Modulation of the Voltage-Gated Potassium Channel Kv1.5 by the SGK1 Protein Kinase Involves Inhibition of Channel Ubiquitination

Christoph Boehmer^{1,2}, Jörg Laufer¹, Sankarganesh Jeyaraj¹, Fabian Klaus¹, Ricco Lindner¹, Florian Lang¹ and Monica Palmada^{1,2}

¹Department of Physiology I, University of Tübingen, ²Department of Molecular Biology, University of Duisburg-Essen

Key Words

Insulin • Voltage-gated potassium channel • Trafficking • Phosphorylation • Nedd4-2

Abstract

The serum and glucocorticoid inducible kinase SGK1 is involved in dexamethasone-induced inhibition of insulin secretion by increasing voltage-gated potassium channel (Kv) activity. SGK1 upregulates the Kv1.5 channel but the precise mechanism underlying the SGK1 dependent regulation of Kv1.5 has not been defined yet. The present study explored the signal transduction processes involved. Expression studies in *Xenopus* oocytes revealed that SGK1 promotes channel activity by interfering with the Nedd4-2 ubiquitination pathway, irrespective of the presence of putative SGK1 phosphorylation sites on Kv1.5. Expression of the ubiquitin ligase Nedd4-2 declined Kv1.5 currents by ubiquitinating and thereby reducing Kv1.5 plasma membrane expression. Increasing concentrations of SGK1 gradually compensated the inhibiting effect of Nedd4-2 on Kv1.5. Enhanced Kv1.5 surface abundance by SGK1 reflects decreased channel internalization as indicated by Brefeldin A experiments. In conclusion, Kv1.5 upregulation by

SGK1 involves inhibition of channel ubiquitination by Nedd4-2 that leads to Kv1.5 stabilization in the plasma membrane. Our results suggest that the kinase might participate in the regulation of insulin secretion in part by controlling Kv1.5 surface abundance.

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Introduction

Glucose induces insulin release by inactivating ATP-sensitive K⁺ channels which leads to cell membrane depolarization and activates voltage-gated Ca²⁺ channels allowing Ca²⁺ influx. The successive increase in intracellular Ca²⁺ triggers insulin secretion by vesicle fusion to the plasma membrane of pancreatic β -cells (rev. in [1]). Several studies have demonstrated the importance of membrane potential regulation by voltage-gated K⁺ (Kv) channels in excitation-secretion coupling in the pancreatic β -cell [2-5]. Blockade of Kv channels promotes glucose-mediated insulin release in the insulin-secreting cell line INS-1 and in pancreatic β -cells whereas transgenic mice overexpressing Kv1.5 show lower serum insulin concentrations [2-5].

Glucocorticoids contribute to the development of diabetes in part by impairing insulin secretion of pancreatic β -cells [6-8]. Dexamethasone-induced inhibition of insulin secretion involves increased glucocorticoid-dependent gene expression since the nuclear glucocorticoid receptor antagonist mifepristone completely abolishes the dexamethasone effect [6, 7]. Glucocorticoid-sensitive genes include the serum- and glucocorticoid-inducible kinase SGK1 [9]. SGK1 transcript and protein levels are elevated in insulin-secreting cells upon dexamethasone treatment, an effect reversed by mifepristone. The kinase has been proposed to account for dexamethasone effects as insulin release is impaired in islets gained from wild-type but not SGK1 null mice [10]. SGK1 is effective at least partially through activation of the voltage-gated K^+ channel Kv1.5. Increased Kv1.5 K^+ channel activity decreases Ca^{2+} entry through voltage-gated Ca^{2+} channels and thus insulin release [10]. The underlying mechanism of SGK1 dependent Kv1.5 modulation remained, however, elusive.

