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Requirement of PDZ Domains for the Stimulation of the Epithelial Ca^{2+} Channel TRPV5 by the NHE Regulating Factor NHERF2 and the Serum and Glucocorticoid Inducible Kinase SGK1

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Key Words

1,25(OH)2D3 • TRPV5 • Calcium transport • Mineralisation • Kidney • Intestine • PDZ domains • Trafficking

Abstract

Renal calcium reabsorption involves the epithelial calcium channel ECaC1 (TRPV5) which is tightly regulated by 1,25(OH)2D3. As shown recently, TRPV5 is activated by the serum and glucocorticoid inducible kinase SGK1, a kinase transcriptionally upregulated by 1,25(OH)2D3. This stimulatory effect is due to enhanced TRPV5 abundance in the plasma membrane and requires the presence of the scaffold protein NHERF2 (sodium hydrogen exchanger regulating factor 2). The present study aims to define the molecular requirements for the interaction of TRPV5 with SGK1 and NHERF2. Pull-down experiments and overlay assays revealed that the TRPV5 C-tail interacts in a Ca^{2+}-independent manner with NHERF2. Deletion of the second but not of the first PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/NHERF2 on TRPV5 protein abundance in the plasma membrane as quantified by chemiluminescence and electrophysiology. Thus, the second PDZ domain in NHERF2 is required for stabilization at or TRPV5 targeting to the plasma membrane. The experiments demonstrate the significance of SGK1 and NHERF2 as TRPV5 modulators which are likely to participate in the regulation of calcium homeostasis by 1,25(OH)2D3.

Introduction

The epithelial Ca^{2+} channel TRPV5/ECaC1 plays a key role in both, intestinal absorption and renal reabsorption of Ca^{2+}[1, 2]. The channel accomplishes Ca^{2+} uptake across the apical membrane of epithelial cells. Subsequently Ca^{2+} is extruded via the basolateral Ca^{2+} ATPase and Na^+/Ca^{2+} exchange [3]. Transcellular transport of Ca^{2+} is modulated by 1,25(OH)2D3 (calcitriol), the active metabolite of vitamin D3, which has further been shown to influence differentiation and proliferation of epithelial cells [4]. The importance of 1,25(OH)2D3 in Ca^{2+} homeostasis of the body is reflected by the development of rickets in patients with mutations in the genes coding for the 1,25(OH)2D3-receptor [5] and for

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1α-hydroxylase [6]. The 1α-hydroxylase controls 1,25(OH)2D3 synthesis from 25-hydroxyvitamin D3 (calcidiol) which is in turn synthesized from vitamin D3 (cholecalciferol) by 25-hydroxylase.

As entry via TRPV5 is the rate limiting step in transcellular transport of Ca^{2+}, TRPV5 activity is decisive for transepithelial transport regulation [7]. The C-terminal tail of TRPV5 contains a PDZ binding motif which may bind to the NHE regulating factors NHERF1 or NHERF2 [8]. NHERF1 and NHERF2 modulate the targeting and trafficking of several proteins including TRPV4 to the plasma membrane [9-11]. TRPV5 [12] and NHERF2 [13] colocalize in principal cells.

Most recent experiments [14] disclosed a role of serum and glucocorticoid dependent kinase SGK1 in the interaction of TRPV5 with NHERF2. The kinase was originally cloned as a glucocorticoid sensitive gene from rat mammary tumor cells [15] and is genomically regulated by 1,25(OH)2D3 [16]. Coexpression studies in *Xenopus* oocytes revealed that TRPV5 conductance is activated by the scaffold protein NHERF2 by increasing the channel abundance at the plasma membrane. This stimulatory effect requires the presence of the kinase SGK1 [14].

The present study has been performed to clarify the role of PDZ domains (named for three proteins in which this domain was first described: postsynaptic density PSD-95/SAP90, the *Drosophila* septate junction protein disc-large, and the tight junction protein ZO-1) in NHERF2 in the regulation of TRPV5 activity.

To this end, cRNA encoding full-length TRPV5 has been injected with or without wild type NHERF2, NHERF2 lacking the first PDZ domain (NHERF2ΔP1) or NHERF2 lacking the second PDZ domain (NHERF2ΔP2) and/or constitutively active ^{S422D}SGK1 into *Xenopus* oocytes. We show here that the interaction of NHERF2 and TRPV5 requires the second PDZ domain of NHERF2 and the C-tail of TRPV5. Deletion of the second but not of the first PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/NHERF2 on TRPV5 activity and abundance at the plasma membrane. Thus, the second PDZ domain in NHERF2 is required for TRPV5 stabilization at or TRPV5 targeting to the plasma membrane.

