

RACK1 Inhibits TRPM6 Activity via Phosphorylation of the Fused α -Kinase Domain

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Summary

Background: The maintenance of the body's Mg^{2+} balance is of great importance because of its involvement in numerous enzymatic systems and its intervention in neuromuscular excitability, protein synthesis, and nucleic acid stability. Recently, the transient receptor potential melastatin 6 (TRPM6) was identified as the gatekeeper of active Mg^{2+} transport and therefore plays a crucial role in the regulation of Mg^{2+} homeostasis. Remarkably, TRPM6 combines a Mg^{2+} channel with an α -kinase domain whose function remains elusive.

Results: Here, we identify the receptor for activated C-kinase 1 (RACK1) as the first regulatory protein of TRPM6 that associates with the α -kinase domain. RACK1 and TRPM6 are both present in renal Mg^{2+} -transporting distal convoluted tubules. We demonstrate that RACK1 inhibits channel activity in an α -kinase activity-dependent manner, whereas small interference (si) RNA-mediated knockdown of RACK1 increases the current. Moreover, threonine¹⁸⁵¹ in the α -kinase domain was identified as an autophosphorylation site of which the phosphorylation state is essential for the inhibitory effect of RACK1. Importantly, threonine¹⁸⁵¹ was crucial for the Mg^{2+} sensitivity of TRPM6 autophosphorylation and channel activity. TRPM6 channel activity was less sensitive to Mg^{2+} when RACK1 was knocked down by siRNA. Finally, activation of protein kinase C by phorbol 12-myristate 13-acetate-PMA prohibited the inhibitory effect of RACK1 on TRPM6 channel activity.

Conclusions: We propose a unique mode of TRPM6 regulation in which the Mg^{2+} influx is controlled by RACK1 through its interaction with the α -kinase and the phosphorylation state of the threonine¹⁸⁵¹ residue.

Introduction

The maintenance of the body's Mg^{2+} balance is of crucial importance for various vital cellular processes [1, 2]. Mg^{2+} homeostasis in mammals depends on the equilibrium between intestinal absorption and renal excretion. The renal distal convoluted tubule (DCT) reabsorbs ~10% of the filtered Mg^{2+} , and the reabsorption rate in this segment defines the final urinary Mg^{2+} concentration [3]. Mg^{2+} reabsorption in

DCT is active and transcellular in nature, but the molecular details and regulation of this pathway remain largely unknown.

Recently, transient receptor potential melastatin 6 (TRPM6) was identified as a pivotal component in active Mg^{2+} (re)absorption, mutations of which cause hypomagnesemia with secondary hypocalcemia (HSH) [4, 5]. TRPM6 is localized along the apical membrane of DCT and intestinal cells. It confines a Mg^{2+} -permeable channel, the activity of which is strongly regulated by the intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) [2]. Expression of TRPM6 is regulated by dietary Mg^{2+} , whereas TRPM7 is unaffected, supporting the idea of an important role of TRPM6 in transepithelial Mg^{2+} transport [6].

Remarkably, TRPM6 and TRPM7 contain a unique carboxyl (C)-terminal serine/threonine protein kinase domain belonging to the α -kinase family [7–9]. This distinctive combination of a channel and an enzyme domain within a single molecule raises still unsolved questions concerning the regulatory role of this catalytic domain on channel activity. While it has been demonstrated that the TRPM7 α -kinase is able to autophosphorylate the channel and downstream substrates such as myosin IIA heavy chain and annexin I [10–13], the role of this α -kinase activity in modulating channel function is far from clear. Previous studies indicated that the α -kinase activity is not essential for TRPM7 activation [11, 13] and that regulation of TRPM7 activity by intracellular Mg^{2+} is dissociated from autophosphorylation or α -kinase activity [13]. Other groups demonstrated regulation of TRPM7 activity by intracellular Mg^{2+} and nucleotides as well as the cAMP/protein kinase A pathway, which required the α -kinase [14, 15]. It was reported that deletion of the TRPM7 α -kinase domain or phosphotransferase-deficient kinase mutants results in nonfunctional channels [7]. Compared to TRPM7, little is known about the α -kinase function of TRPM6. So far, only one study reported that the α -kinase of TRPM6 is capable of crossphosphorylation of TRPM7, but not vice versa, underlying the functional nonredundancy of these two “chanzymes” [12].

The aim of the present study was, therefore, to investigate the role of the α -kinase domain in TRPM6 channel activity through the identification of regulatory proteins specifically interacting with the α -kinase domain.

Results

RACK1 Associates with TRPM6

To identify potential TRPM6-interacting proteins, two independent approaches were used, i.e., the yeast two-hybrid screening of a mouse kidney cDNA library with the complete C-tail of TRPM6 and the combination of a GST pull-down with the α -kinase domain of TRPM6 in mouse kidney lysate followed by Fourier transform ion cyclotron resonance mass spectrometry (FTMS). RACK1, a scaffold protein originally discovered as an adaptor for protein kinase C (PKC) [16], was identified as a TRPM6 interacting protein in both approaches. Figure 1A shows that RACK1 interacted with TRPM6, whereas no interaction was observed in the absence of either RACK1 or the TRPM6 C-tail. To further establish this interaction, GST pull-down binding assays were performed, which demonstrated that RACK1 interacts specifically with

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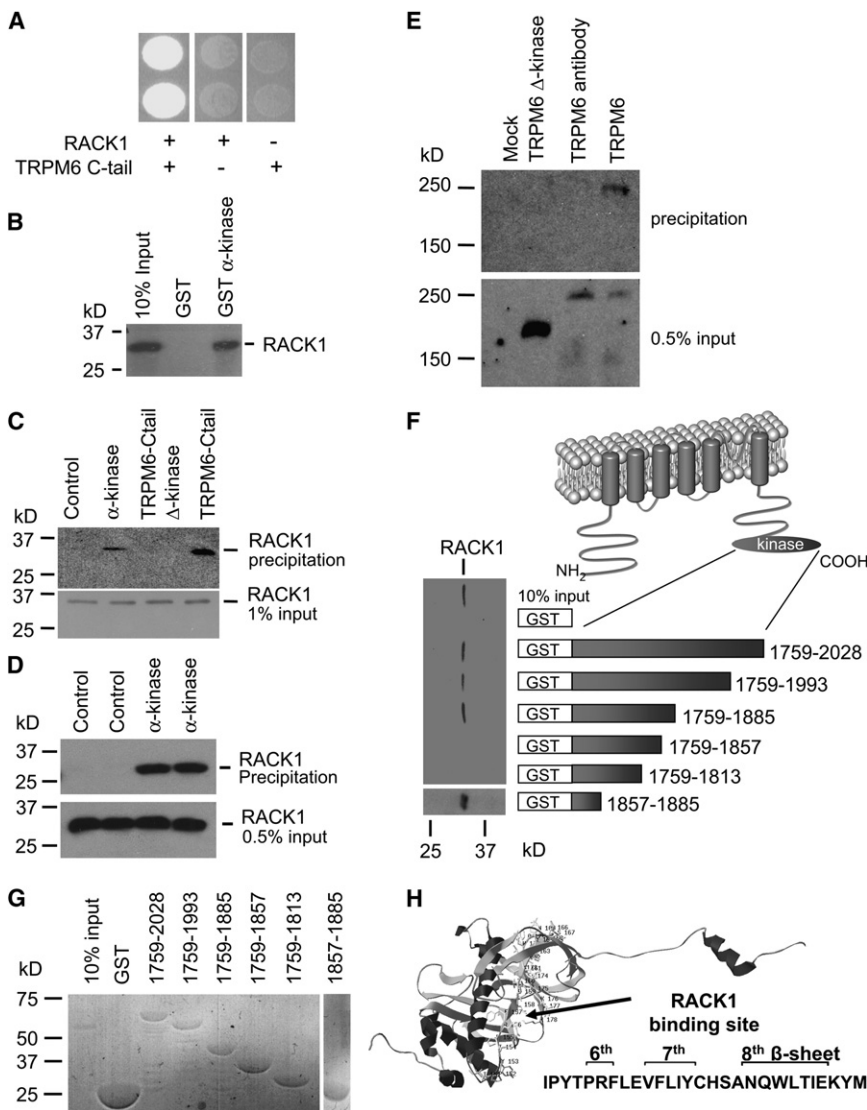


