The C-Terminal PDZ-Binding Motif in the Kv1.5 Potassium Channel Governs its Modulation by the Na⁺/H⁺ Exchanger Regulatory Factor 2

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Key Words

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Abstract

Kv1.5 belongs to the family of voltage-gated potassium (Kv) channels and contains a N- and a C-terminal PDZ-binding motif that might be recognized by PDZ domains on the scaffold proteins NHERF1 and NHERF2. Expression studies in *Xenopus* oocytes demonstrated that NHERF1 and NHERF2 activate Kv1.5, an effect requiring the C-terminal PDZ-binding motif on Kv1.5. NHERF2 enhances Kv1.5 activity and cell surface expression as determined by electrophysiology and immunoassays. NHERF2 elevates Kv1.5 abundance at the plasma membrane by decreasing channel internalization as proven by Brefeldin A experiments. Kv1.5 is stimulated by the serum and glucocorticoid inducible kinase SGK1, a kinase known to interact with the second PDZ domain of NHERF2. This study aims to identify if SGK1 and NHERF2 synergize to increase Kv1.5 currents. Expression of NHERF2 potentiated SGK1-mediated Kv1.5 activation, which was significantly attenuated by deletion of the second PDZ domain in NHERF2.

Specificity of observed effects was verified by evaluating the influence of NHERFs on Kv1.3, a known SGK1 target that contains an internal PDZ binding motif. In summary, our results suggest that NHERFs might participate in the regulation of electrical excitability in part by controlling Kv1.5 surface abundance and by clustering signal transduction molecules to the channel.

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Introduction

The voltage-gated K⁺ channel Kv1.5 is expressed in a wide variety of excitable and non-excitable tissues including heart [1, 2], brain [1], macrophages [3], smooth muscle [4-7], and pancreatic beta cells [8]. Specialized localization of Kv channels is in part attributed to their association with PDZ domain-containing proteins [9-11].

PDZ is a modular protein-protein interaction domain, first identified in the postsynaptic density PSD-95/SAP90, the *Drosophila* septate junction protein disc-large, and the tight junction protein ZO1, which is composed of 80-90 amino acid residues that bind to tetrapeptide sequences located at the C-terminus of the target protein (X-S/T-X-V/L, where X, S, T, V and L stands for any amino acid,

serine, threonine, valine and leucine respectively) [12–16]. Besides the prototypical C-terminal PDZ binding motif, PDZ domains can interact with internal sequences forming a β hairpin finger that resemble a free C-terminus [17, 18]. The voltage-gated potassium channel Kv1.5 and other Kv1-type channels bear both the canonical C-terminal and an internal PDZ binding motif [9].

Cells contain multiple PDZ proteins that are distributed in precise subcellular domains. The Na^+/H^+ exchanger regulatory factors, NHERF1 and NHERF2, are located at or near the cell surface (rev. in [19]) and have been implicated in the targeting and stabilisation of ion channels and carriers at the plasma membrane [20–27]. Several lines of evidence suggest that NHERF proteins play a critical role in the trafficking of membrane proteins to and from the plasma membrane (rev. in [28]). NHERF proteins may participate to direct the endosomal pool of membrane proteins to the plasma membrane or internalize the cell surface proteins and subsequently direct them to lysosomes for degradation and inactivation.

Both proteins contain two tandem PDZ domains and an ERM binding domain at their C-termini that bind all members of the ezrin-radixin-moesin-merlin family of cytoskeletal proteins [28]. Although both PDZ domains in each NHERF isoform share high structural homology they recognize different targets and recruit either one or bridge two or more proteins at the cell surface (rev. in [29]). Moreover, some proteins specifically bind one of the two isoforms [30–34]. NHERF binding to the cytoskeleton allows sorting and localization of membrane proteins. In polarized epithelial cells, apical versus basolateral expression of ion channels/transporters is in part determined by their association with the underlying cytoskeleton [35]. The PDZ binding motif present in CFTR has been reported to bind both NHERF isoforms and functions as an apical membrane polarization signal [36–39]. Loss of this site in CFTR results in distribution of the channel to the basolateral membrane [36].

