RGS2 Inhibits the Epithelial Ca\textsuperscript{2+} Channel TRPV6*

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The epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6 constitute the apical Ca\textsuperscript{2+} entry pathway in the process of active Ca\textsuperscript{2+} (re)absorption. By yeast two-hybrid and glutathione S-transferase pulldown analysis we identified RGS2 as a novel TRPV6-associated protein. RGS proteins determine the inactivation kinetics of heterotrimeric G-protein-coupled receptor (GPCR) signaling by regulating the GTPase activity of \textit{G}_{\alpha}\text{q}. Here we demonstrate that TRPV6 interacts with the NH\textsubscript{2}-terminal domain of RGS2 in a Ca\textsuperscript{2+}-independent fashion and that overexpression of RGS2 reduces the Na\textsuperscript{+} and Ca\textsuperscript{2+} current of TRPV6 but not that of TRPV5-transfected human embryonic kidney 293 (HEK293) cells. In contrast, overexpression of the deletion mutant \textit{ΔN}-RGS2, lacking the NH\textsubscript{2}-terminal domain of RGS2, in TRPV6-expressing HEK293 cells did not show this inhibition. Furthermore, cell surface biotinylation indicated that the inhibitory effect of RGS2 on TRPV6 activity is not mediated by differences in trafficking or retrieval of TRPV6 from the plasma membrane. This effect probably results from the direct interaction between RGS2 and TRPV6, affecting the gating properties of the channel. Finally, the scaffolding protein spinophilin, shown to recruit RGS2 and regulate GPCR-signaling via \textit{G}_{\alpha}\text{q}, did not affect RGS2 binding and electrophysiological properties of TRPV6, indicating a GPCR-independent mechanism of TRPV6 regulation by RGS2.

Recently, the electrophysiological properties of TRPV6 have been studied extensively. When measured in heterologous expression systems, TRPV6 displays characteristic inward rectifying currents carried by Ca\textsuperscript{2+} or monovalent cations when Ca\textsuperscript{2+} is omitted from the extracellular solution. These inward currents are blocked by Mg\textsuperscript{2+} in a voltage-dependent manner (8), which is removed upon neutralization of a single negatively charged amino acid residue, Asp\textsuperscript{241}, within the pore region. The pore diameter of TRPV6 was recently estimated at 5.4 Å by cysteine scanning experiments (9). Together with its closest relative, TRPV5, TRPV6 shares a common feature that is the Ca\textsuperscript{2+}-dependent inactivation of the currents.

TRPV6 is predominantly expressed in placenta, pancreas, small intestine, and colon (6, 10–12). In these tissues TRPV6 plays a major role in Ca\textsuperscript{2+} transport (reviewed in Refs. 13 and 14).

To date, screening and domain mapping of the carboxyl terminus of TRPV6 has revealed proteins associating with TRPV6 and subsequently the regulatory functions of these interacting proteins have been studied, e.g. Rab11a, NHERF4, S100A10 (15–17). In contrast, little is known about the molecular mechanisms involved in regulation of the channel via its intracellular NH\textsubscript{2}-terminal domain. At present, six ankyrin repeats have been predicted in this region (18). Ankyrin repeats have been shown to act as a scaffold in molecular recognition to mediate protein-protein interactions (19). These, or other regions in the NH\textsubscript{2}-terminal domain of TRPV6, may bind regulatory proteins interacting in pathways that mediate the Ca\textsuperscript{2+}-transporting activity of the channel.

