# Stimulation of $Ca^{2+}$-channel Orai1/STIM1 by serum- and glucocorticoid-inducible kinase 1 (SGK1)

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ABSTRACT **$Ca^{2+}$ signaling includes store-operated $Ca^{2+}$ entry (SOCE) following depletion of endoplasmic reticulum (ER) $Ca^{2+}$ stores. On store depletion, the ER $Ca^{2+}$ sensor STIM1 activates Orai1, the pore-forming unit of $Ca^{2+}$-release-activated $Ca^{2+}$ (CRAC) channels. Here, we show that Orai1 is regulated by serum- and glucocorticoid-inducible kinase 1 (SGK1), a growth factor-regulated kinase. Membrane Orai1 protein abundance, $I_{CRAC}$, and SOCE in human embryonic kidney (HEK293) cells stably expressing Orai1 and transfected with STIM1 were each significantly enhanced by coexpression of constitutively active $^{S422D}$SGK1 (by+81, +378, and+136%, respectively) but not by inactive $^{K127N}$SGK1. Coexpression of the ubiquitin ligase Nedd4-2, an established negatively regulated SGK1 target, down-regulated SOCE (by −48%) and $I_{CRAC}$ (by −60%), an effect reversed by expression of $^{S422D}$SGK1 (by +175 and +173%, respectively). Orai1 protein abundance and SOCE were significantly lower in mast cells from SGK1-knockout (*sgk1*$^{-/-}$) mice (by −37% and −52%, respectively) than in mast cells from wild-type (*sgk1*$^{+/+}$) littermates. Activation of SOCE by sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase-inhibitor thapsigargin (2 μM) stimulated migration, an effect significantly higher (by +306%) in $^{S422D}$SGK1-expressing than in $^{K127N}$SGK1-expressing HEK293 cells, and also significantly higher (by +108%) in *sgk1*$^{+/+}$ than in *sgk1*$^{-/-}$ mast cells. SGK1 is thus a novel key player in the regulation of SOCE.—Eylenstein, A., Gehring, E.-M., Heise, N., Shumilina, E., Schmidt, S., Szteyn, K., Münzer, P., Nurbaeva, M. K., Eichenmüller, M., Tyan, L., Regel, I., Föller, M., Kuhl, D., Soboloff, J., Penner, R., Lang, R. Stimulation of $Ca^{2+}$-channel Orai1/STIM1 by serum- and glucocorticoid-inducible kinase 1 (SGK1). *FASEB J.* 25, 2012–2021 (2011). www.fasebj.org**

*Key Words: CRAC · store-operated calcium channel · SOCE · degranulation · migration*

CYTOSOLIC $Ca^{2+}$ ACTIVITY regulates several fundamental processes, such as excitation-contraction, exocytosis, migration, cell proliferation, and cell death (1–4). Increases in cytosolic $Ca^{2+}$ can be accomplished by both release of $Ca^{2+}$ from intracellular stores and $Ca^{2+}$ entry across the cell membrane (5). Emptying of intracellular $Ca^{2+}$ stores stimulates the $Ca^{2+}$-release-activated $Ca^{2+}$ (CRAC) channel (6). The channel is currently thought to consist of tetrameric assemblies (7–9) of the pore-forming units Orai 1, 2, or 3 (10–13), which bind to their regulators STIM 1 or 2 (9, 14, 15). Orai proteins have been shown to function as CRAC current ($I_{CRAC}$) in a wide variety of tissues (16, 17), including lymphocytes (6, 18) and mast cells (19, 20). Orai and STIM have also been proposed to associate with TRPC channels, which might also contribute to store-operated $Ca^{2+}$ entry (SOCE; refs. 21–23).

Orai and STIM are regulated by receptor for activated protein kinase C-1 (RACK1; ref. 23), arachidonic acid (24), reactive oxygen species (25), and lipid rafts (26). STIM1 has been shown to be inhibited by phosphorylation (27). The mechanisms linking growth factor signaling to Orai activity have, however, remained ill-defined.

Previously, SGK1 has been reported to regulate a wide variety of carriers and channels (28). The SGK1-sensitive ion channels include the renal epithelial $Na^+$ channel (ENaC; refs. 29, 30). SGK1 regulates ENaC in part by phosphorylation of the ubiquitin ligase Nedd4-2 (neuronal precursor cells expressed developmentally down-regulated; ref. 31), which otherwise ubiquitinates ENaC and thus prepares the channel protein for clearance from the cell membrane and subsequent degradation (32). Phosphorylation of Nedd4-2 fosters binding of Nedd4-2 to 14-3-3, thus preventing its interaction

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with the target proteins (33, 34). SGK1 has been shown to stimulate the epithelial $Ca^{2+}$ channels TRPV5 (35) and TRPV6 (36). Moreover, lack of SGK1 decreases the antigen-induced $Ca^{2+}$ entry into mast cells (37). The present study explored whether Orai1/STIM1-dependent SOCE and $I_{CRAC}$ are sensitive to the serum- and glucocorticoid-inducible kinase SGK1, a kinase stimulated by growth factors and involved in the regulation of cell survival (28).

## MATERIALS AND METHODS

### DNA constructs, HEK293 cells, and transfection

HEK293 cells stably transfected with Orai1 (38) were cultured in Dulbecco's MEM (Life Technologies, Inc.; Invitrogen, Carlsbad, CA, USA), containing 1 mg/ml glucose and maintained in the presence of G418 (0.5 mg/ml; Gibco, Darmstadt, Germany). The cells were transfected transiently with 1–2 μg DNA encoding STIM1 (14), the constitutively active SGK1 mutant $^{S422D}$SGK1 (hSGK1$^{SD}$ in pIRES-EGFP or pCDNA3.1; ref. 39), the inactive mutant $^{K127N}$SGK1 (hSGK1$^{KN}$ in pIRES-EGFP or pCDNA3.1; ref. 39), or the ubiquitin ligase E3 Nedd4-2 (in pRFP) using FuGene HD transfection reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. SiRNA (10 nM; Life Technologies, Inc.; Ambion, Austin, TX, USA) was transfected according to the manufacturer's instructions using Polyplus Interferin (PeqLab, Erlangen, Germany).

