Original Paper

PIKfyve Sensitivity of hERG Channels

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Key Words

K⁺ channel • Cardiac repolarization • Tumor cells • PKB • AKT • PIKfyve • Electrophysiology

Abstract

Background/Aims: Human ether-a-go-go (hERG) channels contribute to cardiac repolarization and participate in the regulation of tumor cell proliferation. Mutations in hERG channels may cause long QT syndrome and sudden cardiac death due to ventricular arrhythmias. HERG channel activity is up-regulated by the serum- and glucocorticoid-inducible kinase isoforms SGK1 and SGK3. Related kinases are protein kinase B (PKB/Akt) isoforms. SGK's and PKB/Akt's activate phosphatidylinositol-3-phosphate-5-kinase PIKfyve, which in turn up-regulates several carriers and channels. An effect of PIKfyve on hERG channels, has, however, never been shown. The present study thus explored the putative influence of PIKfyve on hERG channel expression and activity. **Methods:** hERG channels were expressed in *Xenopus* oocytes with or without PIKfyve and/or PKB, expression of endogenous and injected hERG quantified by RT-PCR, and hERG channel activity determined utilizing dual electrode voltage clamp. Moreover, hERG protein abundance in the cell membrane was visualized utilizing specific antibody binding and subsequent confocal microscopy and quantified by chemiluminescence. **Results:** Coexpression of wild type PIKfyve increased hERG channel activity in hERG-expressing *Xenopus* oocytes. hERG channel activity was further increased by coexpression of PKB, an effect augmented by additional coexpression of PIKfyve, but not by additional coexpression of PKB/Akt-resistant PIKfyve mutant PIKfyve^{S318A}. Coexpression of PIKfyve increased hERG channel protein abundance in the cell membrane. Inhibition of hERG channel insertion into the cell membrane by Brefeldin A (5 µM) resulted in a decline of current, which was similar in *Xenopus* oocytes expressing hERG together with PIKfyve and in *Xenopus* oocytes expressing hERG alone. **Conclusion:** hERG is up-regulated by PIKfyve, which is in turn activated by PKB/Akt.

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Introduction

The human ether-a-go-go (hERG) channels contribute to cardiac repolarization [1, 2]. Moreover, hERG channels are expressed in several tumor cells and contribute to the regulation of cell proliferation [3-5]. Owing to the role of hERG in tumor cells, hERG inhibitors have been considered for the treatment of malignancy [4-7].

hERG channels are up-regulated by the serum-and glucocorticoid-inducible kinase isoform SGK1 and SGK3 [8, 9]. SGK isoforms [10] and the related PKB/Akt isoforms [11] regulate carrier proteins in part by phosphorylation and activation of phosphatidylinositol-3-phosphate-5-kinase PIKfyve [11-14], also named PIPkIII [15], or PIP5K [16], which is the mammalian ortholog of the yeast Fab1p [17]. Mediated by its FYVE finger domain [18, 19], PIKfyve binds to PtdIns3P localized in membranes of late endocytic compartments [20]. PIKfyve converts PtdIns3P into PtdIns(3,5)P₂ thus regulating a variety of cell functions [21]. PIKfyve participates in the regulation of retrograde trafficking from the early endosome to the trans-Golgi network (TGN) [22] and thus modifies cell membrane protein trafficking [19, 23-26]. By this means, PIKfyve regulates glucose transporters [11-13], creatine transporter [27], glutamate transporters [28-30], glutamate receptors [31], Ca²⁺ channels [32], Cl channels [30, 33] and inward rectifier potassium channels [34].

In view of the effects of SGK1 and SGK3 on hERG channel activity and considering the role of PIKfyve in the actions of PKB/Akt and SGK isoforms, we hypothesized that PIKfyve participates in the regulation of hERG. In order to test this hypothesis, hERG was expressed in *Xenopus* oocytes with or without the expression of PIKfyve, and hERG channel function was determined utilizing dual-electrode voltage clamp and hERG channel protein abundance in the cell membrane by chemiluminescence as well as immunocytochemistry with subsequent confocal microscopy.

Materials and Methods

For generation of cRNA [35], constructs were used encoding hERG channels [8], hERG-HA containing an extracellular hemagglutinin epitope [36], human wild type PKB/AKT1 [37, 38], wild-type PIKfyve [28], and PKB resistant PIKfyve^{S318A} [28]. The cRNA was generated as described previously [39, 40].

