Regulation of KCNQ4 Potassium Channel Prepulse Dependence and Current Amplitude by SGK1 in *Xenopus* oocytes

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Key Words

Voltage gating • Channel gating • Kv7 • Kinase • Kinetics • K⁺ channels

Abstract

The KCNQ gene family comprises voltage-gated potassium channels expressed in epithelial tissues (KCNQ1, KCNQ5), inner ear structures (KCNQ1, KCNQ4) and the brain (KCNQ2-5). KCNQ4 is expressed in inner and outer hair cells of the inner ear where it determines electrical excitability. Accordingly, loss of function mutations of the KCNQ4 gene cause hearing loss. Several K⁺ channels including the closely related KCNQ1/KCNE1 channel are regulated by the serum- and glucocorticoid-inducible kinase (SGK) family. The present study utilized the *Xenopus* oocyte system to explore effects of SGK isoforms on KCNQ4 mediated K⁺-currents: KCNQ4 channels activated in a voltage dependent manner with half maximal activation at -10 mV. The peak channel activity was significantly increased by prepulsing. Coexpression of wild type SGK1 but not coexpression of the inactive mutant ^{K127N}SGK1 significantly increased current amplitudes (by 67 %) and significantly increased the resting potential of

KCNQ4 expressing oocytes. Here we describe for the first time a prepulse dependence of KCNQ4 channels with increased currents after hyperpolarizing prepulses. Coexpression of SGK1 significantly attenuated the effect of prepulsing on peak currents. Mutation of Ser to Asp or Ala in the putative phosphorylation consensus sequence in KCNQ4 significantly decreased the sensitivity to SGK1-coexpression. In conclusion, SGK1 regulates current amplitudes and kinetic properties of KCNQ4 channel activity, an effect sensitive to mutations in the SGK1 consensus sequence of the channel.

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Introduction

K⁺ channels are critically important for the maintenance of cell membrane potential, thus accomplishing repolarisation of action potentials and stabilization of a negative resting membrane potential in excitable cells [1]. Voltage-gated K⁺ channels are composed of a homo- or heterotetrameric assembly of pore-forming α -subunits and additional regulatory β -

subunits (e.g. KCNE, KChIP, Kvbeta). Pore forming channel subunits include KCNQ channels, which comprise a distinct family of voltage dependent potassium channels with five different human members cloned so far. Mutations in specific KCNQ genes can cause cardiac arrhythmia, epilepsy or deafness [2-7]. KCNQ4 was first identified by a homology cloning approach to KCNQ1-3 channels [5]. KCNQ4 maps to chromosome 1p34, the DFNA2 locus for a form of nonsyndromic dominant deafness and mutations were reported to be associated with the disease [5, 8, 9]. Heterologous expression of KCNQ4 channels induced a current with biophysical properties distinct from the $I_{K,n}$ known from outer hair cells [10, 11]. The reasons for this discrepancy remained elusive but may include unknown interacting proteins like scaffolding proteins, KCNEs, or regulation by ions, kinases, PIP2 and/or further signaling mechanisms. Similar to other KCNQ channels, KCNQ4 channels are down-regulated by activation of M1-receptors and upregulated by activation of dopamine D2 receptors [12, 13]. Furthermore, cell volume, the protein kinases PKA and PKC, G proteins and intracellular Ca^{2+} have been reported to regulate KCNQ4 [14]. KCNQ4-mediated currents have been identified in both inner (IHC) and outer (OHC) hair cells [5, 14, 15] where they seem to play different roles. In IHCs, the relatively small KCNQ4 currents set the resting membrane potential to $\sim -68\text{ mV}$ and determine the membrane time constant at rest. In OHCs, the large KCNQ4 currents are the dominating K^+ currents both at rest and at depolarized voltages and thus form the major exit route for K^+ ions entering the OHCs through the transduction channels. Lack of functional KCNQ4 channels in DFNB 2 most likely leads to IHC degeneration and deafness because use-dependent depolarization causes Ca^{2+} influx through voltage-gated Ca^{2+} channels [16] and ultimately excitotoxicity of IHCs and afferent fibres [5].

Regulators of inner ear function include glucocorticoids which were shown to be beneficial in several aspects of hearing impairment [17-22]. Among the glucocorticoid regulated genes is the serum- and glucocorticoid-inducible kinase 1 (SGK1) [23]. SGK1 is activated through a signaling pathway involving phosphatidylinositol 3 (PI3) kinase and the phosphoinositide dependent kinase PDK1 [24]. The kinase regulates a variety of channels and transporters [25, 26-28] including the renal epithelial Na^+ channel ENaC, the epithelial Ca^{2+} channel TRPV5, the voltage gated Na^+ channel SCN5A, the K^+ channels ROMK2, KCNE1/KCNQ1 and Kv1.3, the Na^+/H^+ exchanger NHE3, the dicarboxylate transporter NaDC1,

the glutamate transporter EAAT1, and the Na^+/K^+ -ATPase. Inspection of the KCNQ4 sequence revealed a consensus sequence for phosphorylation by SGK1.