### Animals

Bone marrow was obtained from 6- to 8-wk-old female and male SGK1-knockout (*sgk1*$^{-/-}$) mice and their wild-type (*sgk1*$^{+/+}$) littermates. Generation, breeding, and genotyping of the mice have been described earlier (40).

### Culture of bone marrow-derived mast cells (BMMCs)

Femoral BMMCs from 6- to 8-wk-old *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice were cultured for 4 wk in RPMI 1640 (Gibco) containing 10% fetal calf serum, 1% penicillin/streptomycin, 20 ng/ml interleukin-3 (R&D Systems, Hessen, Germany), and 100 ng/ml of the c-kit ligand stem cell factor (SCF; Peprotech, Rocky Hill, NJ, USA). BMMC maturation was confirmed by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, USA; ref. 37).

### Whole-cell lysates

For total protein analysis, cells were harvested with lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 0.4% β-mercaptoethanol, and protease inhibitor cocktail; Roche) 48 h after transfection. Clarified protein lysate was applied to a polyacrylamide gel and analyzed by Western blot.

### Western blot analysis

Proteins of whole-cell lysates (50 μg) were used for Western blot analysis and incubated with primary antibody against Orai1 (1:1000, Millipore, Bedford, MA, USA; or 1:200, Protein Tech, Chicago, IL, USA), Nedd4-2 (1:1000; Abcam, Cambridge, UK), or tubulin (1:1000; Cell Signaling, Danvers, MA, USA). For detection, a secondary anti-rabbit IgG antibody conjugated with horse radish peroxidase (HRP; 1:2000; Cell Signaling) was used. The blots were stripped and reprobed with tubulin to verify equal loading. Antibody binding was detected with ECL detection reagent (Amersham, Freiburg, Germany). Bands were quantified with Quantity One Software (Bio-Rad, Munich, Germany).

### Biotinylation of cells

After transfection, HEK293 cells were cultured for 48 h, incubated with EZ-Link-Sulfo-NHS-Biotin (Pierce Protein Research Products, Rockford, IL, USA) at a final concentration of 0.5 mg/ml for 30 min and finally solubilized in lysis buffer (20 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 5 mM $Na_2HPO_4$; 0.1% SDS; 1 mM EDTA; 1 mM PMSF; and 80 mM sucrose) containing protease inhibitor cocktail. Labeled protein (800 μg) was collected by rotating the lysates overnight at 4°C with Neutravidin-coated agarose beads (Pierce Protein Research). After elution, samples were analyzed by Western blotting using the affinity purified rabbit anti-Orai1 (intracellular) antibody (1 μg/ml, Millipore).

### Immunofluorescence

BMMCs ($5\times10^4$) were pipetted onto poly-L-lysine (Sigma-Aldrich, Taufkirchen, Germany)-coated microscope slides and incubated at 37°C for 24 h. BMMCs or HEK293 cells were fixated by incubating the cells for 15 min at room temperature in acetone/methanol (1:1), followed by permeabilization with 0.5% Triton X-100/PBS for 10 min. After blocking with 5% BSA/PBS, the cells were incubated overnight at 4°C (BMMCs) or for 2 h at room temperature (HEK293) with goat anti-Orai1 (BMMCs; 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit anti-Orai1 (HEK293 cells; 1:500; Millipore) antibody, respectively. BMMC slides were incubated with the secondary anti-goat-FITC antibody (1:500; Abcam), and HEK293 slides were incubated with FITC-conjugated goat anti-rabbit IgG (H+L; 1:500; Santa Cruz Biotechnology) for 1.5 h at room temperature. Nuclei were stained with DRAQ-5 dye (1:2000; Biostatus, Shepshed, UK) for 5–10 min at room temperature. The slides were mounted with ProLong Gold antifade reagent (Invitrogen). Images were taken on a Zeiss LSM 5 Exciter Confocal Laser Scanning Microscope (Carl Zeiss MicroImaging GmbH, Overkochen, Germany) with a water-immersion Plan-Neofluar ×63/1.3 NA DIC.

### Measurement of intracellular $Ca^{2+}$ by fluorescence spectrometry

HEK293 cells ($5\times10^5$ to $1\times10^6$) or BMMCs ($1\times10^6$) were centrifuged at 1500 rpm for 5 min at room temperature. Afterward, the cells were loaded with Fura-2/AM (5 μM) in the presence of 0.2 μg/ml pluronic acid (Biotium Inc., Hayward, CA, USA). The staining was done for 30 min at 37°C. The $Ca^{2+}$ measurements were performed in initially divalent cation-free solution (HEK293 cells) containing (in mM) 135 NaCl, 10 CsCl, 2.8 KCl, 10 HEPES, 0.1 EGTA, and 10 glucose, pH 7.2 (41) or PBS containing 0.1 mM EGTA (BMMCs) by using a LS 55 fluorescence spectrometer (PerkinElmer, Wellesley, MA, USA). After recording the baseline for 50 s, 5 μM thapsigargin (Invitrogen) and, after 10 min, 2 mM $Ca^{2+}$ were added to the cell suspension. Cells were excited alternatively between 340 and 380 nm, whereas the emission was measured at 509 nm.

### Measurement of intracellular $Ca^{2+}$ concentration by fluorescence microscopy

Fura-2/AM fluorescence was utilized to determine intracellular $Ca^{2+}$ by fluorescence microscopy (42). Cells were excited alternatively at 340 and 380 nm through the objective (Fluar ×40/1.30 oil; Zeiss) of an inverted phase-contrast microscope (Axiovert 100; Zeiss). Emitted fluorescence intensity was recorded at 505 nm. Data were acquired using specialized computer software (Metafluor; Universal Imaging, Burbank, CA, USA). Changes in cytosolic $Ca^{2+}$ concentrations were estimated from the 340/380-nm ratio. HEK293 cells or BMMCs were loaded with Fura-2/AM (5 μM; Molecular Probes; Invitrogen) for 20 min at 37°C, 5% $CO_2$. SOCE was determined by extracellular $Ca^{2+}$ removal and subsequent $Ca^{2+}$ readdition in the presence of 2 μM thapsigargin (43). For quantification of the $Ca^{2+}$ entry, the slope (Δ ratio/s) and peak (Δ ratio) were calculated following readdition of $Ca^{2+}$.