NHERF binding to ezrin also mediates clustering of signalling proteins in microdomains to facilitate cellular signalling. Since ezrin is a protein kinase A anchoring protein, NHERF2 binding to ezrin has been suggested to function as a scaffold protein mechanism localizing protein kinase A in the vicinity of CFTR required for activation of the channel [39]. A similar scaffolding role has been proposed for the modulation of the Na^+/H^+ exchanger 3 (NHE3) by PKA [40, 41]. NHERF2 binds to protein kinases other than PKA, including the serum and glucocorticoid inducible kinase 1 (SGK1) [23, 34], a kinase that has been shown to modulate a variety of ion chan-

nels and transporters (rev. in [42]). SGK1 requires NHERF2 to modulate several of its targets such as the K^+ channel ROMK1 [43] the Ca^{2+} channel TRPV5 [23] or the carrier PEPT2 [21].

Northern blot analysis of NHERF1 and NHERF2 showed broad tissue distribution of the scaffold proteins, including heart and brain [28]. The present work aimed to clarify whether NHERFs modulate the voltage-gated potassium (Kv) channels Kv1.5 and Kv1.3 and to identify the molecular mechanism involved. Here we provide evidence that NHERF1 and NHERF2 isoforms promote Kv1.5 activity. Enhanced Kv1.5 activity and plasma membrane expression by NHERF2 reflects deranged channel internalization as indicated by Brefeldin A treatment. NHERF2 requires the C-terminal PDZ-binding motif in Kv1.5 to activate the channel. Besides, NHERF2 synergizes via its second PDZ domain with the serum and glucocorticoid inducible kinase SGK1 to increase Kv1.5 currents. In summary, our data suggest that NHERF2 might participate in the regulation of electrical excitability in part through controlling Kv1.5 surface abundance and by clustering signal transduction molecules as the SGK1 kinase to the channel.

Materials and Methods

Site directed mutagenesis

The Kv1.5 mutants lacking the N- or C-terminal PDZ binding motif, Kv1.5 Δ N and Kv1.5 Δ C, 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 for deletion of the N-terminal ETQL (aa 123–126) or C-terminal ETDL (aa 598–601) sequence: Kv1.5 Δ N, s: 5' CTC GGG TTT GCG CTT TGG CAC CCT GGC GCA G 3'; Kv1.5 Δ N, as: 5' CTG CGC CAG GGT GCC AAA GCG CAA ACC CGA G 3'; Kv1.5 Δ C, s: 5' CTG GAC ACT AGC CGG TAA AGA GAG ATC GAG 3'; Kv1.5 Δ C, as: 5' CTC GAT CTC TCT TTA CCG GCT AGT GTC CAG 3'. All mutants were sequenced to verify the presence of the desired mutation.

Expression in *Xenopus laevis* oocytes

cRNA encoding wild type or Kv1.5 mutants [44], wild type Kv1.3, wild type NHERF1, wild-type NHERF2, NHERF2 lacking the first PDZ domain (NHERF2 Δ P1) or NHERF2 lacking the second PDZ domain (NHERF2 Δ P2) [45] and constitutively active human ^{S422D}SGK1 [45] have been synthesized as described [46]. Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes has been described in detail elsewhere [46]. To avoid any bias by variability of oocyte batches, comparisons were always made within the same batch of oocytes.

Where not otherwise specified, oocytes were injected with 7.5 ng of ^{8422D}SGK1 cRNA and/or 12.5 ng NHERF1, wild-type or mutants NHERF2 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 were 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 consisting of 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 with the Boltzmann equation to obtain $V_{1/2}$ for channel activation and inactivation.

For quantification of Kv1.5 stability oocytes were collectively preincubated before measurement in Brefeldin A (Sigma, Deisenhofen, Germany) at a concentration of 5 μ M for the time periods indicated in figure 4B. Individual cells were then taken at the respective time points for electrophysiological quantification of Kv1.5 activity.