Materials and Methods

GST-TRPV5 fusion protein and pull-down assays

The amino and carboxyl tails of mouse TRPV5 were amplified by PCR using mouse kidney cDNA:

N tail: forward ccgaattcggatgggggctaaaactccttggatc;
reverse: cgcgctcgagctcaaggctgtccatatttcttccactt;
C tail: forward cccctgggatcc-ggcgacactcactggcgagtggcc;
reverse: acagaagtcgactcagaaatggtagatctcctc,

digested with *Eco*RI and *Xho*I (N tail) or *Bam*HI and *Sal*I (C tail) and cloned into pGEX6p-2 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). pGEX6p-2 construct containing full-length mouse NHERF2 was generously provided by Dr. J. Biber. pGEX6p-2 constructs were transformed in *Escherichia coli* BL21 and glutathione S-transferase (GST) fusion proteins were expressed and purified according to the manufacturer's protocol (Amersham Pharmacia Biotech AB). [^{35}S]methionine-labeled full-length TRPV5 and NHERF2 were prepared using a reticulocyte lysate system in the presence of canine microsomal membranes (Promega Madison, WI) and added to GST or GST-fusion proteins immobilized on glutathione-sepharose 4B beads. The binding buffer contained 20 mM Tris-HCl pH 7.4, 140 mM NaCl, 0.2 % (v/v) Triton-X-100 and 0.2% (v/v) NP-40, supplemented with 1 mM $CaCl_2$ or 2 mM EDTA. After 2 h incubation at room temperature, the beads were washed extensively with binding buffer. Bound proteins were eluted with SDS-PAGE loading buffer, separated on SDS-PAGE gels and visualized by autoradiography.

Overlay assays

Approximately 10 µg GST, GST-TRPV5 C-tail, GST-TRPV6 C-tail, GST-NHERF2 or bovine serum albumin were separated on SDS/PAGE gels and blotted into PVDF membranes. Blots were blocked for 60 min in binding buffer containing 20 mM Tris-HCl pH 7.4, 140 mM NaCl, 1 mM $CaCl_2$, 0.2 % (v/v) Triton-X-100 and 3 % (w/v) BSA. Subsequently, blots were incubated with *in vitro* translated [^{35}S]-labeled NHERF2 or TRPV5 for 2 h at room temperature in binding buffer containing 0.3 % (w/v) BSA, washed extensively and bound proteins were visualized by autoradiography.

Expression in Xenopus laevis oocytes

cRNA encoding rabbit TRPV5 [1], human NHERF2 [17], NHERF2 lacking the first PDZ domain (NHERF2ΔP1) or NHERF2 lacking the second PDZ domain (NHERF2ΔP2) and constitutively active human ^{S422D}SGK1 have been synthesized as described [18]. Oocytes were injected with 2.5 ng TRPV5, 7.5 ng ^{S422D}SGK1 and/or 5 ng NHERF2 or NHERF2ΔP1 or NHERF2ΔP2 cRNA or H_2O. All experiments were performed at room temperature 3 days after injection of the respective cRNAs.

Voltage-clamp analysis

In two-electrode voltage-clamp experiments currents were recorded during a 4 s linear voltage ramp from –150 mV to +50 mV. The intermediate holding potential between the voltage ramps was -50 mV. Data were filtered at 10 Hz and recorded with MacLab digital to analog converter and software for data acquisition and analysis (ADInstruments, Castle Hill, Australia). The bath solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1mM $BaCl_2$,10 µM methoxyverapamil, 5 mM HEPES, pH 7.4 with or without 10 mM $CaCl_2$. Oocytes were kept in modified Barth´s solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM

$NaHCO_3$, 0.8 mM $MgSO_4$, 0.3 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 supplemented with 25 µg/ml gentamycin. The final solutions were 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. The addition of 10 mM $CaCl_2$ induced an inward current ($I_{Cl(Ca)}$) which was created by entry of Ca^{2+} and subsequent activation of Ca^{2+} sensitive Cl^- channels [1]. The peak inward current was taken as a measure for TRPV5 activity. The currents depicted in the figures represent the maximum peak inward current from a voltage ramp ranging from -150 mV to +50 mV in the presence of 10 mM Ca^{2+}. The intermediate holding potential was –50 mV. $I_{Cl(Ca)}$ activity is synchronously triggered by the intracellular calcium concentration close to the membrane determined by the epithelial calcium channel TRPV5. Thus, $I_{Cl(Ca)}$ activity mirrors activation and inactivation kinetics of TRPV5 [19].

