Upregulation of HERG Channels by the Serum and Glucocorticoid Inducible Kinase Isoform SGK3

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Key Words

HERG • Potassium channel • SGK • Protein kinases
• Oocytes

Abstract

Human ether-a-go-go (HERG) channels participate in the repolarization of the cardiac action potential. Loss of function mutations of HERG lead to delayed cardiac repolarization reflected by prolonged QT interval. HERG channels are regulated through a signaling cascade involving phosphatidylinositol 3 (PI3) kinase. Downstream targets of PI3 kinase include the serum and glucocorticoid inducible kinase (SGK) and protein kinase B (PKB) isoforms. The present study has been performed to explore whether SGK1 and SGK3 participate in the regulation of HERG channel activity. HERG was expressed in *Xenopus* oocytes with or without additional expression of SGK1 or SGK3. Chemiluminescence was employed to determine HERG plasma membrane protein abundance. Coexpression of SGK3 but not of SGK1 in *Xenopus* oocytes resulted in an increase of steady state current (I_{HERG}) and enhanced cell membrane protein abundance without affecting gating kinetics

of the channel. Replacement of serine by alanine at the two SGK consensus sites decreased I_{HERG} but neither mutation abolished the stimulating effect of SGK3. In conclusion, SGK3 participates in the regulation of HERG by increasing HERG protein abundance in the plasma membrane and may thus modify the duration of the cardiac action potential.

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Introduction

The serum and glucocorticoid inducible kinase (SGK1) has originally been cloned from rat mammary tumour cells as a glucocorticoid responsive gene [1-3]. Homology screening led to the discovery of the two isoforms SGK2 and SGK3 [4]. Similar to the related protein kinase B (PKB) all three SGK isoforms are activated by phosphorylation through a signaling cascade including phosphatidylinositol 3 (PI3) kinase and the 3-phosphoinositide dependent kinases PDK1 and PDK2 [5-7]. The reported stimulators of SGK include insulin

[6, 8], insulin like growth factor IGF1 [6, 9], depolarization [10], cAMP [10] and oxidation [10].

SGK1 is under transcriptional control of glucocorticoids [11-15], mineralocorticoids [12, 16-22], as well as further hormones and mediators [23-25]. It is heavily expressed in fibrosing tissue [26-30]. SGK2 and SGK3 appear to be constitutively expressed and their transcription has hitherto not been shown to be sensitive to hormonal action [24]. Both, SGK1 [31] and SGK3 [4] are expressed in virtually all tissues tested including the heart. *In vitro* coexpression experiments disclosed the ability of SGK1 and/or SGK3 to activate the voltage-gated Na⁺ channel SCN5A [32] and the K⁺ channel KCNE1/KCNQ1 [33]. The effect of the kinases on KCNE1/KCNQ1 is expected to accelerate the repolarisation thus leading to shortening of the QT interval. As a matter of fact, the QT interval was found to be significantly shorter in carriers of a putative gain of function polymorphism of the SGK1 gene [34].

The effect of the kinases on SCN5A [32] and KCNE1/KCNQ1 [33] is at least partially due to inhibition of the ubiquitin ligase Nedd4 or Nedd4-2. The ubiquitin ligase ubiquitinates target proteins thus preparing them for clearance from the cell membrane [35]. SGK1 phosphorylates Nedd4-2 and thus decreases the affinity of the ubiquitin ligase to its target proteins [36, 37].

Besides KCNE1/KCNQ1 the human ether-a-go-go (HERG) channel contributes to the repolarization of the cardiac action potential. Loss of function mutations of the genes encoding either KCNE1/KCNQ1 or HERG are known to delay cardiac repolarization and thus to cause long QT syndrome, a potentially lethal disorder of cardiac excitation [38, 39].

Recent evidence indicates that HERG channels are regulated by PI3 kinase [40]. Accordingly, pharmacological inhibition of PI3 kinase with Wortmannin decreased, whereas transfection of constitutively active PI3 kinase stimulated HERG activity. Transfection of constitutively active protein kinase B (^{T308D,S473D}PKB) mimicked the effect of PI3 kinase pointing to involvement of this kinase in the regulation of HERG channels [40]. The HERG sequence bears two putative phosphorylation sites for the serum and glucocorticoid inducible kinase isoforms SGK1-3 at position ³³¹Ser and ⁸⁹⁰Ser.

