Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;33:491-500
DOI: 10.1159/000358629
Published online: February 14, 2014
Accepted: January 20, 2014

Karger Open access

1421-9778/14/0332-0491$39.50/0

Original Paper

Up-Regulation of $K_{ir}2.1$ (KCNJ2) by the Serum & Glucocorticoid Inducible SGK3

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Key Words
KCNJ2 • SGK3 • Cell membrane potential • Neurons • Cardiomyocytes • Heart

Abstract
Background/Aims: The serum & glucocorticoid inducible kinase SGK3, an ubiquitously expressed serine/threonine kinase, regulates a variety of ion channels. It has previously been shown that SGK3 upregulates the outwardly rectifying K^+ channel $K_V11.1$, which is expressed in cardiomyocytes. Cardiomyocytes further express the inward rectifier K^+ channel $K_{ir}2.1$, which contributes to maintenance of resting cell membrane potential. Loss-of-function mutations of KCNJ2 encoding $K_{ir}2.1$ result in Andersen-Tawil syndrome with periodic paralysis, cardiac arrhythmia and dysmorphic features. The present study explored whether SGK3 participates in the regulation of $K_{ir}2.1$. **Methods:** cRNA encoding $K_{ir}2.1$ was injected into *Xenopus* oocytes with and without additional injection of cRNA encoding wild type SGK3, constitutively active ^{S419D}SGK3 or inactive ^{K191N}SGK3. $K_{ir}2.1$ activity was determined by two-electrode voltage-clamp and $K_{ir}2.1$ protein abundance in the cell membrane by immunostaining and subsequent confocal imaging or by chemiluminescence. **Results:** Injection of 10 ng cRNA encoding wild type SGK3 and ^{S419D}SGK3, but not ^{K191N}SGK3 significantly enhanced $K_{ir}2.1$-mediated currents. SGK inhibitor EMD638683 (50 μM) abrogated ^{S419D}SGK3-induced up-regulation of $K_{ir}2.1$. Moreover, wild type SGK3 enhanced the channel protein abundance in the cell membrane**.** The decay of $K_{ir}2.1$-mediated currents following inhibition of channel insertion into the cell membrane by brefeldin A (5 μM) was similar in oocytes coexpressing $K_{ir}2.1$ and SGK3 as in oocytes expressing $K_{ir}2.1$ alone, suggesting that SGK3 influences channel insertion into rather than channel retrieval from the cell membrane. **Conclusions:** SGK3 is a novel regulator of $K_{ir}2.1$.

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Introduction

The serum & glucocorticid inducible kinase isoform SGK3 has been shown to stimulate the activity of a wide variety of transporters [1-5] and channels [5-8], including Ca^{2+} channels [8, 9] and voltage-gated K^+ channels [5, 10, 11]. The cardiac channels regulated by SGK3 include $K_V11.1$ channels, which play an important role in the repolarization phase of the cardiac action potential [11].

The resting membrane potential in cardiac myocytes, skeletal muscle and neurons is maintained by inwardly rectifying K^+ channels of the $K_{ir}2.x$ (*KCNJ,* IRK) subfamily [12]. In cardiomyocytes $K_{ir}2.1$ forms heteromeric channels, which generate the inwardly rectifying K^+ current I_{K1} [13-15]. $K_{ir}2.1$ gain-of-function mutations cause atrial fibrillation [15, 16] and $K_{ir}2.1$ loss-of-function mutations result in Andersen-Tawil syndrome (ATS), a rare familial disorder leading to potassium-sensitive periodic paralysis, ventricular arrhythmias and dysmorphic features including syndactyly and altered face shapes [17-20]. $K_{ir}2$ is regulated by mitochondria [21, 22] and $K_{ir}2.1$ contributes to ischemic preconditioning [23]. A wide variety of further $K_{ir}2.1$ regulators have been identified, including arachidonic acid [24], cholesterol [25], phosphatidylinositol 4,5-bisphosphate $PI(4,5)P_2$ [26-29], PKC [30], tyrosine phosphorylation [31, 32], the kinase-anchoring protein AKAP79 [33], Rho [34], TNF-alpha [35], Chapsyn 110 [36], filamin-A [37], PSD95 [38] as well as SAP97, CASK, Veli and Mint1 [39].