Western blotting

The expression of SGK1, wild-type NHERF1, wild-type and NHERF2 mutants 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-NHERF1 antibody (Alomone labs, Jerusalem, Israel, diluted 1:200 in PBS/5 % non-fat dry milk) or a rabbit anti-S-tag-HRP antibody (Merck, Darmstadt, Germany diluted 1:5000 in TBS/0.15 % Tween 20). Secondary peroxidase-conjugated sheep anti-rabbit IgG (diluted 1:2000 in PBS/0.15 % Tween 20/5 % non-fat dry milk) was used for chemiluminescent detection of SGK1 and NHERF1 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 by [47]. Pellets obtained were dissolved in SDS sample

Fig. 1. The scaffold proteins NHERF1 and NHERF2 activate currents encoded by Kv1.5 in *Xenopus* oocytes. (A) *Xenopus* oocytes were injected with wild-type Kv1.5 alone or together with NHERF1 or NHERF2. Oocytes injected solely with NHERF1, NHERF2 or water (n.i.) served as controls. Four days after cRNA injection, Kv1.5 activity was monitored 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. (B) Representative two-electrode voltage-clamp current traces.

buffer, 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% Tween20/3% BSA) was used to detect Kv1.5. Blots were further incubated with anti β -actin-horseradish peroxidase (Cell Signaling Technology, Danvers, MA, USA, diluted 1:10000 in PBS/0.15% Tween 20/5% nonfat dry milk) to ensure lack of cytosolic contamination in the membrane preparation. 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%

Fig. 2. The scaffold proteins NHERF1 and NHERF2 interact with Kv1.5 in *Xenopus* oocytes and mice cardiomyocytes. *Xenopus* oocytes were injected with wild-type Kv1.5 alone or together with NHERF1 or NHERF2. Four days after cRNA injection, Kv1.5 was immunoprecipitated and samples were immunoblotted with Kv1.5, NHERF1 or NHERF2 antibodies (A). Cardiomyocytes from mice were immunoprecipitated with Kv1.5 antibody or equally treated with rabbit IgG (IgG). Samples were immunoblotted with Kv1.5, NHERF1 or NHERF2 antibodies (B).

Tween 20/5% nonfat dry milk) was used for luminescent detection with an enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). Band intensities were quantified using Quantity One® Analysis software (Biorad, Munich, Germany).

Coimmunoprecipitation

For Kv1.5 immunoprecipitation in *Xenopus* oocytes, anti-Kv1.5 antibodies or rabbit IgG (used as a negative control) 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 µl SDS-PAGE sample buffer and heated to 95 °C for 5 min. Immunoprecipitated samples were analyzed by western blotting as indicated above. In experiments from cardiac tissue, samples were homogenized in ice-cold non-denaturing lysis buffer (20 mM HEPES, 150 mM NaCl, 2 mM EDTA, 10 % glycerol, 0.5 % NP-40, 1% hemoglobin, 10 mM iodoacetamide and protease inhibitor cocktail). The lysate was

centrifuged at 1000 g for 10 min at 4°C to pellet nuclei and debris and supernatant was used for coimmunoprecipitation. Anti-Kv1.5 antibodies were pre-bound to protein A/G PLUS-agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany) in non-denaturing lysis buffer overnight at 4°C. The beads were then added to the supernatant and rotated for 1-2 h at 4°C. Beads were washed three times with TSA-T (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 0.1 % Triton X-100), once with TSA (10 mM Tris-HCl pH 8.0, 140 mM NaCl) and once with 50 mM Tris-HCl pH 6.8. Thereafter beads were resuspended in 40 µl SDS-PAGE sample buffer and heated to 95°C for 5 min. Immunoprecipitated samples were analyzed by western blotting as indicated above.

Statistical analysis

Data are provided as means ± 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 examine whether the scaffold proteins NHERF1 and NHERF2 modulate Kv1.5 currents, the channel was

Fig. 3. The C-terminal PDZ-binding motif in Kv1.5 is required for channel modulation by NHERF2. (A) *Xenopus* oocytes were injected with wild-type Kv1.5, Kv1.5ΔN or Kv1.5ΔC alone or together with NHERF2. Four days after cRNA injection, Kv1.5 activity was assessed and NHERF2 expression evaluated by western blotting of whole cell lysates. Arithmetic means $\pm$ SEM of currents normalized to the mean value obtained in oocytes expressing the channel alone. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing Kv1.5 alone. (B) Representative two-electrode voltage-clamp current traces. (C) NHERF2 fails to activate the Kv1.5ΔC mutant at various expression levels tested.

expressed in *Xenopus laevis* oocytes and its activity measured in the presence and absence of the respective scaffold protein. Electrophysiological studies revealed increased Kv1.5 currents by both NHERF1 and NHERF2 coexpression (162.2 ± 17.6 % and 160.5 ± 17.0 % of control, $n = 15$ -16, respectively). No voltage-activated currents were elicited in water injected oocytes or oocytes expressing NHERF1 or NHERF2 alone (Figure 1).