Detection of cell surface expression by chemiluminescence

Defolliculated oocytes were first injected with ^{S422D}SGK1 cRNA (7.5 ng/oocyte) and/or NHERF2, NHERF2ΔP1 or NHERF2ΔP2 cRNA (5 ng/oocyte), and one day later with TRPV5-HA (25 ng/oocyte) which contains an HA (hemagglutinin) epitope extracellularly between amino acid 376 and 377. Oocytes were incubated with 1 µg/ml primary rat monoclonal anti-HA antibody (clone 3F10, Boehringer, Germany), and 2 µg/ml secondary, peroxidase-conjugated affinity-purified $F(ab')_2$ goat anti-rat IgG antibody (Jackson ImmunoResearch, West Grove, USA). Individual oocytes were placed in 20 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, USA), and chemiluminescence was quantified in a luminometer by integrating the signal over a period of 1 s. Results are given in relative light units (RLU).

Statistics

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with at least 3 batches of oocytes from different frogs; in all repetitions qualitatively similar data were obtained. All data were tested for significance using ANOVA, and only results with $P < 0.05$ were considered statistically significant.

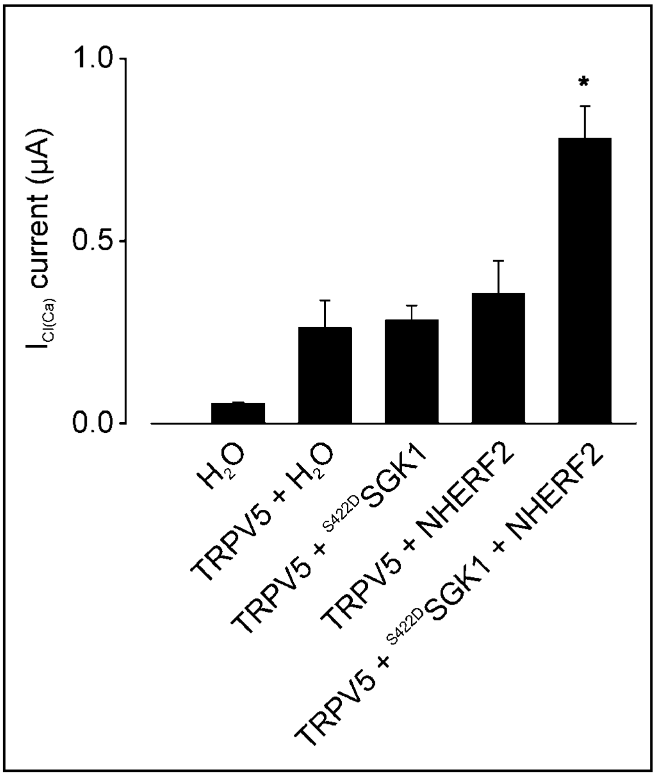

Fig. 1. Stimulation of Ca^{2+} currents by the combined expression of TRPV5, NHERF2 and the ^{S422D}SGK1. TRPV5 mediated calcium currents indirectly activate an endogenous chloride conductance ($I_{Cl(Ca)}$). The depicted $I_{Cl(Ca)}$ is the maximum peak inward current from a voltage ramp ranging from -150 mV to +50 mV in the presence of 10 mM Ca^{2+}. The intermediate holding potential was –50 mV. $I_{Cl(Ca)}$ was stimulated by coexpression of TRPV5 together with NHERF2 and ^{S422D}SGK1. Coexpression of TRPV5 with either NHERF2 or ^{S422D}SGK1 alone did not significantly enhance $I_{Cl(Ca)}$. * denotes significant difference ($p < 0.05$) between *Xenopus* oocytes expressing TRPV5 together with ^{S422D}SGK1 and NHERF2 and oocytes expressing TRPV5 alone. n = 21 – 24 oocytes from 3 animals.