For intracellular calibration purposes, 10 μM ionomycin was applied at the end of each experiment. Experiments were performed with Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $CaCl_2$, 2 $Na_2HPO_4$, 32 HEPES, and 5 glucose, pH 7.4. To reach nominally $Ca^{2+}$-free conditions, experiments were performed using $Ca^{2+}$-free Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 2 $Na_2HPO_4$, 32 HEPES, 0.5 EGTA, and 5 glucose, pH 7.4.

### Measurement of intracellular $Ca^{2+}$ with flow cytometry (FACS)

HEK293 cells were trypsinized and centrifuged, resuspended in Dulbecco's MEM, and loaded with 2.5 μM fura red AM (Invitrogen) for 30 min at 37°C. Then 1 μM fluo3 was added to the cells, followed by incubation for 30 min at 37°C. The cells were subsequently collected and resuspended in $Ca^{2+}$- and $Mg^{2+}$-free PBS containing 2 μM thapsigargin. Cells were analyzed for basal levels of intracellular free $Ca^{2+}$ on a FACSCalibur flow cytometer (BD Biosciences, Erembodegem, Belgium). After 25 s, $Ca^{2+}$ was added to the samples at a final concentration of 2 mM, and intracellular $Ca^{2+}$ levels were monitored for 300 s. The ratio of fluo3 to fura red was analyzed using Cell Quest Pro software (BD Biosciences) and FlowJo software (Tree Star, Inc., Ashland, OR, USA).

### Patch clamp

Patch-clamp experiments were performed at room temperature in voltage-clamp, fast whole-cell mode. Borosilicate glass pipettes (2–4 MΩ tip resistance; GC 150 TF-10; Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with a MS314 electrical micromanipulator (MW; Märzhäuser, Wetzlar, Germany). The currents were recorded by an EPC-9 amplifier (Heka, Freiburg, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY, USA). For $I_{CRAC}$ measurements, whole-cell currents were elicited by 200-ms square-wave voltage pulses from −100 to +80 mV in 20-mV steps, delivered from a holding potential of 0. Alternatively, the currents were recorded with 200-ms voltage ramps from −120 to +100 mV. Leak currents, determined as the currents at the very beginning of each experiment, immediately after reaching the whole-cell mode, were subtracted. All voltages were corrected for a liquid-junction potential of 15 mV. The currents were recorded with an acquisition frequency of 10 kHz and 3-kHz low-pass filtered.

For $I_{CRAC}$ measurements, cells were superfused with a bath solution containing (in mM) 140 NaCl, 5 KCl, 10 $CaCl_2$, 20 glucose, and 10 HEPES/NaOH, pH 7.4. The patch-clamp pipettes were filled with an internal solution containing (in mM) 128 Cs-aspartate, 10 CsBAPTA, 8 $MgCl_2$, 10 HEPES/CsOH, and 0.02 inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], pH 7.2. Cs-BAPTA was from Invitrogen, all other chemicals from Sigma-Aldrich.

### Migration

For migration assays, transwell inserts (BD Falcon; 353097) and BD BioCoat Matrigel Invasion Chambers (354480; BD Biosciences) with a pore diameter of 8 μm were used. The transwells were placed in a 24-well culture plate containing cell culture medium (750 μl) with or without 2 μM thapsigargin in the lower chamber. The upper chambers were filled with 500 μl cell culture medium containing HEK293 cells at a concentration of $5 \times 10^4$ cells/ml, with or without 2 μM thapsigargin. After an incubation time of 32 h at 37°C, migrated cells were analyzed by staining the migrated cells with DAPI. Beforehand, nonmigrated cells were removed by scrubbing with a cotton-tipped swab twice and washing with PBS. After 15 min fixation in 4% PFA, the membranes were removed with a scalpel and mounted with ProLong Gold antifade reagent (Invitrogen). To determine the total number of migrating cells, the slices were then viewed under the microscope, and the number of cells per field in representative areas was counted. Experiments were performed in triplicates.

For the migration of mast cells from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice, $4 \times 10^5$ cells/ml were used with or without 100 μM 2-aminoethoxydiphenyl borate (2-APB) and 2 μM thapsigargin. Cells were incubated for 3 h at 37°C and analyzed as described above.

### Statistics

Data are provided as means ± SE; $n$ = number of independent experiments. All data were tested for significance using Student's unpaired 2-tailed $t$ test, 1-sample $t$ test, or ANOVA (Tukey's test), where applicable. Results with values of $P < 0.05$ were considered statistically significant.

## RESULTS

In HEK293 cells stably expressing Orai1 and transiently transfected with STIM1, addition of the store-depleting SERCA inhibitor thapsigargin (5 μM) in nominally $Ca^{2+}$-free solution was followed by rapid, transient increases in cytosolic $Ca^{2+}$, as measured by fluorescent spectrometry (**Fig. 1*A***). Subsequent addition of $Ca^{2+}$ to the extracellular bath resulted in a rapid and sustained increase in cytosolic $Ca^{2+}$ due to SOCE. Both slope and peak of SOCE were enhanced significantly by transient expression of the constitutively active mutant $^{S422D}$SGK1 but not the inactive mutant $^{K127N}$SGK1 (Fig. 1*B*).

The basal Fura-2 fluorescence ratio, reflecting resting intracellular $Ca^{2+}$ concentration, in Orai1/STIM1-expressing cells was similar in control cells (1.46±0.17, $n$=5, $5\times10^5$ cells/experiment), cells expressing the constitutively active $^{S422D}$SGK1 (1.39±0.04, $n$=6, $5\times10^5$ cells/experiment) and cells expressing the inactive mutant $^{K127N}$SGK1 (1.29±0.06, $n$=6, $5\times10^5$ cells/experiment). Likewise, the increase of the Fura-2 fluorescence ratio due to $Ca^{2+}$ release following thapsigargin (5 μM) treatment in the continued absence of