SGK1 is a downstream kinase in the phosphatidylinositol 3-kinase (PI3K) signalling pathway [11]. To become functional, SGK1 requires its phosphorylation that is triggered by a signalling cascade involving PI3K, the 3-phosphoinositide (PIP3)-dependent kinase (PDK1) and an unidentified PIP3-dependent kinase named as PDK2 or hydrophobic motif (H-motif) kinase [11-14]. The activation of SGK1 requires its phosphorylation at ⁴²²Ser by PDK2/H-motif kinase, followed by the phosphorylation of ²⁵⁶Ser by PDK1 which causes activation [11]. Mutation of ⁴²²Ser to Asp leads to the constitutive phosphorylation of ²⁵⁶Ser and thus to constitutively active SGK1 [11]. Once activated, SGK1 recognizes and phosphorylates Ser/Thr residues within the conserved sequence Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr or Arg-Arg-Xaa-Ser/Thr [14]. Through Ser/Thr phosphorylation of its targets, the kinase modulates a variety of ion channels and transporters (rev. in [15]).

The Kv1.5 sequence bears four putative SGK1 consensus sites at ⁵⁶Thr, ⁸¹Ser, ⁵³⁵Ser and ⁵⁶⁹Ser located at the cytosolic amino (⁵⁶Thr, ⁸¹Ser) and carboxy (⁵³⁵Ser, ⁵⁶⁹Ser) terminus, respectively. The present work aimed to clarify the role of these sites in Kv1.5 regulation by the kinase. To this end, wild type or mutant Kv1.5 lacking each or all SGK1 phosphorylation sites were heterologously expressed in *Xenopus* oocytes and their activity measured upon coexpression of SGK1. Here we provide evidence that SGK1 enhances Kv1.5 activity and surface abundance irrespective of the integrity of putative SGK1 phosphorylation sites. SGK1 is at least partially

effective through inhibition of channel protein retrieval from the cell membrane. SGK1 modulates several membrane proteins via inhibition of the ubiquitin ligase Nedd4-2 [16] including the voltage-gated potassium channel Kv1.3 [17]. In this study we further demonstrate that the ubiquitin ligase inhibits Kv1.5 by ubiquitinating and thereby reducing Kv1.5 plasma membrane expression. Increasing concentrations of SGK1 gradually decreased the inhibiting effect of Nedd4-2 on Kv1.5. In conclusion, Kv1.5 upregulation by SGK1 involves inhibition of channel ubiquitination by Nedd4-2 that culminates in Kv1.5 stabilization in the plasma membrane.

Materials and Methods

Site directed mutagenesis

Kv1.5 mutants 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:

Kv1.5 T56A, s: 5' CGA GGA GGC CCG CAC ACG AGG ATG CGG GC 3'; Kv1.5 T56A, as: 5' GCC CGC ATC CTC GTG TGC GGC GCG CCT CCT CG 3'; Kv1.5 S81A, s: 5' CAG CCT AGAAGG CCA GCT GCT GAG GAT GAG GAG 3'; Kv1.5 S81A, as: 5' CTC CTC ATC CTC AGC AGC TGG CCT TCT AGG CTG 3'. Kv1.5 S535A, s: 5' CAT CCA GAG GCG GGA GGC TGG GCT GGA CAC AGG AG 3'; Kv1.5 S535A, as: 5' CTC CTG TGT CCA GCC CAG CCT CCC GCC TCT GGA TG 3'; Kv1.5 S569A, s: 5' CAG TAT CAG AAG GGG TGC CTG TCC TCT AGAAAA G 3'; Kv1.5 S569A, as: 5' CTT TTC TAG AGG ACA GGCA CCC CTT CTG ATA CTG 3'.

The Kv1.5 4x mutant was generated only with the sense primers by using QuikChange multi site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) as indicated in the manufacturer's instructions. All mutants were sequenced to verify the presence of the desired mutation.

Expression in *Xenopus laevis* oocytes

cRNA encoding wild type Kv1.5, Kv1.5 mutants, wild type Nedd4-2, ^{C962S}Nedd4-2 or ^{S382AS468A}Nedd4-2 and constitutively active ^{S422D}SGK1 have been synthesized as described [18]. Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes has been described in detail elsewhere [18].

Where not otherwise specified, oocytes were injected with 7.5 ng of ^{S422D}SGK1 cRNA and/or 1.25 ng Nedd4-2 cRNA or H₂O on the same day after preparation of the oocytes and subsequently with 0.75 ng Kv1.5 cRNA.