To determine hERG transcripts levels, total RNA was isolated from *Xenopus* oocytes with or without injection of hERG cRNA by using Trifast Reagent (Peqlab Biotechnologie GmbH, Germany) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)₁₂₋₁₈ primers (Invitrogen, Life Technologies GmbH, Germany) and SuperScriptIII Reverse Transcriptase (Invitrogen, Life Technologies GmbH, Germany). cDNA samples were treated with RNaseH (Invitrogen, Life Technologies GmbH, Germany). Quantitative real-time PCR was performed with the iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories GmbH, Germany) and iQTM Sybr Green Supermix (Bio-Rad Laboratories, GmbH, Germany) according to the manufacturer's instructions. The following primers were used (5'→3' orientation): human ERG fw: CAACCTGGGCGACCAGATAG; human ERG rev: GGTGTTGGGAGAGACGTTGC; *Xenopus laevis* Gapdh fw: GACCTGCCGCCTGCAGAAG; *Xenopus laevis* Gapdh rev: GACTAGCAGGATGGGCGAC. The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate, and mRNA fold changes were calculated by the 2^{-ΔΔCt} method using Gapdh as internal reference. Averaging and statistical tests were carried out with the logarithmic values of transcripts levels.

For voltage clamp analysis, *Xenopus* oocytes were prepared as previously described [37]. Where indicated oocytes were injected with water or 10 ng cRNA encoding PKB, PIKfyve and/or PIKfyve^{S318A} and later on the same day with 10 ng cRNA encoding hERG. Standard two electrode voltage clamp recordings were performed 3 days after hERG injection [41, 42]. The oocytes were maintained at 17°C in a solution containing 88.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, tetracycline (Sigma, 0.11mM), ciprofloxacin (Sigma, 4µM), gentamycin (refobacin © 0.2mM) and theophyllin (euphyllong ©, 0.5mM) as well as sodium pyruvate (Sigma, 5mM) were added to the ND96, pH was adjusted to 7.5 by addition of NaOH. The control superfusate contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH was adjusted to 7.4 by addition of NaOH [43].

Pipettes were filled with 3 M KCl and had resistances of 0.3–3.0 M Ω . 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 9.2 (Axon Instruments, Union City, CA, USA) [44]. For data analysis, leak currents were subtracted. Leak currents were estimated from the tail current measured after the preconditioning prepulse to -80 mV. To discriminate between alterations of insertion and retrieval of hERG channel protein from the plasma membrane, the insertion was inhibited by Brefeldin A [45], where indicated. In those experiments, the oocytes were preincubated for 24 and 48 hours before measurement in the presence of Brefeldin A (Sigma, Schnellendorf, Germany) at a concentration of 5 μ M [46].

To visualize the hERG-HA protein abundance in the cell membrane, immunocytochemistry was performed. After 4% paraformaldehyde/PBS fixation for 2 h, oocytes were cryoprotected in 30% sucrose, frozen in mounting medium, and placed on a cryostat. Sections were collected at a thickness of 8 μ m on coated slides and stored at -20°C. For immunostaining, sections were thawed at room temperature, fixed in acetone/methanol (1:1) for 15 min at room temperature, washed in PBS and blocked for 1 hour in 5% bovine serum albumin/PBS. Sections were incubated overnight at 4°C with the primary 1 μ g/mL rat monoclonal anti-HA antibody (clone 3 F10, Roche, Mannheim, Germany). Binding of primary antibody was visualised with fluorescence-labeled secondary Alexa Fluor 488 goat anti-rat IgG (1:200, Invitrogen, UK) for 1 h at room temperature. The slides were mounted with Pro Long Gold antifade reagent (Invitrogen, UK). Images were taken on Zeiss LSM5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Germany) with A-Plan 40x/1.2W. Brightness and contrast settings were kept constant during imaging of all oocytes in each injection series. Due to autofluorescence of the oocyte yolk, unspecific immunofluorescence was observed inside the oocytes.

To determine hERG-HA cell surface expression by chemiluminescence [28], the oocytes were incubated with 1 μ g/mL primary rat monoclonal anti-HA antibody (clone 3 F10, Roche, Mannheim, Germany) and subsequently with secondary HRP-conjugated goat anti-rat IgG antibody (1:1000, Cell Signaling Technology, MA, USA). Individual oocytes were placed in 96 well plates with 20 μ l 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 [28]. Results display normalized relative light units. Integrity of the measured oocytes was assessed by visual control after the measurement to avoid unspecific light signals from the cytosol.