Results

In the presence of Cl^- the Ca^{2+} entry through TRPV5 activated Ca^{2+} sensitive Cl^- channels leading to the appearance of a large Cl^- current ($I_{Cl(Ca)}$). In TRPV5 expressing oocytes, coexpression of NHERF2 together with ^{S422D}SGK1 stimulated $I_{Cl(Ca)}$ from 0.26 ± 0.08 µA in TRPV5 expressing oocytes (n = 24) to 0.78 ± 0.09 µA in oocytes expressing ^{S422D}SGK1/NHERF2/TRPV5 (n = 24, 3 animals) (Fig. 1).

To investigate whether the regulatory effect of NHERF2 and ^{S422D}SGK1 on TRPV5 activity is mediated by protein-protein interaction of NHERF2 and TRPV5, pull-down and overlay assays were performed. To this end, full-length TRPV5 protein was labeled with [^{35}S]methionine by *in vitro* transcription/translation and its interaction with NHERF2 was tested under *in vitro* conditions. As shown in Fig. 2A, TRPV5 interacted with GST-NHERF2 fusion protein, whereas no binding to GST alone was observed, indicating the specificity of the interaction. The binding was identical in the presence or

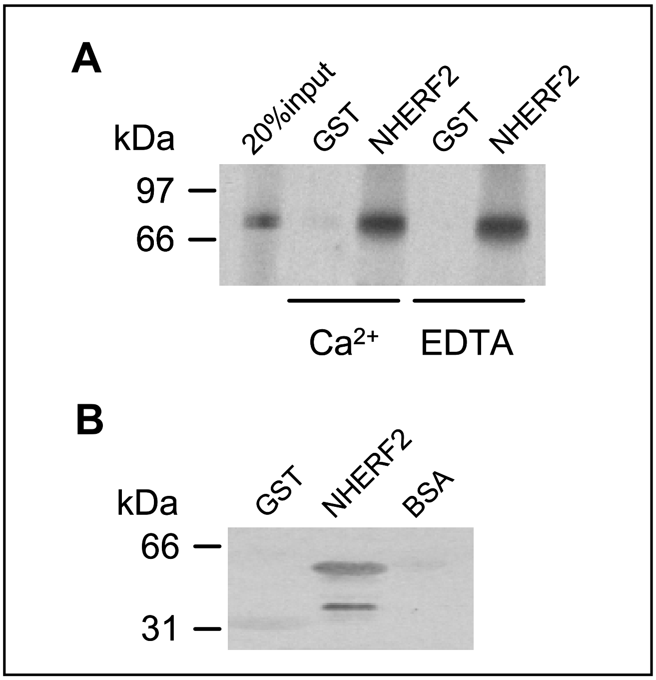

Fig. 2. Interaction of NHERF2 with TRPV5 as demonstrated by GST pull-down and overlay assays. Full-length NHERF2 expressed as GST fusion protein was purified from *E. coli* and immobilized on glutathione-sepharose 4B beads (in A) or blotted onto PVDF membranes (in B) and incubated with *in vitro* translated [^{35}S]methionine-labeled TRPV5. Bound proteins were analyzed by autoradiography. NHERF2 interacts in a Ca^{2+}-independent manner with TRPV5, whereas no binding with GST and BSA was observed.

absence (2 mM EDTA) of Ca^{2+} (1 mM). Results obtained were corroborated by overlay assays. *In vitro* translated [^{35}S]-labeled TRPV5 bound GST-NHERF2, whereas GST alone and bovine serum albumin (BSA) showed no interaction (Fig. 2B).

To demonstrate that the interaction between TRPV5 and NHERF2 ocurred via the carboxyl tail of TRPV5, we amplified the amino and carboxyl tails of mouse TRPV5 by PCR using mouse kidney cDNA. GST fusion proteins encompassing either the amino or carboxyl tail of TRPV5 were incubated with [^{35}S]methionine-labeled NHERF2. NHERF2 interacted specifically with the carboxyl tail, while the amino tail of TRPV5 was unable to bind NHERF2. The binding was again Ca^{2+}-independent (Fig. 3A). To verify the specificity of TRPV5/NHERF2 interaction we performed experiments with the TRPV5 isoform TRPV6. Fig. 3B shows that neither the amino nor the carboxyl tail of TRPV6