The present study explored whether SGK3 participates in the regulation of $K_{ir}2.1$ channels. To this end, inwardly rectifying currents were determined in *Xenopus* oocytes expressing $K_{ir}2.1$ with or without wild type SGK3, constitutively active ^{S419D}SGK3 or inactive ^{K191N}SGK3. Furthermore channel protein abundance in the cell membrane was determined in *Xenopus* oocytes expressing $K_{ir}2.1$ with or without wild type SGK3 by confocal microscopy and chemiluminescence.

Materials and Methods

Constructs

For generation of cRNA [40], constructs were used encoding human wild type $K_{ir}2.1$ [41], and $K_{ir}2.1$-HA containing an extracellular hemagglutinin epitope [42], wild type SGK3 [43], constitutively active ^{S419D}SGK3 [44], and inactive ^{K191N}SGK3 [44]. The constructs were used for the generation of cRNA as described previously [45].

Voltage clamp in Xenopus oocytes

Xenopus oocytes were prepared as previously described [46]. cRNA encoding $K_{ir}2.1$ (10 ng) was injected with or without 1, 3 or 10 ng of cRNA encoding either wild type human SGK3, constitutively active ^{S419D}SGK3 or inactive ^{K191N}SGK3 on the day of preparation of the *Xenopus* oocytes [47]. All experiments were performed at room temperature 3 days after injection. In two-electrode voltage-clamp experiments $K_{ir}2.1$ currents were elicited every 20 s with 1 s pulses from -150 mV to +30 mV applied from a holding potential of -60 mV. The oocytes were maintained at 17°C in a ND96 solution with antibiotics (ND96-A) containing 88.5 mM NaCl (Sigma-Aldrich Chemie, Steinheim, Germany), 2 mM KCl (Carl Roth, Karlsruhe, Germany), 1.8 mM $CaCl_2$ (Sigma-Aldrich Chemie, Steinheim, Germany), 1 mM $MgCl_2$ (Sigma-Aldrich Chemie, Steinheim, Germany), 5 mM HEPES (Carl Roth, Karlsruhe, Germany), 0.11 mM tetracycline (Sigma-Aldrich Chemie, Steinheim, Germany), 4 µM ciprofloxacin (Fresenius Kabi Austria, Austria), 0.22 mM gentamycin (Refobacin, Merck Serono, Darmstadt, Germany), 0.5 mM theophylline (Euphylong, Nycomed, Konstanz, Germany) as well as 5 mM sodium pyruvate (Sigma-Aldrich Chemie, Steinheim, Germany) [48]. The pH was adjusted to 7.4 by addition of NaOH (AppliChem, Darmstadt, Germany). In order to discriminate between enhanced insertion of $K_{ir}2.1$ into the cell membrane and delayed retrieval of $K_{ir}2.1$ from the membrane, experiments were performed utilizing brefeldin A (Sigma-Aldrich Chemie, Steinheim, Germany), which inhibits the trans-Golgi network (TGN) thereby preventing the insertion of newly synthesized channel proteins into the cell membrane [49, 50]. cRNA encoding $K_{ir}2.1$ and SGK3 were injected on the day of oocyte preparation. Brefeldin

A (5 μM) was added to the culture medium 24 h later (for a total 48 h incubation with brefeldin A) or 48 h later (for a total 24 h incubation with brefeldin A). All electrophysiological recordings were performed 72 h after the cRNA injection. Where indicated, the SGK inhibitor EMD638683 (Merck Serono, Merck, Darmstadt, Germany) was used at a concentration of 50 μM for 24 h. The following bath solutions were used as control superfusate in the two electrode voltage clamp experiments at 10 mM K^+ (88 mM NaCl, 10 mM KCl, 1.8 mM $CaCl_2$,1 mM $MgCl_2$ and 5 mM HEPES, titrated to pH 7.4 by NaOH) or at 5 mM K^+ (93 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$,1 mM $MgCl_2$ and 5 mM HEPES, titrated to pH 7.4 by NaOH). The data were filtered at 1 kHz and recorded with a Digidata 1322A A/D-D/A converter and Clampex V.4.2 software for data acquisition and analysis (Axon Instruments, Union City, CA, USA) [51]. The analysis of the data was performed with Clampfit 9.2 (Axon Instruments, Union City, CA, USA) software [52]. For the analysis the inward current conductance was determined between -150 mV and -90 mV from the respective current-voltage relations.