The present study has thus been performed to explore the influence of SGK1 on current properties of KCNQ4. We show that coexpression of SGK1 in *Xenopus* oocytes indeed modulates KCNQ4 kinetics and demonstrate that this regulation by SGK1 coexpression is lost following replacement of Ser444 by Asp in the C-terminal SGK phosphorylation motive of KCNQ4.

Materials and Methods

Molecular Biology

The molecular biological procedures were the same as described before [29]. The KCNQ4 was subcloned in pXOOM dual expression vector [30]. DNA was linearized with XbaI. For synthesis of cRNA, mMessage mMachine T7-polymerase kit (Ambion, Cambridgeshire, UK) was used. Site directed mutagenesis of human *KCNQ4* was performed by PCR using cloned Pfu-polymerase. The construct was confirmed by automated DNA-sequencing.

Oocyte expression

Xenopus laevis frogs were tricaine anesthetised and stage V-VI oocytes were collected. The frogs were humanely killed after the final collection in accordance with the German law. The oocytes were injected with 40 nl of RNA with a nanoliterinjector 2000 (World Precision Instruments, Berlin, Germany) and incubated at 17 °C for 3 to 4 days as previously described [29]. Individual oocytes were injected with 4 ng KCNQ4 RNA or with 4 ng KCNQ4 RNA plus 6 ng SGK1 RNA.

Electrophysiology

Standard two-electrode voltage/current-clamp techniques [31] were used to record whole cell currents and measure resting membrane potentials in *Xenopus* oocytes at room temperature (22-24°C) using a Turbo TEC-10CX amplifier (NPI, Tamm, Germany) combined with Digidata 1322A (Axon Instruments Inc. /Molecular Devices, California, USA). The recording pipettes were filled with 3M KCL and had a tip resistance of 0.4-0.7 MΩ. Large currents were recorded using the amplifiers integrator and controlling the integrity of the recording by the PI-controller. Data were acquired with the Clampex program of pCLAMP 8.0 software (Axon Instruments Inc. /Molecular Devices, California, USA) and analyzed with Clampfit and Origin 6.0 (Microcal, Friedrichsdorf/Ts, Germany). For voltage clamp experiments, oocytes were bathed in ND96 solution containing (in mM): 96 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES; pH 7.6.

Data analysis

The activation and deactivation kinetics of KCNQ4 channels were assessed by analysis of the rising (activating) phase or the tail currents that were fitted to a single exponential

Fig. 1. Effect of SGK isoforms on KCNQ4 channel current amplitude and voltage dependence of activation. Human KCNQ4 was expressed in *Xenopus* oocytes and currents measured in extracellular solution ND96. Currents were elicited from a holding potential of -80 mV by pulses applied in 10 mV increments to potentials ranging from -100 mV to +60 mV (pulse protocol indicated above current traces). These pulses were always followed by a pulse to -120 mV to elicit tail currents (A). The current amplitudes at the end of the test pulses were measured, normalized to the mean values of KCNQ4 at 60 mV and

plotted against the respective voltage (B). The initial tail current amplitude at -120 mV was analysed, normalized to the minimal current at 60 mV and plotted vs. the voltage of the proceeding test pulse (C). The activation of KCNQ4 channels was analyzed by fitting the rising phase of KCNQ4 current traces to a single exponential. Mean results are plotted vs. the respective test pulse voltage (D) ($n = 24-44$). Error bars represent $\pm$ SEM. Filled circle = KCNQ4, open square = KCNQ4/SBK1, diamond = KCNQ4/SBK2 and triangle = KCNQ4/SBK3.

equation of the form

$$I(t) = A \exp(-t/\tau) + A_0, \text{ (Eq. 1)}$$

with a time constant τ and its respective amplitude, A , and a steady-state current, A_0 .

Statistical analysis

Students t- test with independent populations or ANOVA-testing were used to test for statistical significance which was assumed on the basis of $p < 0.05$.

