The serum- & glucocorticoid-inducible kinase 1 (SGK1) influences platelet calcium signaling and function by regulation of Orai1 expression in megakaryocytes

Oliver Borst1,2, Eva-Maria Schmidt1, Patrick Münzer1, Tanja Schönberger2, Syeda T. Towhid1, Margitta Elvers2, Christina Leibrock1, Evi Schmid1, Anja Eylenstein1, Dietmar Kuhl3, Andreas E. May2, Meinrad Gawaz2, Florian Lang1

1Department of Physiology, University of Tübingen, Germany. 2Innere Medizin III, Department of Cardiology and Cardiovascular Medicine, University of Tübingen, Germany. 3Center for Molecular Neurobiology (ZMNH), Institute for Molecular and Cellular Cognition (IMCC), University Medical Center Hamburg-Eppendorf (UKE), Germany.

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Address for correspondence:
Florian Lang
Department of Physiology
University of Tübingen
Gmelinstr. 5
72076 Tübingen
Germany
Phone: +49 7071 29-72194
Fax: +49 7071 29-5618
E-mail: florian.lang@uni-tuebingen.de
Abstract

Platelets are activated upon increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)\(_i\)], accomplished by store operated Ca\(^{2+}\) entry (SOCE) involving the pore forming ion channel subunit Orai1. Here, we show for the first time that the serum- and glucocorticoid-inducible kinase 1 (SGK1) is expressed in platelets and megakaryocytes. SOCE and agonist-induced [Ca\(^{2+}\)\(_i\)] increase are significantly blunted in platelets from SGK1 knockout mice (sgk1\(^{-/-}\)). Similarly, Ca\(^{2+}\)-dependent degranulation, integrin \(\alpha_{IIb}\beta_3\) activation, phosphatidylserine exposure, aggregation and \textit{in vitro} thrombus formation were significantly impaired in sgk1\(^{-/-}\) platelets, while tail bleeding time was not significantly enhanced. Platelet and megakaryocyte Orai1 transcript levels and membrane protein abundance were significantly reduced in sgk1\(^{-/-}\) mice. In human megakaryoblastic cells (MEG-01) transfection with constitutively active \(^{S422D}\)SGK1 but not with inactive \(^{K127N}\)SGK1 significantly enhanced Orai1 expression and SOCE, effects reversed by the SGK1 inhibitor GSK650394 (1\(\mu\)M). Transfection of MEG-01 cells with \(^{S422D}\)SGK1 significantly increased phosphorylation of I\(\kappa\)B kinase (IKK) \(\alpha/\beta\) and I\(\kappa\)B\(\alpha\) resulting in nuclear translocation of NF-\(\kappa\)B subunit p65. Treatment of \(^{S422D}\)SGK1-transfected MEG-01 cells with the IKK inhibitor BMS-345541 (10\(\mu\)M) abolished SGK1-induced increase of Orai1 expression and SOCE. The present observations unravel SGK1 as novel regulator of platelet function, effective at least in part by NF-\(\kappa\)B-dependent transcriptional upregulation of Orai1 in megakaryocytes and increasing platelet SOCE.

\textbf{Key words:} SGK1, calcium, SOCE, platelet activation, Orai1, NF-\(\kappa\)B, megakaryocytes.
Introduction

Platelet adhesion, activation and aggregation are essential for primary hemostasis at sites of vascular injury, but are also critically important for the development of acute thrombotic occlusion at regions of atherosclerotic plaque rupture, the major pathophysiological mechanism underlying myocardial infarction and ischemic stroke.1

Platelet activation is triggered by various agonists, including subendothelial collagen, ADP released from activated platelets, thrombin generated by the coagulation cascade or the collagen receptor GPVI-specific agonists convulxin (CVX) and collagen-related peptide (CRP).2 The agonists lead to platelet granule release, integrin αIIbβ3 activation, phosphatidylserine exposure, aggregation and thrombus formation.2 All those platelet responses depend on an increase of cytosolic Ca2+ concentration ([Ca2+]i),3,4 which is accomplished by inositol-1,4,5-triphosphate (IP3)-mediated Ca2+ release from intracellular stores triggering subsequent stimulation of store operated calcium entry (SOCE) across the plasma membrane.5 Two key players in platelet SOCE have recently been identified: The 4-transmembrane-spanning pore forming calcium release-activated channel (CRAC) moiety Orai1 (CRACM1), which mediates entry of extracellular Ca2+, and stromal interaction molecule 1 (STIM1), an Orai1 regulating Ca2+ sensor expressed predominantly in the endoplasmatic reticulum (ER).6-8 Regulators of Orai1 in other cell types include RACK1 (receptor for activated protein kinase C-1),9 reactive oxygen species10 and lipid rafts11. However, regulation of Orai1 in platelets is poorly understood. Platelet activation has been shown to be regulated in vitro and in vivo by the phosphoinositide 3-kinase (PI3K)/Akt signaling cascade.12,13 Interference with PI3K signaling has previously been shown to compromise Ca2+ influx into platelets.14,15

Signaling molecules regulated by PI3K signaling include the serum- and glucocorticoid-inducible kinase 1 (SGK1), a kinase belonging to the AGC family of serine/threonine protein kinases.16,17 SGK1 has originally been cloned as a glucocorticoid-sensitive gene, but later shown to be regulated by a variety of hormones and other triggers, including thrombin, growth factors as IGF-1 and TGF-β, oxidative stress and ischemia.17

SGK1 has previously been reported to regulate a wide variety of carriers and ion channels including the epithelial Ca2+ channels TRPV5 and TRPV6.17 Most recently, SGK1 has been shown to be critically important for the Ca2+ entry into mast cells following activation of the IgE receptor18, an effect mediated by regulation of Orai119. Furthermore, SGK1 participates in the regulation of renal tubular Na+ reabsorption, salt appetite and thus blood pressure.17 A gain of function SGK1 gene variant, the combined presence of single
nucleotide polymorphism (SNP) in intron 6 (rs1743966) and in exon 8 (rs1057293) is associated with enhanced blood pressure.\textsuperscript{20} The same genetic SGK1-variants are associated with ischemic stroke, an association partially independent of blood pressure and thus due to additional SGK1-dependent mechanisms.\textsuperscript{21}

SGK1 has been shown to foster coagulation by upregulation of tissue factor expression.\textsuperscript{22} But prothrombotic activity is in addition critically dependent on the function of platelets, key players in the initiation of arterial thrombosis and vascular occlusion resulting in ischemic diseases.\textsuperscript{23} Surprisingly, nothing is hitherto known about the influence of SGK1 on platelet function.