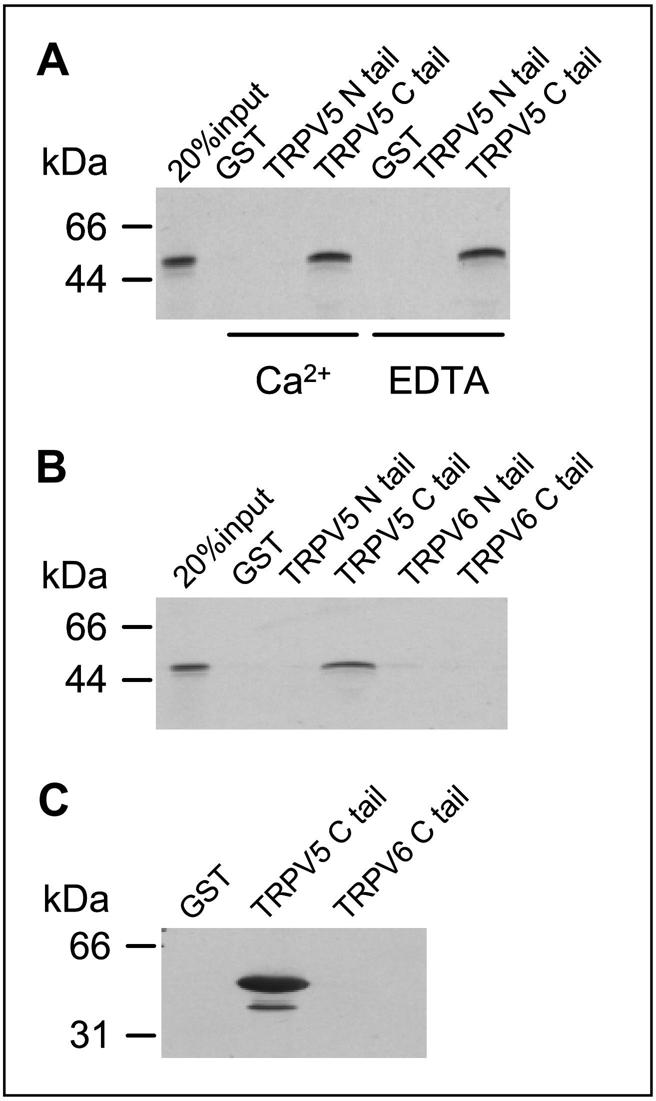

Fig. 3. NHERF2 interacts with TRPV5 through the TRPV5 carboxyl tail. GST fusion proteins encompassing either the amino or carboxyl tail of TRPV5 or TRPV6 were purified from *E. coli* and immobilized on glutathione-sepharose 4B beads (in A, B) or blotted onto PVDF membranes (in C) and incubated with *in vitro* translated [^{35}S]methionine-labeled NHERF2. Bound proteins were analyzed by autoradiography. NHERF2 interacts in a Ca^{2+}- independent manner with TRPV5 C-tail, whereas no binding with GST and TRPV6 is observed indicating the specificity of the interaction.

interacted with [^{35}S]methionine-labeled NHERF2. Overlay assays with *in vitro* translated [^{35}S]-labeled NHERF2 confirmed the selective ability of NHERF2 to bind TRPV5 C-tail (Fig. 3C).

Further studies were performed to identify the PDZ binding domain of NHERF2 required for the stimulating

