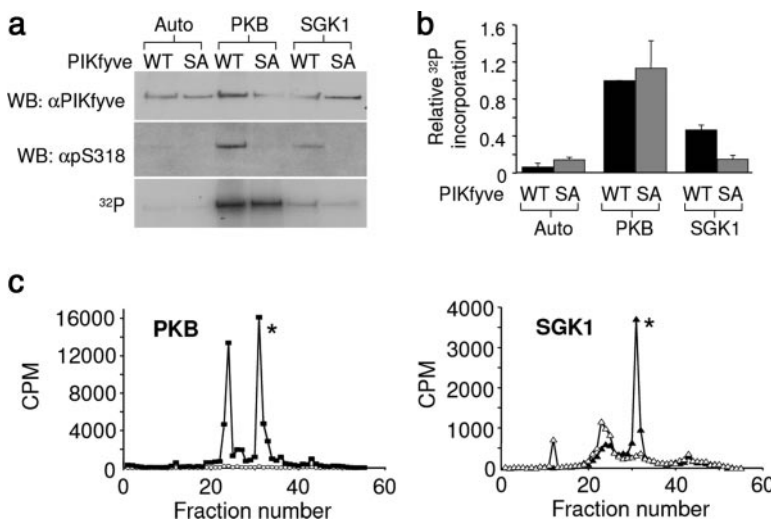


Figure 4. PIKfyve is phosphorylated on Ser318 by SGK1 and on Ser318 and a second unidentified site by PKB. a, Purified recombinant GST-tagged wild-type (WT) or S318A mutant (SA) PIKfyve was incubated with [γ -³²P]ATP and in the absence (Auto) or presence of 1 U of recombinant active PKB or SGK1, as indicated. The figure shows an autoradiograph of the resulting gel (bottom panel) before subsequent Western blotting with a rabbit anti-PIKfyve antibody (α PIKfyve; top panel), followed by stripping and reprobing with a rabbit anti-PIKfyve antibody specific for phosphoserine 318 (apS318; middle panel). b, The level of ³²P incorporation was quantified by densitometric scanning of three independent experiments (mean \pm SEM), with the data in each experiment normalized to the level of phosphorylation achieved for WT GST-PIKfyve in the presence of PKB. c, Purified WT PIKfyve was incubated in the absence (open circles) or presence of PKB (closed squares) and [γ -³²P]ATP. Alternatively, purified WT (closed triangles) or S318A mutant (open triangles) PIKfyve was incubated in the presence of 1 U of SGK1 and [γ -³²P]ATP. The protein was isolated by SDS-PAGE, digested with trypsin and the ³²P-labeled tryptic peptides subjected to reverse phase chromatography. Two major ³²P-labeled tryptic phosphopeptides were found after incubation of PIKfyve in the presence of PKB (closed squares), neither of which were apparent in the absence of PKB (open circles). The more hydrophobic of the two peptides (indicated by an asterisk) was identified as the phosphoserine 318-containing tryptic peptide. Thus, PKB can phosphorylate an alternative site on PIKfyve. SGK1 phosphorylates the alternative PKB site only weakly compared with PKB, which is consistent with the almost complete reduction in phosphorylation of PIKfyve by SGK1 in the presence of [γ -³²P]ATP observed when Ser318 is mutated to Ala (Figure 4a).