Figure 1. RACK1 Interacts with the α -Kinase Domain in TRPM6

(A) The C terminus of *TRPM6* and the empty pAS1 vector were coexpressed with full-length *RACK1* into the Y153 yeast strain and grown on media without tryptophan, leucine, and histidine. (B) GST pull-down assay between [³⁵S]-methionine-labeled *RACK1* protein and GST or GST fused to the α -kinase domain. (C) Coprecipitation studies of GST-kinase, GST-TRPM6-C-tail- Δ -kinase, and GST-TRPM6-C-tail in *RACK1*-expressing HEK293 cells (top panel). *RACK1* input (1%) expression was analyzed by immunoblotting (bottom panel). (D) Coprecipitation studies of GST and GST- α -kinase with endogenous *RACK1* in HEK293 cells (top panel). *RACK1* input (0.5%) expression was analyzed by immunoblotting (bottom panel). (E) Coimmunoprecipitation studies of *RACK1* with GFP-TRPM6, GFP-TRPM6 antibody, or GFP-TRPM6 Δ -kinase in HEK293 cells. Input (0.5%) expression was analyzed by immunoblotting (bottom panel). (F) Mapping of the *RACK1* binding site within the α -kinase domain. Schematic diagram of the GST-fusion proteins containing different truncations of the α -kinase. (G) Coomassie staining of the SDS/PAGE gel. (H) *RACK1* binding site in the predicted 3D structure model of the α -kinase domain.

of the TRPM6-kinase domain bound to *RACK1* (Figure 1F). Therefore, the *RACK1* binding site within TRPM6 is restricted to the region between the positions 1857 and 1885. The integrity and quantity of the GST-fusion proteins was analyzed and confirmed by Coomassie staining (Figure 1G). To explore the *RACK1* binding site in the TRPM6 α -kinase domain, the tertiary structure of TRPM6 α -kinase domain was modeled

the α -kinase domain, but not with GST alone (Figure 1B). Next, the association between TRPM6 and *RACK1* was substantiated in mammalian HEK293 cells. *RACK1* coprecipitated with the GST-kinase and GST-TRPM6-C-tail, but not with GST-TRPM6-C-tail- Δ -kinase and GST alone (Figure 1C). Furthermore, coprecipitation experiments also demonstrated that the endogenously expressed *RACK1* in HEK293 cells is associated with the TRPM6 α -kinase domain (Figure 1D). Finally, coimmunoprecipitation studies of *RACK1* with full-length TRPM6 or TRPM6 Δ -kinase in HEK293 cells showed that wild-type TRPM6 but not TRPM6 Δ -kinase coprecipitates with *RACK1* (Figure 1E).

Mapping of the *RACK1* Binding Site in TRPM6

To determine the *RACK1* binding site within TRPM6, a series of deletion mutants in the α -kinase domain of the channel was constructed and evaluated for their interaction with [³⁵S]-methionine-labeled *RACK1* protein using pull-down experiments (Figure 1F). The interaction between the two proteins was abolished when TRPM6 was truncated at position 1857, whereas truncation at position 1885 had no effect on the interaction with *RACK1*. Moreover, a GST-fusion protein containing only the short stretch between the amino acids 1857 and 1885

by SWISS-MODEL (<http://swissmodel.expasy.org/SWISS-MODEL.html>) (Figure 1H), based on the homology between the TRPM6 α -kinase and the crystallized TRPM7 α -kinase domain [17]. Due to the high homology between the TRPM7 and TRPM6 α -kinase domains (84%), these two α -kinases share a highly conserved secondary structure. Indeed, the GST pull-down assay showed that *RACK1* also interacts with the TRPM7 α -kinase domain (see Figure S1A available online). The surface and accessibility analysis of the *RACK1* binding site (6th, 7th, and 8th β sheets) suggested that, among the 28 amino acids of the *RACK1* binding site, 18 are localized at the surface of the TRPM6 α -kinase domain (see <http://www.cmbi.ru.nl/~hvensela/trpm6/>).

RACK1 and TRPM6 Coexpress in Kidney

To study the localization of *RACK1* in kidney, immunohistochemistry was performed on kidney sections using the thiazide-sensitive Na⁺/Cl⁻ cotransporter (NCC) as a DCT marker. These analyses indicated immunopositive staining for *RACK1* in the NCC-expressing DCT cells (Figure 2A, top panel). TRPM6 completely colocalized with NCC [2] along the apical membrane in DCT, indicating the presence of *RACK1* in the TRPM6-expressing DCT segment (Figure 2A,