Thus, the present study explored the role of SGK1 in the regulation of platelet function. We could show for the first time that SGK1 is strongly expressed in platelets and megakaryocytes, acts as an important regulator of NF-kB-dependent Orai1 expression in megakaryocytes and thus influences Ca\textsuperscript{2+} response, activation and thrombus formation of released platelets.
Materials and Methods

Chemicals and antibodies

Platelets were activated using ADP (Sigma-Aldrich), thrombin (Roche), convulxin (Enzo), collagen (Nycomed), collagen-related peptide (Richard Farndale, University of Cambridge, United Kingdom) and thrombin receptor agonist PAR-4 activating peptide (NH2-AYPGKF, JPT peptide technologies). Fluorophore-labeled antibodies anti-P-selectin-FITC (Wug.E9-FITC, Emfret Analytics), anti-integrin αIIbβ3-PE (JON/A-PE, Emfret Analytics), Annexin V-Fluos (Roche) were used for flow cytometric analysis.

Preparation of human platelets

Blood from healthy volunteers was collected in ACD-buffer and centrifuged at 200 g for 20 minutes. The obtained platelet-rich plasma was added to modified Tyrode-HEPES (N-2-hydroxyethyl-piperazone-N’2-ethanesulfonic acid) buffer (137 mM NaCl, 2.8 mM KCL, 12 mM NaHCO3, 5 mM glucose, 0.4 mM Na2HPO4, 10 mM HEPES, 0.1% bovine serum albumin, pH 6.5). After centrifugation at 900 g for 10 minutes and removal of the supernatant, the resulting platelet pellet was resuspended in Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl2).

Cell culture and transfection

The human megakaryoblastic leukaemia cell line MEG-01 (DSMZ, Braunschweig, Germany) was cultured in RPMI-1640 medium with Glutamax (Gibco), 10 % foetal bovine serum (Gibco) and 1 % penicillin/streptomycin (Gibco) in a humidified atmosphere at 37°C and 5 % CO2. MEG-01 and human embryonic kidney (HEK293) cells were transiently transfected for 48 hours with the constitutively active SGK1 mutant S422DSGK1 (hSGK1SD in pCDNA3.1), which does not require activation by phosphoinositide dependent kinase PDK1, or the inactive mutant K127NSGK1 (hSGK1KN in pCDNA3.1), which lacks catalytic activity. Transfections were performed as described previously using FuGENE HD transfection reagent (Roche) according to manufacturers instructions. For experiments with pharmacological inhibition of SGK1 or IκB kinase (IKK), 1 µM GSK650394 (Solvay) or 10 µM BMS-345541 (Sigma-Aldrich) was added.

Mice

Gene targeted mice lacking functional SGK1 (sgk1−/−) and the corresponding wild type littermate mice (sgk1+/+) were generated and bred as described by Wulff et al. Animals were
genotyped by polymerase chain reaction (PCR). All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities.

**Preparation of mouse platelets**

Platelets were obtained from 10- to 12-week-old sgk1−/− mice and sgk1+/+ mice of either sex. The mice were anesthetized with ether and blood was drawn from the retroorbital plexus into heparinized tubes. Blood parameters were analyzed with pocH-100iv automatic hematology analyzer (Sysmex). Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. To ease platelets apyrase (0.02 U/ml, Sigma-Aldrich) and prostaglandin I₂ (0.5 µM, Calbiochem) were added to the PRP. After two washing steps the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl₂).

**Isolation and culture of murine megakaryocytes**

For the isolation of murine sgk1−/− and sgk1+/+ megakaryocytes, bone marrow cells were harvested by flushing the femurs and tibiae with phosphate-buffered saline as described by Shivdasani et al.²⁶ The obtained cells were separated over Percoll (GE Healthcare) and cultured in specific growth medium (MethoCult®, Stemcell) containing 50 ng/ml thrombopoietin (Invitrogen) as described previously.²⁷ After 5 to 7 days differentiation into megakaryocytes was tested by microscopy and glycoprotein Ib (GPIb) staining.

**RT-PCR analysis**

To determine SGK1 and Orai1 mRNA abundance in platelets and megakaryocytes from sgk1−/− and sgk1+/+ mice as well as in MEG-01 cells mRNA was extracted and quantitative real-time PCR was performed as described in supplemental materials and methods.

**Flow cytometry**

Two-color analysis of mouse platelet activation was conducted using fluorophore-labeled antibodies for P-selectin expression (Wug.E9-FITC) and the active form of α₁bβ₃ integrin (JON/A-PE). Heparinized whole blood was diluted 1:20 in modified Tyrode buffer and washed twice. After adding 1 mM CaCl₂, blood samples were mixed with antibodies and subsequently stimulated with agonists for 15 minutes at room temperature (RT). For analysis of phosphatidylserine exposure washed platelets were diluted in Tyrode buffer containing 2
mM CaCl₂, and activated with convulxin and/or thrombin for 15 minutes and stained with Annexin V-Fluos at RT. For measuring Orai1 surface expression washed platelets were incubated for 60 minutes (37°C) with Orai1 rabbit anti-mouse antibody (Proteintech), washed once in PBS and stained in 1:500 diluted FITC-labeled goat anti-rabbit secondary antibody (Invitrogen) for 30 minutes (37°C). In all approaches reaction was stopped by addition of PBS and samples immediately analyzed on a FACS Calibur flow cytometer (BD Biosciences).

**Platelet aggregometry**

Aggregation experiments were performed in diluted whole blood with electrode impedance aggregometry (Model 700, Chrono-Log). Citrate-anticoagulated whole blood was diluted with physiological saline. Following calibration agonists were added at the indicated concentrations and aggregation was measured for 10 minutes with a stir speed of 1.000 rpm at 37°C. The extent of aggregation was quantified in ohms (Ω) by comparing the deflection of the trace with the calibration mark representing 20 Ω. The data analysis was performed with AGGRO/LINK8 software (Chrono-Log).

**Calcium measurements**

Washed murine platelets were suspended in Tyrode buffer without calcium and loaded with 5 µM Fura-2 acetoxyxymethylester (Invitrogen) in the presence of 0.2 µg/ml Pluronic F-127 (Biotium) at 37°C for 30 minutes. Loaded platelets, washed once and resuspended in Tyrode buffer containing 0.5 mM EGTA (Roth) or 1 mM Ca²⁺, were activated with agonists. Calcium responses were measured under stirring with a spectrofluorimeter (LS 55, PerkinElmer), at alternate excitation wavelength of 340 and 380 nm (37°C). The 340/380 nm ratio values were converted into nanomolar concentrations of [Ca²⁺] by lysis with Triton X-100 (Sigma-Aldrich) and a surplus of EGTA. Harvested MEG-01 cells were centrifuged at 530 g for 5 minutes and then resuspended in RPMI medium (Gibco) and stained as described above. Measurements were performed in Ca²⁺ free Tyrode buffer. After incubation with 5 µM thapsigargin (Invitrogen) for 10 minutes 1 mM Ca²⁺ was added. Isolated human platelets were incubated 60 minutes prior to measurement with the SGK1 inhibitor GSK650394 (1 µM, Solvay), the SOCE inhibitor 2-APB (50 µM, Sigma) or DMSO as solvent control at 37°C.