Most interactions with PDZ domains involve the C-terminal 4 amino acids of the interacting protein but additional (internal) binding mechanisms have also been described [18, 48, 49]. Kv1.5 bears a prototypical PDZ binding motif at its C-terminus (E-T-D-L) and an atypical PDZ binding motif at the N-terminus close to the T1 assembly domain [9]. To address whether the Kv1.5 potassium channel and the scaffold proteins NHERF1 or NHERF2 are in complex with each other co-immunoprecipitation experiments with Kv1.5 antibody were carried out from oocytes expressing the respective proteins. As depicted in Figure 2A, both scaffold proteins were detected in the Kv1.5 immunoprecipitated samples while only marginal expression of intrinsic NHERF1 or NHERF2 could be detected in non-injected oocytes. Furthermore, co-immunoprecipitation experiments from mice cardiomyocytes demonstrate a direct interaction between the channel and scaffold proteins in the native mammalian system (Figure 2B).

To clarify whether the functional interaction between Kv1.5 and NHERF2 depends on the N- or C-terminal PDZ-binding motif of Kv1.5, channel mutants lacking the N- or C-terminal PDZ-binding motif (Kv1.5ΔN and Kv1.5ΔC) were generated and their activity was evaluated when expressed alone or in the presence of NHERF2. Ablation of the N-terminal PDZ-binding motif markedly declined its current to almost undetectable levels (9.8 ± 2.4 % of control, $n = 10$), whilst the Kv1.5

Fig. 4. NHERF2 increases Kv1.5 surface abundance by stabilizing the channel at the plasma membrane. (A) *Xenopus* oocytes were injected with Kv1.5 alone or together with NHERF2. Four days after cRNA injection, Kv1.5 expression was determined from isolated plasma membranes. Blots were further incubated with anti β-actin-horseradish peroxidase to ensure lack of cytosolic contamination in the membrane preparation. In parallel, blots with whole cell lysates were performed as positive controls for the antibody incubations. (B) *Xenopus* oocytes were injected with Kv1.5 alone or together with NHERF2. Four days after cRNA injection, Kv1.5 activity was assessed in oocytes that had been incubated with Brefeldin A (5 μM) for the indicated time points. Arithmetic means ± SEM. * indicates significant difference between expression of Kv1.5 alone and coexpression of Kv1.5 together with NHERF2.

Fig. 5. ^{S422D}SGK1 and NHERF2 synergize to stimulate currents encoded by Kv1.5. *Xenopus* oocytes were injected with Kv1.5 alone or together with ^{S422D}SGK1 with or without NHERF1 or NHERF2. Four days after cRNA injection, voltage-activated currents were measured. Arithmetic means ± SEM of currents normalized to the mean value obtained in oocytes expressing Kv1.5 alone. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 alone, # indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 and ^{S422D}SGK1. (B) Representative two-electrode voltage-clamp current traces.

mutant lacking the C-terminal PDZ-binding motif (Kv1.5ΔC) retained its activity (133.3 ± 22.5 % of control, $n = 15$, Figure 3). NHERF2 was unable to activate Kv1.5ΔC currents (105 ± 17.3 % of control, $n = 15$) despite proper NHERF2 expression as assessed by western blotting of whole cell lysates. Thus, C-terminal PDZ-binding motif in Kv1.5 governs its modulation by NHERF2. The slightly lower increase in Kv1.5 currents upon coinjection of NHERF2 in these series of experiments compared to the respective group in Figure 1 (160.5 ± 17.0 % of control compared to 133.9 ± 8.9 % of con-

trol, $p=0.32$) might reflect variability of exogenous NHERF2 expression between different batches of oocytes. The Kv1.5ΔC mutant showed higher activity than the wild-type Kv1.5 channel that might reflect a saturated expression level of the protein. To exclude that the increased Kv1.5 current might impede further activation by NHERF2, Kv1.5ΔC was injected in varying concentrations together with the scaffold protein. As obvious from the functional data in figure 3D, NHERF2 is not capable to activate Kv1.5ΔC at any concentrations tested.