The present study has been performed to explore whether the cardiac SGK isoforms SGK1 and SGK3 are capable to regulate HERG channels.

Materials and Methods

Site directed mutagenesis of HERG

The mutated HERG channels ^{S331A}HERG, ^{S890A}HERG and ^{S331AS890A}HERG were generated by site-directed mutagenesis

(QuikChange site-directed mutagenesis kit, Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The following primers were used:

S331AHERG_s: 5' CGC TAC CGC ACC ATT GCC AAG ATT CCC CAA ATC 3';

S331AHERG_{as}: 5' GAT TTG GGG AAT CTT GGC AAT GGT GCG GTAGCG 3';

S890AHERG_s: 5' CGC AAG CGC AAG TTG GCC TTC CGC AGG CGC ACG 3';

S890AHERG_{as}: 5' CGT GCG CCT GCG GAA GGC CAA CTT GCG CTT GCG 3'.

All mutants were sequenced to verify the presence of the desired mutation.

Expression in Xenopus oocytes and voltage-clamp analysis

cRNAs encoding wild type HERG, ^{S331A}HERG, ^{S890A}HERG, ^{S331AS890A}HERG, human wild type SGK1 and human wild type SGK3 were synthesized *in vitro* using a commercially available kit (Ambion, Austin, TX, USA) following the manufacturer's instructions.

Handling and injection of *Xenopus* oocytes and synthesis of cRNA have previously been described in detail [41]. Oocytes were first injected with water or 7.5 ng cRNA encoding wild type SGK1 or SGK3 and one day later with 5ng cRNA encoding HERG. Standard two electrode voltage clamp recordings were performed 4 days after HERG injection. Oocytes were superfused continuously with ND-96 buffer containing (mM): NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1 and HEPES 5 (pH 7.4 with NaOH). Pipettes were filled with 3 M KCl and had resistances of 0.5-1.0 MW. Experiments were performed with a Geneclamp 500B amplifier (Axon Instruments, Union City, CA, USA) and a Digidata 1322A interface (Axon Instruments, Union City, CA, USA). Data acquisition was achieved with pCLAMP 9.0 (Axon Instruments, Union City, CA, USA). Activation curves were fitted with a Boltzmann distribution: $G(V) = G_{\max} / (1 - \exp[(V_{1/2} - V)/k])$, where V is the test pulse potential, $V_{1/2}$ is the half-maximal activation potential, and k is the slope of the activation curve. All experiments were repeated with at least three batches of oocytes; in all repetitions, qualitatively similar data were obtained. HERG currents in the histograms were normalized by dividing the current of each oocyte by the mean current of the control group (HERG). For the kinetic analysis HERG currents were normalized by dividing the current of each oocyte at a given test voltage by the current evoked by the same oocyte at the most positive test voltage. Care was taken to use oocytes of the same developmental stage and diameter to minimize variation within the experimental groups.

Fig. 1. SGK3 and PKB are potent stimulators of HERG activity. *Xenopus* oocytes were injected with wild type SGK1 or constitutively active S^{422P} SGK1, wild type SGK2, wild type SGK3 or constitutively active T^{308D},S^{473D} PKB and one day later with water or HERG cRNA. While neither SGK1 nor SGK2 affected HERG activity (A, B), both SGK3 and PKB stimulated HERG currents (B). No effects of protein kinases on background conductances could be detected (C). Four days after ion channel injection, HERG currents were quantified by two electrode voltage clamp. Arithmetic means $\pm$ SEM. * indicates significant difference to cells injected with HERG alone. For analysis, I_{HERG} was taken after depolarizing the membrane potential to 0 mV from -80 mV holding potential. Arithmetic means $\pm$ SEM.

