Regulation of the Epithelial Mg\(^{2+}\) Channel TRPM6 by Estrogen and the Associated Repressor Protein of Estrogen Receptor Activity (REA)*

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The maintenance of the Mg\(^{2+}\) balance of the body is essential for neuromuscular excitability, protein synthesis, nucleic acid stability, and numerous enzymatic systems. The Transient Receptor Potential Melastatin 6 (TRPM6) functions as the gatekeeper of transepithelial Mg\(^{2+}\) transport. However, the molecular regulation of TRPM6 channel activity remains elusive. Here, we identified the repressor of estrogen receptor activity (REA) as an interacting protein of TRPM6 that binds to the 6th, 7th, and 8th β-sheets in its α-kinase domain. Importantly, REA and TRPM6 are coexpressed in renal Mg\(^{2+}\)-transporting distal convoluted tubules (DCT). We demonstrated that REA significantly inhibits TRPM6, but not its closest homologue TRPM7, channel activity. This inhibition occurs in a phosphorylation-dependent manner, since REA has no effect on the TRPM6 phosphotransferase-deficient mutant (K1804R), while it still binds to this mutant. Moreover, activation of protein kinase C by phorbol 12-myristate 13-acetate-PMA (K1804R), while it still binds to this mutant. Moreover, activation of protein kinase C by phorbol 12-myristate 13-acetate-PMA potentiates the inhibitory effect of REA on TRPM6 channel activity. Finally, we showed that the interaction between REA and TRPM6 is a dynamic process, as short-term 17β-estradiol treatment disassociates the binding between these proteins. In agreement with this, 17β-estradiol treatment significantly stimulates the TRPM6-mediated current in HEK293 cells. These results suggest a rapid pathway for the effect of estrogen on Mg\(^{2+}\) homeostasis in addition to its transcriptional effect. Together, these data indicate that REA operates as a negative feedback modulator of TRPM6 in the regulation of active Mg\(^{2+}\) (re)absorption and provides new insight into the molecular mechanism of renal transepithelial Mg\(^{2+}\) transport.

Mg\(^{2+}\) is a central electrolyte important for many biological functions by its intervention in gene transcription, protein synthesis, nucleic acid stability, channel regulation, cell cycle, and numerous enzymatic systems (1–4). In most species, serum Mg\(^{2+}\) levels are kept within a narrow range between 0.8 and 1.1 mM, while the free intracellular Mg\(^{2+}\) concentration [Mg\(^{2+}\)]; has been estimated around 0.5–1.0 mM (5). Regulation of the Mg\(^{2+}\) balance principally resides within the kidney where Mg\(^{2+}\) excretion tightly matches the intestinal absorption of Mg\(^{2+}\) (5). The majority of Mg\(^{2+}\) in the renal ultrafiltrate is reabsorbed passively in the proximal tubule and the thick ascending limb of the loop of Henle, while the final Mg\(^{2+}\) excretion is determined in the distal convoluted tubule (DCT) via an active reabsorption process (5).

The Transient Receptor Potential Melastatin 6 (TRPM6) localizes along the apical membranes of DCT and intestinal cells where it plays a crucial role in active Mg\(^{2+}\) (re)absorption. Mutations in TRPM6 lead to hypomagnesemia with secondary hypocalcemia (HSH) indicating that this channel is important for the maintenance of the Mg\(^{2+}\) balance (6–8). Previous studies demonstrated that expression of TRPM6 is regulated by dietary Mg\(^{2+}\) and its channel activity is strongly inhibited by the intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_{i}\)) (8, 9). Generally, Mg\(^{2+}\)-free solutions are used to measure the characteristic outwardly rectifying TRPM6 currents (8, 10, 11). Remarkably, TRPM6 contains a C-terminal α-kinase domain, of which the regulatory role on channel activity remains elusive (12–14). It has been shown that RACK1 can interact with this domain and inhibit channel activity in a phosphorylation-dependent manner (15). A recent study demonstrated that the ATP binding motif in the α-kinase domain is required for modulation of TRPM6 channel activity by intracellular ATP (16).

Previous studies suggested that Mg\(^{2+}\) uptake in DCT is stimulated by hormones, such as parathyroid hormone, calcitonin, arginine vasopressin, insulin and prostaglandins (17). A recent study reported that epidermal growth factor (EGF) acts as an autocrine/paracrine magnesiotropic hormone via stimulating its receptor on the basolateral membrane of DCT cells, thereby specifically increasing TRPM6 current (18). Further, Groenesteger et al. (9) demonstrated that the renal TRPM6 mRNA level in ovariectomized rats was significantly reduced, whereas 17β-estradiol treatment normalized TRPM6 mRNA levels. Next to this classical transcriptional pathway, accumulating evidence suggests also rapid estrogen effects that occur within minutes via a non-transcriptional route (19–22). Estrogen has the ability to facilitate rapid membrane-initiated signaling cascades

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\(^3\) The abbreviations used are: DCT, distal convoluted tubule; TRPM6, Transient Receptor Potential Melastatin 6; REA, repressor of estrogen receptor activity; GST, glutathione S-transferase; HA, hemagglutinin; ER, estrogen receptor.
through activation of plasma membrane-associated receptors, such as the recently discovered G protein–coupled receptor 30 (GPCR30) (23). In addition, it has been demonstrated that the classical estrogen receptor α (ERα) can be localized to the plasma membrane in response to estrogen or by interaction with adaptor proteins like Shc and p130Cas (24, 25).