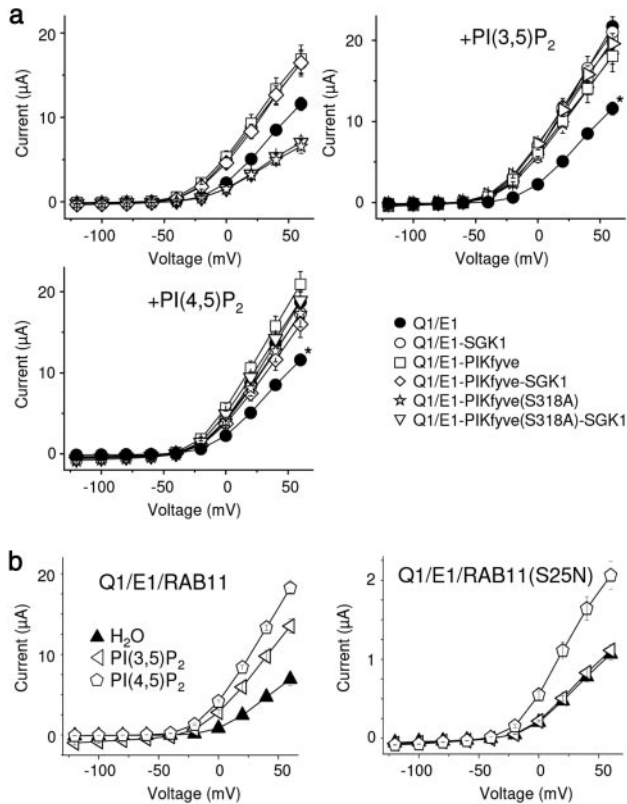


Figure 5. SGK1 phosphorylates PIKfyve at its activation site to produce PIP₂ and to modulate KCNQ1/KCNE1 channels. **a**, Coexpression of KCNQ1/KCNE1 (Q1/E1) with SGK1, PIKfyve or PIKfyve plus SGK1 results in increased current amplitudes at the end of 7 s pulses. Note that the data for these experiments are almost identical and the symbol of KCNQ1/KCNE1/SGK1 is covered by the other two curves. Coexpression of KCNQ1/KCNE1 with inactive mutant PIKfyve(S318A) with or without SGK1 reduces current amplitude (n=12 to 13). Injection of oocytes with PI(3,5)P₂ (n=12 to 13) or PI(4,5)P₂ (n=11 to 13) 30 to 60 minutes before recording abolished 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. **b**, KCNQ1/KCNE1 (Q1/E1) coexpressed with RAB11 or mutant RAB11(S25N) were injected with H₂O or PI(3,5)P₂/PI(4,5)P₂ 30 to 60 minutes before recording and data were analyzed as described above (n=12 to 13).

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Disclosures

None.

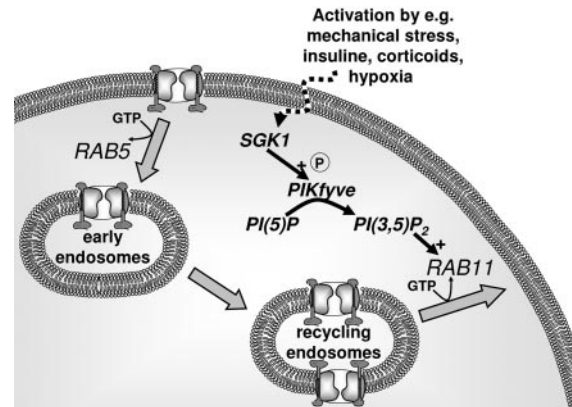


Figure 6. SGK1 activates PIKfyve to stimulate PIP₂-dependent KCNQ1/KCNE1 recycling. SGK1 is an immediate early gene whose expression and function is stimulated by mechanical stress, insulin, IGF-1, glucocorticoids, mineralocorticoids and hypoxia. SGK1 activates the PI(5)kinase PIKfyve and subsequent PI(3,5)P₂ production. PIP₂ may bind to KCNQ1/KCNE1 or other RAB11 regulating factors to increase the rate of RAB11-dependent exocytosis. Thus, SGK1 leads to increased KCNQ1/KCNE1 plasma membrane localization and enhanced whole cell currents.