**Western blot analysis**

MEG-01 or HEK293 cells, freshly isolated human platelets or pooled mouse platelets were centrifuged for 5 minutes at 240 g and the pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Trion-X, 0.5% Na₂HPO₄, 0.4% β-Mercaptoethanol)
containing protease inhibitor cocktail (Sigma-Aldrich). After centrifugation for 30 minutes at 20,000 g and 4°C the supernatant was taken and the protein concentration was measured with Bradford (Biorad). For immunoblotting proteins were electrotransferred onto a nitrocellulose membrane and blocked with 5% nonfat milk or 5% bovine serum albumin at RT for 1 hour. Then, the membrane was incubated with the primary antibody against SGK1 (1:100, Pineda), Orai1 (1 µg/ml, Abcam), phospho-IKKα/β (Ser176/180, 1:1000, Cell Signaling) or phospho-IκBα (Ser32/36 1:1000, Cell Signaling) at 4°C overnight. After washing with TBST the blots were incubated with secondary antibody conjugated with horse radish peroxidase (HRP) (1:2000, Cell Signaling) for 1 hour at RT. After washing antibody binding was detected with the ECL detection reagent (Amersham). Bands were quantified with Quantity One Software (Biorad). Membrane protein extraction was performed as described in supplemental materials and methods.

**Immunofluorescence and confocal microscopy**

Washed platelets were allowed to adhere to a fibrinogen surface (20 µg/ml) on a chamber slide, MEG-01 cells and murine megakaryocytes were adhered to poly-L-lysine (Sigma-Aldrich). Adherent platelets, megakaryocytes, MEG-01 or HEK293 cells were fixed with paraformaldehyde (2%), washed and blocked with 2% bovine serum albumin for 30 minutes, followed by incubation with the primary antibody for 2 hours at RT. Primary antibodies against SGK1 (1:100, Pineda), Orai1 (1:1000, Millipore), NF-κB p65 (1:250, Santa Cruz) and GP1bα (1:200, Emfret) were used. Chamber slides were washed and incubated with a FITC- or Cy3-conjugated secondary antibody (Santa Cruz). The actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen), nuclei were stained with DRAQ-5 dye (1:2000, Biostatus). The slides were mounted with ProLong Gold antifade reagent (Invitrogen). Confocal microscopy was performed using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging) with a A-Plan 63x ocular.

**Flow chamber**

Heparinized whole mouse blood was diluted 1:3 in modified Tyrode buffer and perfused through a transparent flow chamber (slit depth 50 µm) over a collagen-coated surface (200 µg/ml) with a wall shear rate of 1700 s⁻¹ for 5 minutes. After perfusion the chamber was rinsed for 5 minutes by perfusion with Tyrode buffer and pictures were taken from 5 to 6 different microscopic areas (20x, Carl Zeiss). Analysis was done with AxioVision (Carl Zeiss) and the mean percentage value of the covered area was determined.
Bleeding time

Mice were anesthetized and a 3-mm segment of the tail tip was removed with a scalpel. Tail bleeding was monitored by gentle absorption of the blood with filter paper at 20 seconds intervals without making contact with the wound site. When no blood was observed on the paper, bleeding was determined to have ceased. Experiments were stopped after 20 minutes.

Statistical analysis

Data are provided as means ± SD or SEM, \( n \) represents the number of experiments. All data were tested for significance using paired or unpaired Student t-test and one-way ANOVA with Dunnett's post-hoc test. Results with *(p<0.05), **(p<0.01) or ***(p<0.001) were considered statistically significant.
Results

In an initial experiment, expression of the serum- and glucocorticoid-inducible kinase 1 (SGK1) was analysed in human as well as in murine platelets and megakaryocytes. Reverse transcription polymerase chain reaction (RT-PCR) analysis, confocal microscopy and western blot analysis of human platelets revealed strong expression of SGK1 in platelets both at mRNA and protein level (Fig. 1 A, B). The expression level was comparable to that in human embryonic kidney (HEK293) cells.

To study the functional role of SGK1 in regulating platelet function, platelets and megakaryocytes were isolated from mice lacking SGK1 (sgk1⁻/⁻) and respective wildtype littermates (sgk1⁺/⁺). RT-PCR analysis, immunofluorescence staining and western blotting confirmed the absence of sgk1 mRNA and SGK1 protein in sgk1⁻/⁻ platelets (Fig. 1 C, D) and megakaryocytes (Fig. 1 E, F).

Sgk1⁻/⁻ mice appeared healthy and did not exhibit spontaneous bleeding. Blood platelet counts and mean platelet volume (MPV) were similar in sgk1⁺/⁺ and sgk1⁻/⁻ mice (Tab. 1), indicating that SGK1 is not essential for platelet generation. Also no differences were found in other hematological parameters (Tab. 1) or platelet specific glycoproteins (Suppl. Fig. 1).

To elucidate the impact of SGK1 on platelet activation, platelet degranulation, integrin α₁β₃ activation and phosphatidylserine exposure flow cytometric measurements were performed. Degranulation-dependent P-selectin surface exposure was quantified prior to and following activation with ADP (10 µM), thrombin (0.02 U/ml) as well as the specific agonists of the collagen receptor glycoprotein VI (GPVI) convulxin (CVX, 1 µg/ml) and collagen-related peptide (CRP, 5 µg/ml). As illustrated in Fig. 2 A, P-selectin abundance at the platelet surface was significantly lower in sgk1⁻/⁻ platelets than in sgk1⁺/⁺ platelets following stimulation with convulxin and CRP. Degranulation following stimulation with thrombin tended to be lower in sgk1⁻/⁻ platelets than in sgk1⁺/⁺ platelets, a difference, however, not reaching statistical significance.

Following stimulation with convulxin and CRP, surface expression of activated integrin α₁β₃ was significantly lower in sgk1⁻/⁻ platelets than in sgk1⁺/⁺ platelets (Fig. 2 B). Following low dose concentrations of thrombin or ADP the activation of integrin α₁β₃ was not significantly different between sgk1⁻/⁻ and sgk1⁺/⁺ platelets.

For examination of activation-dependent phosphatidylserine exposure, platelets were stimulated with thrombin (1.0 U/ml), CVX (1 µg/ml) or thrombin/CVX (0.05 U/ml/0.5 µg/ml) and annexin positive cells were analyzed by flow cytometry. Following stimulation with thrombin, CVX or the combination (thrombin/CVX), surface exposure of phosphatidylserine was significantly lower in sgk1⁻/⁻ platelets than in sgk1⁺/⁺ platelets (Fig. 2 C).
To determine whether impaired degranulation, integrin α_{IIb}β_{3} activation and phosphatidylserine exposure would translate into functional deficits in $sgk1^{-/-}$ platelets, we performed impedance measurements of platelet aggregation prior to and following activation with low or high concentrations of CRP (1 and 10 µg/ml), collagen (1 and 5 µg/ml), PAR-4 activating peptide (125 and 500 µM) and ADP (2.5 and 10 µM). As illustrated in Fig. 2 D, platelet aggregation following stimulation with low dose concentrations of the GPVI-acting ligands collagen or CRP was significantly less pronounced in $sgk1^{-/-}$ platelets than in $sgk1^{+/+}$ platelets. Only a slight difference was found after stimulation with low-dose PAR-4 activating peptide, an agonist of the principal murine thrombin receptor. The aggregation defect found in $sgk1^{-/-}$ platelets was overcome by increasing the agonist concentration.