Immunocytochemistry

To visualize $K_{ir}2.1$ cell surface expression, the oocytes were fixed in 4% paraformaldehyde for 2 h at room temperature. After washing with PBS, the oocytes were cryoprotected in 30% sucrose, frozen in mounting medium and placed on a cryostat. Sections were collected at a thickness of 8 μm on coated slides and stored at -20°C. For immunostaining, the slides were dried at room temperature, then fixed in aceton/methanol (1:1), washed in PBS and blocked for 1 h in 5% bovine serum albumin in PBS. The primary antibody used was rabbit anti-$K_{ir}2.1$ antibody (1:200, Abcam, Cambridge, UK) incubated overnight at 4°C. Binding of primary antibody was visualised with a FITC-conjugated goat anti-rabbit IgG (2μg/ml, Invitrogen, Molecular Probes, Eugene, OR, USA). Then, oocytes were analyzed by a fluorescence laser scanning microscope (LSM 510, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with A-Plan 40x/0.25 DICIII [53]. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series.

Detection of $K_{ir}2.1$-HA cell surface expression by chemiluminescence

To determine $K_{ir}2.1$-HA cell surface expression by chemiluminescence [54], oocytes were incubated with monoclonal anti-HA antibody conjugated to Horseradish Peroxidase (diluted 1:1000, Miltenyi Biotec, Germany). Individual oocytes were placed in 96 well plates with 20 μl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s [53, 55]. Results display normalized relative light units.

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All oocyte experiments were repeated with at least 3 batches of oocytes; in all repetitions qualitatively similar data were obtained. To avoid any bias due to use of different batches of oocytes comparisons were always made within the same batch of oocytes. Thus, separate controls were generated for each series of experiments. Data were tested for significance using ANOVA, Bonferroni test, and results with $p < 0.05$ were considered statistically significant.

Results

A first series of experiments explored whether the constitutively active serum & glucocorticoid inducible kinase ^{S419D}SGK3 influences the inwardly rectifying current of $K_{ir}2.1$ expressing *Xenopus* oocytes. In $K_{ir}2.1$-expressing, but not in water-injected *Xenopus* oocytes, an inwardly rectifying current (I_{Kir}) was observed (Fig. 1). Experiments were performed with 5 mM K^+ (Fig. 1A-C) and with 10 mM K^+ (Fig. 1D,E) in the bath. In $K_{ir}2.1$ expressing oocytes (10 ng cRNA), the reversal potential was -81.1 ±2.0 mV (n=34) at 5 mM K^+ and -57.1 ± 1.8 mM (n=9) at 10 mM K^+ in the bath. Coexpression of ^{S419D}SGK3 (10 ng cRNA) was followed by a significant increase of I_{Kir} as measured in both 5 mM K^+ (Fig. 1A-C) and 10 mM K^+ (Fig. 1D,E) bath solutions. In the following experiments 10 mM K^+ was used in the bath.