Results

KCNQ4 channels were expressed in *Xenopus* oocytes and studied by the two-electrode-voltage clamp technique. KCNQ4 channels activated in a voltage dependent manner with half maximal activation at -10.2 ± 0.6 mV ($n = 10$). The KCNQ4 current amplitudes were analyzed at the end of pulses to varying voltages each lasting 3 s. A significant increase of KCNQ4 peak currents at 60 mV was observed upon coexpression with SGK1

($67 \pm 34\%$, $n = 11$). SGK2 ($34 \pm 17\%$, $n = 8$) and SGK3 ($20 \pm 14\%$, $n = 8$) tended to increase the peak current, effects, however, not reaching statistical significance (Fig. 1A, B).

To determine the voltage dependence of activation, tail current analysis was performed. The activation of KCNQ4 appeared to be slightly shifted (by about +8 mV, $n = 8 - 11$) by all SGK isoforms (Fig. 1C), a change, however, not reaching statistical significance.

The activation kinetics of KCNQ4 currents can be well described by a single exponential fit to the rising phase of the current traces. Coexpression of the SGKs did not change the activation time constants at any voltage tested (Fig. 1D).

KCNQ4 current deactivation could be fitted by a single exponential at several voltages. The analysis did not reveal any significant differences between water- and SGK-coexpressed KCNQ4 channel currents showing that deactivation of KCNQ4 was not influenced by SGKs (Fig. 2).

Fig 2. Lack of effect of SGK isoforms on KCNQ4 channel deactivation. Currents were elicited by pulses to 40 mV followed by repolarising pulses to voltages from -140 mV to -40 mV in 20 mV increments as indicated by the pulse protocol above. Representative current traces are shown in (A). The tail currents were analyzed by a single exponential fit and the time constants $\tau_{\text{deactivation}}$ were plotted versus the respective voltage (B). Filled circle = KCNQ4, open square = KCNQ4/SGK1, diamond = KCNQ4/SGK2 and triangle = KCNQ4/SGK3.

To explore whether kinase activity was required for the effect of SGK1, KCNQ4 channels were expressed alone, or coexpressed with SGK1, a dominant-negative SGK1 mutant SGK1(K127N) or a constitutively-active SGK1 mutant SGK1(S422D). Only the wild type SGK1 and the constitutively active kinase but not the inactive kinase mutant increased current amplitudes showing that the effects by SGK1 required kinase activity (Fig. 3). Modulation of KCNQ4 current amplitude and activation time constants by SGK1 could be the result of direct phosphorylation or an indirect effect due to interaction partners of KCNQ4. We identified a consensus sequence for SGK phosphorylation (RxxRxxS/T) in the C-terminus of KCNQ4 and mutated the putatively phosphorylated

Fig 3. Stimulation of KCNQ4 depends on the phosphorylation by SGK1. Oocytes were coinjected with KCNQ4 plus H₂O or wild type SGK1, the constitutively active SGK1(S422D) mutant or the inactive SGK1(K127N) mutant. Representative current traces are shown in A. Peak current amplitudes for the respective coinjections were plotted vs. the voltage (B). Pulse protocol like in Fig. 1A. Data are shown with the corresponding $\pm$ SEM and $n = 8-16$. Filled circle = KCNQ4, open square = KCNQ4/SGK1, diamond = KCNQ4/SGK1(S422D) and triangle = KCNQ4/SGK1(K127N).

Ser to Asp or Ala resulting in the channels KCNQ4 S444D (KCNQ4SD) and KCNQ4 S444A (KCNQ4SA). The mutant channels were expressed alone or were coexpressed with SGK1. Current amplitude, voltage of half maximal activation ($V_{1/2}$), activation and deactivation kinetics of KCNQ4SD expressed alone or together with SGK1 were not significantly different (Fig. 4). Activation time constants at higher voltages (0-60 mV) and deactivation time constants of wt KCNQ4 and mutant KCNQ4SD channels were in the same range (Fig. 4). Also, current amplitudes of wt and mutant KCNQ4 channel currents were not significantly different (KCNQ4wt 12.6 ± 1.6 , KCNQ4SD 13.3 ± 1.1 , $n = 10$). Coexpression of KCNQ4 and KCNQ4(SD) restored

Fig 4. Mutation of a putative SGK consensus sequence disrupts modulation of KCNQ4 by SGK1. We identified a putative SGK recognition sequence in the internal C-terminal part of KCNQ4 (A) and mutated the Ser444 to an Asp to study the resulting mutant channel KCNQ4SD. Currents of oocytes expressing KCNQ4SD with or without SGK1 were elicited by test voltage steps from -100 mV to 60 mV in 10 mV increments. Representative KCNQ4SD and KCNQ4SD/SGK1 currents are shown in B. Horizontal scale bar represents 1 sec vertical scale bar represents 5 μ A. Peak current voltage relationships were analyzed and plotted as in Fig. 1B (C). Activation curves were determined as in Fig. 1C (D). The time constants of activation were determined by single exponential fits as in Fig. 2 and plotted vs. the respective voltage (E). The deactivation time constants were determined and plotted as in Fig. 3 (F). Error bars represent $\pm$ SEM. All experiments were done with $n = 8-11$. Filled circles = KCNQ4(SD), open circles = KCNQ4(SD)/SGK1.

