Serum- and glucocorticoid-induced protein kinase 1 (SGK1) is regulated by store-operated calcium entry and mediates cytoprotection against necrotic cell death.

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Running Title: Ca\(^{2+}\) induction of SGK1 inhibits necrosis.

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**Background:** Mechanisms underlying SGK1 activation are incompletely understood in epithelial cells.

**Results:** Store-operated Ca\(^{2+}\) entry upregulates SGK1 thereby modulating the lethal effects of Ca\(^{2+}\) overloading on mitochondrial membrane potential.

**Conclusions:** Ca\(^{2+}\)-induced SGK1 activates cytoprotective signaling and modifies mitochondrial function in epithelial cells.

**Significance:** This work reveals a cytoprotective role for SGK1 in necrosis and has potential relevance for epithelial cell protection and cancer treatment.

**SUMMARY**

Serum and glucocorticoid-regulated kinase 1 (SGK1) encodes a phosphatidylinositol-3-kinase-dependent serine/threonine kinase that is rapidly induced in response to cellular stressors and is an important cell survival signal. Previous studies have suggested that an increase in cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_c\)) is required for increased SGK1 expression, but the subcellular source of Ca\(^{2+}\) regulating SGK1 transcription remains uncertain. Activation of endoplasmic reticulum stress (ERS) with thapsigargin (TG) increased SGK1 mRNA and protein expression in MDA-MB-231 cells. Intracellular Ca\(^{2+}\) imaging revealed that store-operated Ca\(^{2+}\) entry (SOCE) played a prominent role in SGK1 induction by TG. Neither ERS nor release of Ca\(^{2+}\) from the ER was sufficient to activate SGK1. Prolonged elevation of intracellular Ca\(^{2+}\) levels however triggered cell death, with a much greater proportion of the cells undergoing necrosis rather than apoptosis. A relative increase in the percentage of cells undergoing necrosis was observed in cells expressing a short hairpin RNA targeted to the SGK1 gene. Necrotic cell death evoked by cytoplasmic Ca\(^{2+}\) overloading was associated with persistent hyperpolarization of the inner mitochondrial membrane and a modest increase in calpain activation, but did not involve detectable caspase 3 or caspase 7 activation. The effects of cytoplasmic Ca\(^{2+}\) overloading on mitochondrial membrane potential were significantly reduced in cells expressing SGK1 compared to SGK1-depleted cells. Our findings indicate that SOCE regulates SGK1 expression in epithelial cells, and suggest that SGK1-dependent cytoprotective signaling involves effects on maintaining mitochondrial function.
Serum and glucocorticoid-regulated kinase 1 (SGK1) is a transcriptionally-induced serine-threonine kinase of the AGC subfamily of protein kinases that is also regulated by posttranslational modification. The SGK1 gene, an immediate early response gene, was identified from serum- or glucocorticoid-stimulated transcripts in a rat mammary epithelial cell line (1,2). Transcription of SGK1 is also rapidly induced in non-malignant human breast epithelial cells by glucocorticoids, progesterone, or serum (3) and in mouse mammary epithelial cells following oxidative, osmotic and ultraviolet radiation stress (4). Activation of SGK1 can be affected by many kinases including 3-phosphoinositide-dependent protein kinase 1 (PDK1) mTOR and phosphoinositide 3-kinase (PI3K) (6,7). Disruption of SGK1 activation can occur by ubiquitination (8). In contrast to other rapidly degraded protein kinases, neither the catalytic activity of SGK1 nor activation site phosphorylation is required for ubiquitin modification and degradation. Instead, SGK1 degradation requires a lysine-less six-amino-acid (amino acids 19-24) hydrophobic motif (GMVAIL) within the N-terminal domain that also serves to target SGK1 to the endoplasmic reticulum (ER) and mitochondria (9). Interaction with the stress-associated E3 ligase C-terminus of Hsc (heat-shock cognate protein) 70-interacting protein) (CHIP) is also required for ubiquitin modification and rapid proteasomal degradation of SGK1 (10).

Multiple intracellular signal transduction pathways have been implicated in the regulation of SGK1 gene expression (3,11-14). Intracellular Ca\(^{2+}\) regulates SGK1 gene expression in A6 renal cells (15) and SGK1 kinase activity in CHO, CHO-IR and HepG2 cells (16). Activation of SGK1 during cell stress has been shown to be Ca\(^{2+}\)-dependent. Hypotonic stress and Ca\(^{2+}\) overloading increased SGK1 mRNA and protein levels in A6 cells (15). The effects of osmotic stress were attenuated following chelation of intracellular Ca\(^{2+}\) with 1,2-bis (o-aminophenoxo) ethane-N,N,N',N'-tetraacetate (BAPTA) and by N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), an inhibitor of calmodulin. Exposure of CHO, CHO-IR and HepG2 cells to supraphysiological levels of insulin also increased SGK1 kinase activity. The activation of SGK1 by insulin was inhibited by BAPTA and calmodulin antagonists (16). These studies indicate an important role of Ca\(^{2+}\) signaling pathways in SGK1 gene expression and kinase activity. However, the mechanisms underlying Ca\(^{2+}\)-dependent activation of SGK1 remain unresolved.

SGK1 participates in the regulation of a wide range of cellular functions including ion channel activity, Na\(^+\)/H\(^+\) exchange, glucose and amino acid transport, glucose metabolism, gene transcription, hormone secretion, cell volume, proliferation and cell death (17). SGK1 activity maintains electrolyte homeostasis in kidney epithelial cells by regulating epithelial sodium channel (ENaC) and ROMK1 (Kir 1.1) potassium channel expression (18), affects cardiomyocyte Na\(^+\) and K\(^+\) fluxes (19), increases Na\(^+\)/H\(^+\) exchange in renal epithelial cells (20), and modifies carbohydrate metabolism (21). SGK1 also increases embryonic rat hippocampal neurite formation through direct effects on microtubule polymerization (22) and contributes to neuronal plasticity (23). SGK1 phosphorylation and inhibition of B-Raf kinase activity is important for cell cycle regulation in HEK293 cells (24).