In contrast to the constitutively active ^{S419D}SGK3, the inactive ^{K191N}SGK3 failed to significantly modify the $K_{ir}2.1$ current (Fig. 2A). Accordingly, kinase activity was required for the stimulating effect of SGK3 on $K_{ir}2.1$ currents. Similar to expression of ^{S419D}SGK3, expression

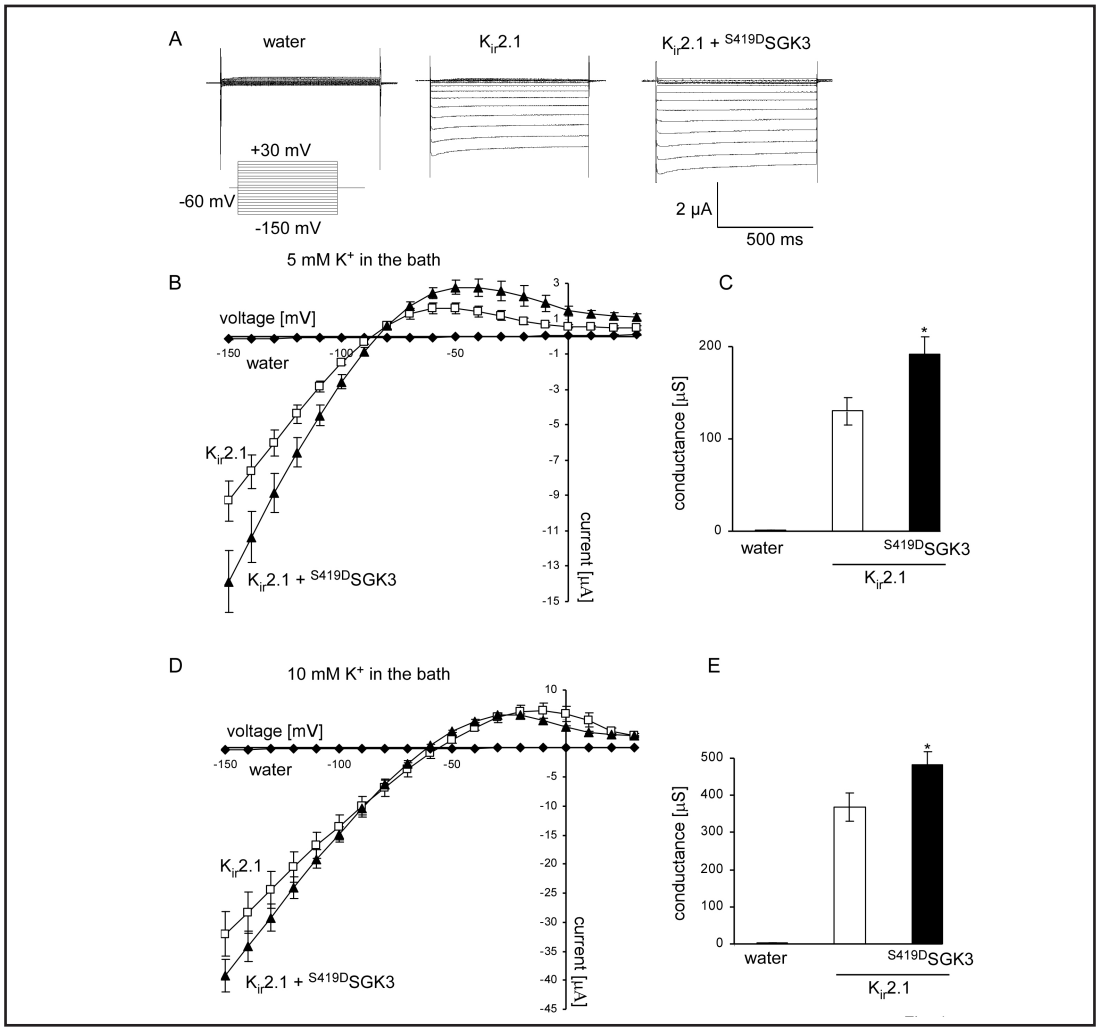

Fig. 1. Coexpression of constitutively active ^{S419D}SGK3 increased inwardly rectifying current in $K_{ir}2.1$-expressing *Xenopus* oocytes. A. Original tracings illustrating currents recorded in 5 mM K^+ containing bath solution determined in *Xenopus* oocytes injected with water (left), or expressing $K_{ir}2.1$ without (middle) or with (right) coexpresion of constitutively active ^{S419D}SGK3. B. Arithmetic means ± SEM (n = 6-34) of the current recorded in 5 mM K^+ containing bath solution as a function of voltage (I/V-curves) in *Xenopus* oocytes injected with water (closed diamonds) or expressing $K_{ir}2.1$ without (open squares) or with (closed triangles) additional expression of ^{S419D}SGK3. C. Arithmetic means ± SEM (n = 6-34) of the inward current conductance analyzed from I/V-curves shown in B between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar) or expressing $K_{ir}2.1$ without (white bar) or with (black bar) additional expression of ^{S419D}SGK3. * indicates statistically significant ($p<0.05$) difference from oocytes expressing $K_{ir}2.1$ alone. D. Arithmetic means ± SEM (n = 3-11) of the current recorded in 10 mM K^+ containing bath solution as a function of voltage (I/V-curves) in *Xenopus* oocytes injected with water (closed diamonds) or expressing $K_{ir}2.1$ without (open squares) or with (closed triangles) additional expression of ^{S419D}SGK3. E. Arithmetic means ± SEM (n = 3-11) of the inward current conductance analyzed from I/V-curves shown in D between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar) or expressing $K_{ir}2.1$ without (white bar) or with (black bar) additional expression of ^{S419D}SGK3. * indicates statistically significant ($p<0.05$) difference from oocytes expressing $K_{ir}2.1$ alone.