Electrophysiology

Two-electrode voltage-clamp recordings were performed at room temperature 4 days after cRNA injection of Kv1.5. The data were recorded with a GeneClamp 500 amplifier, a DigiData 1300 A/D-D/A converter and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA).

The control solution (superfusate / ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4. The final solution was titrated to the pH indicated using HCl or NaOH. The flow rate of the superfusion was 20 ml/min and a complete exchange of the bath solution was reached within about 10 s.

Kv1.5 activation kinetics was determined from peak current quantification during a -60 mV test-pulse following 200 ms pre-pulses from -50 to +50 mV. Determination of Kv1.5 inactivation was based on a two-pulse protocol containing a 1 s pre-pulse from potentials of -60 to +40 mV followed by a 200 ms test-pulse to +70 mV. Normalized test-pulse peak currents were plotted versus pre-pulse voltage and fitted to the Boltzmann equation yielding $V_{1/2}$ for channel activation and inactivation.

Western blotting

The expression of SGK1, phosphorylated and ubiquitinated proteins was analyzed by western blotting. Briefly, 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, 100 μ M sodium orthovanadate and protease inhibitor cocktail (Roche, Mannheim, Germany) at the recommended concentrations. Proteins were separated on an 8 % polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5 % non-fat dry milk in PBS/0.15 % Tween 20 for 1 h at room temperature, blots were incubated overnight at 4 °C with a rabbit anti-SGK1 antibody (Upstate, Waltham, MA, USA, diluted 1:2000 in PBS/0.15 % Tween 20/5 % non-fat dry milk), a rabbit anti-phospho (Ser/Thr) antibody 110B7E (Cell Signaling Technology, Danvers, MA, USA, diluted 1:1000 in TBS/0.15 % Tween 20/5 % BSA) or a mouse anti-ubiquitin antibody (Cell Signaling Technology, Danvers, MA, USA, diluted 1:1000 in TBS/0.15 % Tween 20/5 % non-fat dry milk). Secondary peroxidase-conjugated sheep anti-rabbit IgG or anti-mouse IgG (diluted 1:2000 in PBS/0.15 % Tween 20/5 % non-fat dry milk) was used for chemiluminescent detection of SGK1, phosphorylated or ubiquitinated proteins with enhanced chemiluminescent ECL kit (Amersham, Freiburg, Germany). For determination of Kv1.5 expression in the plasma membrane, plasma membranes were prepared as described previously [19]. Equal protein amount was loaded into a 8 % polyacrylamide gel and transferred to a nitrocellulose membrane at 100 V for 60 min. For immunoblotting, rabbit anti-Kv1.5 antibody (Sigma, St. Louis, MO, USA, diluted 1:200 in PBS/0.15 % Tween 20/3 % BSA) was used to detect Kv1.5. 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 at 4°C overnight. Secondary peroxidase-conjugated sheep anti-rabbit IgG (Amersham, Freiburg, Germany, diluted 1:1000 in PBS/0.15% Tween 20/5% nonfat dry milk) was used for luminescent detection with an enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany).

Detection of Kv1.5 ubiquitination

Kv1.5 channel ubiquitination was analyzed by immunoprecipitation and western blotting as well as by using the UbiQapture™-Q kit according to the manufacturer's

Fig. 1. Kv1.5 is activated by SGK1 (S422D). Deletion of the putative phosphorylation sites does not abolish channel activation by the protein kinase. *Xenopus* oocytes were injected with wild-type or phosphorylation-deficient Kv1.5 mutants alone or together with constitutively active ^{S422D}SGK1. 4 days after cRNA injection, Kv1.5 activity was assessed by the two-electrode voltage clamp technique. Arithmetic means $\pm$ SEM of currents normalized in each batch of oocytes to the mean value obtained in oocytes expressing wild-type Kv1.5 alone. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing the channel alone.