SGK1 is part of a cytoprotective signaling network that inhibits apoptosis (17,25,26). Phosphorylation and inactivation of forkhead receptor-L1 (FKHR-L1 or FOXO3a) has been implicated in SGK1-dependent cell survival signaling (27,28). SGK1 activation of IKK\(\beta\) inhibits breast cancer cell apoptosis (29). In the context of tumor formation and progression, the increased expression of SGK1 associated with invasive breast cancer and myeloma cells suggests that increased SGK1 activity may confer a selective advantage to the survival and proliferation of tumorigenic cells (30, 31). Conversely, the cytoprotective effects of SGK1 signaling may enhance survival of cells following ischemia (17) and therefore could be a novel therapeutic target for treatment of stroke and myocardial infarction.

Ischemic cell death involves apoptosis and necrosis. Unlike apoptosis, necrosis was once thought to be an uncontrolled form of cell death consequent to mechanical or oxidative stress that resulted in irreversible cellular damage to the plasma membrane, bioenergetic capacity and DNA (32). This view of necrotic death has undergone a significant revision in recent years and it is now believed to involve dynamic interplay of multiple death signaling pathways (32-34). Accumulation of cytoplasmic and mitochondrial Ca\(^{2+}\) is critical in the
propagation and execution phases of necrosis (33,35,36). Sustained elevation of intracellular Ca\textsuperscript{2+} (or Ca\textsuperscript{2+} overloading) contributes to necrotic cell death following ischemia in neurons (37,38), hepatocytes (39), and cardiomyocytes (40). Whether SGK1 provides pro-survival signaling in necrotic cell death is not known.

Here, we used a combination of biochemical, pharmacological, and Ca\textsuperscript{2+} imaging approaches to define mechanisms underlying the Ca\textsuperscript{2+} signals that regulate SGK1 expression. In addition, we used short hairpin RNA (shRNA) to define cytoprotective signals requiring SGK1 expression in Ca\textsuperscript{2+}-overloaded cells. Our results suggest that store-operated Ca\textsuperscript{2+} entry (SOCE) and subsequent calmodulin-dependent protein kinase II (CaMKII) activity regulate SGK1 induction in MDA-MB-231 cells. We also discovered that necrotic cell death produced by a sustained increase in intracellular Ca\textsuperscript{2+} was attenuated by SGK1 pro-survival signals associated with maintenance of the mitochondrial membrane potential.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Thapsigargin (TG), 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetate acetoxy-methylester (BAPTA), tunicamycin (TNC), cyclopiazonic acid (CPA), calmidazolium (CMZ), Dithiothreitol (DTT) and ionomycin (IONO) were obtained from Sigma-Aldrich. KN93 and STO609 were purchased from Calbiochem.

**Cell culture**—MDA-MB-231, human breast cancer cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). MCF10A-Myc or HaCaT cells were prepared as previously described (5). Cells were washed one time with 1X PBS, lysed in Laemmli 2X buffer and lysates incubated at 95°C for 5 minutes. Proteins in the lysates were separated on 9% SDS-PAGE gels, electrothoretically transferred to nitrocellulose membrane (Bio-Rad), visualized with Ponceau S Red dye solution, and blocked in a 5%-skim milk/Tris buffer saline/0.1% Tween 20 solution (TBST). Nitrocellulose membranes were exposed to the following antibodies: α-SGK1 (1:1000; Assay Designs), α-P-SGK1 Ser422 (1:1000; Santa Cruz Biotechnology), α-BiP (1:1000, BD Biosciences). α-Fodrin (1:100). Cell Signaling Technology) or α-β-actin (1:5000; Sigma-Aldrich) as a loading control, in TBST for 1 hr (for α-BiP and α-β-actin) or overnight (for α-SGK1 antibodies). The blots were incubated with goat α-rabbit-HRP or α-mouse–HRP conjugated antibodies (1:5000; Santa Cruz Biotechnology) and visualized using an ECL chemiluminescent kit per manufacturer’s instructions (GE Healthcare). Densitometry was performed on the Bio-Rad GS-670 imaging densitometer, analyzed using Quantity One 1D analysis software (Bio-Rad).

**Measurement of intracellular Ca\textsuperscript{2+} concentration**—[Ca\textsuperscript{2+}]\textsubscript{i} in MDA-MB-231 or HaCaT cells was determined as previously described (20,35). Cells were loaded with Fura-2 by applying 1 µM Fura-2 acetoxyethyl ester (Molecular Probes, Inc.) for 15 min at 37 °C in Krebs Ringers Bicarbonate HEPES buffer (pH 7.4) supplemented with 10 mM D-glucose (KRBH10) and 0.015% (v/v) Pluronic F-127 (Molecular Probes, Inc.). Cells were visualized with a Nikon TE-2000U inverted microscope equipped for epifluorescence. Fura-2 dual-excitation ratiometric microspectrophotometry was used to quantitatively measure [Ca\textsuperscript{2+}]\textsubscript{i}, in single cells as described (35). Digitized images were recorded with a Cascade 650 charge-coupled device (Roper Instruments) at 10 sec intervals. Experiments were performed at 37 °C and cells superfused constantly in a microperfusion chamber with KRBH10 containing test reagents. Background-subtracted fluorescence intensity measurements from individual cells were converted to molar values of [Ca\textsuperscript{2+}] using an in vivo calibration method (20). Data are expressed as the fold change in [Ca\textsuperscript{2+}]\textsubscript{i}, normalized to the average basal [Ca\textsuperscript{2+}]\textsubscript{i} recorded during a 60-second period prior to addition of test reagents. OriginPro 7.0 data analyses and graphing software (OriginLab Corp., Northampton, MA) was used for curve integration analysis of the [Ca\textsuperscript{2+}] responses.