of wild type SGK3 (10 ng cRNA) significantly increased the inward current of $K_{ir}2.1$ (10 ng cRNA) expressing oocytes (Fig. 2A). The conductance was not significantly different in oocytes injected with 1 ng cRNA encoding Kir2.1 alone (132 ± 14 µS, n = 9) and in oocytes

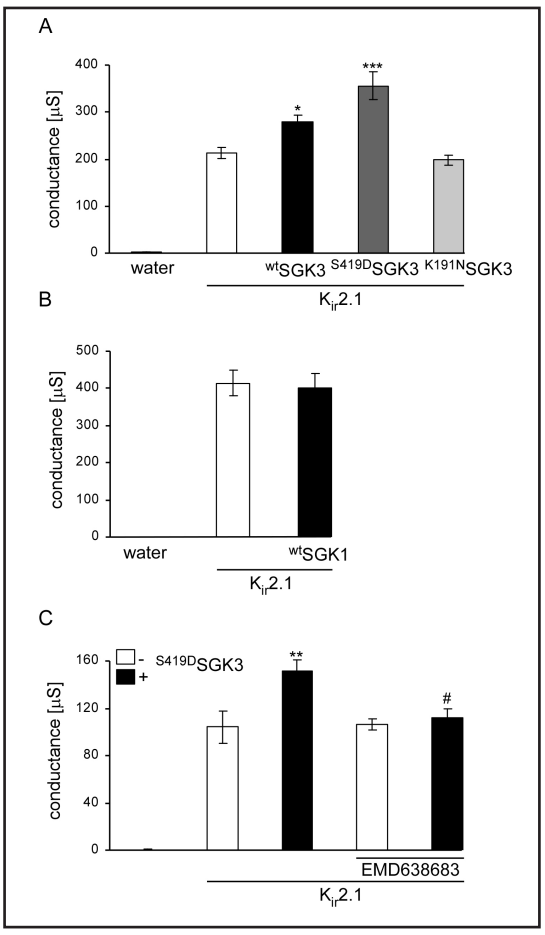

Fig. 2. The effect of inactive ^{K191N}SGK3, wild type SGK3, wild type SGK1 and constitutively active ^{S419D}SGK3 in the presence of the SGK inhibitor EMD638683. A. Arithmetic means ± SEM (n = 10-22) of the inward current conductance analyzed between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar), or expressing $K_{ir}2.1$ without (white bar) or with wild type SGK3 (black bar), constitutively active ^{S419D}SGK3 (dark grey bar) or inactive ^{K191N}SGK3 (light grey bar). *, *** indicate statistically significant ($p<0.05$, $p<0.001$) difference from oocytes expressing $K_{ir}2.1$ alone. B. Arithmetic means ± SEM (n = 11-15) of the inward current conductance analyzed between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar) or expressing $K_{ir}2.1$ without (white bar) or with (black bar) additional expression of wild type SGK1. C. Arithmetic means ± SEM (n = 6-11) of the inward current conductance analyzed between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar), or expressing $K_{ir}2.1$ without (white bars) or with (black bars) constitutively active ^{S419D}SGK3 in the presence (4^{th} & 5^{th} bars) or in the absence (2^{nd} & 3^{rd} bars) of the SGK inhibitor EMD638683 (50 µM, 24 h) ** indicates statistically significant ($p<0.01$) difference from untreated oocytes expressing $K_{ir}2.1$ alone, # indicates statistically significant ($p<0.05$) difference from oocytes coexpressing ^{S419D}SGK3 without inhibitor treatment.