The aim of this study was to identify regulatory proteins that interact with the NH\textsubscript{2}-terminal domain of TRPV6. To this end, we identified RGS2 as a novel binding partner of TRPV6 by yeast two-hybrid screening. RGS2 is a member of the RGS family of proteins that are able to terminate signaling of heterotrimeric G-protein-coupled receptors (GPCRs) by enhancing the GTPase activity of active \textit{G}_{\alpha}\text{q} subunits (20). To date, over 20 different RGS proteins have been identified, and their effector functions extend beyond negative regulation of GPCR signaling. RGS proteins can act as effector antagonists, binding to either the effector protein or the \textit{G}_{\alpha}\text{q} subunit to prevent an operational physical interaction. They have also been shown to interact with a wide variety of auxiliary proteins, such as calmodulin, spinophilin (SPL), and 14-3-3 protein, which can influence their subcellular localization, stability, and function (21). In this respect, the finding of RGS2 as a protein interacting with TRPV6 may lead to altered channel activity by disturbances in protein trafficking or a direct effect at the cell surface. The
putative role of RGS2 in active Ca\(^{2+}\) absorption was established by a comprehensive approach including pulldown assays, electrophysiological analysis, and cell-surface expression of RGS2 interacting with TRPV6.

**EXPERIMENTAL PROCEDURES**

*Constructs and cRNA Synthesis—* The TRPV6, TRPV6 N-tail, and TRPV5 constructs were generated in pGEX 6p-2 and pCINeo/IRES-GFP vectors as described previously (22). The coding sequence of wild-type RGS2 protein was amplified from total mouse kidney cDNA material using primers with a HA tag included and cloned into the PT7Ts vector as BglII-SPel fragment, and subsequently into the pCINeo/IRES-GFP vector as an NheI-XhoI fragment. RGS2 and ΔN-RGS2 (deletion of the NH\(_2\)-terminal domain of RGS2: amino acids 1–82) were sub-cloned from the PT7Ts vector without the HA tag to the pGEX 6p-2 (Amersham Biosciences) vector using forward and reverse primers containing BamHI and XhoI restriction sites, respectively. PT7Ts constructs were linearized, and cRNA was synthesized *in vitro* as described previously (16). In pCINeo/IRES-GFP, ΔN-RGS2 was obtained by *in vitro* mutagenesis of wild-type RGS2. All constructs were verified by sequence analysis. Rat-SPL (in pCMV-myc) and human-RGS2 (in pGex-KG) constructs were described previously (23).

*Yeast Two-hybrid System—* Yeast-two-hybrid experiments were performed as described previously (16). In short, the N-tail of mouse TRPV6 was cloned in the pAS-1 yeast cloning vector using forward and reverse primers containing BamHI and XhoI restriction sites, respectively. PT7Ts constructs were linearized, and cRNA was synthesized *in vitro* as described previously (16). In pCINeo/IRES-GFP, ΔN-RGS2 was obtained by *in vitro* mutagenesis of wild-type RGS2. All constructs were verified by sequence analysis. Rat-SPL (in pCMV-myc) and human-RGS2 (in pGex-KG) constructs were described previously (23).

*Reverse Transcriptase (RT)-PCR Analysis—* Total RNA was isolated from different mouse tissues using TRIzol and subjected to reverse transcription as described (24). For RGS2 RT-PCR, the oligonucleotide primer sequences were as follows: forward primer with BglII restriction site, 5'-GAAGATGCTGCCACCATGCA-AAGTGCCAGTTCCTG-3'; reverse with SpeI restriction site, 5'-GGACATGGTCACTGTAGCATGGGCTCCG-3'; β-actin, 5'-ACAGATTCTCCTCTACGTTG-3' (forward) and 5'-GATGTCCTCCTGCGTACCAC-3' (reverse). For SPL the following primer sequences were as follows: forward primer, 5'-CTTACGAAACACAAAGGCC-3'; reverse primer, 5'-GATGACCTCCAGTTGAGACC-3'. PCR fragments were analyzed by electrophoresis on 1% (w/v) agarose.