Detection of cell surface expression by chemiluminescence

Chemiluminescence has widely been used for quantification of channel protein abundance at the cell surface [42]. Defolliculated oocytes were first injected with water or with human SGK3 cRNA (7.5 ng/oocyte), and one day later with extracellularly hemagglutinin (HA)-tagged HERG (7.5 ng/oocyte) [43]. Oocytes were incubated with 1 μ g/ml primary rat monoclonal anti-HA antibody (clone 3F10, Boehringer, Germany) and 2 μ g/ml secondary, peroxidase-conjugated affinity-purified F(ab')₂ goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA). Individual oocytes were placed in 20 μ l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer by integrating the signal over a period of 1 s. The SuperSignal ELISA Femto Maximum Sensitivity Substrate contains Luminol as the substrate of the chemiluminescence reaction. The emitted light output is linearly dependent on the amount of peroxidase conjugated to the 2nd antibody. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol. As a control, background luminescence was measured from non-injected oocytes.

Western blotting

For determination of HERG expression in whole cell lysates, oocytes were homogenized in lysis buffer containing 50 mM Tris (pH 7.5), 0.5 mM EDTA (pH 8.0), 0.5 mM EGTA, 100 mM NaCl, 1% Triton X-100 and protease inhibitor cocktail (Roche, Penzberg, Germany) at the recommended concentrations. 30 μ g of total protein were separated in a 10% polyacrylamide gel and transferred to a nitrocellulose membrane at 100 V for 90 min. For immunoblotting, rabbit anti-HERG antibody (Alomone Labs, Jerusalem, Israel, diluted 1:400 in PBS/0.15% Tween 20/5% nonfat dry milk) was used to detect HERG protein. After blocking with 5% nonfat dry milk in PBS/0.15% Tween 20 for 1 h at room temperature, blots were incubated with the primary antibody for an additional hour at room temperature. Secondary peroxidase-conjugated sheep anti-rabbit IgG (Amersham, Freiburg, Germany, diluted 1:1000 in PBS/0.15% Tween 20/5% nonfat dry milk) were incubated overnight at 4°C and used for luminescent detection with an

Fig. 2. HERG channel kinetics in the absence and presence of SGK3 or PKB. HERG gating kinetics remained unaffected upon coexpression of SGK3 or T308D, S473D PKB. While coexpression of the respective kinases increased HERG currents ~ 3 fold (A, B) no change in gating behaviour of the channel could be observed (C, D). Peak tail currents were normalized to the maximum current amplitude in cells expressing HERG in A and B. In C and D currents were normalized to the maximum peak tail current in the same group. Arithmetic means $\pm$ SEM. (E) Typical original recordings. N = 6 – 14. (F) Voltage protocol applied for the recordings depicted in E.

enhanced chemoluminescence (ECL) kit (Amersham, Freiburg, Germany).

Data and statistical analysis

Experimental data are expressed as arithmetic means $\pm$ SEM. Statistical significance was tested by using ANOVA, $P < 0.05$ was considered statistically significant.

Results

Heterologous expression of HERG channels in *Xenopus laevis* oocytes led to the induction of a voltage gated current. Coexpression of the constitutively active serum and glucocorticoid inducible kinase isoform 1 (S422D SGK1) or the wild type SGK1 did not significantly alter I_{HERG} (Fig. 1A). In contrast, the wild type SGK3 strongly activated I_{HERG} . As depicted in Fig. 1B, coexpression of SGK3 or constitutively active T308D,S473D PKB, used in this experiment as positive control, increased HERG activity almost fourfold. In contrast, SGK2 did not enhance I_{HERG} . The SGK kinases did not have any effect on intrinsic voltage activated conductances (Fig. 1C).

Conformational changes of voltage activated channels due to phosphorylation may affect the gating kinetics of the protein. To characterize HERG gating, test potentials ranging from -60 mV to +60 mV were applied to trigger activating currents. The test pulses were followed by a 400 ms pulse to -60mV to evoke outward tail currents. Fig. 2 summarizes the current voltage relationship of HERG currents upon coexpression of SGK3 or PKB. The amplitude of the peak tail current was plotted as a function of the preceding test potential. The left panel (A, B) displays the absolute current values to demonstrate potent upregulation of the current by SGK3 and PKB. The right panel (C, D) depicts peak tail currents that were normalized to the maximum peak tail current of the respective group to investigate kinetics. As obvious from C and D, coexpression of neither SGK3 nor PKB led to a shift of the voltage required for half maximal peak tail currents.