References

- Marx SO, Kurokawa J, Reiken S, Motoike H, D'Armiento J, Marks AR, Kass RS. Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science*. 2002;295:496-499.
- Kern W, Perras B, Wodick R, Fehm HL, Born J. Hormonal secretion during nighttime sleep indicating stress of daytime exercise. *J Appl Physiol*. 1995;79:1461-1468.
- Eliot RS, Buell JC. Role of emotions and stress in the genesis of sudden death. *J Am Coll Cardiol*. 1985;5:95B-98B.
- Schwartz PJ, Zaza A, Locati E, Moss AJ. Stress and sudden death. The case of the long QT syndrome. *Circulation*. 1991;83:II71-II80.
- Lang F, Henke G, Embark HM, Waldegger S, Palmada M, Bohmer C, Vallon V. Regulation of channels by the serum and glucocorticoid-inducible kinase - implications for transport, excitability and cell proliferation. *Cell Physiol Biochem*. 2003;13:41-50.
- Lang F, Cohen P. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci STKE*. 2001;2001:RE17.
- Embark HM, Böhrer C, Vallon V, Luft F, Lang F. Regulation of KCNE1 dependent K⁺ current by the serum and glucocorticoid-inducible kinase (SGK) isoforms. *Pflugers Arch*. 2003;445:601-606.
- Barhanin J, Lesage F, Guillemare E, Fink M, Lazdunski M, Romey G. K(V)LQT1 and IsK (minK) proteins associate to form the I(Ks) cardiac potassium current. *Nature*. 1996;384:78-80.
- Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT. Coassembly of K(V)LQT1 and minK (IsK) proteins to form cardiac I(Ks) potassium channel. *Nature*. 1996;384:80-83.
- Busjahn A, Seeböhm G, Maier G, Toliat MR, Nurnberg P, Aydin A, Luft FC, Lang F. Association of the serum and glucocorticoid regulated kinase (sgk1) gene with QT interval. *Cell Physiol Biochem*. 2004;14:135-142.
- Debonneville C, Flores SY, Kamylnina E, Plant PJ, Tauxe C, Thomas MA, Munster C, Chraïbi A, Pratt JH, Horisberger JD, Pearce D, Löffing J, Staub O. Phosphorylation of Nedd4-2 by Sgk1 regulates epithelial Na⁺ channel cell surface expression. *EMBO J*. 2001;20:7052-7059.
- Lang F, Bohmer C, Palmada M, Seeböhm G, Strutz-Seeböhm N, Vallon V. *Physiol Rev*. 2006;86:1151-1178.
- Hoekstra D, Tyteca D, Van IJzendoorn SC. The subapical compartment: a traffic center in membrane polarity development. *J Cell Sci*. 2004;117:2183-2192.
- Marks DL, Pagano RE. Endocytosis and sorting of glycosphingolipids in sphingolipid storage disease. *Trends Cell Biol*. 2002;12:605-613.
- Maxfield FR, McGraw TE. Endocytic recycling. *Nat Rev Mol Cell Biol*. 2004;5:121-132. Miinea CP, Sano H, Kane S, Sano E, Fukuda M, Peranen J, Lane WS, Lienhard GE. AS160, the Akt substrate regulating

- GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J.* 2005;391:87–93.
16. Prekeris R. Rabs, Rips, FIPs, and endocytic membrane traffic. *Scientific-WorldJournal.* 2003;3:870–880.
 17. Ullrich O, Horiuchi H, Bucci C, Zerial M. Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature.* 1994;368:157–160.
 18. Bucci C, Wandinger-Ness A, Lutcke A, Chiariello M, Bruni CB, Zerial M. Rab5a is a common component of the apical and basolateral endocytic machinery in polarized epithelial cells. *Proc Natl Acad Sci U S A.* 1994; 91:5061–5065.
 19. Kessler A, Tomas E, Immler D, Meyer HE, Zorzano A, Eckel J. Rab11 is associated with GLUT4-containing vesicles and redistributes in response to insulin. *Diabetologia.* 2000;43:1518–1527.
 20. Mukhopadhyay A, Funato K, Stahl PD. Rab7 regulates transport from early to late endocytic compartments in *Xenopus* oocytes. *J Biol Chem.* 1997b;272:13055–13059.
 21. Mukhopadhyay A, Barbieri AM, Funato K, Roberts R, Stahl PD. Sequential actions of Rab5 and Rab7 regulate endocytosis in the *Xenopus* oocyte. *J Cell Biol.* 1997a;136:1227–1237.
 22. Gentzsch M, Chang XB, Cui L, Wu Y, Ozols VV, Choudhury A, Pagano RE, Riordan JR. Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol Biol Cell.* 2004;15:2684–2696.
 23. Berwick DC, Dell GC, Welsh GI, Heesom KJ, Hers I, Fletcher LM, Cooke FT, Tavaré JM. Protein kinase B phosphorylation of PIKfyve regulates the trafficking of GLUT4 vesicles. *J Cell Sci.* 2004;117: 5985–5993.
 24. Uhlig M, Passlack W, Eckel J. Functional role of Rab11 in GLUT4 trafficking in cardiomyocytes. *Mol Cell Endocrinol.* 2005;235:1–9.
 25. Loussouarn G, Park KH, Bellocq C, Baro I, Charpentier F, Escande D. Phosphatidylinositol-4,5-bisphosphate, PIP₂, controls KCNQ1/KCNE1 voltage-gated potassium channels: a functional homology between voltage-gated and inward rectifier K⁺ channels. *EMBO J.* 2003;22(20): 5412–21.
 26. Seeböhm G, Pusch M, Chen J, Sanguinetti MC. Pharmacological activation of normal and arrhythmia-associated mutant KCNQ1 potassium channels. *Circ Res.* 2003;93:941–947.
 27. Choudhury A, Dominguez M, Puri V, Sharma DK, Narita K, Wheatley CL, Marks DL, Pagano RE. Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J Clin Invest.* 2002;109:1541–1550.
 28. Sharma DK, Choudhury A, Singh RD, Wheatley CL, Marks DL, Pagano RE. Glycosphingolipids internalized via caveolar-related endocytosis rapidly merge with the clathrin pathway in early endosomes and form microdomains for recycling. *J Biol Chem.* 2003;278:7564–7572.
 29. Kanki H, Kupersmidt S, Yang T, Wells S, Roden DM. A structural requirement for processing the cardiac K⁺ channel KCNQ1. *J Biol Chem.* 2004;279:33976–33983.
 30. Seeböhm G, Strutz-Seeböhm N, Baltaev R, Korniychuk G, Knirsch M, Engel J, Lang F. Regulation of KCNQ4 potassium channel prepulse dependence and current amplitude by SGK1 in *Xenopus* oocytes. *Cell Physiol Biochem.* 2005;16:255–262.
 31. Zerangue N, Schwappach B, Jan YN, Jan LY. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron.* 1999;22:537–548.
 32. Pasqualato S, Senic-Matuglia F, Renault L, Goud B, Salamero J, Cherfils J. The structural GDP/GTP cycle of Rab11 reveals a novel interface involved in the dynamics of recycling endosomes. *J Biol Chem.* 2004; 279:11480–11488.
 33. Van der Sluijs P, Bennett MK, Antony C, Simons K, Kreis TE. Binding of exocytic vesicles from MDCK cells to microtubules in vitro. *J Cell Sci.* 1990;(Pt 4):545–53.
 34. Hong JS, Poisner AM. Effect of low temperature on the release of vasopressin from the isolated bovine neurohypophysis. *Endocrinology.* 1974;94:234–240.
 35. Lagunoff D, Chi EY. Effect of colchicine on rat mast cells. *J Cell Biol.* 1976;71:182–195.
 36. Ullrich S, Berchtold S, Ranta F, Seeböhm G, Henke G, Lupescu A, Mack AF, Chao CM, Su J, Nitschke R, Alexander D, Friedrich B, Wulff P, Kuhl D, Lang F. Serum- and glucocorticoid-inducible kinase 1 (SGK1) mediates glucocorticoid-induced inhibition of insulin secretion. *Diabetes.* 2005;54:1090–1099.

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Guiscard Seeböhm, Nathalie Strutz-Seeböhm, Ria Birkin, Ghislaine Dell, Cecilia Bucci, Maria R. Spinosa, Ravshan Baltaev, Andreas F. Mack, Ganna Korniyshuk, Amit Choudhury, David Marks, Richard E. Pagano, Bernard Attali, Arne Pfeufer, Robert S. Kass, Michael C. Sanguinetti, Jeremy M. Tavaré and Florian Lang