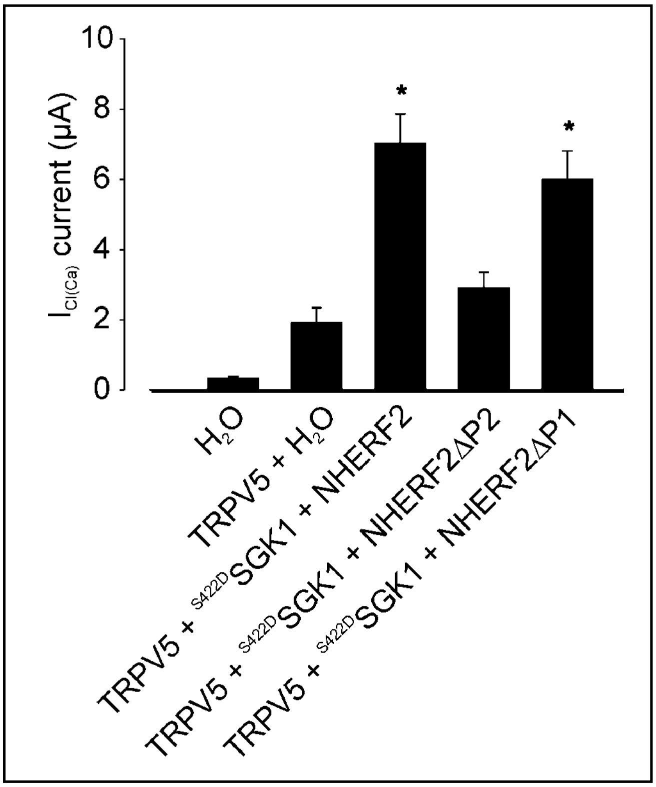

Fig. 4. Requirement of the second PDZ domain of NHERF2 for the stimulation of TRPV5 by constitutively active ^{S422D}SGK1. *Xenopus laevis* oocytes were injected with water or cRNA encoding TRPV5 alone or with ^{S422D}SGK1 and either wild-type NHERF2, NHERF2 lacking the second PDZ domain (NHERF2ΔP2) or NHERF2 lacking the first PDZ domain (NHERF2ΔP1). Only the combined coexpression of ^{S422D}SGK1 and wild-type NHERF2 or NHERF2ΔP1 increases $I_{Cl(Ca).}$ The depicted $I_{Cl(Ca)}$ is the maximum peak inward current from a voltage ramp ranging from -150 mV to +50 mV in the presence of 10 mM Ca^{2+}. The intermediate holding potential was –50 mV. * indicate significant difference between expression of TRPV5 alone and of TRPV5 coexpressed with ^{S422D}SGK1 and NHERF2. n = 14 – 15 oocytes from 3 animals.

effect of ^{S422D}SGK1 and NHERF2 on TRPV5 activity. As shown in Fig. 4, TRPV5 channel activity was enhanced with both, ^{S422D}SGK1 and wild-type NHERF2 (from 1.90 ± 0.44 µA to 7.04 ± 0.83 µA, n = 15), but not by coexpression of TRPV5 together with ^{S422D}SGK1 and NHERF2 lacking the second PDZ domain NHERF2ΔP2. In contrast, coexpression of ^{S422D}SGK1 together with a NHERF2 mutant deficient of the first PDZ domain (NHERF2ΔP1) increased TRPV5 activity similar to coexpression of ^{S422D}SGK1 together with wild type NHERF2 (from 1.90 ± 0.44 µA to 6.0 ± 0.81 µA, n = 14, 3 animals). Thus, the second but not the first PDZ domain is essential for upregulation of TRPV5 activity. This requirement for the second PDZ domain is consistent with the interaction of SGK1 with the second but not first PDZ domain of NHERF2.

The PDZ domains of NHERF2 are thought to link NHERF2 and associated proteins to the cytoskeleton and thus to accomplish the targeting to or stabilization of transport proteins at the cell membrane. Therefore we examined TRPV5 surface expression, using a chemiluminescence assay in parallel with electrophysiological measurements, upon coexpression of ^{S422D}SGK1 and NHERF2 mutants. Fig. 5 demonstrates that both surface expression and current are increased in oocytes expressing ^{S422D}SGK1/NHERF2/TRPV5 compared to TRPV5 control oocytes. Co-expression of ^{S422D}SGK1/NHERF2 enhanced TRPV5 surface labelling by 4.4 fold and TRPV5-mediated currents from 1.87 ± 0.41 µA in TRPV5 expressing oocytes (n = 21) to 7.01 ± 1.27 µA in oocytes expressing ^{S422D}SGK1/NHERF2/TRPV5 (n = 20). TRPV5 surface expression was also stimulated up to 3.5 fold upon coexpression of ^{S422D}SGK1 and NHERF2 lacking the first PDZ domain (NHERF2ΔP1). However, co-expression of NHERF2 lacking the second PDZ domain failed to increase TRPV5 expression and TRPV5-mediated currents 1.87 ± 0.41 µA in TRPV5 expressing oocytes (n = 21) versus 2.42 ± 0.41 µA in oocytes expressing ^{S422D}SGK1/NHERF2ΔP2/TRPV5 (n = 20, 3 animals).