To elucidate the relevance of SGK1 in pathologic thrombus formation, we examined platelet adhesion to collagen-coated surfaces under flow at high arterial shear rates (1700 s⁻¹). As illustrated in Fig. 2 E, $sgk1^{+/+}$ platelets formed massive and dense thrombi after 5 minutes perfusion, whereas $sgk1^{-/-}$ platelets formed only some smaller single thrombi with a significantly reduced thrombus surface coverage. To test whether the defect in $sgk1^{-/-}$ platelets impaired hemostasis, we measured tail bleeding time. As shown in Fig. 2 F, the bleeding time was not significantly different in $sgk1^{-/-}$ mice compared to $sgk1^{+/+}$ mice.

Platelet activation including integrin α_{IIb}β_{3} activation, granule release, phosphatidylserine exposure, aggregation and thrombus formation are directly dependent on an increase in the intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ). Spectrofluorimetric measurements were thus employed to elucidate the impact of SGK1 on the increase of cytosolic Ca²⁺ activity following platelet activation by thrombin (0.02 U/ml), CVX (1 µg/ml), CRP (10 µg/ml) and ADP (10 µM).

To discriminate between intracellular Ca²⁺ store release and Ca²⁺ influx from extracellular space, we performed measurements of activation-dependent changes in [Ca²⁺]ᵢ in presence (1 mM Ca²⁺) and absence (0.5 mM EGTA) of extracellular Ca²⁺ (Fig. 3 A, B). Prior to stimulation, the cytosolic Ca²⁺ concentration was similar in $sgk1^{+/+}$ and $sgk1^{-/-}$ platelets. In presence of extracellular Ca²⁺ all agonists triggered an increase of cytosolic Ca²⁺ activity in $sgk1^{+/+}$ and $sgk1^{-/-}$ platelets. The increase of cytosolic Ca²⁺ activity was, however, less pronounced in $sgk1^{-/-}$ platelets than in $sgk1^{+/+}$ platelets, a difference reaching statistical significance after stimulation with convulxin and CRP (Fig. 3 A, B). In the absence of extracellular Ca²⁺ the increase of cytosolic Ca²⁺ activity following stimulation with either agonist was similar in $sgk1^{-/-}$ and $sgk1^{+/+}$ platelets.
The impaired agonist induced Ca\(^{2+}\) response in the presence of extracellular Ca\(^{2+}\) pointed to an impaired store operated Ca\(^{2+}\) entry (SOCE) in SGK1-deficient platelets. To test this hypothesis, SOCE was induced in \(sgk1^{+/+}\) and \(sgk1^{-/-}\) platelets with SR/ER Ca\(^{2+}\) ATPase (SERCA) pump inhibitor thapsigargin (TG). In the absence of extracellular Ca\(^{2+}\) thapsigargin (5 \(\mu\)M) triggered a Ca\(^{2+}\) release from intracellular stores (store release) which was similar in \(sgk1^{+/+}\) platelets and in \(sgk1^{-/-}\) platelets (Fig. 3 C). The subsequent addition of extracellular Ca\(^{2+}\) in the continued presence of thapsigargin triggered a SOCE, which was significantly less pronounced in \(sgk1^{-/-}\) platelets than in \(sgk1^{+/+}\) platelets. These results demonstrate for the first time that SGK1 is a regulator of platelet SOCE contributing to the regulation of cytosolic Ca\(^{2+}\) activity in platelets and platelet activation.

To test if the decreased SOCE could have been due to a decreased cell membrane protein abundance of the major platelet SOCE-mediating channel Orai1, we compared the Orai1 protein expression in \(sgk1^{+/+}\) and \(sgk1^{-/-}\) platelets. As shown in Fig. 4 A-D, western blotting, immunofluorescence/confocal microscopy and FACS analysis all disclosed that Orai1 protein (membrane) abundance was significantly lower in \(sgk1^{-/-}\) platelets than in \(sgk1^{+/+}\) platelets, whereas STIM1 expression was not significantly different.

Immunofluorescence/confocal microscopy revealed that Orai1 protein abundance was again significantly lower in \(sgk1^{-/-}\) megakaryocytes compared to \(sgk1^{+/+}\) megakaryocytes (Fig. 4 E). Further experiments were performed in the human megakaryocytic cell line MEG-01, an extensively used model of human megakaryocytes. According to confocal microscopy and western blot analysis, the protein abundance of Orai1 in the plasma membrane of MEG-01 cells was significantly increased following transfection with the constitutively active mutant \(S422DSGK1\) but not following transfection with the inactive mutant \(K127NSGK1\) (Fig. 5 A, B). Addition of the store-depleting SERCA inhibitor thapsigargin (5 \(\mu\)M) in nominally Ca\(^{2+}\)-free solution was followed by rapid, transient increase in cytosolic Ca\(^{2+}\) in MEG-01 cells (Fig. 5 C). Subsequent addition of Ca\(^{2+}\) to the extracellular medium resulted in a rapid and sustained increase in cytosolic Ca\(^{2+}\) due to store operated Ca\(^{2+}\) entry (SOCE). Both, slope and peak of MEG-01 SOCE were significantly enhanced by transient expression of the constitutively active mutant \(S422DSGK1\) but not of the inactive mutant \(K127NSGK1\) (Fig. 5 C).

The basal fura-2 fluorescence ratio, reflecting resting intracellular Ca\(^{2+}\) concentration, was similar in MEG-01 cells transfected with control plasmid, in MEG-01 cells transfected with \(S422DSGK1\) and in MEG-01 cells transfected with \(K127NGSK1\). Moreover, the Ca\(^{2+}\) release from intracellular stores following thapsigargin (5 \(\mu\)M) treatment was not significantly different between MEG-01 cells transfected with control plasmid, MEG-01 cells transfected
with \textsuperscript{S422D}SGK1 and MEG-01 cells transfected with \textsuperscript{K127N}SGK1 (Fig. 5 C). The specific SGK1 inhibitor GSK650394 (1 µM) abolished the \textsuperscript{S422D}SGK1-induced upregulation of Orai1 expression and SOCE (Fig. 5 D, E).

Quantitative RT-PCR revealed that Orai1 mRNA levels were significantly lower in \textit{sgk1}^{-/-} platelets and megakaryocytes than in \textit{sgk1}^{+/+} platelets and megakaryocytes (Fig. 6 A). Transfection of MEG-01 cells with \textsuperscript{S422D}SGK1, but not with \textsuperscript{K127N}SGK1 significantly increased Orai1 mRNA levels (Fig. 6 B). Those observations pointed to SGK1-sensitive transcriptional regulation of Orai1 in megakaryocytes.