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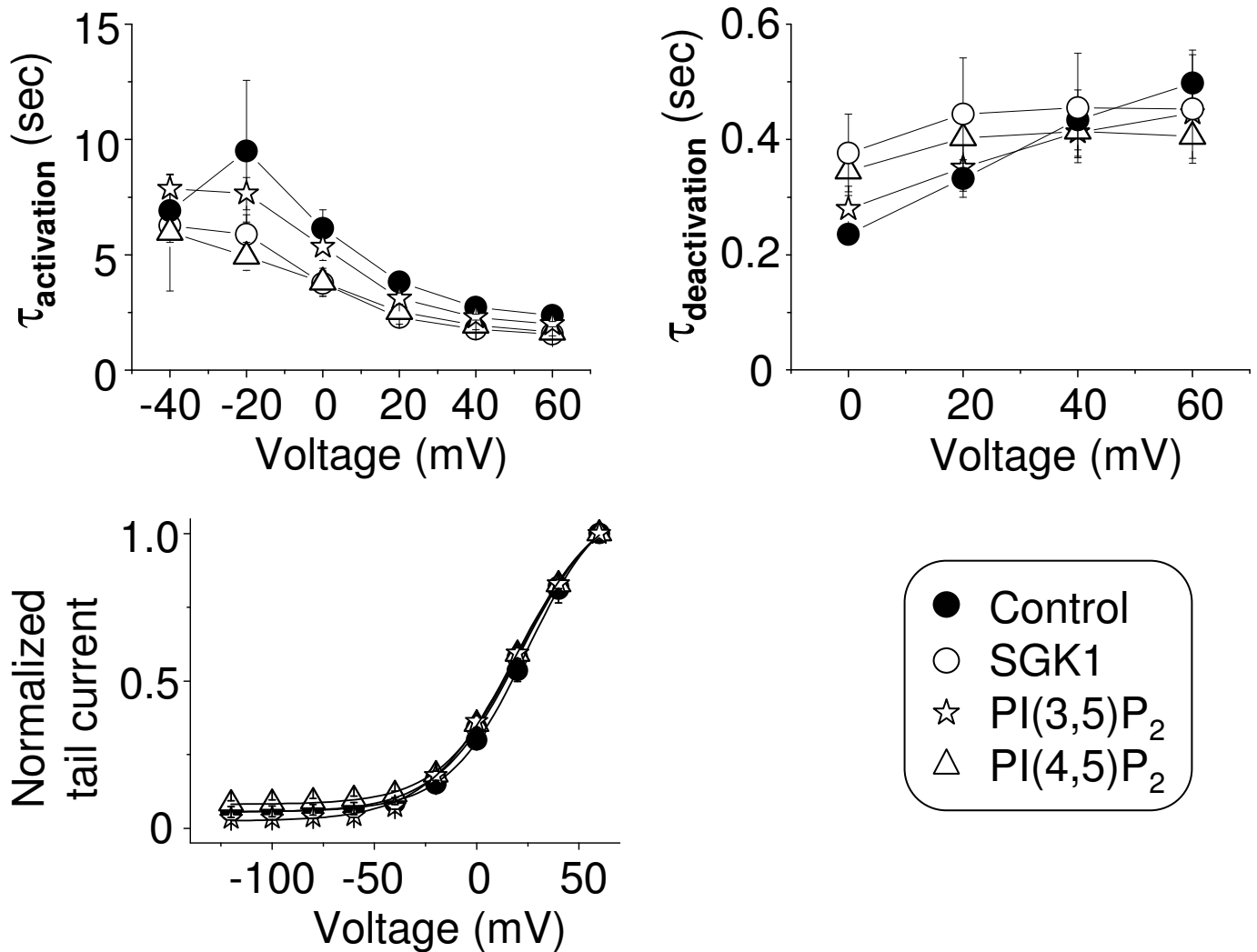
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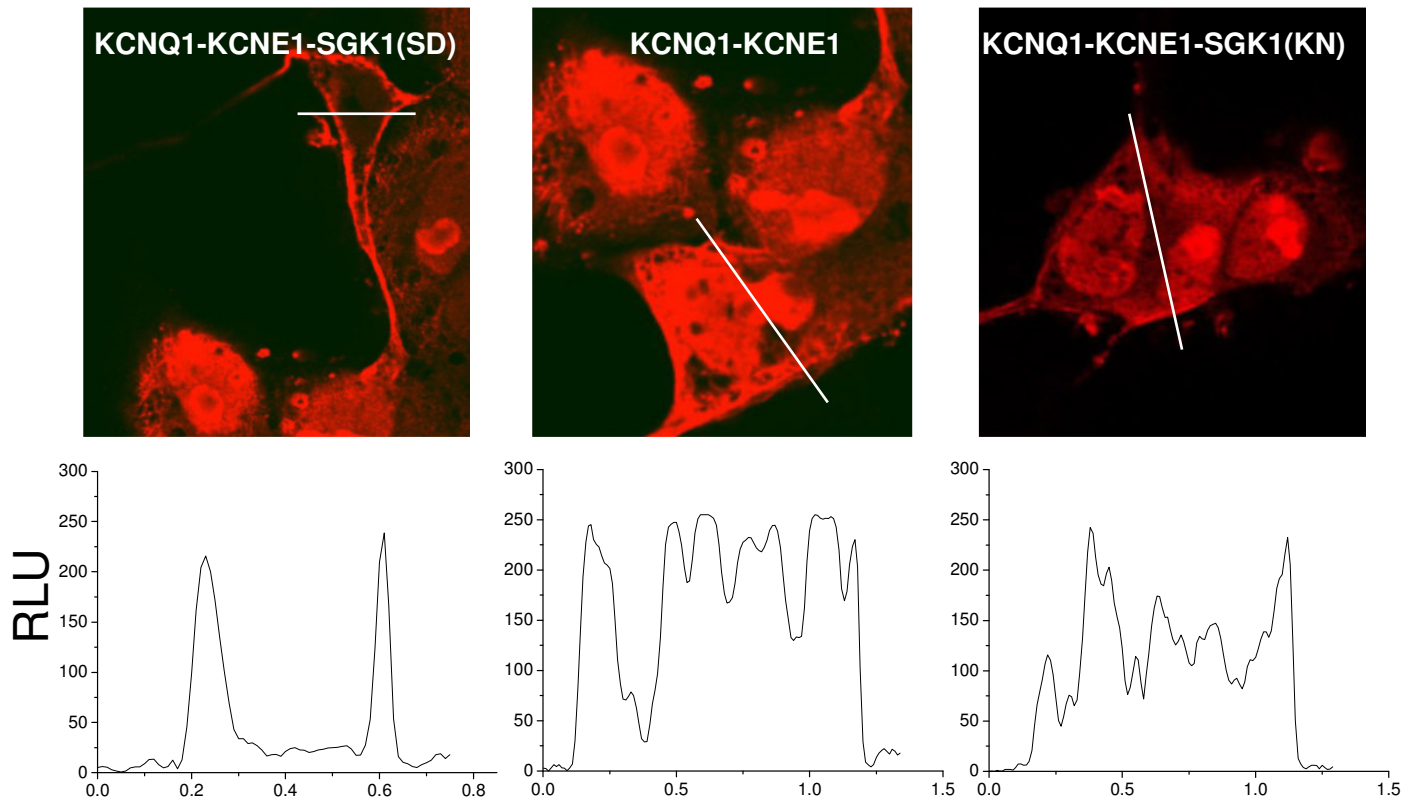
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Supplementary figure a