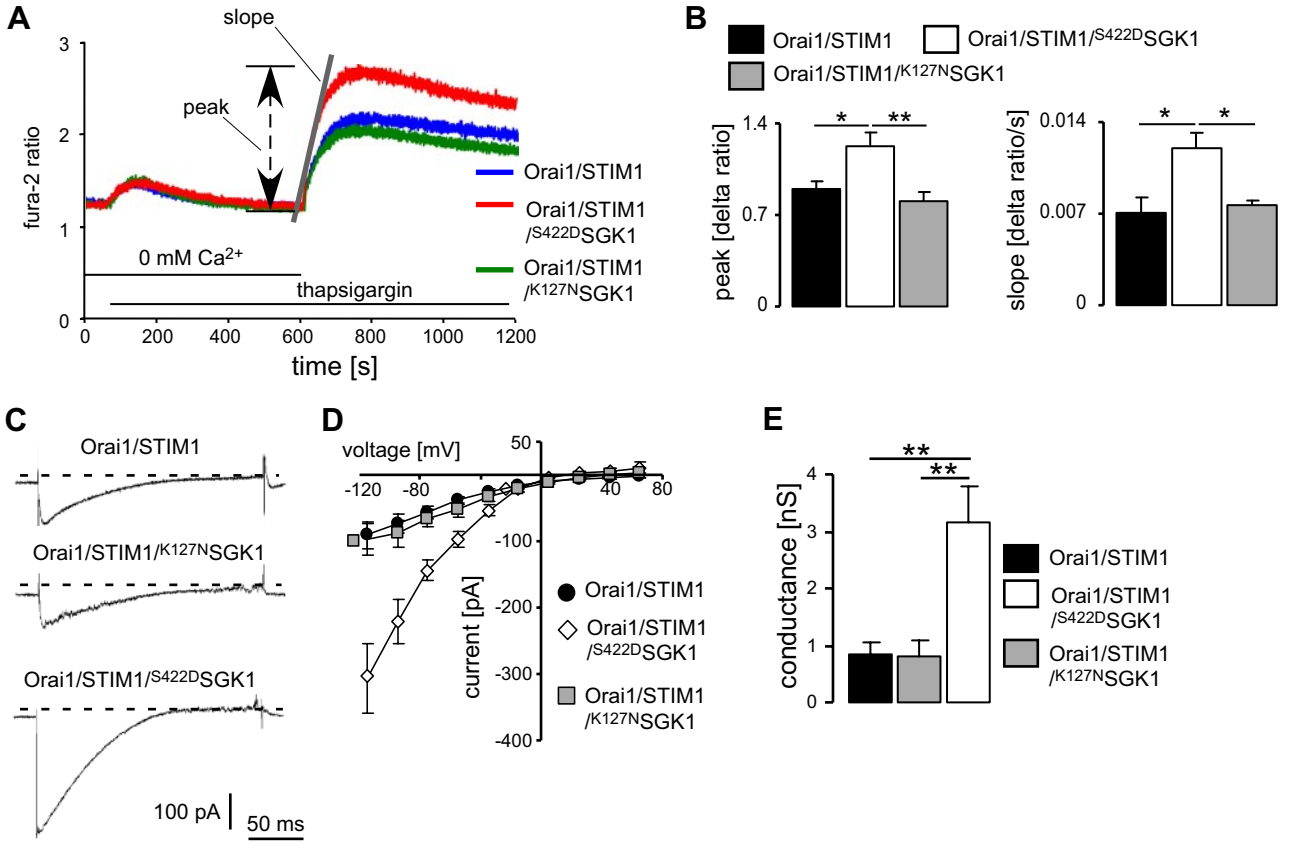


**Figure 1.** Effect of SGK1 on SOCE and $I_{CRAC}$ in HEK293 cells expressing Orai1 and STIM1. *A*) SOCE in HEK293 cells. Representative tracings of Fura-2 fluorescence ratio in fluorescence spectrometry during and after $Ca^{2+}$ depletion and thapsigargin (5 μM) addition in HEK293 cells stably expressing Orai1, transfected with STIM1 (blue), STIM1/$^{S422D}$SGK1 (red), and STIM1/$^{K127N}$SGK1 (green). Slope (Δ ratio/s) and peak (Δ ratio) following readdition of $Ca^{2+}$ were determined. *B*) Arithmetic means ($n$=5–6 independent experiments; $5\times10^5$ cells/experiment) of peak and slope of $Ca^{2+}$ increase in HEK293 cells following readdition of $Ca^{2+}$, determined by fluorescence spectrometry. *C*) Original ramp currents in HEK293 cells expressing Orai1 and transfected with STIM1, STIM1/$^{K127N}$SGK1, or STIM1/$^{S422D}$SGK1. *D*) Mean *I/V* relationships constructed from the currents elicited by the step pulses ($n$=5–6) of $I_{CRAC}$ in HEK293 cells. *E)* Mean conductance ($n$=5–6) calculated by linear regression of *I/V* curves between −115 and −35 mV in HEK293 cells. Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$; ANOVA.

extracellular $Ca^{2+}$ was similar in Orai1/STIM1-expressing cells (Δ ratio=0.52±0.09, $n$=5, $5\times10^5$ cells/experiment), Orai1/STIM1-expressing cells additionally expressing $^{S422D}$SGK1 (Δ ratio=0.51±0.12, $n$=6, $5\times10^5$ cells/experiment) and Orai1/STIM1-expressing cells additionally expressing $^{K127N}$SGK1 (Δ ratio=0.65±0.06, $n$=6, $5\times10^5$ cells/experiment).

Similar results were obtained in a second series of experiments utilizing Fura-2 fluorescence imaging (data not shown). Both slope and peak of the increase in cytosolic $Ca^{2+}$ activity following readdition of $Ca^{2+}$ after store depletion with thapsigargin (2 μM) and exposure to $Ca^{2+}$-free extracellular fluid were enhanced by transient expression of STIM1. The additional expression of $^{S422D}$SGK1 led again to a significant further enhancement of $Ca^{2+}$ entry. In contrast, the coexpression of $^{K127N}$SGK1 again failed to significantly modify $Ca^{2+}$ entry.

The alterations of SOCE were paralleled by corresponding changes in $I_{CRAC}$ (Fig. 1*C–E*). $I_{CRAC}$ in HEK293 cells expressing both STIM1 and Orai1 was increased significantly by coexpression of $^{S422D}$SGK1, but not by $^{K127N}$SGK1.