partially the amplitude effects of SGK1 (SGK1 currents increased by 61%, $p = 0.016$, $n = 10-11$). KCNQ4(S444A) channels were similarly not stimulated by SGK1 coexpression (KCNQ4(SA) = $12.8 \pm 1.3 \mu\text{A}$ vs. KCNQ4(SA)/SGK1 = 13.7 ± 1.5 , $p = 0.65$, $n = 15$). In summary, in contrast to KCNQ4wt the mutant channels were not regulated by SGK1 and behaved similar to wtKCNQ4 in the absence of SGK1.

Pulsing to different potentials can have dramatic effects on the availability of channels with closed state inactivation. Here, we identify a novel prepulse-dependence of KCNQ4 channel activity (Fig. 5A). However, the inactivation of the hyperpolarisation dependent channel fraction was variable in different batches of oocytes. This variation made it impossible to analyze the macroscopic inactivation in detail (Fig. 5A). The effect of prepulsing on peak current was relative robust, i.e. prepulsing was followed by a significant

increase of the peak current. We tested the effect of SGK1 on the peak currents of WT and mutant KCNQ4 channels and pooled data from 2 batches of experiments (Fig. 5B). The prepulse dependence of WT and mutant KCNQ4 channels was comparable but coexpression with SGK1 decreased the prepulse dependence in WT KCNQ4 and to a smaller degree in KCNQ4(SA) and KCNQ4(SD) channels (Fig. 5B).

To determine, whether the stimulation of KCNQ4 by SGK1 could influence the cell membrane potential in non-depolarized cells, we expressed KCNQ4wt channels alone or together with SGK1 in *Xenopus* oocytes and recorded the resting membrane potential (V_r) of each oocyte. In oocytes expressing KCNQ4 alone the resting membrane potential V_r approached $-72.5 \pm 1.8 \text{ mV}$ ($n = 7$). In oocytes coinjected with KCNQ4 and SGK1 V_r was $-82.7 \pm 2.7 \text{ mV}$ ($n = 7$), in those coinjected with SGK2 $-76.1 \pm 2.7 \text{ mV}$ ($n = 8$) and in those coinjected with SGK3

Δ

Fig. 5. Identification and modulation of prepulse dependence of KCNQ4 by SGK1. Currents were elicited by pulses to +40 mV following conditioning prepulses to voltages from -140 mV to -50 mV in 30 mV increments as indicated by the pulse protocol in A. Representative current traces from KCNQ4wt channels expressed in different sets of oocytes harvested from two different individuals show a prepulse dependence of current amplitude and variable amounts of inactivation (A). B, the currents of KCNQ4 channels expressed alone (closed symbols) or together with SGK1 (open symbols) were elicited with the same pulse protocol as in A. The peak currents were analyzed, values were normalized to the peak current amplitude after -140mV prepulses and were plotted versus the respective prepulse voltage. The representative current traces were scaled to the same size to allow better comparison. Error bars represent $\pm$ SEM. All experiments were done with $n = 6$ -13. Circle = KCNQ4, triangle = KCNQ4(SA), square = KCNQ4(SD), filled symbol without SGK1, open symbol with SGK1.

V_r was -72.6 ± 2.8 mV ($n = 9$). KCNQ4SD/SGK1 injected oocytes had a resting membrane potential of -73.9 ± 2.7 mV ($n = 13$). The data are summarized in Fig. 6. The cell membrane was significantly ($p = 0.012$) more polarized in oocytes expressing KCNQ4 together with SGK1 than in oocytes expressing KCNQ4 alone.

Discussion

The present observations reveal a novel function of the serum- and glucocorticoid- inducible kinase, i.e. the regulation of KCNQ4. To our knowledge this is the first observation pointing to an influence of SGK1 on the kinetic properties of voltage dependent potassium channels.

It is possible that regulation by SGK1 occurs indirectly via a modulatory protein of KCNQ4.

Fig. 6. Effects of KCNQ4 and SGK isoforms on the resting membrane potential. *Xenopus* oocytes were injected with KCNQ4 either alone or together with one of the SGK isoforms. The resting membrane potentials of oocytes were determined in the two electrode current clamp mode. Mean values are shown with error bars representing $\pm$ SEM. All experiments were done with $n = 7$ -13.