*Pulldown Assays—* RGS2 pGex-KG and HA-RGS2, HA-ΔN-RGS2, and empty pGex 6p-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 Biosciences). [\(^{35}\)S]Methionine-labeled TRPV6 N-tail was prepared using a reticulocyte lysate system (Promega, Madison, WI) and analyzed for pull-down with GST, GST-HA-RGS2, or GST-HA-ΔN-RGS2 fusion proteins immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) as described previously (16). After extensive washing, bound proteins were subjected to SDS-PAGE, and visualized by Coomassie staining and autoradiography. For pull-down with hRGS2, TRPV6/mock, SPL/mock, and TRPV6/SPL were transiently transfected in human embryonic kidney 293 (HEK293) cells and after 72-h extracts from lysed cells were adjusted to equal input concentrations and analyzed for pull-down with GST or GST-HRGS2 fusion proteins immobilized on glutathione-Sepharose 4B beads. The beads were washed extensively and pulled proteins were analyzed by SDS-PAGE and immunoblotting.

*Electrophysiology and Solutions—* The full-length cDNAs encoding TRPV5, TRPV6, RGS2, and ΔN-RGS2 were transiently transfected in HEK293 cells and patch clamp experiments were performed in the tight-seal whole-cell configuration at room temperature using an EPC-9 patch clamp amplifier controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany), as described previously (25). Cells were held at 20 mV, and voltage ramps of 450 ms, ranging from −100 to +100 mV, were applied every 5 s. Cell capacitance and access resistance were monitored continuously using the automatic capacitance compensation of the Pulse software. Na\(^{+}\) current densities were obtained by dividing the current amplitude measured at −80 mV during the ramp protocols by the cell capacitance. Ca\(^{2+}\)-dependent inactivation was studied using a 3-s voltage step to −100 mV from a holding potential of +70 mV. From this step protocol, Ca\(^{2+}\) peak values were extracted by dividing the current at −100 mV by the cell capacitance. The standard extracellular solution (Krebs) contained: 150 mM NaCl, 6 mM CsCl, 1 mM MgCl\(_2\), 10 mM HEPES/NaOH, and 10 mM glucose, pH 7.4. The concentration of Ca\(^{2+}\) ranged between 1 and 10 mM. Divalent-free (DFV) solutions did not contain added divalent cations, whereas trace amounts of divalent cations were removed with 100 μM EDTA. The standard internal (pipette) solution contained: 20 mM CsCl, 100 mM Cs-aspartate, 1 mM MgCl\(_2\), 4 mM Na\(_2\)ATP, 10 mM BAPTA, 10 mM HEPES/CsOH, pH 7.2. Cells were kept in a nominal Ca\(^{2+}\)-free medium to prevent Ca\(^{2+}\) overload and exposed for a maximum of 5 min to a Krebs solution containing 1.5 mM Ca\(^{2+}\) before sealing the patch pipette to the cell. The analysis and display of patch clamp data were performed using Igor Pro software (WaveMetrics, Lake Oswego, OR).

*Biotinylation—* TRPV6, HA-RGS2, and empty pCINeo/IRES- GFP constructs were transfected transiently in HEK293 cells and subjected to cell surface biotinylation as described previously (26). Biotinylated proteins were eluted with SDS-PAGE loading buffer, separated on 10% (w/v) SDS-PAGE gel, and analyzed by immunoblotting with rabbit antiserum against TRPV6 (1:2000) (11).

*Statistical Analysis—* In all experiments, the data are expressed as mean ± S.E. Overall statistical significance was determined by analysis of variance. In case of significance (p < 0.05), individual groups were compared using a Student’s t test.
Identification of RGS2 as a TRPV6-associated Protein—To identify proteins that interact with TRPV6, the NH2-terminal tail of TRPV6 was used to screen a library of mouse kidney cDNA by the yeast two-hybrid technique. RGS2 was identified as a positive clone and belongs to the R4 subfamily of regulator of G-protein signaling proteins (27). To confirm the interaction, the full-length cDNA sequence of RGS2 was re-analyzed in a yeast two-hybrid with the N-tail of TRPV6 as bait. The γ-subunit of the epithelial Na+ channel (γENaC) was used as a negative control. TRPV6 and RGS2 displayed a strong interaction, whereas TRPV6 and γENaC did not interact (data not shown).