The HERG protein contains two putative SGK phosphorylation sites on its sequence at ³³¹Ser and ⁸⁹⁰Ser. We generated the three mutations ^{S331A}HERG, ^{S890A}HERG and ^{S331A, S890A}HERG to investigate whether PKB and SGK3 modulate HERG by phosphorylating the channel directly. Replacement of serine by alanine in the first phosphorylation site in ^{S331A}HERG decreased I_{HERG} by some 64% (Fig. 3). The activity of ^{S890A}HERG was only some 14% of the current mediated by the wild type

Fig. 3. Destruction of the putative SGK phosphorylation sites on the HERG protein does not prevent channel upregulation by SGK3. Comparable to the effect on wtHERG, SGK3 stimulated current activity of the ^{S331A}HERG, ^{S890A}HERG and ^{S331A, S890A}HERG ~ 3 fold. Currents evoked by the respective mutant HERG constructs were significantly lower compared to the wild type channel (Clamp protocol as in Fig. 1). Arithmetic means $\pm$ SEM.

channel. Mutation of both phosphorylation sites on the ^{S331A, S890A}HERG decreased the currents to 65% of the current mediated by the wild type channel (Fig. 3). Destruction of the putative phosphorylation sites did not prevent channel activation by SGK3. SGK3 coexpression did not only increase the current carried by wild type HERG (by 209%) but similarly increased the current carried by the ^{S331A, S890A}HERG double mutant (166%), the ^{S331A}HERG mutant (by 354%) and the ^{S890A}HERG mutant (by 143%).

As depicted in Fig. 4, destruction of the putative phosphorylation sites did not change HERG activation kinetics. Channel activation was neither affected in the HERG double nor single mutants. Also, channel kinetics were unaffected by coexpression of the SGK3 protein kinase. The upper panel (A) depicts current values normalized to the maximum peak tail currents of cells expressing HERG without SGK3. In panel B current values were plotted normalized to the maximum peak tail currents of the same group. Thus, activation of HERG currents by SGK3 is not due to a change in activation kinetics.

Fig. 4. Upregulation of $S331A$ HERG, $S890A$ HERG and $S331A, S890A$ HERG currents by SGK3 does not affect channel kinetics. While SGK3 induced HERG currents evoked by the SGK phosphorylation site mutants $S331A$ HERG, $S890A$ HERG and $S331A, S890A$ HERG (A), the channel kinetics remained unaffected (B). The slight shift of the curve observed with the double mutated $S331A, S890A$ HERG was not significantly different compared to control (Clamp protocol as in Fig. 1). Arithmetic means $\pm$ SEM. $n = 8 - 15$. (C) Typical original recordings.

Although HERG tail currents and activation kinetics are of major physiological importance, HERG phosphorylation may also affect steady state deactivation of the channel in the *Xenopus* oocyte expression system. Deactivation kinetics are summarized in Fig. 5. Coexpression of SGK3 did not affect the deactivation time constant τ (A) while absolute HERG currents were significantly activated by the SGK protein kinase (B).

To quantify HERG protein cell surface abundance, we generated a hemagglutinin tagged HERG construct. Proper expression of this HA-tagged HERG was demonstrated by Western blotting of whole cell lysates

(Fig. 6 A). HERG/GAPDH band intensity quantification indicated that total HERG protein abundance was not significantly affected by SGK3. Coexpression of SGK3 led to an enhancement of HERG plasma membrane expression by 151% as monitored by chemiluminescence of extracellularly HA-tagged HERG proteins (B). As demonstrated by electrophysiology, the insertion of the HA tag did not affect channel function. The coexpression of SGK3 increased the HERG(HA) induced current significantly (C). Similar to what has already been shown for the wild type channel, HERG(HA) kinetics were not affected by SGK3 (D).

Fig 5. HERG channel deactivation kinetics in the absence and presence of SGK3. HERG deactivation kinetics remained unaffected upon coexpression of SGK3 (A) while SGK3 absolute currents were increased upon coexpression of the kinase as depicted in the original recordings in B. (C) Voltage protocol applied for the recordings shown in B. $n = 8 - 11$.