Alternatively, SGK1 could directly phosphorylate KCNQ4. We analysed the KCNQ4 sequence and identified the sequence RIRMGS fulfilling the requirements of a putative SGK consensus sequence [32]. Mutation of this putative SGK consensus sequence disrupted the modulatory effects by SGK1, which could suggest that SGK1 mediates its effects via this site. As phosphorylation introduces negative charge at the phosphorylation site, the mutational introduction of a negatively charged Asp at the site of the Ser could have been expected to mimic the effects of phosphorylation. However, KCNQ4 mutation of Ser444 to Asp was not sufficient to slow activation kinetics and increase macroscopic currents compared to wtKCNQ4. Thus, introduction of a negative charge is apparently not sufficient for the full effect of SGK1 on KCNQ4. In Western blots, an anti-phospho-threonine antibody failed to detect phosphorylated threonine at the

expected size of KCNQ4 in homogenates of oocytes injected with KCNQ4 or KCNQ4 & SGK1 (data not shown). Thus, we cannot safely conclude that SGK1 directly phosphorylates KCNQ4 and that this phosphorylation accounts for the regulation of the channel.

The SGK isoforms have been shown to increase the functional expression of several membrane integral proteins like transport proteins and ion channels. In some cases, this increase in functional expression was the result of increased protein level at the cell surface. In the case of the epithelial sodium channel, this effect resulted from decreased ubiquitination with subsequent decreased protein degradation [27, 28, 33]. In this case, the ubiquitin ligase Nedd4-2 was phosphorylated and was less effective in ubiquitinating the sodium channel. A similar mechanism was found for the voltage gated Na⁺ channel SCN5A [34] and the voltage gated K⁺ channel Kv1.3 [35]. Ubiquitination normally occurs at a specific PY motif of the sequence PPxY that is absent in KCNQ4. We further coexpressed the ubiquitin ligase NEDD4-2 with wtKCNQ4 and could not find a significant difference in current amplitudes of KCNQ4 with or without NEDD4-2 (data not shown). Thus it is not likely that the stimulation of KCNQ4 by SGK1 is due to interaction with Nedd4-2.

A further effect of SGK1 on KCNQ4 was the influence on the prepulse dependence. However, coexpression with SGK1 decreases the prepulse dependence in KCNQ4wt and to a smaller degree in KCNQ4(SA) and KCNQ4(SD) channels (Fig. 5B). Thus, the mutations affected but did not disrupt the prepulse dependence completely. The increased channel availability by SGK1 as a result of less channels being inactivated could contribute to the effect of SGK1 on channel activity.

The physiological role of KCNQ4 in hair cells is to set the resting membrane potential [10]. Here we show that KCNQ4 expressed in *Xenopus* oocytes is also largely affecting the resting membrane potential. Furthermore, we show that KCNQ4 coexpressed with SGK1 further

hyperpolarizes the cell membrane. This observation indicates that SGK1 influences the resting membrane potential via KCNQ4. We could detect abundant SGK1 mRNA in murine inner hair / supporting cells and outer hair cells by RT-PCR (data not shown) which could point to expression of the kinase in those cells. However, we presently do not know whether the protein is expressed and whether SGK1 indeed interacts with KCNQ4 in hair cells. If, so, then SGK1 could participate in the effects of glucocorticoids on the inner ear. In patients with moderate hearing loss therapeutic use of glucocorticoids has been suggested [20, 22]. Indeed, glucocorticoids have proven effective in several disorders associated with hearing loss or vertigo [17, 19, 21, 36]. The mechanisms underlying the beneficial effect of glucocorticoids has remained ill-defined and could besides other effects include the up-regulation of SGK1 [23] and subsequent regulation of KCNQ4. The marked effects of KCNQ4 on the resting membrane potential of oocytes and the additional effects of SGK1 may support the proposed importance of KCNQ4 currents in the hair cells [5]. Further studies will have to explore the putative functional significance of SGK1 dependent regulation of hair cells in the inner ear.

In conclusion, the serum- and glucocorticoid-inducible kinase 1 is a regulator of KCNQ4 prepulse dependence and current amplitude in *Xenopus* oocytes and thus is able to modify the cell membrane potential of KCNQ4 expressing cells.

Acknowledgement

The support by stipends from Gottlieb Daimler- und Karl Benz-Stiftung, the Erwin-Riesch-Stiftung, the DFG La315/4-5 and DFG En 294/2-3 (to J.E.) is gratefully acknowledged. We thank T. Jespersen, University of Copenhagen, Denmark for providing the hKCNQ4 cDNA and Sylvia Kasperek for expert technical assistance.

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