The RGS2 Gene Is Ubiquitously Expressed—The expression of RGS2 in different mouse tissues was analyzed by RT-PCR. RGS2 was detected ubiquitously (Fig. 1, upper panel). All tissues expressed β-actin that was used as a positive control to confirm the integrity of the cDNAs (Fig. 1, lower panel). As negative controls, the primer sets for RGS2 and β-actin were screened in the absence of cDNA (depicted as H2O samples in Fig. 1).

TRPV6 Interacts with the NH2-terminal Domain of RGS2—The interaction between TRPV6 and the binding domain of RGS2 was further established using GST pulldown assays. To this end, RGS2 and ΔN-RGS2 were expressed as GST fusion proteins immobilized on glutathione-Sepharose 4B beads. Subsequently, these fusion proteins were tested for their interaction with in vitro translated [35S]methionine-labeled N-tail of TRPV6 in the presence or absence of 1 mM Ca2+. As shown in Fig. 2, the N-tail of TRPV6 associated with GST-fused RGS2 in a Ca2+-independent fashion whereas no binding between the N-tail of TRPV6 and GST-fused ΔN-RGS2 or GST alone was observed, neither in the presence nor absence of Ca2+.

RGS2 Inhibits Specifically TRPV6 Activity—To study the functional effect of RGS2 binding to TRPV6 the electrophysiological properties of the TRPV6 channel were investigated in the presence of RGS2. Steeply inward rectifying currents carried by Na+ could be measured in HEK293 cells co-expressing TRPV6 and empty vector (mock) and in cells expressing both TRPV6 and RGS2 (Fig. 3, A and B). The current carried by Na+ was significantly reduced in cells expressing both TRPV6 and
RGS2 inhibits TRPV6

**FIGURE 4. Activity of TRPV5 is not affected by co-expressing RGS2.**

HEK293 cells were transiently transfected with TRPV5/mock (control (CTRL)) or TRPV5/RGS2 and analyzed using immunoblotting (A) and by whole-cell patch clamp. B, I/V relations measured from ramp protocol in nominally DVF solution in control (solid trace) or TRPV5/RGS2-transfected cells (dotted trace). C, average Na⁺ current density at −80 mV in nominally DVF solution was 495 ± 130 pA/pF for control cells compared with 600 ± 100 pA/pF for TRPV5/RGS2 co-expressing cells (n = 15 cells for each condition). D, averaged Ca²⁺ currents measured with 10 mM Ca²⁺ in the extracellular solution using the step protocol in control cells (solid trace) and TRPV5/RGS2-expressing cells (dotted trace). E, average density of the Ca²⁺ peak current measured as in D was 210 ± 50 pA/pF for control compared with 201 ± 35 pA/pF for cells co-transfected with TRPV5 and RGS2 (n = 15 cells for each condition).

Inward rectifying currents carried by Na⁺ could be measured in HEK293 cells co-transfected with TRPV6 and ∆N-RGS2 or RGS2 or with TRPV6 and mock (Fig. 5, A and B). The Na⁺ currents were significantly reduced in cells co-expressing TRPV6 with RGS2, whereas cells co-expressing ∆N-RGS2 and TRPV6 did not show this reduction (Fig. 5, A and B, n = 10 cells, p < 0.001). Likewise, RGS2 significantly reduced the Ca²⁺ current in response to a hyperpolarizing voltage step in TRPV6-expressing cells (Fig. 5, D and E, n = 10 cells, p < 0.001), whereas co-expression of ∆N-RGS2 with TRPV6 had no effect on the TRPV6-mediated current amplitude.