Further experiments aimed to disclose the underlying mechanism of SGK1-dependent transcriptional regulation of Orai1. Transfection with \textsuperscript{S422D}SGK1 but not transfection with \textsuperscript{K127N}SGK1 significantly increased phosphorylation of the NF-κB regulating kinases IκB kinase (IKK) α/β (Fig. 6 C) and IκBα (Fig. 6 D) in MEG-01 cells. The upregulation of Orai1 mRNA 48 hours following \textsuperscript{S422D}SGK1 transfection was abrogated following a 24 hours incubation with the highly selective IKK inhibitor BMS-345541 (10 µM) or the SGK1 inhibitor GSK650394 (1 µM) (Fig. 6 E). Furthermore the \textsuperscript{S422D}SGK1-induced upregulation of SOCE could be inhibited by treatment with BMS-345541 (10 µM) as described above (Fig. 6 F). Finally, as evident from immunofluorescence/confocal microscopy, the nuclear translocation of the NF-κB subunit p65 reflecting transcriptional activity was significantly less pronounced in \textit{sgk1}^{-/-} megakaryocytes than in \textit{sgk1}^{+/+} megakaryocytes (Fig. 6 G). Consistent with that finding we found an increased expression of p65 in isolated nuclear fractions of MEG-01 cells transfected with \textsuperscript{S422D}SGK1 compared to untransfected MEG-01 cells or to cells transfected with \textsuperscript{K127N}SGK1 (Suppl. Fig. 5).
Discussion

Platelets play a central role in the pathogenesis of arterial thrombosis and the mechanisms regulating the adhesive functions of platelets are thus of pivotal importance for occlusive cardiovascular disease.\(^1,2,3\) The present study unravels a novel regulator of platelet function, the phosphoinositide-3-kinase (PI3K) pathway downstream effector serum- and glucocorticoid-inducible kinase 1 (SGK1). SGK1 belongs to the AGC family of serine-threonine kinases and shares a relatively high degree of homology with Akt in its catalytic domain.\(^1,6,17\) Previous work has identified a critical role for PI3K\(^1,3-15\) and some of its downstream effectors (e.g. the AGC family members Akt\(^1,2,9\) and protein kinase C\(^3,0\)) in promoting and maintaining platelet activation including activation-dependent Ca\(^{2+}\) signaling. But the molecular targets of the products of PI3K in their respective functions have been incompletely defined.\(^29\) In this study we could show for the first time that SGK1 is a novel important regulator of cytosolic Ca\(^{2+}\) and function of platelets and megakaryocytes.

Similar to observations in platelets completely lacking Orai1\(^7\) or expressing a functional inactive Orai1 mutant (\(^{R93W}\)Orai1)\(^6\), the impact of SGK1 deficiency on activation-dependent degranulation or integrin \(\alpha_{IIb}\beta_3\) activation is more pronounced following application of GPVI agonists than following administration of thrombin or ADP. Possibly, thrombin or ADP stimulate in addition Orai1 independent Ca\(^{2+}\) entry, whereas Orai1-mediated Ca\(^{2+}\) entry is particularly important for GPVI-ITAM-mediated cell activation.\(^31\)

The decreased activation of integrin \(\alpha_{IIb}\beta_3\) in platelets lacking SGK1 paralleled a deficit in the ability of these platelets to undergo aggregation at low dose concentrations of agonists. Increasing the agonists concentrations dissipated the differences between \(sgk1^{-/-}\) and \(sgk1^{+/+}\) platelets, indicating that SGK1 deficiency enhances the sensitivity of platelets to activating agonists but does not modify the maximal effect following full activation. Apparently, the abundance of Orai1 is limiting following moderate but not following full platelet activation. Clearly, SGK1 deficiency does not abrogate SOCE and Ca\(^{2+}\)-sensitive platelet function but decreases the sensitivity of the platelets to stimulators of Ca\(^{2+}\) entry. In the absence of SGK1 apparently other signaling molecules such as Akt maintain basic platelet function. Thus, in contrast to the severe phenotype of Orai1-deficient mice\(^7\), the phenotype of \(sgk1^{-/-}\) mice is mild.

Rupture of an atherosclerotic lesion leads to endothelial denudation and exposure of the thrombogenic subendothelial collagen to circulating platelets, initiating platelet recruitment to the injured vessel wall.\(^32\) According to the present study, lack of SGK1 decreases collagen-
triggered thrombus formation under high shear stress. Platelet responses including degranulation, integrin αIIbβ3 activation, thrombus formation and especially phosphatidylserine exposure, which collectively accomplish platelet procoagulant activity, critically depend on an increase in [Ca2+]i. An increase of [Ca2+]i may result from release of intracellular Ca2+ compartmentalized in endoplasmic reticulum (ER) and entry of extracellular Ca2+ triggered by the depletion of SR Ca2+ stores, the store operated Ca2+ entry (SOCE). SGK1 deficiency markedly decreased platelet SOCE. While the filling and release of the intracellular Ca2+ stores were unaffected in sgk1−/− platelets, SOCE was markedly reduced in platelets of SGK1 deficient mice. Furthermore sgk1−/− platelets and megakaryocytes expressed less Orai1 Ca2+ channel protein in their cell membrane. Orai1 is a plasma membrane protein and the pore forming unit for the store-operated Ca2+ entry (SOCE). Orai1 is regulated by STIM1. STIM1 senses the Ca2+ content of the intracellular stores in platelet endoplasmic reticulum (dense tubular system) and activates the plasma membrane Orai1 upon store depletion. Both, Orai1 and STIM1 have previously been shown to be critically important for proper function of platelets. Besides the decreased Orai1 protein abundance in sgk1−/− platelets, SGK1 sensitivity of Orai1 expression was apparent from transfections with the constitutively active mutant (S422D)SGK1 or the inactive mutant (K127N)SGK1 of SGK1 in the human megakaryoblastic cell line MEG-01, cells with many properties in common with normal human megakaryocytes at an early stage of maturation. MEG-01 cells regulate the cytosolic Ca2+ concentration in response to activation or store depletion via the same mechanisms, which are operative in platelets and megakaryocytes. Furthermore MEG-01 cells highly express the store-operated Ca2+ channel Orai1 as well as the store Ca2+ sensor STIM1 and thus represent an ideal model for studying mechanisms regulating SOCE in human megakaryocytes. Transfection with the constitutively active mutant S422D SGK1, but not transfection with the inactive mutant K127N SGK1, significantly increased Orai1 membrane abundance and SOCE, an effect abrogated in the presence of GSK650394 (1 µM), a specific SGK1 inhibitor. Accordingly, SGK1 regulates membrane expression of Orai1 and SOCE in megakaryocytes.