Data are provided as arithmetic means $\pm$ SEM or geometric mean $\pm$ SEM as indicated in the figure legends; n represents the number of oocytes investigated. All oocyte experiments were repeated with at least three batches of oocytes; in all repetitions, qualitatively similar data were obtained. All data were tested for significance by using one way ANOVA with Tukey's post-hoc test and $p < 0.05$ was considered statistically significant.

Results

The present study explored whether the phosphatidylinositol-3-phosphate-5-kinase PIKfyve influences the function of human ether-a-go-go (hERG) channels. To this end, cRNA encoding hERG was injected either with or without cRNA encoding PIKfyve. Quantitative RT-PCR was employed to test for hERG expression. As illustrated in Fig. 1A, ERG transcript levels are detectable in *Xenopus* oocytes injected with water or with PIKfyve cRNA alone. Thus, *Xenopus* oocytes express low levels of endogenous ERG channels. Coinjection of hERG cRNA significantly increased hERG transcripts levels in *Xenopus* oocytes. HERG-mediated current was determined utilizing dual electrode voltage clamp. The channel activity was analyzed by depolarization from -80 mV holding potential to different voltages followed by a 500 ms pulse to -60 mV. In *Xenopus* oocytes expressing hERG but not in water-injected oocytes or PIKfyve-injected oocytes the maneuver resulted in outward tail currents (Fig. 1B,C). In hERG-expressing *Xenopus* oocytes, the additional expression of wild type PIKfyve significantly increased the tail current (Fig. 1B,C). Fig. 1D illustrates the current-voltage relationship of hERG currents with or without coexpression of wild type PIKfyve. The amplitude of the peak tail current was plotted as a function of the preceding test potential. As illustrated in Fig. 1D,

Fig. 1. Coexpression of PIKfyve increased hERG current in *Xenopus* oocytes. **A.** Geometric means $\pm$ SEM ($n = 5$) of hERG transcripts levels in *Xenopus* oocytes injected with water, with cRNA encoding PIKfyve alone, with cRNA encoding hERG alone, or with cRNA encoding hERG and PIKfyve. ***($p < 0.001$) indicates statistically significant difference from *Xenopus* oocytes injected with water. **B.** Original tracings recorded in *Xenopus* oocytes injected with water (a), with cRNA encoding PIKfyve alone (b), with cRNA encoding hERG alone (c) or with cRNA encoding hERG together with PIKfyve (d). The *Xenopus* oocytes were depolarized from -80 mV holding potential to different voltages followed by a 500 ms pulse to -60 mV evoking outward tail currents. **C.** Arithmetic means $\pm$ SEM ($n = 13$ -20) of the normalized outward tail current following a depolarization to $+70$ mV, recorded in *Xenopus* oocytes injected with water, with cRNA encoding PIKfyve alone, with cRNA encoding hERG alone, or with cRNA encoding hERG and PIKfyve. ***($p < 0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing hERG channels alone. **D.** Arithmetic means $\pm$ SEM ($n = 16$ -21) of the peak tail current as a function of voltage in *Xenopus* oocytes injected with water (white diamonds), with cRNA encoding PIKfyve alone (white squares), with cRNA encoding hERG alone (white circles) or with cRNA encoding hERG and PIKfyve (black circles). **E.** Arithmetic means $\pm$ SEM ($n = 17$ -21) of the normalized peak tail current as a function of voltage in *Xenopus* oocytes injected with cRNA encoding hERG alone (white circles) or with cRNA encoding hERG and PIKfyve (black circles).

the absolute current values were up-regulated by coexpression of wild type PIKfyve. Following normalization to the maximum peak tail current for each group, no significant differences were apparent between *Xenopus* oocytes expressing hERG together with PIKfyve and *Xenopus* oocytes expressing hERG alone. Along those lines, the voltage required for half maximal peak tail currents was similar in oocytes expressing hERG alone and in oocytes expressing hERG together with PIKfyve (Fig. 1E).