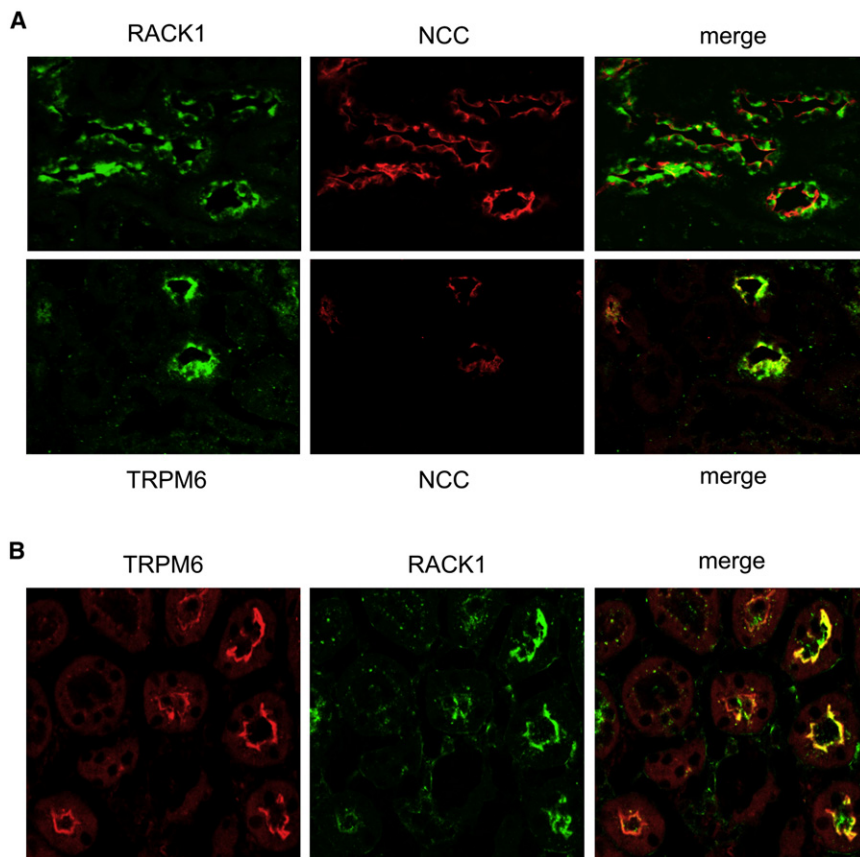


Figure 2. Localization of RACK1 in Kidney

(A) Immunohistochemical analysis of RACK1 (green) and NCC (red) in kidney sections (top panel). Kidney sections were costained with antibodies against TRPM6 (green) and NCC (red). (B) Immunohistochemical analysis of TRPM6 (red) and RACK1 (green) in rat kidney sections.

Small Interference RNA-Mediated RACK1 Silencing Stimulates TRPM6 Activity

To further elucidate the molecular mechanism of the RACK1 inhibitory effect, siRNA technology was applied. Immunoblot analysis revealed a significant downregulation of RACK1 protein abundance in HEK293 cells expressing RACK1-targeted siRNA(1) and siRNA(2) in comparison to mock-transfected cells (Figures 3F and 3G). siRNA(1) and siRNA(2) for RACK1 induced a significant increase of the TRPM6 current amplitude [356 ± 31 pA/pF, -18 ± 1 pA/pF and 397 ± 25 pA/pF, -33 ± 6 pA/pF for RACK1 siRNA(1) and siRNA(2) at +80 and -80 mV, respectively, in comparison to 275 ± 26 pA/pF, -13 ± 2 pA/pF for TRPM6 and empty vector, respectively] (Figure 3H and Figures S2C and S2D). The siRNA for luciferase, siRNA(luc), did not significantly affect TRPM6 current amplitudes. Importantly, RACK1 siRNA(1), RACK1 siRNA(2), and siRNA(luc) did not alter the current amplitude of the TRPM6 Δ -kinase mutant (Figure 3I and Figures S2E and S2F).

bottom panel). Subsequent immunohistochemical stainings confirmed the colocalization of RACK1 and TRPM6 in the DCT (Figure 2B).

RACK1 Inhibits TRPM6 Channel Activity

The functional role of RACK1 on TRPM6 current was investigated in HEK293 cells by whole-cell recordings. In mock-transfected HEK293 cells, dialyzed with a Mg^{2+} -free pipette solution, around 40% of the cells developed small albeit significant currents, generally referred to as the Mg^{2+} -inhibited cation current (MIC current) [18, 19]. This endogenous current was approximately ten times smaller and developed over a longer time period compared to the TRPM6 current (Figure S1D). We demonstrated that coexpression of RACK1 with TRPM6 significantly inhibited the TRPM6-mediated current amplitude in a dose-dependent manner. TRPM6 current was reduced by $56\% \pm 4\%$ and $88\% \pm 3\%$ compared to TRPM6 alone when 0.1 μ g or 0.5 μ g RACK1 cDNA, respectively, was cotransfected (Figures 3A–3C and Figure S2A). Furthermore, RACK1 also inhibited the TRPM7 channel activity (Figures S1B and S1C). However, the inhibitory effect of RACK1 on TRPM6/7 current was specific, since RACK1 did not significantly affect the current in TRPV5-expressing HEK293 cells ($-11\% \pm 3\%$, $-16\% \pm 3\%$, and $-9\% \pm 2\%$ at -80 mV in divalent-free, EDTA, and 10 mM Ca^{2+} solutions, respectively, $n = 10$ –12 cells as described previously [20]). Importantly, RACK1 coexpression with an α -kinase-deleted TRPM6 mutant (TRPM6 Δ -kinase) failed to suppress the TRPM6 Δ -kinase-mediated current at both +80 and -80 mV (Figures 3D and 3E and Figure S2B).

RACK1-Mediated Inhibition of TRPM6 Is Kinase Activity Dependent

Next, the role of the kinase-mediated autophosphorylation was investigated. TRPM6 α -kinase autophosphorylation activity was first assessed by an in vitro protein kinase assay. Figure 4A indicates that TRPM6 is autophosphorylated, whereas deletion of its α -kinase or the addition of alkaline phosphatase prevents channel autophosphorylation. Next, we constructed a phosphotransferase-deficient mutant ($K^{1804}R$) according to the homologous TRPM7 phosphotransferase-deficient mutant ($K^{1648}R$) [11]. The in vitro phosphorylation assay showed that the kinase activity of the $K^{1804}R$ mutant was abolished, while the mutant was equally expressed compared to wild-type TRPM6 (Figure 4B). $K^{1804}R$ mutant was still able to bind RACK1, as demonstrated by GST pull-down assay (Figure S3A). $K^{1804}R$ mutant displayed current amplitude and current development similar to the wild-type channel. Importantly, RACK1 was unable to inhibit the $K^{1804}R$ mutant-mediated current (Figure 4C and Figures S3B and S3C).

Autophosphorylation of Threonine¹⁸⁵¹ in the Kinase Domain Is Essential for the Inhibitory Effect of RACK1

To address the autophosphorylation site(s) in TRPM6 that are essential for the regulatory effect of RACK1, the purified GST-TRPM6 α -kinase protein was subjected to an in vitro phosphorylation assay and subsequently analyzed by FTMS.