The decreased Orai1 protein abundance in sgk1−/− platelets could have resulted from decreased Orai1 transcription or accelerated degradation of Orai1 protein. As sgk1−/− platelets and megakaryocytes contain significantly less Orai1 mRNA than sgk1+/+ platelets and megakaryocytes SGK1 is at least partially effective by upregulating Orai1 transcription. Similarly, Orai1 mRNA levels in MEG-01 cells were increased by transfection with S422D SGK1 but not by transfection with K127N SGK1. Apparently, SGK1 does not directly
influence SOCE, agonist-induced Ca\(^{2+}\) entry or Ca\(^{2+}\)-dependent platelet activation, as
degranulation or integrin \(\alpha_{IIb}\beta_3\) activation were not directly modified by SGK1 inhibitor
GSK650394 (Suppl. Fig. 2 and 3).

Further experiments addressed the mechanism mediating SGK1-dependent Orai1
transcription. SGK1 is known to regulate transcription by up-regulating nuclear factor \(\kappa B\)
(NF-\(\kappa B\)) activity through phosphorylation and activation of IkB kinase (IKK) \(\alpha/\beta\).\(^{16,40}\) Thus
SGK1 enhances the ability of IKK\(\alpha/\beta\) to phosphorylate endogenous IkBa.\(^{41}\) The majority of
NF-\(\kappa B\) family members, including the regulatory IkB and IKK, are expressed in both MEG-
01 cells and human megakaryocytes.\(^{42}\) Functionally NF-\(\kappa B\) has been shown to regulate
megakaryocytic differentiation.\(^{43}\) Thus, there is evolving evidence pointing to a decisive role
of NF-\(\kappa B\) in regulating gene expression in megakaryocytes. Nevertheless, megakaryocytic
proteins which are under transcriptional control of NF-\(\kappa B\) and NF-\(\kappa B\)-activating mechanisms
in megakaryocytes remain to be clarified. In the present study, transfection with \(S^{422D}\)SGK1
but not with \(K^{127N}\)SGK1 significantly increased IKK\(\alpha/\beta\) and IkBa phosphorylation in
megakaryocytic MEG-01 cells. NF-\(\kappa B\) activation could be disrupted by small-molecule
inhibitors of IKK\(\beta\).\(^{44}\) Accordingly, the highly selective IkB kinase (IKK) inhibitor BMS-
345541 (10 \(\mu M\)), which shows greater than tenfold selectivity for IKK\(\beta\) than for IKK\(\alpha\),\(^{45}\)
abrogated the upregulation of Orai1 mRNA induced by transfection of MEG-01 cells with
\(S^{422D}\)SGK1 and abolished the SGK1-induced increase of SOCE (Fig. 6 F).

NF-\(\kappa B\) is held latent in the cytoplasm as a complex bound with unphosphorylated IkB,
thereby blocking the nuclear translocation of NF-\(\kappa B\).\(^{46}\) Phosphorylation of IkB by IKK leads
to proteosomal degradation of IkB liberating the NF-\(\kappa B\) dimers (mostly p50-p65 dimers) to
translocate into the nucleus and initiate transcription of target genes.\(^{47}\) Accordingly, nuclear
translocation of p65 was less pronounced in \(sgk1^{-/-}\) megakaryocytes than in \(sgk1^{+/+}\)
megakaryocytes and transfection of MEG-01 cells with \(S^{422D}\)SGK1 increased nuclear
expression of p65.

IKK\(\beta\)-dependent NF-\(\kappa B\) activation plays a key role in inflammation and inflammatory
signaling pathways in metabolic diseases\(^{48}\) as diabetes or the metabolic syndrome which are
classically associated with platelet hyperresponsiveness and atherothrombotic complications,
such as myocardial infarction or ischemic stroke\(^{49}\). Hyperglycemia, glucose-induced AGEs
and oxidative stress are powerful stimulators of SGK1\(^{17}\) and its downstream target NF-\(\kappa B\)
\(^{48}\). Platelets from patients suffering from type 2 diabetes show increased degranulation, adhesion
and aggregation of platelets\(^{49}\), which could be a result of an increased Orai1 expression and
SOCE found in these platelets\(^{50}\). As SGK1 is strongly upregulated in diabetic
hyperglycemia, we speculate that an increased stimulation of SGK1 in these patients could contribute to megakaryocytic NF-κB induction, stimulation of Orai1 expression resulting in enhanced SOCE and increased activation-dependent responsiveness of their platelets. In view of the present observations gain of function SGK1 polymorphisms could increase platelet responsiveness thus predisposing the carriers to thrombotic complications. In a recent study a common gain of function SGK1 gene variant indeed has been identified to be associated with ischemic stroke.

As a result of the strong effect of SGK1 on Orai1 expression and the similarity between the phenotype of Orai1-deficient and SGK1-deficient platelets, it appears safe to conclude that the SGK1-dependent regulation of Orai1 protein abundance substantially contributes to SGK1-sensitive platelet function. However, it must be kept in mind that SGK1 regulates a variety of further carriers and channels, enzymes and transcription factors, which, at least in theory, could participate in the modulation of platelet function independently of regulating Orai1.

In conclusion, the present observations identify SGK1 as a novel transcriptional regulator of Orai1 in megakaryocytes which is at least partially effective through activation of NF-κB. Thus, SGK1-dependent Orai1 regulation in megakaryocytes can influence SOCE and activation-dependent Ca²⁺ entry as well as Ca²⁺-dependent mechanisms such as degranulation, aggregation and thrombus formation in released platelets.
Acknowledgments

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Authorship Contributions

O.B. performed experiments, analyzed data, designed research and contributed to the writing of the manuscript; E.-M.S., P.M., T.S., S.T., M.E., C.L. E.S. and A.E. performed experiments and analyzed data; A.M., D.K., M.G. and F.L. analyzed data, designed research and contributed to the writing of the manuscript.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.
References


Table

Table 1: Blood count of sgk1<sup>+/+</sup> and sgk1<sup>−/−</sup> mice.

Figures

Fig. 1: SGK1 mRNA and protein expression in human as well as in murine platelets and megakaryocytes.

A. Quantitative RT-PCR of mRNA encoding SGK1 in human platelets and HEK293 cells.
B. Western blot and confocal microscopy of SGK1 abundance in human platelets, non-transfected and S422D SGK1-transfected HEK293 cells. Red – actin, green – SGK1. Scale bar equals 5 µm.
C. Arithmetic means ± SEM (n = 4) of mRNA encoding SGK1 in platelets from sgk1<sup>−/−</sup> mice expressed in % of the respective value in platelets from sgk1<sup>+/+</sup> mice. ***(p<0.001) indicates statistically significant difference.
D. Representative western blot analysis of SGK1 protein abundance in platelets from sgk1<sup>+/+</sup> and sgk1<sup>−/−</sup> mice.
E. Arithmetic means ± SEM (n = 4) of mRNA encoding SGK1 in megakaryocytes from sgk1<sup>−/−</sup> mice expressed in % of the respective value in megakaryocytes from sgk1<sup>+/+</sup> mice. ***(p<0.001) indicates statistically significant difference.
F. Confocal microscopy of SGK1 protein abundance in megakaryocytes from sgk1<sup>+/+</sup> (top) and sgk1<sup>−/−</sup> (bottom) mice. Red – glycoprotein Ib, green – SGK1, blue – nuclei. Scale bar equals 10 µm.