Discussion

The present results demonstrate that the serine and threonine kinase SGK3 upregulates HERG channel currents by enhancing the channel abundance in the plasma membrane whereas it has no effect on intrinsic conductances of the *Xenopus laevis* oocyte. The unchanged total amount of HERG protein indicates that the observed increase of channel cell surface abundance is due to posttranslational rather than translational regulation. This observation casts new light on the functional significance of SGK3. The kinase has previously been shown to upregulate KCNE1/KCNQ1 [33], the other key potassium channel involved in cardiac repolarization [39]. Thus, SGK3 is an excellent candidate signalling molecule in the regulation of cardiac repolarization.

SGK1 failed to regulate HERG channels. This finding may be considered surprising, given that a certain variant of the SGK1 gene is associated with a decrease of the QT interval [34]. The influence of SGK1 on the QT

interval is probably the result of its effect on KCNE1/KCNQ1 [33]. As SGK3 stimulates both KCNE1/KCNQ1 [33] and HERG, its influence on cardiac repolarization may be even more potent than that of SGK1. However, the present *in vitro* results cannot be translated into the *in vivo* situation without reservations and presently no polymorphisms of SGK3 are known which could be correlated with the QT interval. In any case, all three kinases, SGK1 [31], SGK3 [4] and PKB [40] are expressed in cardiac tissue and all three kinases could participate in the regulation of cardiac ion channels. Their relative activity and thus their potential impact on ion channels, however, remain uncertain. Moreover, the relative contribution may depend on the functional condition. SGK3 appears to be constitutively expressed and less sensitive to transcriptional regulation [24]. In contrast SGK1 is under strong transcriptional control of cell volume [44, 45], glucocorticoids [1-3], or mineralocorticoids [12, 16-18, 20, 21]. Thus, SGK1 may allow adaptation to certain physiological and pathophysiological challenges whereas the

Fig. 6. Current amplitudes and plasma membrane abundance of HA-tagged HERG channels upon coexpression of SGK3. *Xenopus* oocytes were injected with water or SGK3 cRNA and one day later with HA-tagged HERG cRNA. Western blotting of whole cell lysates was performed to verify proper HERG expression (A), $n = 3$. Four days after HERG injection, plasma membrane abundance (B) and HERG mediated currents (C, D) were measured ($n = 8 - 14$). Arithmetic means $\pm$ SEM, * indicates significant difference to cells injected with HERG alone. Enhanced channel activity upon SGK3 coexpression correlated with enhanced channel abundance in the plasma membrane. A representative Western blot is shown demonstrating HERG and SGK3 expression.

regulation by SGK3 may be less variable.

The present observations suggest that SGK3 is not effective through direct phosphorylation of the HERG channel. The replacement of the serines by alanines in the SGK consensus sequences affects HERG channel activity but does not abrogate the stimulating effect of the kinase. Furthermore, the co-immunoprecipitation experiments suggest that the kinase does not bind to the HERG channel directly. In previous reports from our laboratory we demonstrated that SGK may regulate some cell membrane transporters and ion channels through direct phosphorylation of the membrane protein [46] or

through indirect modulation via the ubiquitin ligase Nedd4-2 [36, 47, 48]. SGK phosphorylates Nedd4-2 thus decreasing its substrate affinity and preventing ubiquitination and degradation of the transporter or ion channel [36]. Both mechanisms may occur in parallel. Compelling evidence suggests, however, the involvement of further mechanisms in addition to direct phosphorylation of the respective transport system and in addition to Nedd4-2 [49, 50]. Like these transporters, HERG channel activity and expression is not affected by Nedd4-2 (own observation) and similarly seems not to be directly targeted by SGK phosphorylation. Thus, the kinase may

be effective through phosphorylation of a hitherto unknown intermediate protein.

In conclusion, SGK3 modulates the cardiac HERG channel activity by enhancing HERG abundance in the plasma membrane without affecting HERG activation kinetics. Thus the kinase may participate in the regulation of cardiac repolarization under physiological and pathophysiological conditions.

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