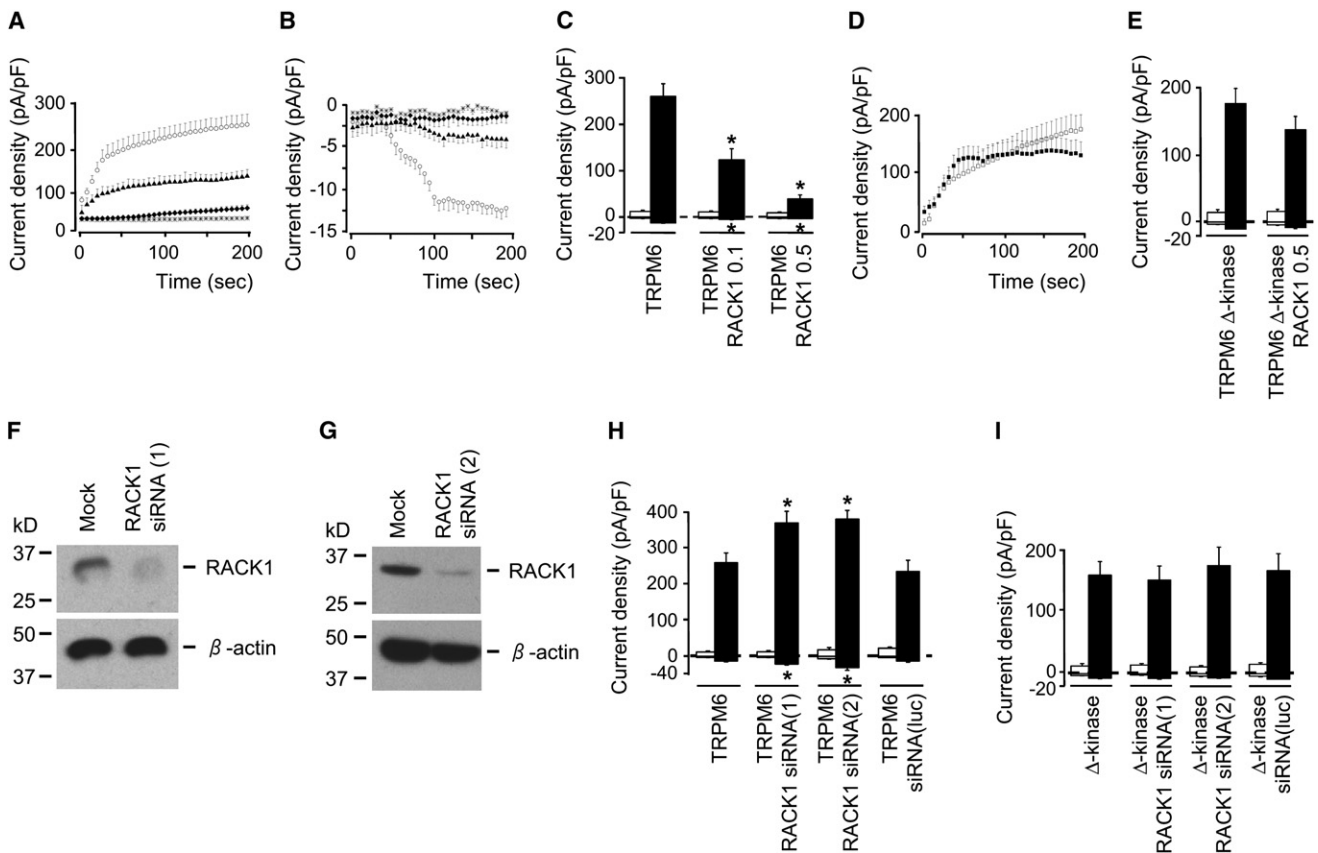


Figure 3. Functional Effect of RACK1 on TRPM6 Activity

(A) Time course of the current density (pA/pF) at +80 mV of TRPM6 (\square), TRPM6 and RACK1 (0.5 μ g, \blacklozenge), TRPM6 and RACK1 (0.1 μ g, \blacktriangle), and mock TRPM6 (*) transfected HEK293 cells.
 (B) Corresponding time course at -80 mV.
 (C) Averaged values of the current density at +80 and -80 mV after 200 s of mock TRPM6 (n = 10), TRPM6 (n = 22), mock TRPM6 and RACK1 (0.1 μ g, n = 11), TRPM6 and RACK1 (0.1 μ g, n = 18), mock TRPM6 and RACK1 (0.5 μ g, n = 13), and TRPM6 and RACK1 (0.5 μ g, n = 24). Open bars correspond to cells transfected with mock-transfected DNA, and closed bars to cells transfected with TRPM6 in the absence and presence of RACK1. *p = 0.001 compared to TRPM6 current.
 (D) Time course of the current density at +80 mV of TRPM6 Δ -kinase (\square) and TRPM6 Δ -kinase and RACK1 (0.5 μ g, \blacksquare) transfected HEK293 cells.
 (E) Averaged values of the current density at +80 and -80 mV after 200 s of mock TRPM6 Δ -kinase (n = 9), TRPM6 Δ -kinase (n = 22), mock TRPM6 Δ -kinase and RACK1 (0.5 μ g, n = 10), and TRPM6 Δ -kinase and RACK1 (0.5 μ g, n = 19) transfected HEK293 cells. Open and closed bars correspond to cells transfected with mock TRPM6 Δ -kinase and TRPM6 Δ -kinase, respectively, in the absence and presence of RACK1.
 (F and G) Immunoblot analysis of RACK1 and β -actin expression in mock, siRNA-RACK1(1), and siRNA-RACK1(2) transfected HEK293 cells.
 (H) Averaged values of the current density at +80 and -80 mV after 200 s of mock TRPM6 (n = 9), TRPM6 (n = 19), mock TRPM6 and siRNA-RACK1(1) (0.5 μ g, n = 17), TRPM6 and siRNA-RACK1(1) (0.5 μ g, n = 21, p = 0.029), mock TRPM6 and siRNA-RACK1(2) (0.1 μ g, n = 18), TRPM6 and siRNA-RACK1(2) (0.1 μ g, n = 23, p = 0.001), mock TRPM6 and siRNA(luc) (0.5 μ g, n = 13), and TRPM6 and siRNA(luc) (0.5 μ g, n = 17, p = 1.000). *p < 0.05 compared to the TRPM6 current. Open bars correspond to cells that are transfected with mock DNA, whereas closed bars to cells transfected with TRPM6 in the absence and presence of siRNA-RACK1 or luciferase.
 (I) Similarly to Figure 3H, averaged values of mock TRPM6 Δ -kinase (n = 10), TRPM6 Δ -kinase (n = 23), mock TRPM6 Δ -kinase and siRNA-RACK1(1) (0.5 μ g, n = 12), TRPM6 Δ -kinase and siRNA-RACK1(1) (0.5 μ g, n = 19, p = 1.000), mock TRPM6 Δ -kinase and siRNA-RACK1(2) (0.1 μ g, n = 17), TRPM6 Δ -kinase and siRNA-RACK1(2) (0.1 μ g, n = 22, p = 1.000), mock TRPM6 Δ -kinase and siRNA(luc) (0.5 μ g, n = 18), and TRPM6 Δ -kinase and siRNA(luc) (0.5 μ g, n = 25, p = 1.000) current densities. p values are relative to the TRPM6 Δ -kinase current. Values are expressed as mean \pm SEM.

Figure 5A revealed a single autophosphorylation site within the α -kinase domain corresponding to the threonine residue at position 1851 (T^{1851}). Interestingly, in the tertiary structure model of the TRPM6 α -kinase domain, T^{1851} is located at the end of the fourth α helix adjacent to the RACK1 binding site (Figure 5B). Subsequently, T^{1851} was either point mutated into an alanine, resulting in a nonphosphorylated residue ($T^{1851}A$), or into an aspartate, mimicking the constitutive autophosphorylated threonine residue ($T^{1851}D$). Both $T^{1851}A$ and $T^{1851}D$ mutations significantly decreased TRPM6 autophosphorylation in comparison to the wild-type channel

(Figure 5C and Figure S4A), while GST pull-down assays demonstrated that RACK1 binding to $T^{1851}A$ and $T^{1851}D$ mutants was not altered (Figure S4B). Importantly, the expression of the (mutated) TRPM6 proteins was equal (Figure 5C, lower panel). Furthermore, both mutants ($T^{1851}A$ and $T^{1851}D$) displayed currents similar to the wild-type channel (Figures 5D and 5E and Figures S4C–S4F). However, the $T^{1851}A$ mutation prevented the inhibitory effect of RACK1 (Figure 5D and Figures S4C and S4D), whereas the RACK1-induced inhibition remained unaltered when coexpressed with the $T^{1851}D$ mutant (Figure 5E and Figures S4E and S4F).