instructions (BIOMOL, Hamburg, Deutschland). For Kv1.5 immunoprecipitation in *Xenopus* oocytes, anti-Kv1.5 antibodies were pre-bound to protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany) in oocyte lysis buffer (120 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5 % NP-40 supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany)) overnight at 4°C. The beads were then added to oocyte lysates and rotated for 1-2 h at 4°C. Beads were washed three times in washing buffer I (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 500 mM LiCl, 0.5 % NP-40), and twice in washing buffer II (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5 % NP-40). Thereafter beads were resuspended in 40 μ l SDS-PAGE sample buffer and heated to 95°C for 5 min. Immunoprecipitated samples were analyzed by western blotting as indicated above. For the isolation of ubiquitinated proteins with the UbiQapture™ -Q Kit, a total sample protein of 50 μ g was allowed to bind to the affinity UbiQapture™ -Q matrix overnight at 4 °C. After collecting the matrix by centrifugation, the matrix was washed twice in washing buffer consisting of 9 volumes of PBS to 1 volume of oocyte lysis buffer. The ubiquitin-protein conjugate was eluted from the matrix by addition of 80 μ l PBS and 25 μ l 5x SDS/PAGE-loading buffer. Eluated samples were analyzed by western blotting as indicated above.

Fig. 2. Dexamethasone and insulin stimulate the activating effect of wtSGK1 on Kv1.5 activity. *Xenopus* oocytes were injected with wild-type Kv1.5 together with wt SGK1. 4 days after cRNA injection, Kv1.5 activity was assessed by the two-electrode voltage clamp technique. Arithmetic means $\pm$ SEM of currents normalized in each batch of oocytes to the mean value obtained in oocytes expressing wild-type Kv1.5 together with wt SGK1. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 together with wt SGK1.

Fig. 3. SGK1 does not phosphorylate Kv1.5. *Xenopus* oocytes were injected with wild-type Kv1.5 or the Kv1.5 mutant lacking the consensus site for phosphorylation by SGK1 alone or together with constitutively active S^{422D} SGK1. Representative Western Blot (A). Quantification of band intensities by densitometry (B).

Detection of Kv1.5 phosphorylation

Kv1.5 channel phosphorylation was analyzed by immunoprecipitation and western blotting by using a rabbit anti-phospho (Ser/Thr) antibody as indicated above.

Statistical analysis

Data are provided as means $\pm$ SEM, n represents the number of oocytes investigated. All data were tested for significance using ANOVA, and only results with $P < 0.05$ were considered as statistically significant.

Results

To characterize the mechanism of Kv1.5 modulation by SGK1, the channel was expressed in *Xenopus laevis* oocytes and voltage-gated currents were measured in the presence and absence of the protein kinase.

Electrophysiological studies revealed an increase in Kv1.5-mediated currents upon coexpression of the constitutively active S^{422D} SGK1 (192.8 ± 14.2 % of control, $n = 28$). No voltage-activated currents were elicited in water injected oocytes or in oocytes expressing S^{422D} SGK1 alone. Kv1.5 contains four putative SGK1 phosphorylation sites within its sequence at ^{56}Thr , ^{81}Ser , ^{535}Ser and ^{569}Ser present at the cytosolic amino (^{56}Thr , ^{81}Ser) and carboxy (^{535}Ser , ^{569}Ser) terminus, respectively. From all sites, ^{569}Ser is conserved in most species including human, mouse, rat and rabbit while the other three putative phosphorylation sites are less conserved. To determine whether SGK1 stimulates Kv1.5 by phosphorylating the channel directly, each single or all putative phosphorylation sites together on Kv1.5 were mutated into alanine ($T^{56A}\text{Kv1.5}$, $S^{81A}\text{Kv1.5}$, $S^{535A}\text{Kv1.5}$, $S^{569A}\text{Kv1.5}$, Kv1.5 4x mutant ($T^{56A}S^{81A}S^{535A}S^{569A}\text{Kv1.5}$))