**Western immunoblotting**—Lysates of MDA-MB-231, MCF10A-Myc or HaCaT cells were prepared with 1X PBS, lysis in Laemmli 2X buffer and lysates incubated at 95°C for 5 minutes. Proteins in the lysates were separated on 9% SDS-PAGE gels, electrophoretically transferred to nitrocellulose membrane (Bio-Rad), visualized with Ponceau S Red dye solution, and blocked in a 5%-skim milk/Tris buffer saline/0.1% Tween 20 solution (TBST). Nitrocellulose membranes were exposed to the following antibodies: α-SGK1 (1:1000; Assay Designs), α-P-SGK1 Ser422 (1:1000; Santa Cruz Biotechnology), α-BiP (1:1000, BD Biosciences). α-Fodrin (1:100). Cell Signaling Technology) or α-β-actin (1:5000; Sigma-Aldrich) as a loading control, in TBST for 1 hr (for α-BiP and α-β-actin) or overnight (for α-SGK1 antibodies). The blots were incubated with goat α-rabbit-HRP or α-mouse–HRP conjugated antibodies (1:5000; Santa Cruz Biotechnology) and visualized using an ECL chemiluminescent kit per manufacturer’s instructions (GE Healthcare). Densitometry was performed on the Bio-Rad GS-670 imaging densitometer, analyzed using Quantity One 1D analysis software (Bio-Rad).

**Measurements of gene expression**—Cells were washed in 1X PBS and total RNA was isolated using the RNeasy MiniRNAeasy kit (Qiagen). Total RNA
(1 µg) was then used for cDNA synthesis using TaqMan reverse transcription kit (Applied Biosystems) in a GeneAmp PCR system 9700 (Applied Biosystems). Reverse transcribed RNA was combined with SYBR PCR master mix green (Applied Biosystems) and primer pairs for SGK1 or GAPDH. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the ABI Prism 7300 (Applied Biosystems) and the data analyzed as described previously (44).

Flow cytometry—MDA-MB-231 cells stably expressing scrambled sequence (ss) control or SGK1 shRNA cells were treated with ionomycin or DMSO vehicle in 2.5% FCS media for 2 and 8 hours. Flow cytometry was used to determine the extent and mechanism of cell death of these cells by measuring phosphatidylserine externalization by annexin A5 (conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI) DNA staining. Both vital stains were applied according to manufacturer’s instructions (FITC-Annexin-5 apoptosis detection kit I, BD Biosciences). The Amnis ImageStream® multispectral imaging flow cytometer was used to analyze the different stages of cell death based on staining and morphology. Fluorescence emission from the stained cells was detected in the 488-nm and 785-nm channels for annexin A5 and PI, respectively. Data analysis (n> 2,500 cells) was performed using IDEAS software. Live cells (annexin A5+/PI−), early apoptotic (annexin A5+/PI−), late apoptotic (annexin A5+/PI−) or necrotic (annexin A5+/PI+) were distinguished based on staining. Apoptotic and necrotic cell death were further distinguished based on morphology as described (45).

Mitochondrial membrane potential assay—Dynamic changes in mitochondrial membrane potential (ΔΨm) were measured using a modification of an assay described previously (46). Rhodamine 123 (Rh123), a fluorescent lipophilic cationic dye that specifically partitions into negatively charged mitochondrial membranes was loaded into cells by incubation with 10 µg/ml Rh123 in KRBH10 for 20 min at 37 °C. As ΔΨm increases (hyperpolarization), Rh123 accumulates in the mitochondrial membrane that consequently caused self-quenching of the Rh123 fluorescence. Depolarization of ΔΨm on the other hand, causes redistribution of Rh123 from mitochondria into the cytosol which relieves intermolecular quenching and generates an increase of Rh123 fluorescence. Cells were placed into a microperfusion chamber mounted on an inverted microscope equipped for epifluorescence as described above for the Ca2+ imaging studies. Fluorescence emission (530 nm) from Rh123-loaded cells was excited at 490 nm. Fluorescence intensity measurements from single cells were recorded at 10-s intervals. Data were normalized to baseline Rh123 fluorescence intensity 60-s prior to application of test reagents, and analyzed using curve integration (Origin 7.0).

RESULTS

SERCA (sarco/endoplasmic reticulum Ca2+-ATPase) inhibition results in increased expression of SGK1 protein. SGK1 is transcriptionally induced and SGK1 enzymatic activity is stimulated by oxidative and osmotic stress (17). To test the hypothesis that SGK1 expression could be induced by ERS, we measured SGK1 protein levels in MDA-MB-231 breast cancer cells following exposure to three well-characterized activators of ERS. TG (500 nM), TNC (10 µg/ml) and dithiothreitol (DTT; 5 mM) were applied for 1, 2, 4 and 8 h. TG rapidly increased the expression of SGK1 (Fig. 1A). Increased SGK1 was evident as early as 1-h and continued increasing 2-8 h after administration of TG. Neither TNC nor DTT significantly increased SGK1 expression (Fig. 1A). Induction of ERS in MDA-MB-231 cells was confirmed by measuring for the presence the 78-kDa glucose-regulated protein (GRP78), also known as immunoglobulin heavy-chain binding protein (BiP), a member of the 70-kDa heat shock protein family and a protein chaperone within the ER lumen. Consistent with stimulation of ERS, TG, TNC, and DTT treatment increased BiP protein expression 8 h after treatment (Fig. 1B). Induction of SGK1 protein expression was remarkably sensitive to low concentrations of TG: a 4-h exposure to 10 nM TG increased SGK1 (Fig. 1C).

Posttranslational modification of SGK1 is also an important regulator of SGK1 activity. Phosphorylation of SGK1 at residues Thr-256 and Ser-422 regulates its activation and downstream pro-survival signaling (6,47). Phosphorylation of the Ser422 site of SGK1 was detected 2 h after TG treatment of MDA-MB-231 cells (Fig. 1D). We found the same effects of TG on SGK1 total protein
expression and SGK1 Ser422 phosphorylation in keratinocyte HaCaT cells (Fig. 1E). These results are consistent with previous observations suggesting that SGK1 Ser422 phosphorylation and subsequent activation is associated with peak steady-state levels of SGK1 (7).

**Thapsigargin treatment results in increased SGK1 mRNA expression.** Since SGK1 activation is initiated by transcriptional upregulation, we measured the effects of DTT, TNC, and TG on SGK1 mRNA expression levels. One hour after application of 500 nM TG, SGK1 mRNA steady-state levels in MDA-MB-231 cells were significantly increased relative to control cells (Fig. 2). TG-induced SGK1 mRNA was significantly greater than levels evoked by TNC or DTT (p < 0.05) (Fig. 2A, left panel). SGK1 steady-state mRNA levels were also significantly increased in the MCF10A-Myc breast epithelial cell line 4 h following application of 500 nM TG (Fig. 2B, right panel).