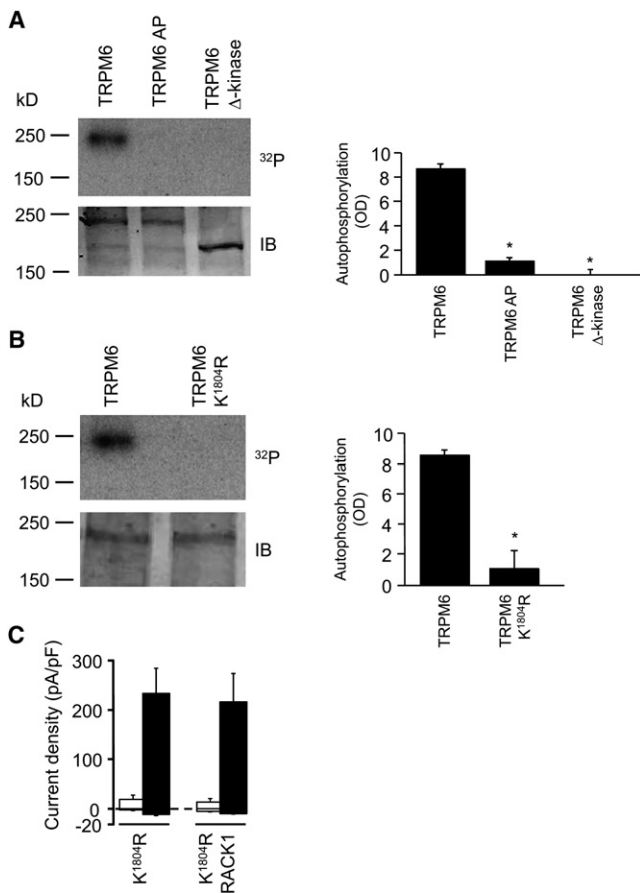


Figure 4. Inhibition of TRPM6 by RACK1 Depends on Phosphorylation of the α -Kinase

(A) In vitro protein kinase assay of TRPM6 and the TRPM6 Δ -kinase mutant (^{32}P , top panel). Addition of alkaline phosphatase (AP) was used as a negative control. Immunoblotting (IB, bottom panel) and densitometry quantification (right panel) of TRPM6 and TRPM6 Δ -kinase expression. * $p = 0.001$, $n = 3$.

(B) In vitro protein kinase assay of TRPM6 and the K^{1804}R mutant (^{32}P , top panel). Immunoblotting (IB, bottom panel) and densitometry quantification (right panel) of TRPM6 and K^{1804}R expression. * $p = 0.001$, $n = 3$.

(C) Summary of the current density at +80 and -80 mV of K^{1804}R ($n = 22$), K^{1804}R and RACK1 (0.5 μg , $n = 24$; $p = 1.000$ compared to the K^{1804}R current), mock K^{1804}R ($n = 15$), and mock K^{1804}R and RACK1 ($n = 16$). Open bars correspond to mock conditions, whereas closed bars to cells transfected with K^{1804}R in the absence and presence of RACK1.

Values are expressed as mean \pm SEM.

Threonine¹⁸⁵¹ and RACK1 Play an Important Role in Mg^{2+} -Dependent Channel Activity

To explore whether RACK1 and the T^{1851} residue in the α -kinase domain are important for the inhibitory effect of Mg^{2+} on TRPM6 current, the influence of $[\text{Mg}^{2+}]$ on TRPM6 and T^{1851}A mutant autophosphorylation was investigated. TRPM6 autophosphorylation activity was strongly dependent on the Mg^{2+} concentration and steadily increased in the physiological range of 0.1–1.0 mM Mg^{2+} (Figure 5F). In contrast, the autophosphorylation activity of the T^{1851}A mutant was not affected by the Mg^{2+} concentration (Figure 5G). Both TRPM6 and T^{1851}A mutant channel activities were significantly inhibited by Mg^{2+} in the patch-pipette. However, the T^{1851}A mutant channel was less sensitive to the intracellular Mg^{2+} concentration compared to wild-type TRPM6 (IC_{50} 0.72 \pm 0.07 mM versus IC_{50} 0.51 \pm 0.06 mM,

respectively) (Figure 5H). Figure 5I showed that knockdown of endogenous RACK1 by siRNA significantly reduces the Mg^{2+} -dependent inhibition of the TRPM6 current. Together, these data suggest that the T^{1851} phosphorylation-dependent inhibitory effect of RACK1 may play an important role in the suppression of the TRPM6 current by intracellular free Mg^{2+} .

Coexpression of RACK1 Does Not Affect Expression of TRPM6 at the Plasma Membrane

The influence of RACK1 on the amount of TRPM6 channels expressed at the plasma membrane was investigated by cell-surface biotinylation experiments. As shown in Figure 6 (upper panel), coexpression of RACK1 did not affect the amount of TRPM6 channels expressed at the plasma membrane. Of note, TRPM6 was equally expressed in all tested conditions, as determined in the total cell lysates (Figure 6, bottom panel).

RACK1 Inhibits TRPM6 in a PKC-Dependent Manner

Next, the effect of PKC activation by phorbol 12-myristate 13-acetate-PMA (PMA) [21] on the regulatory effect of RACK1 was studied. PKC activation by preincubation with 100 nM PMA for 5 min prevented the inhibitory effect of RACK1 on the TRPM6 current (Figure 7B and Figures S5C and S5D). The current amplitude in the presence of PMA was, however, not modified in cells expressing TRPM6 only (Figure 7A and Figures S5A and S5B). To confirm the specificity of PMA, HEK293 cells coexpressing TRPM6 and RACK1 were incubated with 5 μM chelerythrine chloride, a specific PKC inhibitor [22], for 20 min prior to PMA treatment. As shown in Figure 7B and Figures S5C and S5D, the stimulatory action of PMA was counteracted by chelerythrine chloride, suggesting that the PMA effect is indeed due to PKC activation. To further elucidate the PKC effect, the TRPM6 C-tail was coexpressed with RACK1 in HEK293 cells, subjected to PKC activation by PMA or vehicle, and subsequently precipitated using glutathione Sepharose 4B beads. The interaction of RACK1 with the TRPM6 C-tail was significantly reduced when PKC was activated (Figure 7C, upper panel). Importantly, RACK1 was equally expressed in all situations (Figure 7C, lower panel).

Discussion

In the present study, we identified RACK1 as the first TRPM6-associated protein and demonstrated that RACK1 inhibits TRPM6 channel activity depending on the phosphorylation state T^{1851} in the α -kinase domain. First, RACK1 binds to the TRPM6 α -kinase domain and shares a similar expression pattern with TRPM6 in the kidney. Second, overexpression of RACK1 inhibits TRPM6 activity, and conversely, silencing of RACK1 by siRNA increases the TRPM6 current. Third, the RACK1-mediated inhibition of TRPM6 is prevented by deletion of the α -kinase domain. Fourth, inactivation of the α -kinase abrogates the inhibitory effect of RACK1. Fifth, T^{1851} is identified as an important autophosphorylation site in TRPM6-modulating channel activity by RACK1. Sixth, T^{1851} was crucial for the Mg^{2+} sensitivity of TRPM6 autophosphorylation and channel activity. Finally, knockdown of endogenous RACK1 by siRNA significantly reduces the Mg^{2+} -dependent inhibition of the TRPM6 current.