injected in addition with low levels (3 ng) of SGK3 (112 ± 10 µS, n = 7). Moreover, following injection of lower amounts of cRNA encoding Kir2.1 (1 ng) and ^{S419D}SGK3 (3 ng), the conductance was similar in *Xenopus* oocytes expressing $K_{ir}2.1$ alone (287 ± 30 µS, n = 19) and in oocytes expressing both $K_{ir}2.1$ and ^{S419D}SGK3 (311 ± 30 µS, n = 17). Similarly, injection of cRNA encoding SGK1 (10 ng) did not modify I_{Kir} (Fig. 2B).

Further experiments were performed to test whether the $K_{ir}2.1$ activity in ^{S419D}SGK3 expressing oocytes could be modified by the SGK inhibitor EMD638683 [56]. As shown in Figure 2C, treatment with EMD638683 (50 µM, 24 h) abrogated the SGK3-mediated upregulation of $K_{ir}2.1$. EMD638683 had no effect on $K_{ir}2.1$ current in the absence of ^{S419D}SGK3 (Fig. 2C).

A second series of experiments explored whether the effect of SGK3 on $K_{ir}2.1$ currents resulted from an effect of the kinase on $K_{ir}2.1$ protein abundance in the cell membrane. The protein abundance was visualized by confocal microscopy and quantified by chemiluminescense. As shown in Figure 3, the cell surface expression of the $K_{ir}2.1$ channel protein in *Xenopus* oocytes injected with cRNA encoding $K_{ir}2.1$ or tagged $K_{ir}21$-HA was indeed increased by the coexpression of wild type SGK3.

The increased channel protein abundance in the cell membrane could result from either, accelerated insertion of channel protein into the cell membrane or delayed retrieval of channel protein from the cell membrane. To discriminate between those two possibilities, voltage clamp experiments were performed in the absence and presence of brefeldin A (5 µM). *Xenopus* oocytes expressing either $K_{ir}2.1$ alone or together with wild type SGK3 were treated with 5 µM brefeldin A, which blocks the insertion of new carrier protein into the