Our findings indicated that TG, TNC and DTT trigger ERS in MDA-MB-231 cells. However, ERS alone was not sufficient to increase SGK1 expression because TNC and DTT did not affect SGK1 protein induction. This led us to examine other signaling pathways activated by TG that might underlie SGK1 activation. TG has long been known to be a specific inhibitor of SERCA. ER Ca\(^{2+}\) homeostasis is critically dependent on SERCA activity. Irreversible inhibition of SERCA by TG causes depletion of ER Ca\(^{2+}\) stores and an elevation of [Ca\(^{2+}\)]\(_c\). This raised the possibility that inhibition of SERCA and changes in [Ca\(^{2+}\)]\(_c\), rather than ERS itself, contributed to the observed rapid induction of SGK1 by TG. To test this hypothesis, we determined the effect of cyclopiazonic acid (CPA), a SERCA inhibitor that is structurally distinct from TG, on SGK1 expression. CPA (50-150 µM) rapidly and transiently increased expression of SGK1 (Fig. 3, top panel). The initial CPA-induced increase in SGK1 was observed within 1-2 h. Maximum levels occurred within 4 h and then declined after 8 h. CPA effects were reversible as soon as 1 h following washout (Fig. 3, bottom panel).

**SGK1 expression is regulated by Ca\(^{2+}\) influx from the extracellular milieu.** In many cell types, inhibition of SERCA increases [Ca\(^{2+}\)]\(_c\). We used Fura2, a fluorescent Ca\(^{2+}\) indicator and microspectrophotometry to measure the effects of TG on [Ca\(^{2+}\)]\(_c\) in MDA-MB-231 cells. Baseline [Ca\(^{2+}\)]\(_c\) was 59 ± 5 nM (n = 129 cells). Application of 100 nM TG rapidly increased [Ca\(^{2+}\)]\(_c\), within 90 sec; after 15 min, maximum levels were increased 20 ± 4 fold (n = 29 cells) over baseline values (Fig. 4A). The increase in [Ca\(^{2+}\)]\(_c\) during a 15-min exposure to TG was decreased by 85% in cells in which the cytoplasm had been loaded with BAPTA, a Ca\(^{2+}\) chelator (Fig. 4A). The area under the [Ca\(^{2+}\)]\(_c\) response curve in BAPTA-loaded cells was 1,400 ± 465 compared with 9,147 ± 1,310 in control cells. Intracellular accumulation of BAPTA did not affect baseline [Ca\(^{2+}\)]\(_c\) (62 ± 6 nM; n = 34 cells). Consistent with a role of increased [Ca\(^{2+}\)]\(_c\) in the induction of SGK1, we found that chelation of intracellular [Ca\(^{2+}\)]\(_c\) with BAPTA significantly decreased TG-induced SGK1 mRNA (Fig. 4B) and protein (Fig. 4C) accumulation. SGK1 protein expression was also increased following a 4-h exposure of cells to ionomycin (1-2.5 µM), a Ca\(^{2+}\)-ionophore (Fig. 4D). Taken together, these findings suggest that an increase in [Ca\(^{2+}\)]\(_c\) is sufficient for induction of SGK1.

We next determined the source of Ca\(^{2+}\) that triggered increased SGK1 expression following TG exposure. TG-induced elevation of [Ca\(^{2+}\)]\(_c\), is caused by Ca\(^{2+}\) leak into the cytoplasm following SERCA inhibition and Ca\(^{2+}\) influx through plasma membrane store-operated Ca\(^{2+}\) channels. The increase in [Ca\(^{2+}\)]\(_c\) following a 15-min application of 100 nM TG was reduced 93% in cells incubated in a Ca\(^{2+}\)-free solution (Fig. 5A). The average ± SEM area under the [Ca\(^{2+}\)]\(_c\) response curve was 931 ± 57 (n = 29 cells) in a Ca\(^{2+}\)-free buffer compared with 13,826 ± 1,687 in an extracellular solution containing 2.5 mM Ca\(^{2+}\) (n = 42 cells). In Ca\(^{2+}\)-free solutions, 135 ± 9 s after administration of TG, [Ca\(^{2+}\)]\(_c\) increased transiently to a maximum amplitude 2.7 ± 0.2 times the baseline resting level (Fig. 5A). These results suggest that influx of extracellular Ca\(^{2+}\) via store-operated Ca\(^{2+}\) entry is the major source of the rise in [Ca\(^{2+}\)]\(_c\) evoked by TG. Parallel experiments demonstrated that Ca\(^{2+}\) influx was required for induction of SGK1. In the absence of extracellular Ca\(^{2+}\), TG treatment did not induce detectable levels of SGK1 protein (Fig. 5B). Our findings suggest that influx of extracellular Ca\(^{2+}\) through store-operated Ca\(^{2+}\) channels underlies Ca\(^{2+}\)-mediated activation of SGK1 in MDA-MB-231-mediated cell lines.
Calmodulin-mediated signaling is required for Ca\(^{2+}\)-mediated SGK1 induction. Calmodulin and CaMKII are important downstream effectors of intracellular Ca\(^{2+}\) signal transduction and have been shown to play a role in the induction of SGK1 mRNA expression in A6 renal cells following hypotonic shock (11). We used pharmacological agents to probe the role of calmodulin, CaMKII, and CaMKK in TG-induced SGK1 expression in MDA-MB-231 cells. Application of calmidazolium (CMZ), a well-characterized inhibitor of calmodulin, inhibited TG-stimulated SGK1 protein induction by 98% (Fig. 6A). Short-term SGK1 mRNA expression in MDA-MB-231 cells treated with TG was also inhibited by KN93 and STO609, inhibitors of CaMKII and CaMKK, respectively (Fig. 6B) – both reagents significantly decreased the effect of TG on SGK1 induction within 1 h. However, while inhibition of TG-induced SGK1 mRNA by STO609 was transient, in the presence of a combination of KN93 and STO609 the effect of TG on SGK1 mRNA induction was significantly decreased at all time points studied (Fig. 6B). TG-induced SGK1 protein levels were reduced by 86% in cells pretreated with KN93 (Fig. 6C). SGK1 protein levels were actually higher in STO609-pretreated cells. These data suggest that CaMKII, and not CaMKK, is required for mediating TG-induced SGK1 expression.