TRPM6 forms the Mg^{2+} influx channel involved in intestinal and renal Mg^{2+} (re)absorption and is so far the only known molecular identity in the process of active transepithelial

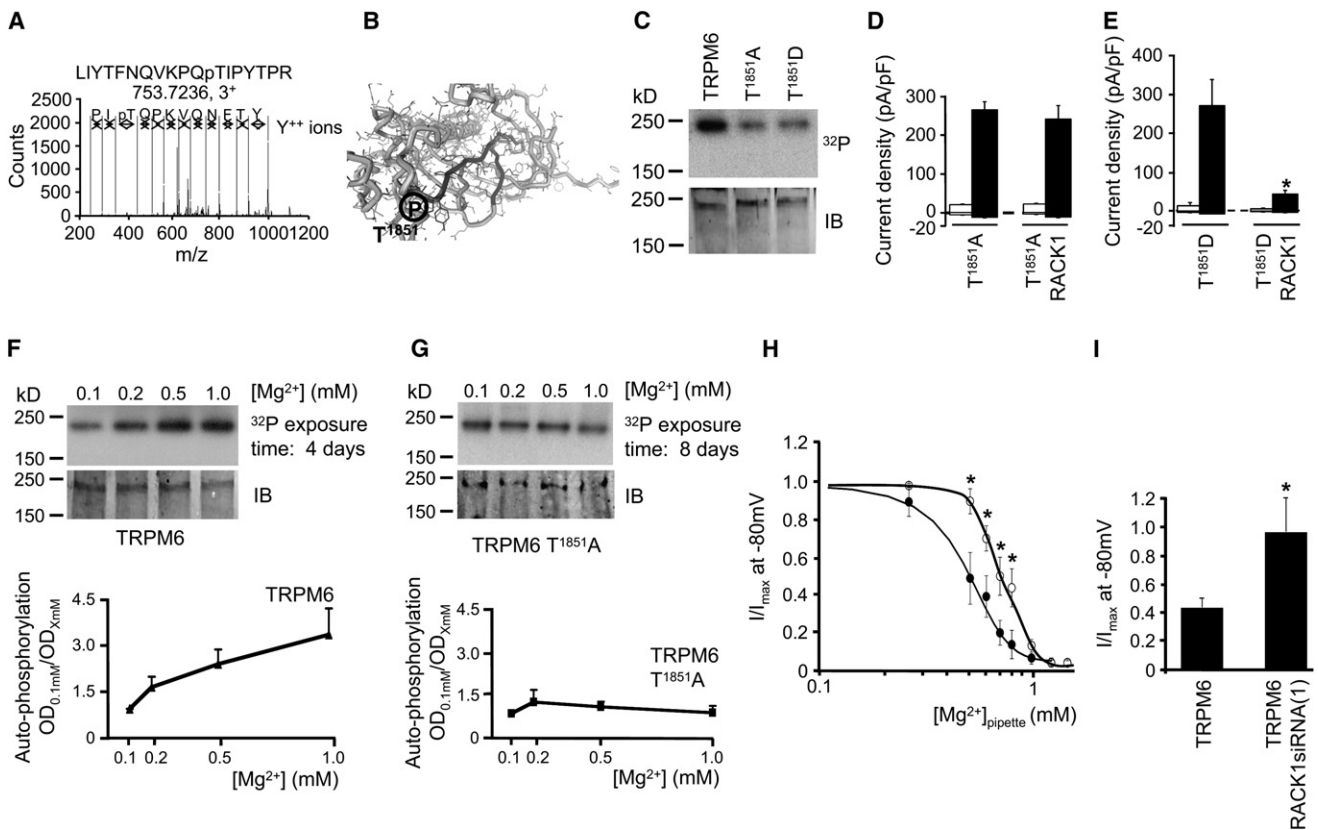


Figure 5. Mapping and Function of the Autophosphorylation Site in the TRPM6 α -Kinase Domain

(A) Analysis of phosphorylated tryptic peptide by LTQ-FT ICR mass spectrometer. For detailed information, see [Experimental Procedures](#).
 (B) Autophosphorylated threonine at position 1851 in the putative 3D structure of the TRPM6 α -kinase domain, in which the RACK1 binding site is highlighted.
 (C) In vitro protein kinase assay (^{32}P , top panel) and immunoblotting analysis (IB, bottom panel) of TRPM6, T¹⁸⁵¹A, and T¹⁸⁵¹D mutants.
 (D and E) Averaged values of the current density at +80 and -80 mV of T¹⁸⁵¹A with (n = 23; p = 1.000 compared to T¹⁸⁵¹A) or without RACK1 (n = 25) (D) and T¹⁸⁵¹D with (n = 27; p = 0.001 compared to T¹⁸⁵¹D) or without RACK1 (n = 23) (E). Open and closed bars correspond to cells transfected with mock DNA and TRPM6 mutants, respectively, in the absence or presence of RACK1.
 (F and G) In vitro phosphorylation of TRPM6 (F) and TRPM6 T¹⁸⁵¹A (G) at different Mg²⁺ concentrations (^{32}P , top panels). Autoradiograms were exposed 4 days for TRPM6 and 8 days for TRPM6 T¹⁸⁵¹A. TRPM6 and TRPM6 T¹⁸⁵¹A expression was determined by immunoblotting (IB, bottom panels). The phosphorylated proteins were quantified and plotted against 0.1 mM [Mg²⁺] (n = 3 for each; P^{0.2 mM [Mg²⁺]} = 0.120; P^{0.5 mM [Mg²⁺]} = 0.042; P^{1.0 mM [Mg²⁺]} = 0.049 for TRPM6 and P^{0.2 mM [Mg²⁺]} = 0.442; P^{0.5 mM [Mg²⁺]} = 0.370; P^{1.0 mM [Mg²⁺]} = 0.973 for TRPM6 T¹⁸⁵¹A).
 (H) Effect of the pipette Mg²⁺ concentration on the inhibition of the inward Na⁺ current at -80 mV of TRPM6 (●, n = 8–11) and the T¹⁸⁵¹A-transfected HEK293 cells (○, n = 9–13). *p < 0.05, compared to TRPM6 current in 0 mM [Mg²⁺].
 (I) Effect of the pipette Mg²⁺ concentration on the inhibition of the inward Na⁺ current at -80 mV of TRPM6 (n = 10) and TRPM6 and siRNA-RACK1(1) (0.5 μg , n = 10, p = 0.03) transfected HEK293 cells.
 Values are expressed as mean \pm SEM.

Mg²⁺ transport. However, TRPM6 regulation remains poorly understood. In this respect, the identification of RACK1 as a TRPM6 regulator provides the first molecular insight into the control of renal Mg²⁺ entry and, therefore, into the maintenance of Mg²⁺ balance. The interaction between RACK1 and TRPM6 was confirmed by GST pull-down assay and coimmunoprecipitation. Unfortunately, similar studies investigating an interaction between endogenous TRPM6 and RACK1 in kidney DCT cells were unsuccessful, possibly due to the low protein abundance of the channel. In addition, we demonstrated that endogenously expressed RACK1 in HEK293 cells interacts with the TRPM6 α -kinase domain. At the molecular level, our results suggest that RACK1 specifically binds to the 6th, 7th, and 8th β sheets of the α -kinase domain. Eighteen amino acids of the RACK1 binding site are located on the peripheral region of the tertiary structure of the TRPM6 α -kinase domain, which may facilitate the association between RACK1 and TRPM6.