Discussion

The present experiments confirm the previous observations disclosing a completely novel mechanism regulating TRPV5 activity, i.e. the regulation by the NHE regulating factor NHERF2 and the serum and glucocorticoid inducible kinase SGK1 [14].

As shown in this study, NHERF2 binds to the carboxyl tail of TRPV5 and requires its second PDZ domain to be effective. NHERF2 is thought to link membrane proteins to cytoskeletal proteins through its PDZ domains [17, 20]. The binding to the cytoskeleton may serve to target or stabilize the protein at the cell membrane. We demonstrate that deletion of the second but not of the first PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/NHERF2 on TRPV5 protein abundance at the plasma membrane. Thus, the second PDZ domain in NHERF2 is required for TRPV5 stabilization at or TRPV5 targeting to the plasma membrane.

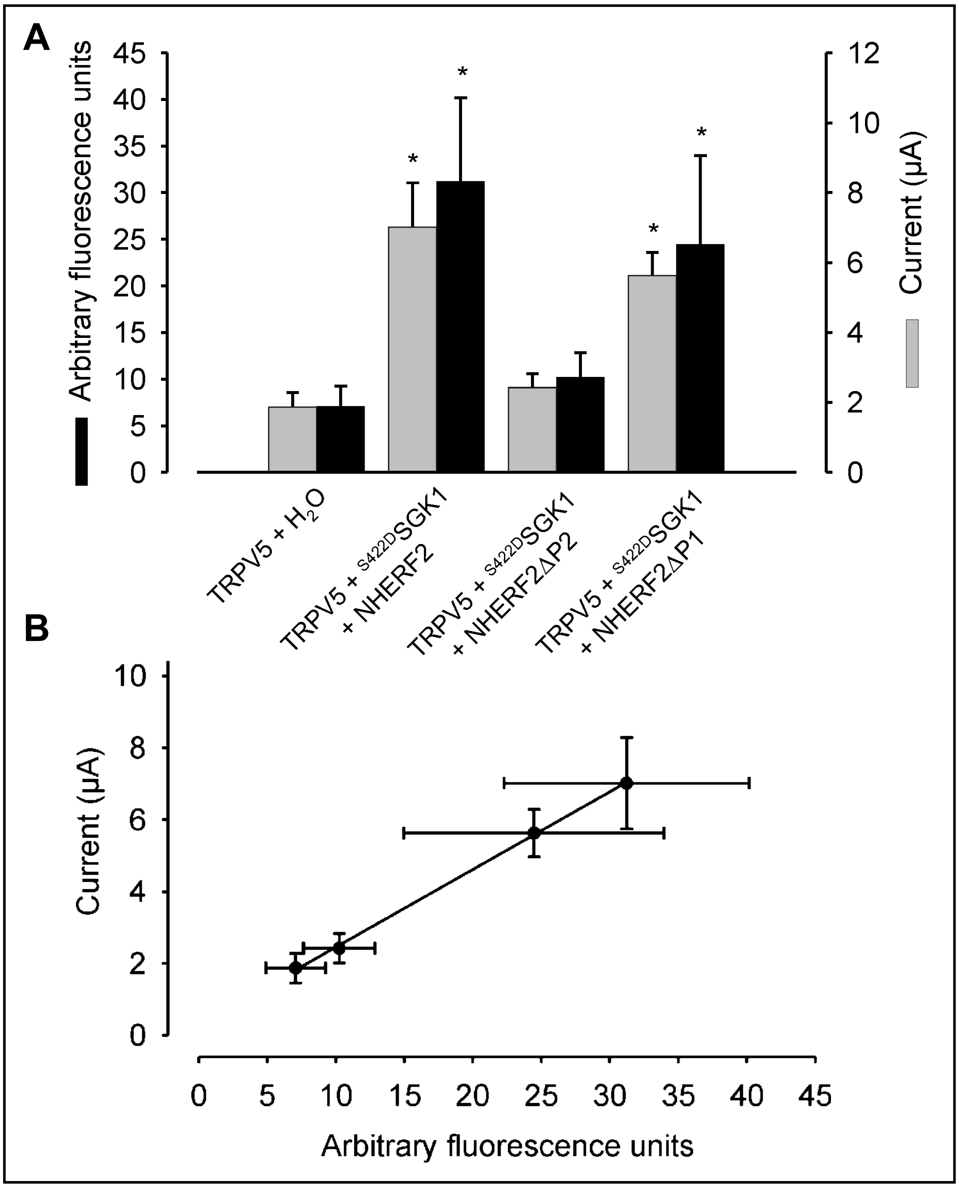

Fig. 5. Requirement of the second PDZ domain of NHERF2 for the stimulation of TRPV5 cell surface expression by constitutively active ^{S422D}SGK1. *Xenopus laevis* oocytes were injected with water or cRNA encoding TRPV5 alone or with ^{S422D}SGK1 and either wild-type NHERF2, NHERF2 lacking the second PDZ domain (NHERF2ΔP2) or NHERF2 lacking the first PDZ domain (NHERF2ΔP1). A) Only the combined coexpression of ^{S422D}SGK1 and wild-type NHERF2 or NHERF2ΔP1 increases TRPV5 cell surface expression. * indicate significant difference between expression of TRPV5 alone and of TRPV5 coexpressed with ^{S422D}SGK1 and NHERF2. n = 20 - 29 oocytes from 3 animals. B) Cell surface expression correlates with TRPV5 whole cell current. n = 20 - 29 oocytes from 3 animals.

NHERF2 and SGK1 modulate TRPV5 and not the Ca^{2+}-activated Cl^- channels, since intrinsic NPPB sensitive chloride current remains unaffected upon SGK1 and/or NHERF2 coexpression [14]. The effect of SGK1 depends on an intact catalytic subunit as the inactive mutant ^{K127N}SGK1 did not influence TRPV5 even in the presence of NHERF2 [14]. Thus, the kinase is obviously effective through phosphorylation of the target protein. SGK1 has been reported to bind NHERF2 [8]. However, SGK1 cannot phosphorylate NHERF2 (unpublished observations). TRPV5 possess a putative SGK1 phosphorylation site in its sequence. Hence, SGK1 might phosphorylate and thereby stimulate TRPV5 activity. Nevertheless this stimulatory effect requires NHERF2 which, similar to what has been described for the modulation of NHE3 by PKA [8], might bind SGK1 to bring the kinase in close proximity to TRPV5 or an associated protein.