SGK1 expression attenuates cell death induced by Ca\(^{2+}\) overloading. We next explored the role of [Ca\(^{2+}\)]\(_{\text{cyt}}\)-induced SGK1 expression in maintaining cell viability. Prolonged elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), or calcium overloading is a pathway for initiating cellular demise. We reasoned that Ca\(^{2+}\)-dependent induction of SGK1 expression might contribute to mitigating Ca\(^{2+}\)-induced cell death. We selected MDA-MB-231 cells stably expressing SGK1 shRNA (7) and exhibiting no detectable SGK1 despite either ionomycin (Fig. 7A right panel) or TG (data not shown) treatment. In contrast, cells stably expressing a control scrambled sequence (ss) shRNA, SGK1 protein levels increased 1-4 h following the application of 2.5 µM ionomycin (Fig. 7A, left panel).

Diminished SGK1 expression in the SGK1 shRNA cells was also associated with increased cell death following Ca\(^{2+}\) overload. Flow cytometric detection of annexin A5 and PI staining was used to characterize the type of cell death induced by exposure to ionomycin for 2 and 8 h. Cells undergoing early stages of apoptosis stain positive for annexin A5 by binding to phosphatidylserine on the cell surface. Late apoptotic and necrotic cells lose membrane integrity and lack an ability to exclude PI, a fluorescent cationic DNA binding molecule. A 2 h exposure to ionomycin did not evoke statistically significant increased cell death in SGK1 shRNA cells vs. control (data not shown). Application of ionomycin for 8 h however induced cell death in 31% and 50% of control and SGK1 shRNA cells, respectively (Fig 7B). Brightfield imaging of the early apoptotic cells (annexin A5+/PI\(^{–}\)) indicated that they retained a relatively normal cellular morphology, but late apoptotic cells (annexin A5+/PI\(^{+}\)) were irregularly shaped. Necrotic cells (annexin A5+/PI\(^{+}\)) maintained normal cell morphology. We estimated the type of cell death based on staining of annexin A5 and PI (Fig 7C). At 8-h, ionomycin did not significantly induce cell death characteristic of early apoptosis (annexin A5+/PI\(^{+}\)). Nevertheless, the percentage of cells undergoing late apoptosis was significantly increased in the SGK1 shRNA cells (1.1% following vehicle treatment compared with 8.6% after ionomycin exposure). These findings suggest that ionomycin-induced apoptosis plays a modest role in MDA-MB-231 cell death consequent to Ca\(^{2+}\) overloading.

In contrast to the relatively low percentage of early and late apoptotic cells following Ca\(^{2+}\) overloading, necrotic cell death appeared to be the predominant form of cellular demise evoked by ionomycin. After an 8-h exposure to ionomycin, necrotic cell death (reflected by annexin A5+/PI\(^{–}\) staining cells) was observed in 23% and 40% of the control ss shRNA and SGK1 shRNA cells, respectively. These data suggest that SGK1 plays an important cytoprotective role in Ca\(^{2+}\)-induced necrotic cell death.

SGK1 expression attenuates the effects of cytoplasmic Ca\(^{2+}\) overloading on mitochondrial membrane potential. Our findings suggest that SGK1 pro-survival signals affect the biochemical and molecular events that regulate necrotic cell death. Caspases, a family of proteolytic enzymes, are key executioners of cell death (48) and can be activated by cytoplasmic Ca\(^{2+}\) overloading (49). We used flow cytometry and Western analysis to measure the effects of cytoplasmic Ca\(^{2+}\) overloading
on caspase activation. A 24-h application of ionomycin did not activate caspase 3 or caspase 7 in either control or SGK1-depleted cells as detected by either flow cytometry or Western analysis (data not shown). This suggested that cytoplasmic Ca\(^{2+}\) overloading likely triggered MDA-MB-231 cell death by a caspase–independent mechanism.

Caspase-independent cell death has been associated with sustained elevation of intracellular Ca\(^{2+}\) following application of ionomycin (50). Multiple cellular proteins can contribute to Ca\(^{2+}\)-mediated cell death including proteases, Ca\(^{2+}\)-dependent kinases and phosphatases, and endonucleases (48). Calpains, cysteine proteases that are activated by an increase in intracellular Ca\(^{2+}\), play an important role in neuronal cell death (33,51,52). To determine whether calpain activation was involved in MDA-MB-231 cell death induced by sustained elevation of cytoplasmic Ca\(^{2+}\), we next measured relative cleavage of alpha-fodrin, a calpain substrate, by Western blot following ionomycin treatment. Modest fodrin cleavage was observed in control and SGK1 shRNA cells following ionomycin treatment (unpublished observations). Using densitometric assessment of immunoreactive cleaved fodrin normalized to actin, the extent of alpha-fodrin cleavage was only slightly (1.4-fold) greater in SGK1 shRNA expressing cells compared to ss shRNA control cells (unpublished observations). This modest increase in fodrin cleavage in SGK1-depleted cells suggests that SGK1 expression does not primarily inhibit Ca\(^{2+}\)-mediated necrosis through blockade of calpain activation.