The observed increases in $Ca^{2+}$ entry could be due to either activation of $Ca^{2+}$ channels or increases in channel protein abundance. According to confocal microscopy, the protein abundance of Orai1 in the plasma membrane of Orai1-expressing HEK293 cells was increased significantly following transfection with STIM1. A significant further increase of Orai1 protein abundance was observed following additional transfection with $^{S422D}$SGK1 but not following additional transfection with $^{K127N}$SGK1 (**Fig. 2*A*, *B***). Western blot analysis revealed that total Orai1 protein abundance was enhanced by transfection with $^{S422D}$SGK1, but not by $^{K127N}$SGK1 (Fig. 2*C*). Furthermore, biotinylation studies revealed that $^{S422D}$SGK1 increased the Orai1 protein abundance in the plasma membrane of HEK293 cells (Fig. 2*D*).

We suggest that the increased Orai1 protein abundance resulted from decreased retrieval and degradation of channel proteins. SGK1 is known to phosphor-

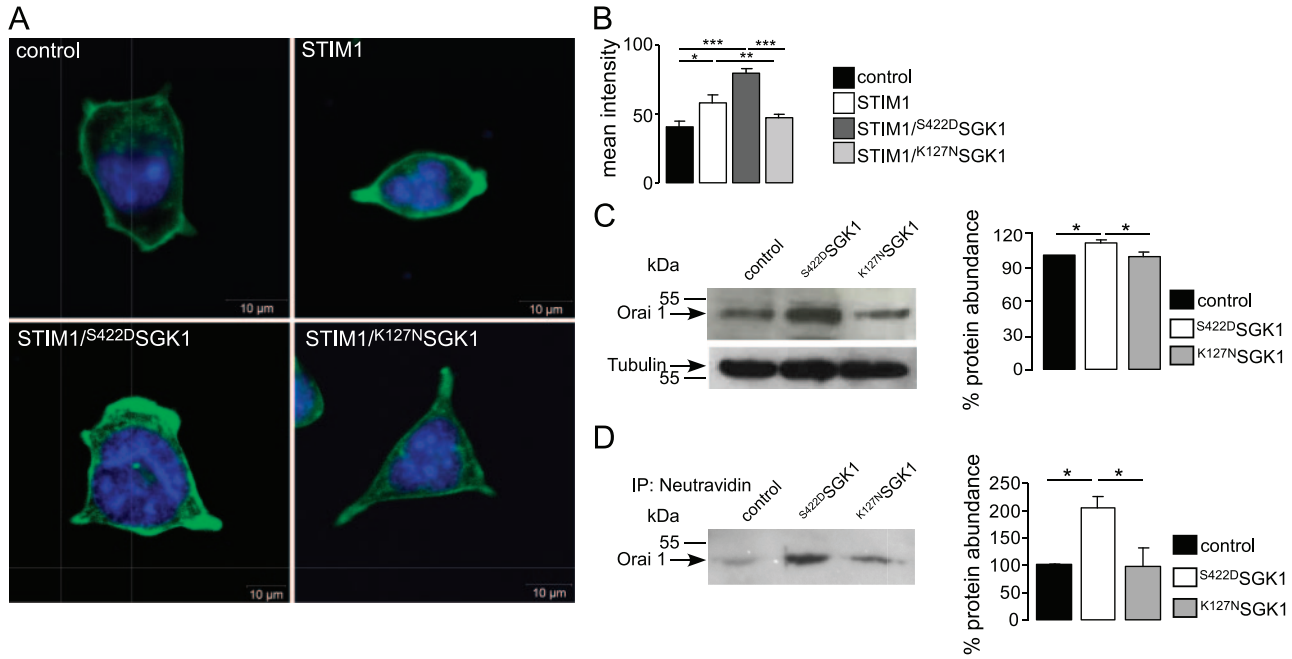


**Figure 2.** Effect of SGK1 on Orai1 protein abundance of HEK293 cells expressing Orai1 and STIM1. *A*) Confocal microscopy of Orai1 abundance in HEK293 cells stably expressing Orai1. Shown are Orai1-expressing HEK293 cells transfected with control plasmid (control), with STIM1, STIM1 and the active mutant $^{S422D}$SGK1, or STIM1 and the inactive mutant $^{K127N}$SGK1. Nuclei are stained with DRAQ5 (blue); Orai1 with FITC-conjugated secondary antibody (green). *B*) Arithmetic means of Orai1 protein abundance in the cell membrane, estimated by confocal microscopy, in Orai1-expressing HEK293 cells transfected with control plasmid, STIM1, STIM1/$^{S422D}$SGK1, or STIM1/$^{K127N}$SGK1 ($n$=5 independent experiments; 13 cells/experiment). *C*) Western blot analysis of whole-cell lysate protein of Orai1. Left panel: representative experiment showing Orai1 band and after stripping the Tubulin control band. Right panel: arithmetic means ($n$=4). *D*) Western blot analysis of biotinylated Orai1 in HEK293 cells. Orai1 was precipitated by NeutrAvidin beads binding to Biotin. Arrow indicates Orai1 band. Left panel: representative experiment. Right panel: arithmetic means ($n$=6). Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; ANOVA.

ylate the ubiquitin ligase Nedd4-2, which ubiquitinates several channel and carrier proteins in the plasma membrane and thus initiates their degradation (44, 45). SGK1-dependent phosphorylation results in binding of Nedd4-2 to the scaffolding protein 14-3-3, which prevents the interaction of Nedd4-2 with target proteins (44). Therefore, we examined the relationship between Orai1 and Nedd4-2. To this end, the Orai1 protein abundance of HEK293 cells stably expressing Orai1 was determined following transfection with control plasmid, with Nedd4-2, and with Nedd4-2 and $^{S422D}$SGK1 (**Fig. 3**). Nedd4-2 transfection led to a marked decrease in Orai1 protein abundance, an effect that was reversed by additional expression of $^{S422D}$SGK1 (Fig. 3*A*, *B*). Conversely, silencing of Nedd4-2 by siRNA, which decreased Nedd4-2 protein abundance by up to 60%, significantly elevated the expression of Orai1 (Fig. 3*C*). Furthermore, expression of Nedd4-2 led to a decrease in Orai1 abundance, an effect reversed by coexpression with $^{S422D}$SGK1 (Fig. 3*D*). These changes in Orai1 protein abundance were paralleled by corresponding alterations in SOCE.