Importantly, we showed that RACK1 is abundantly present in TRPM6-expressing tubules, which further suggests the physiological relevance of the interaction between these proteins in the kidney.

RACK1, a member of the WD-40 family of proteins, is regarded as a scaffolding protein in multiple intracellular signal transduction pathways, including ion channel proteins such as the IP₃ receptor, NMDA receptor, and GABA_A receptor channels [23–25]. RACK1-mediated decline of the TRPM6 current was dose dependent, and depletion of endogenously expressed RACK1 in HEK293 cells resulted in increased TRPM6 currents. The inhibitory effect of RACK1 requires the α -kinase domain with functional phosphotransferase activity. This was supported by the analysis of the TRPM6 Δ -kinase and the phosphotransferase-deficient (K¹⁸⁰⁴R) mutants, which were both insensitive to RACK1. While the autophosphorylation activity of the α -kinase domain has been well documented

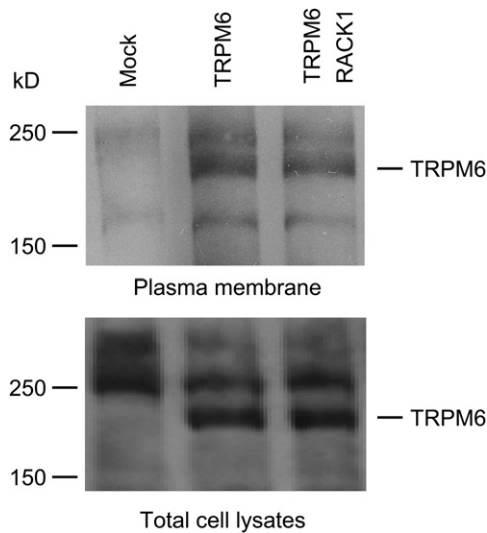


Figure 6. Coexpression of RACK1 Does Not Affect the Plasma Membrane Expression of TRPM6

TRPM6 and RACK1-transfected HEK293 cells were subjected to cell-surface biotinylation analysis. TRPM6 expression was analyzed by immunoblotting for the plasma membrane fraction (top panel) and in total cell lysates (bottom panel). As negative control, mock cells were used.

[10–13], the regulatory role of this catalytic domain on channel activity remains elusive. Previously, it has been demonstrated that the balance between phosphorylation and dephosphorylation operates as a key mechanism to regulate ion channel activity [26, 27]. However, the phosphotransferase-deficient $K^{1804}R$ mutant displayed currents indistinguishable from wild-type TRPM6. Interestingly, the mutants $T^{1851}A$ and $T^{1851}D$ displayed currents similar to the wild-type channel but exhibited a significant albeit reduced autophosphorylation, while the autophosphorylation of mutant $K^{1804}R$ was fully abolished. These data suggest that, besides T^{1851} , additional autophosphorylation sites are present in TRPM6. Our findings support

the conclusion that TRPM6 α -kinase autophosphorylation events are not vital for TRPM6 channel activation. Consistently, previous studies showed that TRPM7 phosphotransferase activity is not essential for channel activation [12, 13]. In contrast, the inhibitory action of RACK1 requires functional phosphotransferase activity. This notion is supported by the fact that the current of the phosphorylation-impaired mutants ($K^{1804}R$ and $T^{1851}A$) is insensitive to RACK1. Furthermore, the constitutive phosphorylated mutant $T^{1851}D$ was still inhibited by RACK1. Based on these observations, an increased channel activity for the phosphorylation-impaired mutants ($K^{1804}R$ and $T^{1851}A$) and the TRPM6 Δ -kinase mutant was expected. However, functional analysis of these mutated channel proteins did not indicate elevated channel activity. It has been demonstrated that the α -kinase domain can regulate TRPM7 channel activity via other signaling pathways besides RACK1. For instance, previous studies [11, 14, 15] indicated that the TRPM7 activity controlled by intracellular Mg^{2+} , nucleotides, and the cAMP/protein kinase A pathway requires the α -kinase domain or kinase activity. Therefore, it is conceivable that removing the α -kinase domain or suppressing its phosphotransferase activity may abolish associated regulation(s) of TRPM6 channel activity. This could compensate a potential upregulation of TRPM6 activity caused by removal of the inhibitory RACK1 action. In reminiscence of the adjacent location of the autophosphorylation site in the α -kinase and the RACK1 binding site, we hypothesize that the phosphorylation state of T^{1851} plays a key role in controlling channel activity via RACK1. Together, our results convincingly demonstrate a novel indirect model of regulation in which RACK1 functions as a mediator linking autophosphorylation of the α -kinase domain to channel activity. In this way, TRPM6 channel activity is controlled efficiently to manage constitutively active epithelial Mg^{2+} channels. Furthermore, cell-surface biotinylation experiments demonstrated that coexpression of RACK1 does not affect the amount of TRPM6 channels expressed at the plasma membrane. These data suggest that the inhibitory effect of RACK1 on TRPM6 is likely due to channel gating. Association of RACK1 and TRPM6 seems a dynamic process that can be

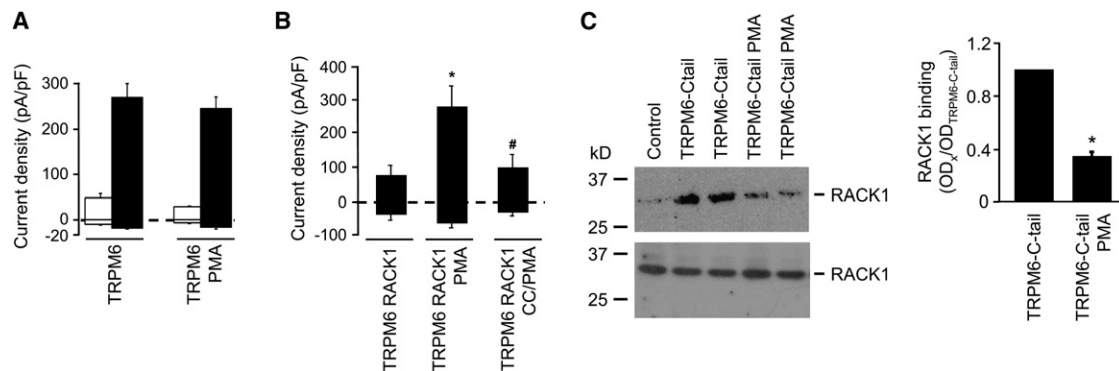


Figure 7. The Inhibitory Effect of RACK1 Is PKC Dependent

(A) Effect of PKC activation by PMA (100 nM, 5 min pretreatment) on the current density at +80 and –80 mV of mock TRPM6 ($n = 14$) and TRPM6 ($n = 22$). Open bars correspond to cells transfected with mock DNA, whereas closed bars to cells transfected with TRPM6.