**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 V_{1/2} 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.

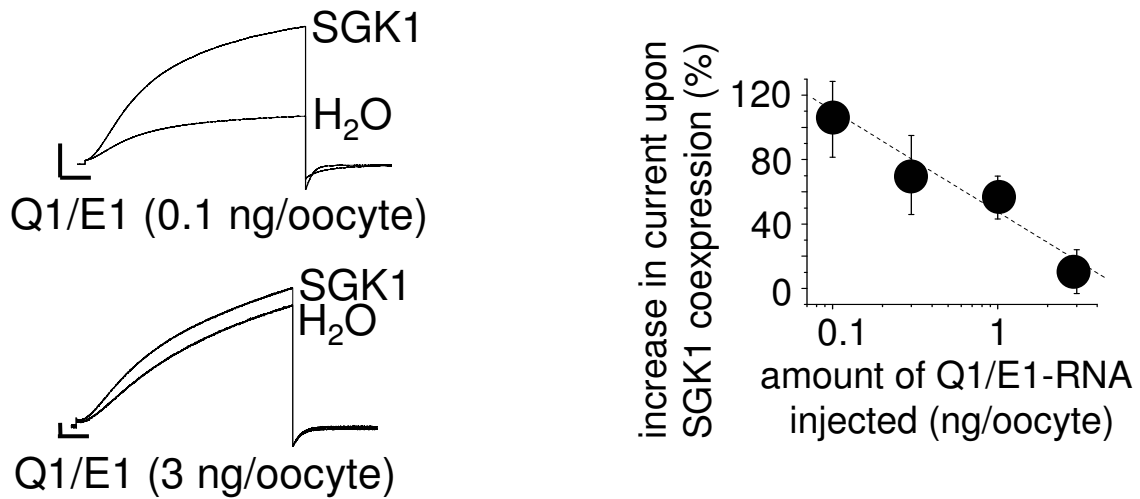
Supplementary figure b



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



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.