Fig. 6. NHERF2ΔP1 is also capable to further increase the stimulating effect of ^{S422D}SGK1 on Kv1.5 currents. *Xenopus* oocytes were injected with Kv1.5 alone or together with NHERF2ΔP1 or ^{S422D}SGK1 with or without NHERF2ΔP1. Four days after cRNA injection, voltage-activated currents were measured. Arithmetic means ± SEM of currents normalized to the mean value obtained in oocytes expressing Kv1.5 alone. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 alone, # indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 and ^{S422D}SGK1. (B) Representative two-electrode voltage-clamp current traces. (C) Typical current-voltage plots.

Fig. 7. NHERF2ΔP2 is not capable to further increase the stimulating effect of ^{S422D}SGK1 on Kv1.5 currents. *Xenopus* oocytes were injected with Kv1.5 alone or together with NHERF2ΔP2 or ^{S422D}SGK1 with or without NHERF2ΔP2. Four days after cRNA injection, voltage-activated currents were measured. Arithmetic means ± SEM of currents normalized to the mean value obtained in oocytes expressing Kv1.5 alone. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.5 alone. (B) Representative two-electrode voltage-clamp current traces.

In addition to its scaffolding function, NHERF2 regulates endocytosis and/or exocytosis. Results shown in Figure 4A demonstrate that NHERF2 modulates Kv1.5 activity by increasing channel expression at the plasma

membrane. NHERF2 could enhance plasma membrane Kv1.5 expression by increasing channel insertion or blocking their internalization. To distinguish between both mechanisms, Kv1.5 currents were recorded in oocytes

Fig. 8. Neither NHERF1 nor NHERF2 regulate Kv1.3 activity in *Xenopus* oocytes. *Xenopus* oocytes were injected with Kv1.3 alone or together with ^{S422D}SGK1, NHERF1 or NHERF2 4 days after cRNA injection, (A) voltage-activated currents were measured and (B) NHERF isoforms expression analyzed by western blotting of whole cell lysates. Arithmetic means ± SEM. * indicates statistically significant difference to currents in *Xenopus* oocytes expressing wild-type Kv1.3 alone. Currents were normalized to the mean value obtained in oocytes expressing the channel alone.

expressing Kv1.5 alone or Kv1.5 together with NHERF2 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 μM) led to a continuing decrease of channel activity, which was significantly faster in oocytes expressing Kv1.5 alone as compared to oocytes expressing Kv1.5 together with NHERF2 (Figure 4B). These data suggest that NHERF2 stabilizes cell surface expression of Kv1.5.

Further experiments were performed to determine whether, in addition to its effect on Kv1.5 protein abundance in the cell membrane, NHERF2 modulates Kv1.5 inactivation and activation kinetics. As shown in Table 1, coexpression of the NHERF2 protein did not affect Kv1.5 kinetics in the *Xenopus* system.

Kv1.5 is modulated by the serum and glucocorticoid inducible kinase SGK1 [8]. The kinase is known to interact with NHERF2 but not NHERF1 [34] which is based on protein-protein interaction between its C-terminal PDZ binding motif and the second PDZ domain in NHERF2 [34]. Thus the possibility that SGK1 and NHERF2 synergize to stimulate Kv1.5 was considered. To this end, constitutively active ^{S422D}SGK1 was used to avoid varying levels of kinase activity in the *Xenopus* oocytes expression system. The current increase upon coexpression of the constitutively active ^{S422D}SGK1 (285.5 ± 21.8 % of control (oocytes expressing Kv1.5 alone), n = 15) or NHERF2 (160.5 ± 17.0 % of control, n = 16) in oocytes injected with Kv1.5 was additive upon simultaneous coexpression of the protein kinase and the scaffold protein together with the channel (459.1 ± 44.4 % of control, n = 15). Expression of NHERF1 was similarly capable to stimulate Kv1.5 currents (162.2 ± 17.6 % of control, n = 15), however the protein did not exert a significant additive effect on top of the effect of ^{S422D}SGK1 on the channel (334.2 ± 57.9 % of control, n = 15, Figure 5).

cRNA injected	V1/2 inactivation	V1/2 activation	n
Kv1.5	-16.3 ± 2.3 mV	-1.3 ± 1.4 mV	16
Kv1.5 + NHERF2	-17.1 ± 3.1 mV	-0.2 ± 1.7 mV	12

Table 1. Summary of Kv1.5 current kinetics. Arithmetic means ± SEM of the voltage required ($V_{1/2}$) for half activation and half inactivation of the channel (n = number of oocytes tested).