(B) Effect of PKC activation by PMA and PKC inhibition by chelerythrine chloride (CC, 5 μM , 20 min pretreatment prior to PMA treatment) on the current density at +80 and –80 mV of TRPM6 and RACK1 (0.5 μg , $n = 7$ –12) transfected HEK293 cells. * $p = 0.01$ compared to TRPM6 and RACK1 without pretreatment, and # $p = 0.02$ compared to TRPM6 and RACK1 pretreated with PMA.

(C) Effect of PKC activation on RACK1 binding. After PMA or vehicle preincubation, RACK1 was coprecipitated with GST-fused C-tail, separated by SDS/PAGE, and visualized using the anti-RACK1 antibody (top panel). Immunoblotting (bottom panel) and densitometry quantification (right panel) of TRPM6-C-tail and RACK1 binding. * $p = 0.001$ compared to TRPM6-C-tail. Values are expressed as mean \pm SEM.

abolished by PKC activation, thereby preventing the inhibitory effect of RACK1. An increased TRPM6 current upon PMA treatment in TRPM6-overexpressed HEK293 cells was anticipated, since PKC activation prevents the RACK1 inhibitory effect in TRPM6 and RACK1-overexpressing HEK293 cells. However, this inhibitory effect was not observed, which might be due to the fact that the amount of endogenous RACK1 is insufficient to inhibit the overexpressed TRPM6.

TRPM6 and TRPM7 confine a Mg^{2+} -permeable channel of which the activity is strongly regulated by $[Mg^{2+}]_i$ [2, 7, 13, 14, 28]. Channel inactivation by elevated $[Mg^{2+}]_i$ may function as negative-feedback machinery to balance the Mg^{2+} homeostasis. Matsushita and coworkers postulated that regulation of TRPM7 channel activity by $[Mg^{2+}]_i$ is disassociated from its α -kinase activity [13], whereas other studies suggested the involvement of the α -kinase domain [11, 14]. In our study, the T¹⁸⁵¹A mutant is less sensitive to $[Mg^{2+}]_i$, which is in agreement with previous studies showing that TRPM7 phosphotransferase activity influences the Mg^{2+} -dependent inhibition of channel activity [11, 14]. This shift in Mg^{2+} sensitivity could have significant impact on TRPM6 activity during transepithelial Mg^{2+} transport because the channel is tightly controlled by $[Mg^{2+}]_i$ [2]. The T¹⁸⁵¹A mutant is still inhibited by high $[Mg^{2+}]_i$, suggesting that additional Mg^{2+} -sensing site(s) are present within TRPM6. Our data indicated that wild-type TRPM6 autophosphorylation activity is gradually enhanced by increasing the Mg^{2+} concentration, in contrast to autophosphorylation of the T¹⁸⁵¹A mutant for which the Mg^{2+} sensitivity is abolished. Thus, residue T¹⁸⁵¹ seems crucial in the Mg^{2+} sensitivity of TRPM6 autophosphorylation. In line with our study, Minagawa and coworkers demonstrated that the Mg^{2+} sensitivity of PhoQ phosphorylation, vital for *Escherichia coli* Mg^{2+} homeostasis, relies on a single residue, D¹⁷⁹ [29]. Over the last decade, the idea was that the free $[Mg^{2+}]_i$ is more or less constant. However, more recent studies provided evidence that the free $[Mg^{2+}]_i$ concentration varies significantly [30, 31]. Enzymes like adenylate kinases and TRPM7 kinase are dependent on the $[Mg^{2+}]_i$ [11, 32, 33]. Moreover, we showed that siRNA-mediated downregulation of endogenous RACK1 significantly reduced the inhibition of the TRPM6 current by Mg^{2+} . These findings suggest that RACK1 is involved in the Mg^{2+} sensitivity of TRPM6 channel activity. Together, we suggest that TRPM6-mediated Mg^{2+} influx induces phosphorylation of T¹⁸⁵¹ located in the α -kinase domain. Subsequently, the phosphorylation of T¹⁸⁵¹ will activate the inhibitory effect of RACK1. This latter step may act as an intracellular feedback regulation to control TRPM6-mediated Mg^{2+} influx and avoid Mg^{2+} overload during renal epithelial Mg^{2+} transport. Our data contribute to a further understanding of molecular mechanisms involved in renal Mg^{2+} handling and to the α -kinase-dependent regulation of TRPM6 channel activity.

Experimental Procedures

Cell Culture and Transfection

HEK293 cells were grown and transfected as described previously [34].

DNA Constructs and cRNA Synthesis

Information about the TRPM6 and RACK1 constructs used is available on-line.

Yeast Two-Hybrid System

Yeast two-hybrid experiments were performed as previously described [35].

GST-Fusion Proteins and Pull-Down Assay

TRPM6 α -kinase GST fusion protein was purified as previously described [35]. Please see Supplemental Data for detailed information.

Electrophysiology

Whole-cell recordings with standard pipette and extracellular solutions were performed as already described [34]. The information on pipette solutions used for the intracellular Mg^{2+} experiments is available online.

Coprecipitation, Coimmunoprecipitation, and Immunoblotting

The supernatants of cell lysates were incubated overnight with glutathione Sepharose 4B beads at 4°C. The anti-RACK1 antibody (IgM) was labeled with biotin and precipitated by neutravidin-agarose beads (Pierce) and then incubated overnight with the supernatants of the lysates. After extensive washing with lysis buffer, the bound proteins were eluted and subjected to SDS-PAGE followed by western blotting analysis. The immunoblotting experiments were performed as previously described [35]. Please see Supplemental Data for detailed information.

In Vitro Phosphorylation and Phosphatase Assays

The precipitated TRPM6 was incubated in kinase reaction buffer and 2 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 30 min at 30°C. For the in vitro phosphatase assay, 15 units alkaline phosphatase (Roche, Mannheim, Germany) were added to TRPM6 immunoprecipitates before in vitro phosphorylation assays. The reaction was terminated by three washing steps with phosphorylation washing buffer. Phosphorylation was analyzed after gel electrophoresis by autoradiography. Please see Supplemental Data for detailed information.

Statistical Analysis

Please see Supplemental Data for detailed information.

Supplemental Data

Additional information about the Experimental Procedures and five figures, can be found online at <http://www.current-biology.com/cgi/content/full/18/3/168/DC1/>.

Acknowledgments

This work was supported by the Netherlands Organization for Scientific Research (Zon-Mw 016.006.001, ZonMW 9120.6110, TOPCW 05.B.012), EURYI, Human Frontiers Science Program (RGP32/2004), the Dutch Kidney foundation (C03.6017), and the EMBO fellowship (ALTF 727-2005). We would like to thank Dr. S. van de Graaf for valuable discussions and Mr. R. Janssen, Mr. M. de Graaf, Mr. D. van den Berg, and Mr. M. Prinz for technical assistance. The pAS-1 yeast expression vector was kindly provided by Dr. S. Elledge (Baylor College of Medicine, Houston, Texas, USA). The authors would like to thank Drs. H. Venselaar and G. Vriend (Centre for Molecular and Biomolecular Informatics, Nijmegen, The Netherlands) for surface and accessibility analysis of the TRPM6 α -kinase domain.

Received: August 20, 2007

Revised: November 23, 2007

Accepted: December 21, 2007

Published online: February 7, 2008

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