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**FIGURE 5. Inhibition of TRPV6-mediated Na⁺ and Ca²⁺ currents by RGS2 but not ∆N-RGS2.**

HEK293 cells were transiently transfected with TRPV6/mock (control (CTRL)) or TRPV6 and RGS2 or ∆N-RGS2 and analyzed using immunoblotting (A) and by whole-cell patch clamp. B, I/V relations measured from ramp protocol in nominally DVF solution from control (solid trace), TRPV6/RGS2-transfected cells (dotted trace), and TRPV6/∆N-RGS2-transfected cells (dashed trace). C, average Na⁺ current density at −80 mV in nominally DVF solution was 268 ± 40 pA/pF for control, 30 ± 5 pA/pF for cells transfected with TRPV6 and RGS2, and 280 ± 45 pA/pF for TRPV6/∆N-RGS2-transfected cells (n = 10 cells for each condition). D, averaged Ca²⁺ currents measured with 10 mM Ca²⁺ in the extracellular solution using the step protocol in control cells (solid trace), TRPV6/RGS2-expressing cells (dotted trace), and TRPV6/∆N-RGS2 co-expressing cells (dashed trace). E, average density of the Ca²⁺ peak current measured as in D was 200 ± 50 pA/pF for control (CTRL), 55 ± 10 pA/pF for TRPV6/RGS2 co-expressing cells, and 196 ± 55 pA/pF for TRPV6/∆N-RGS2 co-expressing cells (n = 10 cells for each condition). * indicates significant difference (p < 0.001).

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RGS2 (Fig. 3, B and C, n = 15 cells, p < 0.001). Likewise, RGS2 co-expression significantly reduced the Ca²⁺ current in response to a hyperpolarizing voltage step in TRPV6-expressing cells (Fig. 3, D and E, n = 15 cells, p < 0.001), while the Ca²⁺-dependent inactivation of the currents remained unchanged. In addition, the inhibitory effect of RGS2 on TRPV6-mediated Na⁺ and Ca²⁺ currents was studied for its closest relative, TRPV5, during identical experimental conditions. In contrast to TRPV6, RGS2 had no significant effect on Na⁺ and Ca²⁺ currents recorded from TRPV5-expressing HEK293 cells (Fig. 4).

**TRPV6 Activity Is Inhibited by the NH₂-terminal Domain of RGS2**—To test the physiological significance of the NH₂-terminal domain of RGS2 on TRPV6 activity, the electrophysiological properties of TRPV6-mediated currents in HEK293 cells co-expressing either RGS2 or ∆N-RGS2 were determined.
**RGS2 Does Not Affect TRPV6 Abundance at the Plasma Membrane**—To investigate whether RGS2 influences trafficking of TRPV6, cell surface expression of TRPV6 in HEK293 cells co-expressing RGS2 and TRPV6 was examined using Sulfo-NHS-LC-LC-Biotin. TRPV6 was detected in total cell lysates of TRPV6-expressing cells as well as in the biotinylated plasma membrane fractions of TRPV6 and TRPV6/RGS2 co-expressing cells (Fig. 6). TRPV6 expression in the biotinylated membrane fraction was not significantly altered by co-expression of RGS2.

**TRPV6 Is Independently Regulated from the Scaffolding Protein SPL**—The effect of SPL on the interaction of RGS2 with TRPV6 was investigated by GST pulldown assay. Total cell lysates of HEK293 cells transiently transfected with TRPV6, SPL, or TRPV6 and SPL were prepared, and equal amounts of protein were analyzed for the interaction with RGS2. As indicated in Fig. 7, TRPV6 associated with RGS2 both in the presence or absence of SPL. Next, the electrophysiological properties of TRPV6-mediated currents were examined by whole-cell patch clamp in HEK293 cells co-expressing TRPV6 and SPL or TRPV6 and mock. Co-expression of SPL and TRPV6 had no significant effect either on Na\(^+\) or Ca\(^{2+}\) currents (n = 12 cells for each condition) as depicted in Fig. 8.