To identify the PDZ domain required for Kv1.5 activation, we made use of two NHERF2 mutants lacking either the first or second PDZ domain. Simultaneous coexpression of the protein kinase together with NHERF2 lacking the first PDZ domain (NHERF2ΔP1) (495.7 ± 62.5 % of control, n = 15) augmented the stimulatory effect of ^{S422D}SGK1 (285.5 ± 21.8 % of control, n = 15) or NHERF2ΔP1 (235.7 ± 34.0 % of control, n = 15) on Kv1.5 (Figure 6).

In contrast, coexpression of NHERF2 lacking the second PDZ domain (NHERF2ΔP2) did not further enhance the stimulating effect of ^{S422D}SGK1 on Kv1.5 currents (312.5 ± 24.9 % of control, n = 15, Figure 7).

Several Kv1 potassium channels hold a N-terminal internal PDZ binding motif that might be recognized by PDZ domain containing proteins. Kv1 channels that have been reported to bind PDZ domain containing proteins include the voltage-gated potassium channel Kv1.3 [9]. In a previous study we demonstrated that Kv1.3 is activated by SGK1 [50]. Thus, NHERF1 and/or NHERF2 might influence Kv1.3 currents as well as promote SGK1 effects on Kv1.3. Consistent with our previous study, Kv1.3 currents increased upon ^{S422D}SGK1 coexpression (410.8 ± 61.9 % of control, n = 45, Figure 8A). However, neither NHERF1 nor NHERF2 regulated Kv1.3 activity significantly (135.0 ± 30.1 % and 88.6 ± 11.1 % of control, respectively, n = 23), suggesting a specific effect of NHERF1 and NHERF2 isoforms on Kv1.5 but not on Kv1.3 (Figure 8A). Proper expression of NHERF1 and NHERF2 was proven by immunoblotting (Figure 8B).

Discussion

The present study aimed to clarify the role of the PDZ proteins NHERF1 and NHERF2 in Kv1.5 regulation and to define the molecular mechanism involved. Several reports have suggested that NHERF2 has a distinct binding specificity and physiological function different from NHERF1 (rev. in [29]). In the present study we identified Kv1.5 as a target of both, NHERF1 and NHERF2, isoforms. The two PDZ domains in NHERF1 have distinct peptide-binding affinity. The first PDZ domain of NHERF1 binds to the C-terminal motif D-S/T-X-L, whilst the second PDZ domain interacts with the C-terminal amino acid sequence S-S/T-W-L [13, 25]. The binding specificity of NHERF2 PDZ domains has not been revealed yet, but several NHERF2 targets bind to only one of either of the PDZ domains (rev. in [29]). According to our results NHERF2 activates Kv1.5 through both PDZ domains (Figure 6 and 7).

The voltage-gated potassium channel Kv1.5 bears two PDZ binding motifs, a canonical C-terminal (E-T-D-L) and an internal motif that localizes to the Kv1.5 T1 region [9]. The T1 (tetramerization 1) domain is a conserved sequence present in the N-terminal cytoplasmatic region of voltage-gated K⁺ channels. This domain is required for the assembly of voltage-gated K⁺ channel β subunit proteins into a tetramer and formation of functional channels [51]. Disruption of the internal PDZ binding site drastically reduced Kv1.5 currents to almost undetectable levels which might be explained by reduced channel expression. The PDZ protein SAP97 increases

Kv1.5 current through an indirect interaction with the N-terminus [10]. Thus, the Kv1.5 mutant might be expressed to a lesser extent due to the disrupted regulation by intrinsic SAP97. The N-termini of Kv channels contain sequences that are essential for the assembly of channel subunits [52] or association with regulatory proteins such as β -actinin2 [53]. Therefore deletion of the internal PDZ binding site might affect expression and channel gating. Ablation of the C-terminal PDZ binding motif slightly increased absolute Kv1.5 currents as observed by Eldstrom and collaborators [9]. Currents elicited by this mutant were not elevated upon NHERF2 coexpression, indicating that NHERF2 activates the channel via its C-terminal PDZ binding motif. The C-terminal PDZ binding motif in Kv1.5 is involved in the regulation of the PDZ domain-containing protein PSD95 as well [9]. However PSD95 increases Kv1.5 currents only when the N-terminus of the channel is deleted [9].