Fig. 2: Activation-dependent platelet degranulation, α<sub>IIb</sub>β<sub>3</sub> integrin activation, phosphatidylinerine exposure and aggregation as well as in vitro thrombus formation and tail bleeding time.

A. Flow cytometric analysis of degranulation dependent P-selectin exposure in platelets from sgk1<sup>+/+</sup> (black bar) and sgk1<sup>−/−</sup> (grey bar) mice in response to 10 µM ADP, 0.02 U/ml thrombin, 1 µg/ml convulxin (CVX) and 5 µg/ml collagen related peptide (CRP). Arithmetic means ± SEM (n = 6) are shown. *(p<0.05) and ***(p<0.01) indicate statistically significant difference.
B. Flow cytometric analysis of α<sub>IIb</sub>β<sub>3</sub> integrin activation in platelets from sgk1<sup>+/+</sup> (black bar) and sgk1<sup>−/−</sup> (grey bar) mice in response to 10 µM ADP, 0.02 U/ml thrombin, 1 µg/ml convulxin (CVX) and 5 µg/ml collagen related peptide (CRP). Arithmetic means ± SEM (n = 6) are shown. *(p<0.05) and ***(p<0.01) indicate statistically significant difference.
C. Flow cytometric analysis of phosphatidylserine exposure in platelets from $sgk1^{+/+}$ (black bar) and $sgk1^{-/-}$ (grey bar) mice in response to 1.0 U/ml thrombin, 1 µg/ml convulxin (CVX) and 0.05 U/ml thrombin + 0.5 µg/ml CVX. (Top) Arithmetic means ± SEM (n = 9) are shown, *(p<0.05) indicates statistically significant difference. (Bottom) Representative overlays of Annexin-positive $sgk1^{+/+}$ (black line) and $sgk1^{-/-}$ (grey line) platelets, light grey panels represent isotype controls.

D. Impedance aggregometry after stimulation with different concentrations of CRP (1 and 10 µg/ml), collagen (1 and 5 µg/ml), PAR-4 activating peptide (125 and 500 µM) and ADP (2.5 and 10 µM). Representative aggregation tracings of $sgk1^{+/+}$ (black line) and $sgk1^{-/-}$ (grey line) mice are shown (n = 4).

E. Thrombus formation in vitro. Whole blood from $sgk1^{+/+}$ and $sgk1^{-/-}$ mice was perfused over a collagen-coated surface for 5 minutes at a shear rate of 1700 s⁻¹. Arithmetic means ± SEM (n = 6) (top) and representative phase contrast images (bottom) of surface coverage are shown. **(p<0.01) indicates statistically significant difference. Scale bar equals 50 µm.

F. Tail bleeding time measured after amputating the tail tip of $sgk1^{+/+}$ and $sgk1^{-/-}$ mice. Each dot represents one individual, black bar represents the mean value.

Fig. 3: Agonist-induced Ca²⁺ response and impaired store operated Ca²⁺ entry (SOCE) in $sgk1^{-/-}$ and $sgk1^{+/+}$ platelets.

A. Representative tracings of Fura-2-fluorescence reflecting cytosolic Ca²⁺ concentration $[\text{Ca}^{2+}]_i$ of $sgk1^{+/+}$ (black line) and $sgk1^{-/-}$ (grey line) platelets prior to and following stimulation with thrombin (0.02 U/ml), CVX (1 µg/ml), CRP (10 µg/ml) and ADP (10 µM) in the absence (0.5 mM EGTA, left) or presence (1 mM Ca²⁺, right) of extracellular Ca²⁺.

B. Arithmetic means of maximal $\Delta[\text{Ca}^{2+}]_i$ ± SD (n = 4 per group) are shown. **(p<0.01) indicates statistically significant difference.

C. Fura-2-fluorescence reflecting cytosolic Ca²⁺ concentration $[\text{Ca}^{2+}]_i$ of $sgk1^{+/+}$ (black line) and $sgk1^{-/-}$ (grey line) platelets following exposure to 5 µM thapsigargin in the nominal absence of extracellular Ca²⁺ for 10 minutes and subsequent addition of 1 mM extracellular Ca²⁺. Representative tracings (left) and arithmetic means (right) of maximal $\Delta[\text{Ca}^{2+}]_i$ ± SD (n = 6 per group) before and after addition of 1 mM Ca²⁺ are shown. **(p<0.01) indicates statistically significant difference.

Fig. 4: Orai1 protein abundance in $sgk1^{-/-}$ and $sgk1^{+/+}$ platelets and megakaryocytes.
A. Confocal microscopy of Orai1 protein abundance in platelets from \textit{sgk1}\textsuperscript{+/-} (left panels) and \textit{sgk1}\textsuperscript{-/-} (right panels) mice. Red – actin, green – Orai1. Scale bar equals 10 µm.

B. Flow cytometric analysis of Orai1 surface expression in platelets from \textit{sgk1}\textsuperscript{+/-} (black bar) and \textit{sgk1}\textsuperscript{-/-} (grey bar) mice. (Bottom) Arithmetic means ± SEM (n = 7) are shown, **(p<0.01) indicates statistically significant difference. (Top) Representative overlays of Orai1-positive \textit{sgk1}\textsuperscript{+/-} (black line) and \textit{sgk1}\textsuperscript{-/-} (grey line) platelets, light grey panel represents isotype control.

C. Western blot analysis of whole cell lysate protein of Orai1 and STIM1 from \textit{sgk1}\textsuperscript{+/-} and \textit{sgk1}\textsuperscript{-/-} platelets. Arithmetic means ± SEM (n = 7) of Orai1 protein abundance in \textit{sgk1}\textsuperscript{+/-} (black bar) and \textit{sgk1}\textsuperscript{-/-} (grey bar) platelets. **(p<0.01) indicates statistically significant difference.

D. Western blot analysis of isolated membrane protein and cytosolic fraction of Orai1 from \textit{sgk1}\textsuperscript{+/-} and \textit{sgk1}\textsuperscript{-/-} platelets. Arithmetic means ± SEM (n = 4) of Orai1 protein abundance in \textit{sgk1}\textsuperscript{+/-} (black bars) and \textit{sgk1}\textsuperscript{-/-} (grey bars) platelets. **(p<0.01) indicates statistically significant difference.

E. Confocal microscopy of Orai1 protein abundance in megakaryocytes cultivated from bone marrow of \textit{sgk1}\textsuperscript{+/-} (left panels) and \textit{sgk1}\textsuperscript{-/-} (right panels) mice (top). Red – glycoprotein Ib, green – Orai1, blue – nuclei. Scale bar equals 10 µm. Statistical analysis of Orai1 immunofluorescence abundance (bottom), **(p<0.01) indicates statistically significant difference (n = 4).