Fig. 4. Expression of the ubiquitin ligase Nedd4-2 reduces Kv1.5 activity by ubiquitinating and thereby inhibiting Kv1.5 plasma membrane expression. *Xenopus* oocytes were injected with wild-type Kv1.5 alone or together with the ubiquitin ligase Nedd4-2. Four days after cRNA injection, voltage-activated currents were measured and normalized in each batch of oocytes to the mean value obtained in oocytes expressing wild-type Kv1.5 alone (Arithmetic means $\pm$ SEM, * indicates statistically significant difference to currents in *Xenopus* oocytes expressing Kv1.5 alone) (A). In addition, Kv1.5 ubiquitination was assessed in whole cell lysates (B) and in isolated plasma membrane fraction after channel immunoprecipitation (C, upper panel) or by using the UbiQapture™-Q kit (C, lower panel). Kv1.5 protein abundance was determined by western blotting of isolated plasma membranes (D).

and their activity was evaluated with or without coexpression of the kinase (figure 1). Disruption of any of the putative SGK1 phosphorylation sites did not abrogate Kv1.5 stimulation by S^{422D} SGK1 (190.3 ± 15.1 % of control, $n = 8$; 165.7 ± 10.5 % of control, $n = 7$; 144.3 ± 17.3 % of control, $n = 7$ and 132.0 ± 8.6 % of control, $n = 19$, in oocytes expressing T^{56A} Kv1.5, S^{81A} Kv1.5, S^{535A} Kv1.5, S^{569A} Kv1.5, respectively). Moreover, coexpression of S^{422D} SGK1 stimulated the current mediated by the Kv1.5 4x mutant $T^{56A}S^{81A}S^{535A}S^{569A}$ Kv1.5 lacking all four putative SGK1 phosphorylation sites (88.8 ± 5.6 % of control, $n = 21$). Thus, phosphorylation of the Kv1.5 channel protein at the putative phosphorylation sites is not required for the stimulating effect of SGK1.

In a further series of experiments dexamethasone and insulin were applied to activate SGK1 at the transcriptional and posttranslational level, respectively (rev. in [15]). Preincubation of oocytes with dexamethasone (1 μ M) or insulin (1 μ M) for 24h further augmented the stimulating SGK effect on the potassium channel in Kv1.5/wtSGK1 expressing oocytes to an extent comparable to oocytes expressing the constitutive active S^{422D} SGK1 (155.5 ± 6.2 % of control, $n = 10$ and 161.7 ± 8.8 % of control, $n = 10$, in oocytes preincubated with dexamethasone or insulin respectively, figure 2).

The observed activating effect of SGK1 on Kv1.5 activity was independent of channel phosphorylation. As depicted in figure 3, the extent of Kv1.5 phosphorylation

Fig. 5. ^{S422D}SGK1 blunts the effect of Nedd4-2 on Kv1.5 activity. *Xenopus* oocytes were injected with Kv1.5 alone or together with wild-type Nedd4-2 (A) or ^{S382AS468A}Nedd4-2 (B) and/or ^{S422D}SGK1 at the indicated concentrations. 4 days after cRNA injection, Kv1.5 activity was studied. Arithmetic means $\pm$ SEM. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing Kv1.5 alone. + indicates statistically significant difference to currents in *Xenopus* oocytes expressing Kv1.5 together with Nedd4-2. Current values were normalized to the mean value obtained in oocytes expressing Kv1.5 alone.

was not significantly different in oocytes expressing Kv1.5 alone compared to cells expressing Kv1.5 together with the protein kinase. Furthermore deletion of all four putative SGK phosphorylation sites on the channel protein did not reduce the extent of Kv1.5 phosphorylation.