As PIKfyve is stimulated by protein kinase PKB, additional experiments were performed to elucidate the effect of PKB on hERG current. As shown in Fig 2A,B, the current

Fig. 2. Coexpression of PKB up-regulated hERG current, an effect augmented by PIKfyve but not by PKB-resistant PIKfyve^{S318A}. **A.** Original tracings recorded in *Xenopus* oocytes injected with water (a), with cRNA encoding hERG alone (b), with cRNA encoding hERG and PIKfyve (c), with cRNA encoding hERG and PIKfyve^{S318A} (d), with cRNA encoding hERG and PKB (e), with cRNA encoding hERG and PIKfyve + PKB (f) or with cRNA encoding hERG, PKB and PIKfyve^{S318A} (g). The oocytes were depolarized from -80 mV holding potential to different voltages followed by a 500 ms pulse to -60 mV evoking outward tail currents. **B.** Arithmetic means $\pm$ SEM ($n = 7-48$) of the normalized outward tail current following a depolarization to +70 mV recorded in oocytes injected with water (water), with cRNA encoding hERG alone (hERG) with cRNA encoding hERG and PIKfyve (hERG+PIKfyve), with cRNA encoding hERG and PKB-resistant PIKfyve^{S318A} (hERG+PIKfyve^{S318A}), with cRNA encoding hERG and protein kinase B (hERG + PKB), with cRNA encoding hERG, PKB and PIKfyve (hERG+PKB+PIKfyve) or with cRNA encoding hERG, PKB and PIKfyve^{S318A} (hERG+PKB+PIKfyve^{S318A}). * ($p < 0.05$), *** ($p < 0.001$) indicate statistically significant difference from *Xenopus* oocytes expressing hERG channels alone; # ($p < 0.05$) indicate statistically significant difference from *Xenopus* oocytes co-expressing hERG and PKB.

in hERG-expressing *Xenopus* oocytes was significantly increased by the coexpression of PKB. Additional expression of wild type PIKfyve was followed by a significant further increase of the current. In contrast, the additional co-expression of PKB with PKB resistant PIKfyve^{S318A} was not followed by a significant additional increase of the tail current.

The up-regulation of hERG activity following coexpression of PIKfyve could have resulted from an increase of channel protein abundance in the plasma membrane. Immunocytochemistry and confocal microscopy have been applied to visualize the hERG-HA channel protein abundance in the cell membrane. As illustrated in Fig. 3A, the co-expression of PIKfyve in hERG-HA expressing oocytes was followed by an increase of hERG protein abundance within the *Xenopus* oocyte cell membrane. In order to quantify hERG-HA protein abundance in the cell membrane of *Xenopus* oocytes, chemiluminescence was employed. As illustrated in Fig. 3B, the co-expression of PIKfyve was again followed by a significant increase of hERG-HA protein abundance within the *Xenopus* oocyte cell membrane.

The increase of hERG protein abundance in the cell membrane following coexpression of PIKfyve could have been due to enhanced channel protein stability due to delayed clearance of channel protein from the cell membrane. To determine the clearance of channel protein from the cell membrane, *Xenopus* oocytes expressing either hERG alone or together

Fig. 3. Coexpression of PIKfyve increased hERG-HA protein abundance at the surface of hERG-expressing *Xenopus* oocytes. A. Confocal images of hERG-HA protein cell surface expression in *Xenopus* oocytes injected with water (left panel), with cRNA encoding hERG-HA alone (middle panel) or with cRNA encoding hERG-HA and PIKfyve (right panel). B. Arithmetic means $\pm$ SEM (n = 34-66) of hERG-HA protein abundance in the cell membrane measured by chemiluminescence in *Xenopus* oocytes injected with water (left bar), with cRNA encoding hERG-HA alone (middle bar), or cRNA encoding hERG-HA and wild type PIKfyve (right bar). ***($p < 0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing hERG channels alone.

with PIKfyve were treated with 5 µM Brefeldin A, which blocks the insertion of new carrier protein into the cell membrane. As shown in Fig. 4, Brefeldin A treatment was followed by a decline of hERG current. The decline was similarly rapid in the oocytes coexpressing hERG and PIKfyve as in *Xenopus* oocytes expressing hERG alone.

Discussion

The present study identifies a novel mechanism of human ether-a-go-go (hERG) channel regulation. HERG channel protein abundance and thus hERG-mediated currents are up-regulated by phosphatidylinositol-3-phosphate-5-kinase (PIKfyve).

PIKfyve phosphorylates phosphatidylinositol 3-phosphate (PtdIns3P) leading to the production of phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂) [21]. Phosphatidylinositides contribute to the regulation of membrane trafficking [24, 47-49]. PIKfyve presumably contributes to Rab-mediated endosomal transport. PIKfyve has been shown to associate with the Rab9 effector molecule p40 [50, 51] leading to regulation of Rab9-mediated processes, such as transport from late endosome to the trans-Golgi network [22, 52]. PIKfyve is phosphorylated by protein kinase B PKB/Akt leading to activation of the enzyme [11]. Treatment of cells with insulin is followed by PIKfyve phosphorylation, an effect depending on PI3-kinase-activity [11]. Phosphorylation of PIKfyve by PKB increases PtdIns(3,5)P₂ generation on the cytosolic surface of the PIKfyve containing intracellular vesicles and thus impacts on vesicle trafficking [11]. PIKfyve is localized in a subpopulation of highly dynamic vesicles containing the glucose carrier GLUT4 [11]. PIKfyve modifies the trafficking of those vesicles and thus participates in the regulation of GLUT4 trafficking [11].