**Fig. 5: SGK1-dependent Orai1 membrane abundance and store-operated Ca\textsuperscript{2+} entry (SOCE) in megakaryocytic cell line MEG-01.**

A. Confocal microscopy of Orai1 membrane abundance in non-transfected (control plasmid), \textit{S422D}SGK1-transfected and \textit{K127NSGK1}-transfected MEG-01 cells (left). Red – actin, green – Orai1, blue – nuclei. Scale bar represents 10 µm. Statistical analysis of Orai1 immunofluorescence membrane abundance (right), *(p<0.05) and **(p<0.01) indicate statistically significant difference (n = 4).

B. Western blot analysis of Orai1 membrane abundance in non-transfected (control plasmid), \textit{S422D}SGK1-transfected and \textit{K127NSGK1}-transfected MEG-01 cells. Arithmetic means ± SEM (n = 7) of Orai1 protein abundance. *(p<0.05) and **(p<0.01) indicate statistically significant difference.

C. SOCE in SGK1-transfected MEG-01 cells. Fura-2-fluorescence reflecting cytosolic Ca\textsuperscript{2+} concentration [Ca\textsuperscript{2+}]\textsubscript{i} of MEG-01 cells transfected with control-plasmid (grey), \textit{S422D}SGK1 (black) or \textit{K127NSGK1} (red) following exposure to 5 µM thapsigargin (Ca\textsuperscript{2+} store depletion) in the nominal absence of extracellular Ca\textsuperscript{2+} for 10 minutes and subsequent addition of 1 mM
extracellular Ca\(^{2+}\). Representative tracings (top) and arithmetic means (bottom) of maximal $\Delta[Ca^{2+}]_i \pm$ SEM (n = 15 per group) before and after addition of 1 mM Ca\(^{2+}\) are shown. **(p<0.01) indicates statistically significant difference.

D. Western blot analysis of Orai1 membrane abundance in non-transfected (control plasmid) MEG-01 cells and in $\text{S}42\text{D}$SGK1-transfected MEG-01 cells treated with the SGK1 inhibitor GSK650394 (1 $\mu$M) or DMSO as solvent control. Arithmetic means $\pm$ SEM (n = 4) of Orai1 protein abundance. **(p<0.01) indicates statistically significant difference.

E. SOCE in MEG-01 cells treated with the specific SGK1 inhibitor GSK650394. Fura-2 fluorescence reflecting cytosolic Ca\(^{2+}\) concentration $[Ca^{2+}]_i$ of MEG-01 cells transfected with control plasmid (grey) or $\text{S}42\text{D}$SGK1-transfected MEG-01 cells after treatment with GSK650394 (1 $\mu$M, red) or DMSO (black) as solvent control. Representative tracings (top) and arithmetic means (bottom) of maximal $\Delta[Ca^{2+}]_i \pm$ SEM (n = 9 per group) following exposure to 5 $\mu$M thapsigargin (Ca\(^{2+}\) store depletion) in the nominal absence of extracellular Ca\(^{2+}\) for 10 min and subsequent addition of 1 mM extracellular Ca\(^{2+}\). **(p<0.01) indicates statistically significant difference.

**Fig. 6: SGK1-sensitive NF-κB-dependent transcription in MEG-01 cells und primary megakaryocytes.**

A. Arithmetic means $\pm$ SEM (n = 4) of mRNA encoding Orai1 in platelets (left) and megakaryocytes (right) from $sgk1^{+/+}$ (black bar) and $sgk1^{-/-}$ mice (grey bar). **(p<0.01) indicates statistically significant difference.

B. Arithmetic means $\pm$ SEM (n = 6) of mRNA encoding Orai1 in non-transfected (control plasmid), $\text{S}42\text{D}$SGK1-transfected and $\text{K}12\text{N}$SGK1-transfected MEG-01 cells of Orai1 mRNA abundance. **(p<0.01) indicates statistically significant difference.

C. Western blot analysis of phospho-IKKα/β in non-transfected (control plasmid), $\text{S}42\text{D}$SGK1-transfected and $\text{K}12\text{N}$SGK1-transfected MEG-01 cells. Arithmetic means $\pm$ SEM (n = 5) of IKKα/β phosphorylation. **(p<0.01) indicates statistically significant difference.

D. Western blot analysis of phospho-IκBα in non-transfected (control plasmid), $\text{S}42\text{D}$SGK1-transfected and $\text{K}12\text{N}$SGK1-transfected MEG-01 cells. Arithmetic means $\pm$ SEM (n = 5) of IκBα phosphorylation. **(p<0.01) indicates statistically significant difference.

E. Arithmetic means $\pm$ SEM (n = 6) of mRNA encoding Orai1 in non-transfected (control plasmid) and $\text{S}42\text{D}$SGK1-transfected MEG-01 cells incubated with SGK1 inhibitor GSK650394 (1 $\mu$M), IκB kinase (IKK) inhibitor BMS-345541 (10 $\mu$M) or DMSO as solvent control. **(p<0.01) indicates statistically significant difference.
F. SOCE in MEG-01 cells treated with the highly specific IKK inhibitor BMS-345541. Fura-2-fluorescence reflecting cytosolic Ca\(^{2+}\) concentration \([\text{Ca}^{2+}]_i\) of MEG-01 cells transfected with control-plasmid (grey) or \(S^{422}\text{SGK1}\)-transfected MEG-01 cells treated with the IKK inhibitor BMS-345541 (10 \(\mu\text{M}, \text{red}\)) or DMSO as solvent control (black) following exposure to 5 \(\mu\text{M}\) thapsigargin (Ca\(^{2+}\) store depletion) in the nominal absence of extracellular Ca\(^{2+}\) for 10 min and subsequent addition of 1 mM extracellular Ca\(^{2+}\). Representative tracings (left) and arithmetic means (right) of maximal \(\Delta[\text{Ca}^{2+}]_i\) ± SEM (\(n = 8\) per group) before and after addition of 1 mM Ca\(^{2+}\) are shown. **(p<0.01) indicates statistically significant difference.

G. Confocal microscopy of nuclear translocation of the NF-\(\kappa\)B subunit p65 (RelA) in murine megakaryocytes cultivated from bone marrow of \(sgk1^{+/+}\) (top) and \(sgk1^{-/-}\) (bottom) mice. Red – glycoprotein Ib, green – p65, blue – nuclei. White arrows point to nuclear translocated p65. Scale bar equals 10 \(\mu\text{m}\).
Figure 1
Figure 2
Figure 3
Figure 5
Figure 6
The serum- and glucocorticoid-inducible kinase 1 (SGK1) influences platelet calcium signaling and function by regulation of Orai1 expression in megakaryocytes

Oliver Borst, Eva-Maria Schmidt, Patrick Münzer, Tanja Schönberger, Syeda T. Towhid, Margitta Elvers, Christina Leibrock, Evi Schmid, Anja Eylenstein, Dietmar Kuhl, Andreas E. May, Meinrad Gawaz and Florian Lang