In a previous study we demonstrated that the voltage gated K⁺ channel Kv1.3 is inhibited by Nedd4-2 [17], an ubiquitin ligase that mediates the clearance of channel proteins from the cell membrane [16, 20, 21]. Nedd4-2 itself is a target of SGK1 which phosphorylates and thus inactivates the ubiquitin ligase [16, 22]. To delineate whether SGK1 promotes Kv1.5 activity through interference of the Nedd4-2 ubiquitination pathway, Kv1.5 activity was determined in the presence and absence of the ubiquitin ligase. Indeed, Nedd4-2 coexpression significantly decreased Kv1.5 currents (22.3 ± 7.2 % of control, $n = 15$, figure 4A). In additional experiments, channel ubiquitination was determined in whole cell lysates and in the immunoprecipitate from oocyte plasma membrane preparations. The immunoprecipitate was obtained by using an anti-Kv1.5 antibody (Sigma, St. Louis, MO, USA) and the precipitated protein was then analyzed by western blotting with antibodies against the channel protein or ubiquitin (Cell Signaling Technology, Danvers, MA, USA). As depicted in figure 4 (upper panel), ubiquitin staining in oocyte whole cell lysates (figure 4B) and in Kv1.5-immunoprecipitated samples (figure 4C) was already apparent in Kv1.5 injected oocytes, probably due to the presence of intrinsic xNedd4-2. Kv1.5 ubiquitination was further demonstrated by the UbiQapture™-Q kit that utilizes the affinity UbiQapture™-Q matrix capable to bind ubiquitinated proteins. Oocyte plasma membrane preparations were incubated with the beads and the precipitated protein was then analyzed using western blot with an antibody against the channel protein. Kv1.5 was

detected in the bound fraction from oocytes injected with Kv1.5 together with Nedd4-2 but not in non-injected oocytes, verifying Kv1.5 ubiquitination (figure 4C, lower panel). Nedd4-2 mediated ubiquitination resulted in reduced Kv1.5 expression at the plasma membrane as demonstrated by western blotting of isolated plasma membranes (figure 4D).

To assess the ability of SGK1 to modify the effects of Nedd4-2, the kinase was injected at different concentrations in oocytes expressing Kv1.5 alone or with the ubiquitin ligase. As illustrated in figure 5A, increasing concentrations of ^{S422D}SGK1 gradually decreased the effect of Nedd4-2 on Kv1.5 activity. Ablation of the SGK1 phosphorylation sites in the Nedd4-2 protein (^{S382AS468A}Nedd4-2, [23]) significantly blunted the effect of the kinase on Nedd4-2 (figure 5B).

Further experiments were performed to pursue whether SGK1 modulates Kv1.5 inactivation and

Fig. 6. S^{422D} SGK1 expression does not affect Kv1.5 kinetics. *Xenopus* oocytes were injected with Kv1.5 alone or together with S^{422D} SGK1. 4 days after cRNA injection, Kv1.5 inactivation/activation kinetics was studied as indicated in the material and methods section. Arithmetic means $\pm$ SEM.

activation kinetics and/or plasma membrane abundance. As depicted in figure 6, no shift in Kv1.5 kinetics occurred upon coexpression of the SGK1 protein kinase in the *Xenopus* oocyte system.

Kv1.5 plasma membrane abundance examined by immunoassays revealed that Kv1.5 expression is augmented by S^{422D} SGK1 (figure 7A). SGK1 could enhance plasma membrane Kv1.5 expression by increasing channel insertion, decreasing its removal or a combination of the two mechanisms. To distinguish between these possibilities, Kv1.5 currents were

Fig. 7. S^{422D} SGK1 increases Kv1.5 cell surface abundance by stabilizing the channel in the plasma membrane. (A) Surface Kv1.5 expression was assessed by western blotting of isolated plasma membranes of oocytes expressing the channel alone or together with S^{422D} SGK1. (B) *Xenopus* oocytes were injected with wild-type Kv1.5 alone or together with S^{422D} SGK1. Three days after cRNA injection, Kv1.5 activity was assessed in oocytes that had been incubated with Brefeldin A (BFA, 5 μ M) for 12h. Current ratios reflects current of cells incubated in BFA versus current of cells incubated in drug-free ND96. Arithmetic means $\pm$ SEM.

monitored in oocytes exposed to Brefeldin A, which blocks cellular secretory mechanisms by inhibiting vesicle formation at the Golgi apparatus. Incubation of oocytes expressing Kv1.5 with Brefeldin A (5 μ M) led to a gradual decrease of channel activity, which was significantly more pronounced after 12 h incubation of oocytes expressing Kv1.5 alone as compared to oocytes expressing Kv1.5 together with S^{422D} SGK1 (figure 7B). This result points to a stabilizing effect of SGK1 on Kv1.5 channel protein in the plasma membrane.