Changes in mitochondrial membrane potential (Δψ\(_{m}\)) accompany the initiation of apoptosis (35) while persistent hyperpolarization of mitochondria is associated with necrotic cell death (53-56). We measured time-dependent changes in Δψ\(_{m}\) using rhodamine 123, a fluorescent potentiometric indicator. Under resting conditions, baseline Δψ\(_{m}\) in the control and SGK1 shRNA cells was not significantly different (p = 0.42, n = 246 and 122 control and SGK1 shRNA cells, respectively). A 60-min application of 2.5 μM ionomycin produced two patterns of Δψ\(_{m}\) response in the control and SGK1 shRNA cells (Fig. 8). In 62% of control cells (44/71 cells) expressing a scrambled sequence (ss) shRNA, Ca\(^{2+}\) overloading evoked a rapid and transient depolarization of Δψ\(_{m}\). The maximum level of mitochondrial depolarization in the control cells occurred within 10 minutes and was sustained over the next 25 minutes. Subsequently, Δψ\(_{m}\) hyperpolarized (Fig. 8). This pattern of response was observed in only 14% of the SGK1-depleted cells (12/76 cells). In 86% of the SGK1-shRNA cells (64/76 cells), Δψ\(_{m}\) rapidly hyperpolarized in the presence of the Ca\(^{2+}\) ionophore. After 20 minutes, Δψ\(_{m}\) in the SGK1-depleted cells reached a maximum level of hyperpolarization that remained until the end of the experiment (Fig. 8). In contrast, the persistent Δψ\(_{m}\) hyperpolarization that occurred during Ca\(^{2+}\) overloading was detected in only 38% of the control cells (27/71 cells). These results suggest that a primary mechanism whereby SGK1 induction decreases Ca\(^{2+}\)-dependent necrosis through preventing persistent hyperpolarization of the mitochondrial membrane.

**DISCUSSION**

In this study, we found that only a subset of chemicals causing ER stress (ERS) are capable of inducing SGK1 expression in human epithelial cell lines. The mechanism of SGK1 induction in this subset is specific to a Ca\(^{2+}\)-dependent signal transduction mechanism. The application of those chemical inducers of ERS that inhibit ER Ca\(^{2+}\)-ATPases both activated transcription of the SGK1 gene and greatly increased steady-state SGK1 protein levels. SGK1 induction did not occur in cells loaded with a Ca\(^{2+}\) chelator or maintained in a Ca\(^{2+}\)-free extracellular solution, but was observed in cells treated with ionomycin, a Ca\(^{2+}\) ionophore. Our findings are in accord with results in Chinese hamster ovary (CHO) cells (16) and *Xenopus laevis* A6 renal epithelial cells (15) suggesting a role for increased intracellular Ca\(^{2+}\) in inducing SGK1 expression. However, we show for the first time that an increase in [Ca\(^{2+}\)]\(_{c}\) stemming from Ca\(^{2+}\) entry through store-operated Ca\(^{2+}\) channels upregulates SGK1 expression, and that SGK1 upregulation attenuates mitochondrial membrane hyperpolarization and necrotic cell death induced by Ca\(^{2+}\) overloading.

Acute induction of ERS results from leakage of intraluminal ER Ca\(^{2+}\) following, for example, SERCA inhibition. Subsequently, altered protein folding and post-translational modification of nascent polypeptides triggers the unfolded protein response (UPR). This signaling cascade facilitates
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restoration of normal cellular function and viability following ERS \((57,58)\). The transient activation of the UPR maintains cellular homeostasis in the presence of acute stress by relieving ERS through reducing translation, upregulating the expression of genes encoding ER chaperones, and decreasing misfolded or mutant proteins through ER-associated degradation (ERAD) \((59,60)\). Additional cellular signals that may participate in the recovery from ERS have not been completely defined; the stress-responsive SGK1 gene presented an excellent candidate gene for participating in the recovery from ERS, especially in light of SGK1’s role in regulating cation flux \((18)\) and its mitochondrial and ER subcellular localization \((9)\).

We tested the hypothesis that ERS would increase SGK1 expression. Since ERS can be caused by different experimental conditions, we determined the effects of three chemical reagents that induce ERS by distinct mechanisms. As expected, TG, TNC, and DTT rapidly increased BiP levels, an indicator of ERS and the UPR. However, induction of ERS per se was insufficient to upregulate SGK1 transcription. TG, a sesquiterpene lactone compound derived from the root of the Thapsia garganica plant and CPA, a mycotoxin produced by Penicillium cyclopium, stimulated robust expression of SGK1. Neither TNC, a product of Streptomyces lyosuperficus that inhibits GlcNAc phosphotransferase and post-translational modification of glycoproteins, nor DTT (or Cleland’s reagent) which interferes with the protein folding within the ER, induced SGK1. This suggests that signals downstream of the UPR do not in themselves contribute to SGK1 induction.

SERCA transports Ca\(^{2+}\) from the cytosol into the ER lumen and is the central regulator of ER Ca\(^{2+}\) homeostasis. TG and CPA specifically inhibit SERCA, and in many cell types, increase [Ca\(^{2+}\)]\(_c\) by a combination of release of Ca\(^{2+}\) sequestered within the ER lumen and influx of extracellular Ca\(^{2+}\) through store-operated Ca\(^{2+}\) channels (SOCCs) \((61,62)\). Application of TG to MDA-MB-231 cells resulted in the rapid and irreversible increase in [Ca\(^{2+}\)]\(_c\). However, in the absence of Ca\(^{2+}\) in the extracellular medium, an experimental condition that excludes any contribution of SOCCs to the influx of Ca\(^{2+}\), we observed a modest increase in Ca\(^{2+}\) levels 2 min following TG treatment. This increase in [Ca\(^{2+}\)]\(_c\) likely stems from ER store leakage, but did not affect SGK1 expression compared to the influx of extracellular Ca\(^{2+}\), suggesting that the main mechanism of SGK1 induction following SERCA inhibition is extracellular Ca\(^{2+}\) rather than the release of Ca\(^{2+}\) from ER stores. This observation is consistent with findings in other cell systems demonstrating control of gene expression specifically by Ca\(^{2+}\) entry through plasmalemmal Ca\(^{2+}\) channels \((63)\).

SERCA inhibition causes a rapid depletion of ER Ca\(^{2+}\) stores \((64)\). Persistent depletion of ER Ca\(^{2+}\) stores has been associated with induction of ERS and apoptosis \((65,66)\). We investigated the role of ER Ca\(^{2+}\) depletion in SGK1 induction using BAPTA. BAPTA, a chelator of Ca\(^{2+}\), blocked the TG-induced increase in SGK1 expression and [Ca\(^{2+}\)]\(_c\). This suggests that the rise in [Ca\(^{2+}\)]\(_c\) was necessary and sufficient for upregulation of SGK1 expression following SERCA inhibition. Our findings also suggest that depletion of ER Ca\(^{2+}\) stores alone is not sufficient to upregulate SGK1 transcription.