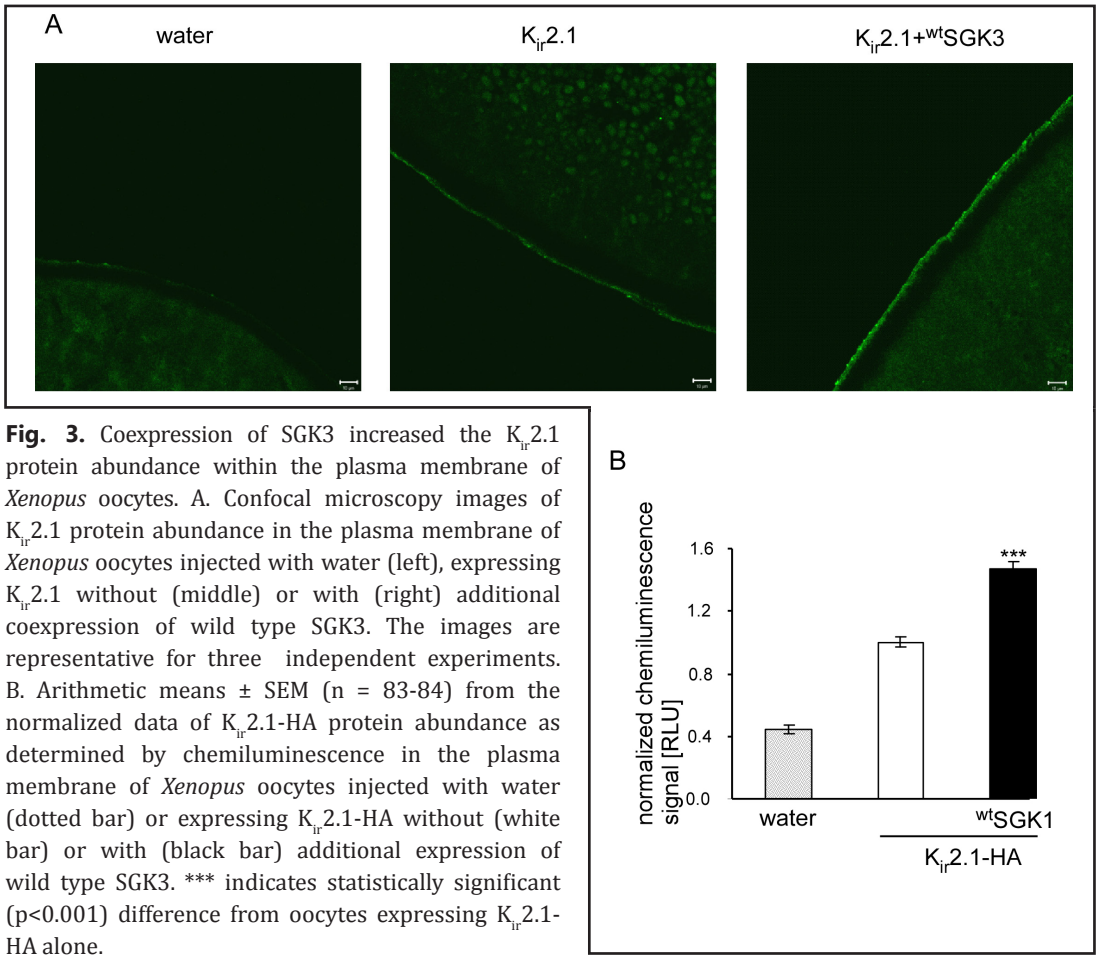

Fig. 3. Coexpression of SGK3 increased the $K_{ir}2.1$ protein abundance within the plasma membrane of *Xenopus* oocytes. A. Confocal microscopy images of $K_{ir}2.1$ protein abundance in the plasma membrane of *Xenopus* oocytes injected with water (left), expressing $K_{ir}2.1$ without (middle) or with (right) additional coexpression of wild type SGK3. The images are representative for three independent experiments. B. Arithmetic means ± SEM (n = 83-84) from the normalized data of $K_{ir}2.1$-HA protein abundance as determined by chemiluminescence in the plasma membrane of *Xenopus* oocytes injected with water (dotted bar) or expressing $K_{ir}2.1$-HA without (white bar) or with (black bar) additional expression of wild type SGK3. *** indicates statistically significant ($p<0.001$) difference from oocytes expressing $K_{ir}2.1$-HA alone.