In analogy hERG is similarly up-regulated by coexpression of PKB. Additional coexpression of wild type PIKfyve but not additional coexpression of the PKB-resistant mutant

Fig. 4. Effects of Brefeldin A on *Xenopus* oocytes expressing hERG with or without PIKfyve. Arithmetic means $\pm$ SEM ($n = 27$ -33) of hERG current measured in *Xenopus* oocytes injected with cRNA encoding hERG without (white bars) and with (black bars) cRNA encoding PIKfyve and exposed to 5 μ M Brefeldin A for 0 hours, 24 hours or 48 hours prior to the measurements. * ($p < 0.05$), *** ($p < 0.001$) indicate statistically significant difference from *Xenopus* oocytes expressing hERG channels in the absence of PIKfyve.

Fig. 5. Putative model illustrating the SGK/PKB dependent regulation of hERG by PIKfyve and Nedd4-2. PKB/Akt (PKB) and/or SGK isoforms phosphorylate (P = phosphate) and thus activate PIKfyve (P5). PIKfyve in turn phosphorylates phosphatidylinositol 3-phosphate (P3P) leading to the production of phosphatidylinositol 3,5-bisphosphate (P3,5P₂), which in turn influences trafficking of channel bearing vesicles. SGK further phosphorylates Nedd4-2 thus fostering binding of Nedd4-2 to 14-3-3. Unphosphorylated Nedd4-2 ubiquitinates hERG thus preparing the channel for degradation.

PIKfyve^{S318A} augmented the effect of PKB, indicating that PKB is at least partially effective through phosphorylation of PIKfyve. The present observations did not address PIKfyve independent mechanisms involved in the effects of PKB/Akt or SGK isoforms on hERG channels. hERG has recently been shown to be regulated by the ubiquitin ligase Nedd4-2, which ubiquitinates target proteins thus preparing them for degradation [9, 53]. SGK and PKB isoforms may phosphorylate Nedd4-2 leading to binding of the ubiquitin ligase to the scaffolding protein 14-3-3, which interferes with the interaction of Nedd4-2 with its target proteins [54, 55]. Accordingly, the kinases could disrupt Nedd4-2-sensitive degradation of several ion channel proteins [54, 55] including hERG [9, 53].

SGK isoforms have further been shown to regulate expression of channel proteins by up-regulating the transcription factor NF κ B [56]. However, to the best of our knowledge nothing is known about transcriptional regulation of hERG by NF κ B.

The PIKfyve-sensitive regulation of hERG channels may be relevant for cardiac repolarization [1, 2]. Moreover, up-regulation of hERG channels by PIKfyve may be relevant for tumor cells, as hERG channel activity has been shown to participate in the regulation of tumor growth [3-5]. It should be pointed out, however, that regulation of channels by coexpressed signaling molecules in *Xenopus* oocytes does not allow predicting the impact of the respective signaling molecule on channel activity in the heart or in tumor cells. The expression level of the channel and the signaling molecule may be different in oocytes and any given mammalian cells. Moreover, the impact of the coexpressed signaling molecule on channel activity may be modified by additional signaling pathways expressed differently in *Xenopus* oocytes and mammalian cells. Nevertheless, the coexpression experiments may disclose interactions of signaling molecules with ion channels, which could be further tested in mammalian systems. Along those lines, the effect of SGK isoforms on ion channels prompted the analysis of QT intervals in individuals expressing a gain of function polymorphism of SGK1 in humans [57]. As a result, the polymorphism was indeed associated with a shortened QT interval, a finding underscoring the impact of SGK isoforms on repolarizing cardiac ion channels [57].

Lack of PIKfyve is apparently not compatible with survival, an observation highlighting the functional significance of PIKfyve-dependent regulation [58]. PIKfyve is obviously required for early embryonic development [58].

In conclusion, the present study demonstrates that PIKfyve is a powerful regulator of voltage-gated K⁺ channel hERG and thus may participate in the shaping of the cardiac action potential and in the regulation of tumor growth.

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