Signal transduction by intracellular Ca\(^{2+}\) is mediated through a wide range of effector proteins that undergo Ca\(^{2+}\)-dependent conformational changes and consequently activate distal Ca\(^{2+}\)-dependent physiological processes. For example, calmodulin (CaM), a ubiquitous Ca\(^{2+}\)-binding protein, has no intrinsic enzymatic activity, but integrates Ca\(^{2+}\) signaling by transducing changes in [Ca\(^{2+}\)]\(_c\) to downstream enzymes such as Ca\(^{2+}\)/CaM-activated protein kinases, CaM-kinase kinase (CaMKK), CaMKI, CaMKII and CaMKIV. Previous studies have demonstrated that phosphorylation and activation of SGK1 following stimulation of CHO-IR cells is regulated by CaMKK in a PI3K-independent manner \((16)\). In addition, calmodulin-dependent signaling was implicated in upregulation of SGK1 mRNA expression in A6 cells following exposure to hypotonic stress conditions \((15)\). Interestingly, our data suggest that calmodulin/CaMKII activity is necessary for Ca\(^{2+}\)-dependent induction of SGK1 mRNA and protein expression in MDA-MB-231 human breast epithelial cells exposed to TG. Taken together with our Ca\(^{2+}\) flux measurements, the aggregate data suggest that an influx of Ca\(^{2+}\) through SOCCs is coupled with activation of CaMKK and CaMKII signaling cascades that in turn upregulate SGK1 gene
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expression leading to increased SGK1 steady-state protein levels and activity.

Because SGK1 expression plays a role in mitigating cell death following exposure to many stressors including chemotherapy and growth factor withdrawal, we speculated that increased SGK1 expression might promote cell survival following Ca\(^{2+}\) influx. Indeed, we found that ionomycin treatment induced a higher percentage of MDA-MB-231 cells undergoing late apoptotic and necrotic cell death in cells in which SGK1 expression was diminished by an SGK1-specific shRNA. Ionomycin-induced necrosis was partially inhibited in SGK1-expressing cells after 8 hours of ionomycin treatment. Necrosis and apoptosis are independent biological processes and apoptosis is not (always) a requisite step for necrosis. We do not yet understand the precise mechanisms linking Ca\(^{2+}\) overload, SGK1 induction, cell survival, and preservation of mitochondrial membrane potential. Though it has long been appreciated that overloading mitochondria with Ca\(^{2+}\) leads to apoptotic and necrotic cell death, whether SGK1 plays a specific role in inhibiting accumulation of Ca\(^{2+}\) in mitochondria and/or attenuating loss of mitochondrial membrane potential during Ca\(^{2+}\) influx remains uncertain. Additional studies will be necessary to define the interrelationship between SGK1 expression, maintenance of mitochondrial membrane potential, and protection from Ca\(^{2+}\) overload-induced cell death.

In summary, we have demonstrated that contrary to our initial hypothesis that all ERS and/or UPR signals would result in increased expression of the stress-associated protein SGK1, SGK1 induction appears to respond specifically to increased [Ca\(^{2+}\)]\(_e\). Among ER stressors, only compounds also causing increased epithelial cell [Ca\(^{2+}\)]\(_e\) stimulated a rapid and robust induction of SGK1 mRNA and protein levels followed by phosphorylation at the SGK1 active site Ser422. Furthermore, our findings suggest that Ca\(^{2+}\)-dependent activation of SGK1 transcription is caused by opening of store-operated Ca\(^{2+}\) channels in the plasma membrane and the influx of extra-cellular Ca\(^{2+}\) from the extracellular milieu rather than from the release of Ca\(^{2+}\) from the intraluminal ER. Moreover, we demonstrate that Ca\(^{2+}\)-dependent SGK1 induction and activation contribute to attenuating changes in mitochondrial membrane polarity, and are independent of caspase activation. Taken together, these observations suggest that SGK1 could function as a central responder to calcium-mediated mitochondrial membrane dysfunction and subsequent cell death. Future studies will address the hypothesis that SGK1 induction and/or increased activity may specifically regulate cation flux within the mitochondria, thereby attenuating cell death.
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FOOTNOTES

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The abbreviations used are:
BAPTA 1,2-bis (o-aminophenoxy) ethane-N,N,N',N'-tetraacetate acetoxy-methylester
CaMKK calmodulin-dependent protein kinase kinase
CaMKII Ca\(^{2+}\)/camodulin-dependent protein kinase II
CMZ calmidazolium
CPA cyclopiazonic acid
DTT dithiothreitol
ENaC epithelial sodium channel
ERS endoplasmic reticulum stress
IONO ionomycin
SERCA sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase
SGK1 serum and glucocorticoid-regulated kinase 1
shRNA short hairpin RNA
SOCE store-operated Ca\(^{2+}\) entry
ss scramble sequence
TG thapsigargin
TNC tunicamycin

FIGURE LEGENDS

FIGURE 1. Thapsigargin treatment, but not other ER stressors, induces SGK1 protein expression in MDA-MB-231 human breast cancer cells. (A) MDA-MB-231 cells treated with thapsigargin (TG) (500 nM), tunicamycin (10 µg/ml), or dithiothreitol (5 mM), vehicle (DMSO or H\(_2\)O) for the indicated times and examined by Western blot. β-ACTIN as a loading control. (B) MDA-MB-231 cells were treated with TG (500 nM), tunicamycin (10 µg/ml), or dithiothreitol (5 mM) for a longer time period from 4 h to 32 h and protein lysates were examined for total SGK1 and BiP protein expression (C) MDA-MB-231 cells were treated with various concentration of TG (from 0.1 nM to 500 nM) for 4 h. (D) MDA-MB-231 cells were treated with 500 nM TG for 2 h and P-SGK1 Ser422 and total SGK1 were measured. (E) Immortalized keratinocytes (HaCaT) treated with thapsigargin for the indicated times, the P-SGK1 Ser422 and total SGK1 were detected by Western blot. All data are representative of at least three independent experiments. The asterisks represent a background band seen with the total SGK1 antibody; β-actin was detected as a loading control.