$Ca^{2+}$ entry into HEK293 cells stably expressing Orai1 was significantly enhanced by additional transfection with STIM1 and was significantly decreased by additional expression of Nedd4-2 (Fig. 3*E*). The effect of SOCE was confirmed by patch-clamp analysis showing decreased $I_{CRAC}$ after cotransfection of Nedd4-2 in Orai1- and STIM1-expressing HEK293 cells. $I_{CRAC}$ conductances assessed between −115 and −35 mV were significantly ($P$<0.002) lower in Orai1/STIM1/Nedd4-2-expressing cells (0.83±0.11 nS, $n$=26) than in cells expressing Orai1/STIM1 alone (2.09±0.46 nS, $n$=22). Cotransfection of $^{S422D}$SGK1 with Nedd4-2 partially restored $I_{CRAC}$ (conductance 1.44±0.17 nS, $n$=21; significantly different from Orai1/STIM1/Nedd4-2-expressing cells, $P$<0.004). Down-regulation of SOCE following transfection of Nedd4-2 was also observed in FACS analysis (Fig. 3*F*).

To determine whether SGK1-dependent regulation is relevant for native Orai1 protein abundance in nontransfected cells, Orai1 expression of BMMCs was determined by immunocytochemistry. As illustrated in **Fig. 4*A*, *B***, Orai1 protein abundance was significantly lower in BMMCs from *sgk1*$^{-/-}$ mice than in BMMCs from *sgk1*$^{+/+}$ mice. Orai1 expression was not uniform in mast cells, but the average abundance of Orai1 was clearly and significantly weaker in *sgk1*$^{-/-}$ than in *sgk1*$^{+/+}$ BMMCs. Accordingly, the rate of $Ca^{2+}$ entry following store depletion was significantly smaller in *sgk1*$^{-/-}$ BMMCs than in *sgk1*$^{+/+}$ BMMCs (Fig. 4*C*, *D*).

A further series of experiments assessed the effect of SGK1-dependent regulation of Orai1 on cell migration, which was estimated from translocation of cells from one chamber to another across a membrane with a pore diameter of 8 μm. In Orai1-transfected HEK293