**DISCUSSION**

The present study demonstrates a novel regulatory function of RGS2 by direct inhibition of TRPV6 channel activity at the plasma membrane. This conclusion is based on the following experimental data. First, interaction of RGS2 with TRPV6 specifically inhibits both Na\(^+\) and Ca\(^{2+}\) currents through TRPV6 whereas no effect on TRPV5 activity was observed. Second, deletion of the NH\(_2\)-terminal domain of RGS2 disrupts the binding with TRPV6 and restores the original electrophysiological properties of the channel. Third, RGS2 expression does not interfere with TRPV6 trafficking from and toward the plasma membrane (to mediate its inhibitory effect on channel activity). Fourth, the multidomain scaffolding protein SPL does not affect binding and electrophysiological properties of TRPV6 in the presence of RGS2. Therefore, our data indicate that TRPV6 activity is regulated by RGS2 without the involvement of GPCR signaling.

We showed that the NH\(_2\)-terminal tail of RGS2 is essential for the interaction with and the activity of TRPV6. The NH\(_2\)-terminal domain has already been described to be an important regulatory site for RGS2. Together with the "RGS-box" domain, it is necessary for its effector protein interactions, e.g. GPCRs and/or auxiliary proteins such as SPL (23, 28, 29). In addition,
the membrane-targeting sequence has also been predicted in this domain (30, 31).

Patch clamp analysis of HEK293 cells transfected with TRPV6 or TRPV5 revealed that specifically TRPV6 activity is inhibited by co-expression of RGS2. Interestingly, an analogous inhibitory mechanism has only been found for calmodulin and TRPV6 (32). Since both Na\(^+\) and Ca\(^{2+}\) currents through TRPV6 were inhibited by RGS2, and thus affecting not specifically the Ca\(^{2+}\) conductance of the channel, we expected a disturbed TRPV6 trafficking toward the cell surface. In contrast, cell surface biotinylation in TRPV6-expressing HEK293 cells demonstrated that RGS2 does not affect channel expression in the biotinylated fractions. Therefore, the inhibitory effect of RGS2 is presumably mediated directly at the plasma membrane.

RGS2 needs to be recruited toward the cell surface to affect the gating properties of TRPV6. Roy et al. (33) showed that G-proteins recruit RGS proteins toward the plasma membrane. In their study, the abundance of RGS2 and RGS4 proteins at the plasma membrane was enhanced when the number of G proteins was increased or when they interacted with their GPCR. Also SPL has been shown to be involved in RGS protein recruitment (23). Investigating the role of SPL provided us with additional evidence for a direct effect of RGS2 on TRPV6 activity. Wang et al. (23) showed that SPL is a scavenger of RGS2 which forms a SPL-RGS2 protein complex that regulates Ca\(^{2+}\) signaling by binding the third intracellular domain of GPCRs. In their study, the binding of RGS2 with the \(\alpha\)-adrenergic receptor was investigated by GST pulldown analysis. The interaction between RGS2 and the \(\alpha\)-adrenergic receptor was significantly increased in the presence of SPL. Furthermore, they observed that the inhibitory effect of RGS2 on Ca\(^{2+}\)-activated Cl\(^{-}\) currents in parotid duct cells was increased in the presence of SPL. For this reason, we anticipated that SPL could increase the inhibitory effect of endogenously present RGS2 on TRPV6 activity in HEK293 cells when acting via a Go\(_{\alpha}\)-coupled GPCR. RGS2 expression was confirmed in HEK293 cells by RT-PCR (data not shown). First, it was demonstrated that SPL does not change TRPV6 expression (Fig. 7, top panel). Second, TRPV6-mediated Na\(^+\) and Ca\(^{2+}\) currents were not affected by SPL as well (Fig. 8). Therefore, we conclude that the inhibitory effect of RGS2 on TRPV6 activity is independently regulated of a GPCR but is rather mediated by direct interaction of RGS2 and TRPV6.

In conclusion, we have identified RGS2 as a novel regulatory protein specifically inhibiting the activity of the epithelial Ca\(^{2+}\) channel TRPV6. Our findings may provide insight in the direct regulation of different TRP channels by interacted proteins at the plasma membrane.

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