FIGURE 2. Thapsigargin induces SGK1 mRNA in mammary epithelial cells (A) MDA-MB-231 cells were treated with dithiothreitol (DTT), tunicamycin (TNC), thapsigargin (TG) or vehicle and qRT-PCR
was used to detect $SGK1$ steady-state mRNA levels. (B) MCF10A-Myc cells were treated with TG (500 nM), and qRT-PCR was used to detect the $SGK1$ mRNA levels. The fold change of $SGK1$ mRNA was normalized to GAPDH mRNA and all data are representative of three independent experiments.

**FIGURE 3. Increased $SGK1$ expression is dependent on SERCA pump inhibition.** Western analysis of $SGK1$ expression following treatment with the reversible SERCA pump inhibitor cyclopiazonic acid (CPA). Upper panel: time course Western blot analysis of $SGK1$ following treatment with CPA (50-150 µM). Bottom panel: $SGK1$ expression in cells treated with CPA (50 µM) for 2 or 4h followed by a PBS washout and subsequent recovery in DMEM lacking CPA for an additional 1-4 h.

**FIGURE 4. The calcium chelator, BAPTA, inhibits thapsigargin-induced $SGK1$ expression.** MDA-MB-231 cells were pretreated with either 20 µM BAPTA for 2 h followed by thapsigargin (TG) or vehicle. (A) Intracellular Ca$^{2+}$ was measured over time using Fura-2-AM following TG treatment (100 nM) of MDA-MB-231 cells pretreated with BAPTA (n = 41) or vehicle control (n = 47). (B) MDA-MB-231 cells treated with BAPTA alone, pretreated with BAPTA and then treated with TG, or TG alone for 4 h. $SGK1$ mRNA levels were measured by qRT-PCR. The fold changes in $SGK1$ mRNA levels in treated samples were normalized to GAPDH expression and then compared to vehicle. (C) Western blot analysis of $SGK1$. (D) MDA-MB-231 cells treated with ionomycin or DMSO vehicle for $SGK1$ expression by Western blotting. Data representative of three independent experiments are presented.

**FIGURE 5. $SGK1$ induction by thapsigargin (TG) requires extracellular calcium.** (A) MDA-MB-231 cells were maintained in Ca$^{2+}$-free DMEM under low serum conditions (2.5% FCS) and pretreated with 20 µM BAPTA or DMSO vehicle control for 2 h, loaded with Fura-2AM and subsequently stimulated with TG (100 nM). To monitor intracellular Ca$^{2+}$ release, Fura-2 fluorescence was measured over time. (B) $SGK1$ protein expression was examined by Western blot analysis of total cell lysates. β-Actin was used as a loading control.

**FIGURE 6. The calmodulin/CamKII pathway is required for calcium-mediated $SGK1$ induction.** (A) MDA-MB-231 cells were treated with thapsigargin (TG) alone or pretreated with calmidizum chloride (CMZ) prior to TG (CMZ/TG) and total $SGK1$ detected by Western blot. (B) Total RNA was isolated from cell treated with TG alone, or pretreated with either KN93 or STO609 or both. qRT-PCR was performed to detect steady-state $SGK1$ mRNA levels and then normalized to GAPDH levels. (C) $SGK1$ expression following pretreatment with either TG alone, co-treatment with KN93 and TG, co-treatment with STO609 and TG, co-treatment with KN93, STO609 and TG, DMSO vehicle control or untreated control. Data shown are representative of three independent experiments.

**FIGURE 7. $SGK1$ induction contributes to protection from apoptosis and necrosis.** (A) MDA-MB-231 cells stably expressing $SGK1$ shRNA or a control “scrambled sequence” short hairpin (sh)RNA were treated with ionomycin (2.5 µM) for the indicated time. (B) $SGK1$ or control short hairpin (sh) RNA-expressing MDA-MB-231 cells were treated with 2.5 µM ionomycin for 8 h in the presence of media containing 2.5% serum, stained with Annexin A5 (A5) and PI, and analyzed by flow cytometry. (C) The different stages of cell death were delineated based on whether they were healthy (A5+/PI$^-$), early apoptotic (A5$^+$/PI$^-$), late apoptotic (A5$^+$/PI$^+$) or necrotic (A5$^+$/PI$^+$). Inset shows representative microscopic images of these categories from the ImageStream flow cytometer. B.F = bright field. A5 = Annexin A5. For both B and C the graph represents the average cell death of triplicate control or $SGK1$ shRNA samples treated with ionomycin or vehicle. The error bars represent the standard error of the mean (SEM). *p<0.05 compared to vehicle treated control or $SGK1$ shRNA. **p<0.05 compared to ionomycin treated control shRNA. The two-tailed Student’s $t$-test was used to determine significance.
**FIGURE 8. SGK1 prevents Ca\(^{2+}\) overloading-induced persistent hyperpolarization of mitochondria.**

Time course of Δψ\(_m\) response in MDA-MB-231 cells after application of ionomycin (2.5 μM). Cells were loaded with the cationic fluorescent dye, rhodamine 123 (Rh123). Data are expressed as normalized Rh123 fluorescence (F/F\(_0\)), where F is the Rh123 measured at each time point and F\(_0\) is the average Rh123 fluorescence measured in the cells during the 60-s period prior to the addition of ionomycin. As mitochondria hyperpolarize relative to baseline levels, Rh123 accumulates within the mitochondria. Intermolecular crowding of Rh123 causes fluorescence quenching and a decrease in fluorescence emission. Conversely, as mitochondria depolarize, Rh123 is released from the mitochondria into the cytosol which relieves quenching and increases Rh123 fluorescence. Note that ionomycin evokes persistent hyperpolarization of mitochondria in MDA-MB-231 cells that do not express SGK1 (SGK1 shRNA). In control cells expressing a scrambled sequence shRNA that does not target any known gene (darker grey line), the Ca\(^{2+}\) ionophore causes transient depolarization of Δψ\(_m\) that repolarizes to suprabasal levels. Results depict mean ± SEM of 71 control and 76 SGK1 shRNA cells.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Serum- and Glucocorticoid-induced Protein Kinase 1 (SGK1) is Regulated by Store-Operated Ca2+ Entry and Mediates Cytoprotection Against Necrotic Cell Death

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