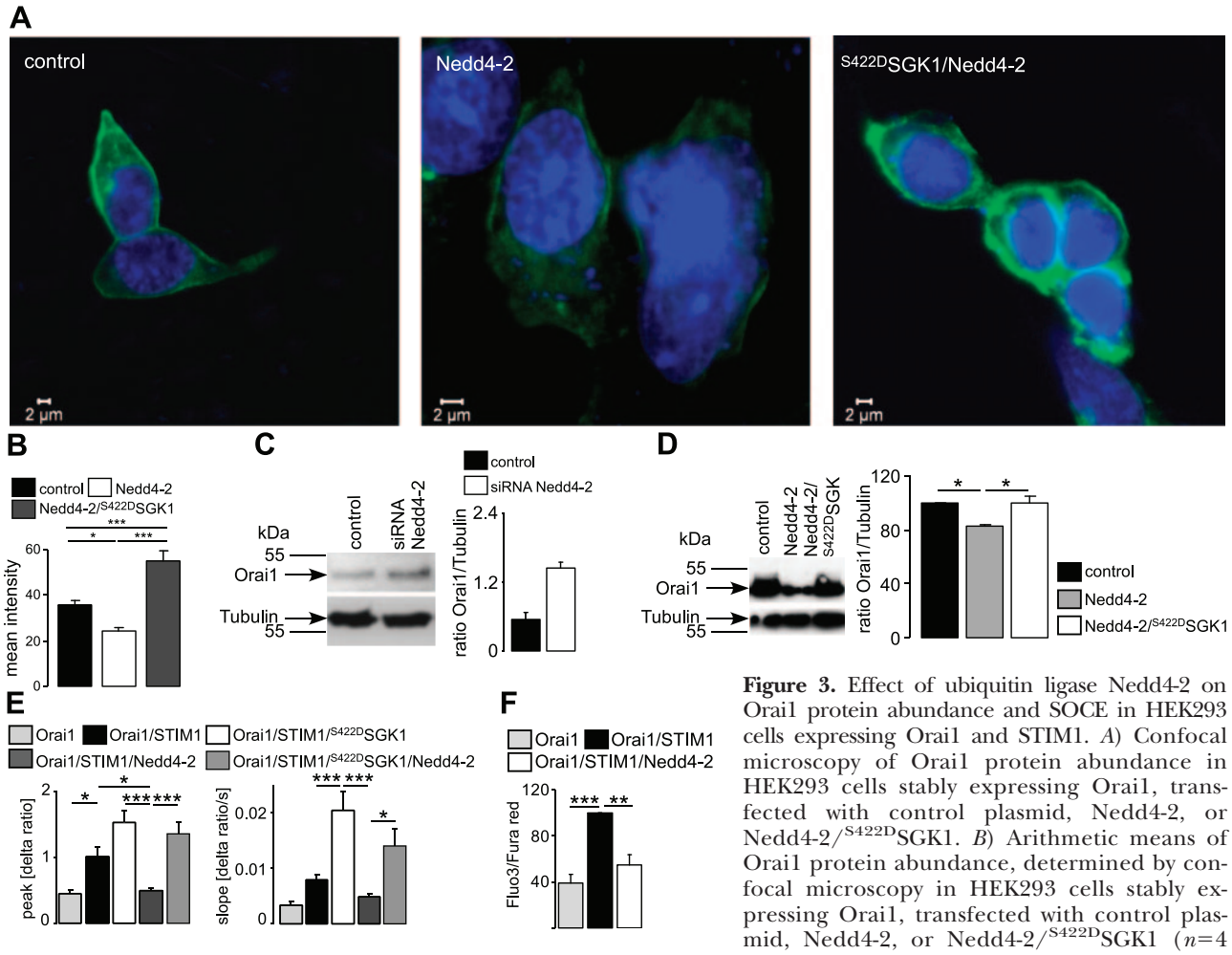


**Figure 3.** Effect of ubiquitin ligase Nedd4-2 on Orai1 protein abundance and SOCE in HEK293 cells expressing Orai1 and STIM1. *A*) Confocal microscopy of Orai1 protein abundance in HEK293 cells stably expressing Orai1, transfected with control plasmid, Nedd4-2, or Nedd4-2/$^{S422D}$SGK1. *B*) Arithmetic means of Orai1 protein abundance, determined by confocal microscopy in HEK293 cells stably expressing Orai1, transfected with control plasmid, Nedd4-2, or Nedd4-2/$^{S422D}$SGK1 ($n$=4 independent experiments; 9 cells/experiment). *C*) Orai1 protein abundance of whole-lysate protein in HEK293 cells transfected with scrambled siRNA (control) or siRNA for Nedd4-2. Left panel: representative blot. Right panel: arithmetic means ($n$=4). *D*) Orai1 protein abundance of whole-cell lysate protein in HEK293 cells transfected with control plasmid, Nedd4-2, or Nedd4-2 and $^{S422D}$SGK1. Left panel: representative blot. Right panel: arithmetic means ($n$=5). *E*) Arithmetic means ($n$=14–27) of peak and slope increase of Fura-2 fluorescence ratio in cells expressing Orai1 alone, Orai1/STIM1, Orai1/STIM1/$^{S422D}$SGK1, Orai1/STIM1/Nedd4-2, or Orai1/STIM1/Nedd4-2/$^{S422D}$SGK1. *F*) Geometric means ($n$=6) of change in the fluo3/fura red fluorescence ratio for HEK293 control cells (light shaded bar), STIM1-transfected cells (solid bar), and cells transfected with STIM1 and Nedd4-2 (dark shaded bar). Values are means ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; ANOVA.

cells stably expressing STIM1, migration was stimulated by thapsigargin (2 µM). Both, prior to and following thapsigargin treatment, the number of migrated cells was significantly higher when cells were cotransfected with $^{S422D}$SGK1 (171±26 and 323±30, respectively) compared to cotransfection with $^{K127N}$SGK1 (39±7 and 80±19, respectively) or even without transfection (75±10 and 221±26, respectively; **Fig. 5*A***). Similar observations were made in HEK293 cells stably expressing Orai1 after transfection with STIM1, STIM1/$^{S422D}$SGK1, and STIM1/$^{K127N}$SGK1 (data not shown).

The effect of SGK1 on migration was further determined by analysis of *sgk1*$^{-/-}$ and *sgk1*$^{+/+}$ BMMCs. Both prior to and following the administration of thapsigargin, significantly fewer *sgk1*$^{-/-}$ BMMCs (10±1 and 24±1, respectively) crossed the membrane in transwell chambers than *sgk1*$^{+/+}$ BMMCs (21±2 and 49±5, respectively; Fig. 5*B*). After adding the SOCE inhibitor 2-APB, the migration rate was significantly reduced (22±1 in *sgk1*$^{+/+}$ and 10±1 in *sgk1*$^{-/-}$). Migration of *sgk1*$^{+/+}$ BMMCs was similar to migration of HEK cells with transfection of either STIM1 or Orai1 alone, but was less than the migration of HEK cells overexpressing both Orai1 and STIM1.

## DISCUSSION

The present study reveals a powerful influence of the serum- and glucocorticoid-inducible kinase SGK1 on store-operated $Ca^{2+}$ entry. We show that transfection of HEK293 cells with constitutively active $^{S422D}$SGK1, but not with inactive $^{K127N}$SGK1, enhances SOCE by increasing the CRAC current. Orai1 protein abundance in the cell membrane and SOCE were significantly lower in BMMCs *sgk1*$^{-/-}$ mice compared to the corresponding *sgk1*$^{+/+}$ mice.

Additional studies disclose the underlying mecha-

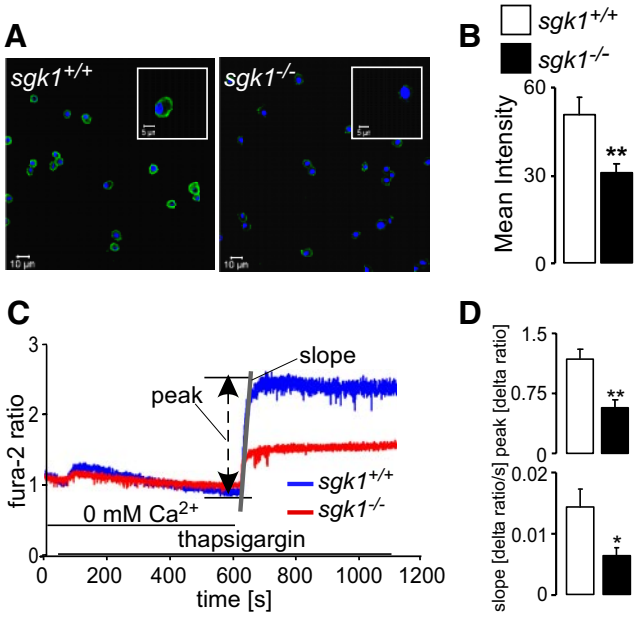


**Figure 4.** Orai1 protein abundance and SOCE in BMMCs from *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ mice. *A*) Confocal microscopy of Orai1 protein abundance in *sgk1*$^{+/+}$BMMCs (left panel) and *sgk1*$^{-/-}$ BMMCs (right panel). Nuclei are blue (DRAQ5); Orai1 is green (FITC-conjugated antibody). *B*) Arithmetic means of Orai1 protein abundance, estimated by confocal microscopy ($n$=5 independent experiments; 16 cells/experiment). *C*) Representative tracings of Fura-2 fluorescence ratio in *sgk1*$^{+/+}$BMMCs and *sgk1*$^{-/-}$BMMCs during and after $Ca^{2+}$ depletion in the presence of thapsigargin (5 μM). *D*) Arithmetic means ($n$=5 independent experiments; $5\times10^5$ cells/experiment) of peak (Δ ratio) and slope (Δ ratio/s) of Fura-2 fluorescence ratio increase following readdition of $Ca^{2+}$ in *sgk1*$^{+/+}$BMMCs (open bars) and *sgk1*$^{-/-}$BMMCs (solid bars), measured by fluorescence spectrometry. Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$; $t$ test.

nism. We demonstrate that Orai1 is a target of the ubiquitin ligase Nedd4-2, which previously has been shown to ubiquitinate, and thus regulate, the ENaC (45–47) and further channels and carriers (48–51). As shown in other cells, SGK1 phosphorylates Nedd4-2, leading to binding of the phosphorylated protein to a heterodimeric protein complex composed of 14-3-3β and 14-3-3ε (52). Nedd4-2 bound to 14-3-3 is thus unable to ubiquitinate its targets (53, 54). Accordingly, phosphorylation of Nedd4-2 by SGK1 is followed by decreased ubiquitination and subsequent degradation of the channel protein.