Discussion

The present study aimed to define the signal transduction involved in Kv1.5 regulation by the serum and glucocorticoid inducible kinase SGK1 at the molecular level. To date several mechanisms of SGK1 action have been reported that result in altered function of various ion channels and transporters. The kinase may modulate its target by direct phosphorylation [24, 25] or by phosphorylation of the ubiquitin ligase Nedd4-2 which otherwise ubiquitinates and induces degradation of its target proteins [16, 21-23, 26]. Furthermore, SGK1 may be recruited by scaffold proteins such as the Na⁺/H⁺-exchanger regulatory factor (NHERF) 2 via its C-terminal PDZ binding motif [26-28].

Kv1.5 contains four putative SGK1 consensus sites that are conserved among species suggesting that these sites might play an important role in channel regulation by the kinase. However, our data provide evidence that the kinase enhances Kv1.5 activity independently of the putative SGK1 phosphorylation sites on Kv1.5. Phosphorylation processes are known to modulate Kv1.5 activity. Another protein kinase regulating Kv1.5 currents is protein kinase A (PKA) [29-32]. Similar to SGK1, PKA modulates the channel indirectly since pharmacological inhibition of the kinase reduces currents of a mutant Kv1.5 lacking the C-terminal PKA phosphorylation sites. To regulate the channel, the kinase requires an intact actin cytoskeleton and α -actinin-2 [31]. PKA activates Kv1.5 by slowing channel macroscopic inactivation when coexpressed with the Kv β subunit, Kv β 1.3. The kinase is effective through phosphorylation of a PKA consensus site in the N-terminus of Kv β 1.3 [33]. A direct phosphorylation of Kv β 1.3 by SGK1 appears unlikely, as the Kv β subunit does not bear any SGK1 consensus site on its sequence.

As mentioned above, SGK1 affects membrane transport through Nedd4-2 phosphorylation that inhibits target ubiquitination [16]. Numerous studies have implicated ubiquitination as a mechanism to regulate expression of cytosolic proteins (rev. in [34]) or to degrade misfolded or improperly assembled protein complexes. Transmembrane proteins can similarly undergo ubiquitination, which tags them for internaliza-

tion and subsequent degradation by the endosomal/lysosomal pathway (rev. in [35]). A recent study [36] identified ubiquitination of functional membrane-bound ENaC by Nedd4-2. The ubiquitin ligase reduces channel expression at the cell surface without modifying the amount of immature ENaC. Here we demonstrate that SGK1 activates Kv1.5 through interference of Nedd4-2 ubiquitination pathway. The ubiquitin ligase inhibits Kv1.5 by ubiquitinating surface Kv1.5 and thereby reducing Kv1.5 plasma membrane abundance. The ubiquitination of Kv1.5 has previously been reported [37]; however, the identity of the enzyme involved remained unknown. In the present report we demonstrate that Nedd4-2 is an ubiquitin ligase capable to downregulate Kv1.5. The inhibiting effect of the ubiquitin ligase appears more profound than the stimulating effect of the protein kinase. Possibly, endogenous Nedd4-2 activity is low and its inactivation by exogenous SGK1 might be less effective than the effect of exogenous Nedd4-2.

Increasing concentrations of SGK1 gradually decreased the effect of Nedd4-2 on Kv1.5. This mechanism of SGK1 action on Kv1.5 is shared by other ion channels like the Na⁺ channel ENaC [16] and the chloride channels ClCKa/Barttin and ClC-2 [21, 38]. The SGK1 dependence of Kv1.5 ubiquitination and degradation explains the delayed decline of channel activity in the presence of Brefeldin A. A delayed channel protein turnover in the plasma membrane has been shown previously [28].

In summary, this study indicates that SGK1 modulates Kv1.5 function and expression by blunting Nedd4-2 mediated channel ubiquitination. This mechanism leads to Kv1.5 stabilization in the plasma membrane. Kv1.5 ubiquitination represents a novel form of posttranslational modification additional to phosphorylation, glycosylation and S-acylation that culminate in the regulation of channel function/surface abundance [29-32, 39, 40].

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