NHERF2 binding to the cytoskeleton allows sorting and localization of membrane proteins. An additional function of NHERF2 is to mediate clustering of signalling proteins to specific domains to facilitate cellular signalling. NHERF2 acts as a scaffold protein to localize protein kinases in the vicinity of ion channels/transporters to activate them [23, 34, 38-41, 43]. NHERF2 enhances the activity of K⁺ channel ROMK and Ca²⁺ channel TRPV5 by scaffolding the SGK1 kinase allowing phosphorylation and thereby activation of the channels [23, 43, 54]. This report demonstrates that NHERF2 synergizes with SGK1 to increase Kv1.5 currents. However, in contrast to the aforementioned studies, the present observations reveal that the kinase is capable of modulating Kv1.5 in the absence of NHERF2. Western blotting of oocyte plasma membranes revealed a robust increase in Kv1.5 channel abundance at the plasma membrane compared to the modest increase in current density. Thus, a pool of channels trafficked by NHERF2 to the plasma membrane might need further activation by accessory proteins (i.e. SGK1). As a matter of fact, the NHERF2-mediated increase in Kv1.5 currents was further augmented upon coexpression of the protein kinase SGK1, suggesting that the kinase might also stimulate Kv1.5 channels present at the plasma membrane (Figure 4).

Several C-terminal PDZ binding motifs contain a serine and/or threonine (X-S/T-X-V/L) that might be phosphorylated by Ser/Thr kinases. Phosphorylation at this site might affect target affinity for NHERF isoforms [55]. The C-terminal PDZ binding motif in Kv1.5 (E-T-D-L) contains a threonine that lies within a putative

SGK1 consensus site [56]. The Kv1.5 sequence contains three further putative SGK1 phosphorylation sites. Deletion of all putative phosphorylation sites did not abrogate the stimulating SGK1 effects on Kv1.5. These findings suggest a model of Kv1.5 regulation in which NHERF2 brings SGK1 close to the Kv1.5 protein where the kinase does not phosphorylate the potassium channel directly but a *hitherto* unknown regulatory protein.

Mutations in cardiac voltage-gated K⁺ channels are related to long QT syndrome, cardiac arrhythmias and sudden cardiac death [57]. Kv1.5 gene transfer in a long QT phenotype mouse model normalizes action potential duration and shortens QT interval [58]. Thus, enhanced expression of Kv1.5 channels by NHERF2 and/or SGK1 may lead to short QT syndrome. As a matter of fact, a SGK1 gene variant (E8CC/CT-I6CC) conferring enhanced SGK1 expression [59, 60], is indeed associated with a shortened QT interval [61]. SGK1 dependent regulation of Kv1.5 further participates in the inhibitory action of glucocorticoids on insulin release from pancreatic beta cells [8]. Notably, the same SGK1 gene variant (E8CC/CT-I6CC) conferring enhanced SGK1

expression is associated with enhanced risk to develop diabetes mellitus. SGK1/NHERF2 dependent regulation of Kv1.5 may participate in but presumably does not fully account for decreased QT interval and diabetes in the carriers of this gene variant. At least in theory SGK1 dependent regulation of other channels or carriers [42, 61-65] could be accomplished by similar mechanisms.

In conclusion, this study indicates that NHERF2 enhances Kv1.5 function and expression by stabilizing Kv1.5 in the plasma membrane. In order to activate the channel, NHERF2 requires the C-terminal PDZ-binding motif in Kv1.5. Additionally, NHERF2 synergizes with SGK1 to increase Kv1.5 currents. Our data disclose a novel mechanism regulating Kv1.5 channel activity, which may indeed participate in the control of the pleiotropic Kv1.5 channel functions.

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