SGK1 may modify cytosolic $Ca^{2+}$ activity not only by up-regulation of Orai1. SGK1 has previously been shown to increase the activity of the $Ca^{2+}$-permeable TRP channels TRPV5 (55) and TRPV6 (56). Moreover, SGK1 up-regulates a wide variety of $K^+$ channels (57, 58). Activation of those channels is expected to hyperpolarize the cell membrane and thus enhance the driving force for $Ca^{2+}$ entry through several $Ca^{2+}$ channels.

SGK1 expression (59, 60) and activity (61) are stimulated by increased cytosolic $Ca^{2+}$ activity. Thus, at least in theory, SGK1 could serve as an amplifier of $Ca^{2+}$ entry.

SGK1 is functionally relevant for cell migration, which has previously been shown to critically depend on $Ca^{2+}$ signaling (62). Cell migration is stimulated by emptying intracellular $Ca^{2+}$ stores with thapsigargin, and this is augmented by expression of Orai1, STIM1, and constitutively active SGK1, but not by coexpression of inactive SGK1. We further show that thapsigargin stimulates migration in BMMCs from *sgk1*$^{+/+}$ mice, an effect markedly decreased in BMMCs from *sgk1*$^{-/-}$ mice. The residual difference of migration between *sgk1*$^{+/+}$ and *sgk1*$^{-/-}$ BMMCs in the presence of the $Ca^{2+}$ channel blocker 2-APB may point to some additional, Orai1-independent effect of SGK1 on migration.

SGK1-dependent regulation of SOCE might further be functionally relevant for degranulation. As shown previously (37), antigen-induced $Ca^{2+}$ entry, activation of $Ca^{2+}$-activated $K^+$ channels, and degranulation were all markedly depressed in BMMCs from *sgk1*$^{-/-}$ mice. Moreover, the anaphylactic reaction, which critically depends on mast cell function (63), was strongly impaired in those mice (37). However, those earlier studies did not define the SGK1-sensitive $Ca^{2+}$ entry mechanism involved.

The role of SGK1 in the regulation of Orai1/STIM1 is presumably not restricted to degranulation or migration. SGK1 is ubiquitously expressed (28), and Orai1

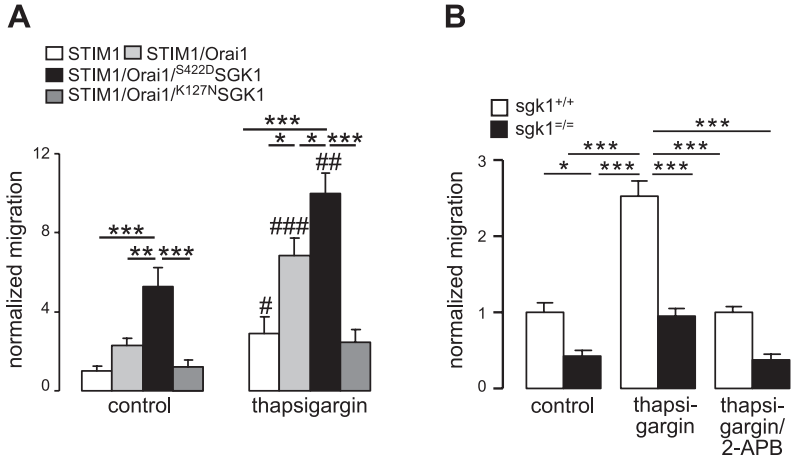


**Figure 5.** Influence of SGK1 on the migration of Orai1-expressing HEK293 cells and BMMCs. *A*) Arithmetic means ($n$=6) of the normalized migration of HEK293 cells expressing STIM1 alone; STIM1 and Orai1; STIM1, Orai1, and $^{S422D}$SGK1; or STIM1, Orai1, and $^{K127N}$SGK1 in the absence or presence of 2 μM thapsigargin. *B*) Arithmetic means ($n$=3–4 mice/group) of normalized migration of mast cells from *sgk1*$^{-/-}$ mice and from their *sgk1*$^{+/+}$ littermates in the absence or the presence of 2 μM thapsigargin or additional treatment with the SOCE inhibitor 2-APB. Values are means ± SE. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between indicated groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* corresponding control group; ANOVA.

and STIM1 have been identified in a wide variety of tissues (16, 17, 64, 65) including those of the immune system (6, 18, 66, 67).

SGK1 activity is triggered by PI3 kinase and phosphoinositide-dependent kinase PDK1 signaling (28), which is stimulated by growth factors (68–70). Along those lines, $Ca^{2+}$ entry into mast cells is stimulated similarly by PDK1 (71). Thus, the SGK1-dependent regulation of Orai1 establishes a previously unknown link between growth factor receptor-mediated signaling and control of SOCE. SGK1 might be up-regulated in tumor cells (28, 48, 72, 73) and Orai1 (74–76) as well as SGK1 (77–79) have been shown to be critically important in the regulation of cell proliferation. The SGK1-dependent regulation of Orai1 might thus participate in the machinery underlying tumor cell proliferation and migration.

In summary, SGK1 is of critical importance for the regulation of Orai1/STIM1, thereby indirectly modulating the numerous $I_{CRAC}$-dependent functions, including excitation, exocytosis, migration, cell proliferation, and cell death. In the present work, this role was specifically demonstrated for migration. However, since STIM1, Orai1, and SGK1 are ubiquitously expressed, our findings are presumably relevant for diverse functions in multiple cell types. In fact, SGK1 has been shown to participate in a multitude of physiological and pathophysiological mechanisms (28), which may in part prove to be secondary to Orai regulation. FJ

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