A well known function of SGK1 is its participation in the regulation of the epithelial Na^+ channel ENaC [21-30]. SGK1 is effective by increasing the abundance of the ENaC protein within the cell membrane [21, 30, 31]. Most recently, SGK1 has been shown to upregulate the renal epithelial K^+ channel ROMK1 [32]. Similar to SGK-dependent regulation of TRPV5, the regulation of ROMK1 by SGK1 has been shown to depend on NHERF2 and on the PDZ binding motif present in ROMK1. TRPV5, as well as ROMK1, display an exquisite H^+ sensitivity [1, 16, 33, 34]. TRPV5 activity is markedly reduced by lowering of the ambient pH. Thus, NHERF2 could participate in the link between TRPV5 activity and acid base balance. The sensitivity of renal tubular Ca^{2+} transport to H^+ is of physiological significance, since on the one hand mineralization of bone depends on the deposition of highly alkaline Ca^{2+} salts [35] and on the other hand precipitation of Ca^{2+} phosphate salts is favoured by alkalinization of urine [36].

In contrast to the regulation of TRPV5, NHE3 [37] and ROMK1 [32], the regulation of ENaC does not require the participation of NHERF2. SGK1 stimulates ENaC in part by phosphorylating the ubiquitin protein ligase Nedd4-2 in a PY motif-dependent manner [38, 39]. The phosphorylation impedes the binding of Nedd4-2 to ENaC [38]. The ENaC sequence does not include a PDZ motif, suggesting that NHERF2 can not directly interact with ENaC.

In conclusion, TRPV5 is the target of a complex regulating mechanism involving both, the NHE regulating factor NHERF2 and the serine/threonine kinase SGK1. The concerted action of NHERF2 and the kinase markedly upregulates the activity and plasma membrane abundance of this key channel in the regulation of Ca^{2+} homeostasis. The stimulatory effect requires the second PDZ domain of NHERF2 and the carboxyl tail of TRPV5.

Abbreviations

GST (glutathione S-transferase); SGK (serum and glucocorticoid inducible kinase); PVDF (Polyvinylidene Fluoride); NHERF (sodium hydrogen exchanger regulating factor); Nedd (Neuronal cell expressed developmentally downregulated ubiquitin ligase).

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