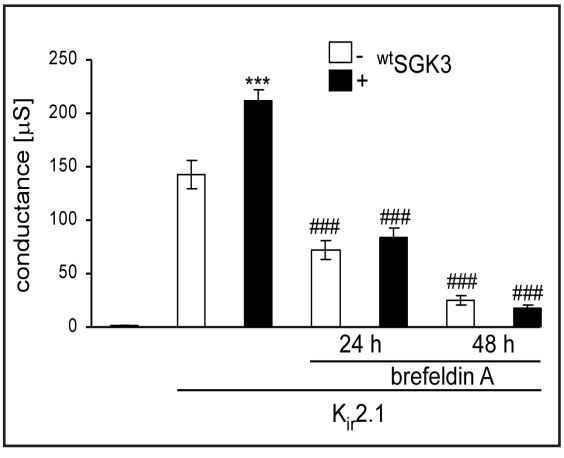

Fig. 4. Effect of brefeldin A in $K_{ir}2.1$ expressing *Xenopus* oocytes with or without SGK3 coexpression. Arithmetic means ± SEM (n = 10-22) of the inward current conductance analyzed between -150 mV and -90 mV in *Xenopus* oocytes injected with water (dotted bar), or expressing $K_{ir}2.1$ without (white bars) or with (black bars) additional expression of wild type SGK3 prior to (left bars) or following a 24 h (middle bars) or a 48 h (right bars) treatment with 5 µM brefeldin. *** indicates statistically significant ($p<0.001$) difference from the expression of $K_{ir}2.1$ alone; ### indicates statistically significant ($p<0.001$) difference from the absence of brefeldin A.

cell membrane. As illustrated in Figure 4, brefeldin A treatment was followed by a decay of the current, which was similar in *Xenopus* oocytes expressing $K_{ir}2.1$ together with wild type SGK3 and in *Xenopus* oocytes expressing $K_{ir}2.1$ alone. Thus SGK3 apparently does not delay the channel retrieval from the cell membrane.

Discussion

The present observations provide evidence for the ability of the serum & glucocorticoid inducible kinase SGK3 to up-regulate the inwardly rectifying current generated by the K^+ channel $K_{ir}2.1$. According to confocal microscopy and chemiluminescence, SGK3 increases the channel protein abundance in the cell membrane presumably by stimulating channel insertion into the cell membrane. The effect was only apparent following injection of 10 ng cRNA encoding the channel and the kinase. Similar to what has been observed earlier [57], injection of lower amounts of cRNA did not yield a significant regulation of the channel. Thus, SGK3 possibly affects $K_{ir}2.1$ only, when it is highly expressed. Accordingly, the present observations do not allow the prediction of SGK3 sensitive $K_{ir}2.1$ activity for any individual cell type. Nevertheless, the present observations disclose the ability of SGK3 to up-regulate $K_{ir}2.1$, an effect possibly contributing to channel regulation at conditions of strong SGK3 expression.

The effect of SGK3 could be reversed by the SGK inhibitor EMD638683. At the concentrations used, the substance inhibits all three SGK isoforms [56]. Far higher concentrations are needed to inhibit further kinases [56].

The percentage increase of current following coexpression of SGK3 was not identical in the different series of electrophysiological experiments (compare Figs.1 and 2). Moreover, the percentage increase of protein abundance following coexpression of SGK3 was not identical to the percentage increase of current following coexpression of SGK3. It must be kept in mind that the expression of proteins may vary between different batches of oocytes and thus, statistical comparisons were always made between oocytes from the same batch. Comparisons between different series of experiments done in different batches of oocytes do not allow safe conclusions.

The decay of current following treatment of the oocytes with brefeldin A was similar in oocytes expressing $K_{ir}2.1$ together with SGK3 and in *Xenopus* oocytes expressing $K_{ir}2.1$ alone. The decay of the current in the presence of brefeldin A reflects the retrieval of channel protein by inactivation, degradation or internalization of the channel protein. The data in Fig.4 reveals that expressed $K_{ir}2.1$ does not remain functional but that its function is a matter of gradual decay. The actual steady state expression level of $K_{ir}2.1$ is a function of channel insertion into and channel retrieval from the cell membrane. As SGK3 increases the steady state expression level of $K_{ir}2.1$, an effect not explained by delayed channel retrieval, the observations suggest that SGK3 stimulates the insertion of novel channel protein into the cell membrane.

Conclusion

The present observations show that $K_{ir}2.1$ channels are regulated by the serum & glucocorticoid inducible kinase isoform SGK3, an effect expected to modify the function and survival of a wide variety of cells.

Conflict of Interests

This study was supported by the Deutsche Forschungsgemeinschaft (GK 1302). The authors of this manuscript declare that they have neither financial nor any other conflicts of interests.

Acknowledgements

The authors acknowledge the technical assistance of E. Faber. The manuscript was meticulously prepared by L. Subasic and S. Rübe.

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