The aim of the present study was to investigate the regulation of the α-kinase domain on TRPM6 channel activity by identification of proteins specifically interacting with the α-kinase domain. Using a combined approach including biochemical, immunohistochemical, mass spectrometry, and electrophysiological analyses, we demonstrated a novel operation mode for rapid estrogen regulation on TRPM6 channel activity via attenuating the inhibitory effect of the TRPM6-associated protein, repressor of estrogen receptor activity (REA).

**EXPERIMENTAL PROCEDURES**

Cell Culture and Transfection—HEK293 cells were grown and transfected as previously described (26), and electrophysiological recordings were performed 48-h post-transfection.

**DNA Constructs and cDNA Synthesis**—The kinase domain of mouse (1759–2028) and human (1750–2022) TRPM6 was cloned into pGEX6p-2 (Amersham Biosciences, Uppsala, Sweden) by PCR using mouse kidney cDNA or human TRPM6 in pCINeo/IRES-GFP (8) as template. The α-kinase domain (1750–2022) of human TRPM6 was subcloned into the pEBG vector (27). Full-length mouse REA cDNA was cloned into pT7Ts, pCB7 and pEBG by PCR using mouse kidney cDNA. Wild-type TRPM6 in the pCINeo/IRES-GFP vector was HA-tagged at the N-terminal tail as described previously (27). GST-α-kinase truncants (TRPM6 1759–1993, 1759–1885, 1759–1857, 1857–1885, and 1759–1813) mutants, TRPM6 phosphotransferase-deficient mutant (K1804R) and GST-α-kinase K1804R mutant were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. All constructs were verified by sequence analysis. REA cRNA was synthesized in vitro using T7 RNA polymerase as described previously (28).

**Identification of Proteins by Nano Liquid Chromatography Tandem Mass Spectrometry**—After SDS-PAGE, GST, and GST-TRPM6 α-kinase interacting proteins were treated with dithiothreitol and iodoacetamide, and digested in-gel by trypsin as previously described (29). Peptide identification experiments by liquid chromatography tandem mass spectrometry were performed using a nano-HPLC Agilent 1100 nanoflow system connected online to a 7-Tesla linear quadrupole ion trap-Fourier Transform Ion Cyclotron Resonance (LTQ-FT) mass spectrometer (Thermo Electron, Bremen, Germany) as described previously (29). Peptides and proteins were identified using the Mascot 2.1 (Matrix Science, Boston, MA) algorithm to search a local version of the Uniprot data base. First ranked peptides were parsed from Mascot data base search html files with MSQuant to generate unique first ranked peptide lists and internal calibration of measured ion masses.

**GST Fusion Proteins and Pull-down Assay**—TRPM6 α-kinase GST fusion protein was purified as previously described (30). REA protein was labeled with [35S]methionine using a reticulocyte lysate system (Promega, Madison, WI) and added to purified GST fusion proteins, immobilized on glutathione-Sepharose 4B beads. After 2 h of incubation at room temperature, beads were washed extensively with pull-down buffer (10 mM Tris-HCl, pH 7.4), 150 mM NaCl, 0.33% (v/v) Triton X-100. The bound proteins were eluted with SDS-PAGE loading buffer, separated on SDS-PAGE gel and visualized by autoradiography.

**Sequence Analysis and Structure Modeling**—The structural model of TRPM6 α-kinase domain was built based on the crystal structure of TRPM7 α-kinase domain (31) (Protein Data Bank under ID codes: 11A1 (apo), 11AH (ADP complex), and 11A9 (AMP-PNP complex)) using SWISS-MODEL and analyzed by DeepView Swiss-PdbViewer (Version 3.7) and Yasara.

**RT-PCR**—Total RNA isolation from mouse tissue and reverse transcription were performed as described previously (30). REA and β-actin were amplified by PCR and subsequently analyzed by agarose gel electrophoresis.

Electrophysiology—Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature using an EPC-10 patch clamp amplifier computer controlled by the Patchmaster software (HEKA Elektronik, Lambrecht, Germany). Electrode resistances were 2–5 MΩ, and capacitance and access resistance were monitored continuously. A ramp protocol, consisting of linear voltage ramps from −100 to +100 mV (within 450 ms), was applied every 2 s from a holding potential of 0 mV. Current densities were obtained by normalizing the current amplitude to the cell membrane capacitance. The time course of current development was determined by measuring the current at +80 and −80 mV. I/V relations were established from the ramp protocols. The analysis and display of patch clamp data were performed using Igor Pro software (WaveMetrics, Lake Oswego). The standard pipette solution contained 150 mM NaCl, 10 mM EDTA, and 10 mM HEPES-NaOH, pH 7.2. The extracellular solutions contained 150 mM NaCl, 10 mM HEPES-NaOH, and 1 mM Ca2+. pH 7.4. To investigate the effect of purified GST-REA or GST on TRPM6-mediated currents, proteins were added to the standard pipette solution and thereby infused via the patch pipette for 7 min before starting the recordings.

**Co-precipitation**—HEK293 cells were transiently co-transfected with REA and pEBG–TRPM6 α-kinase or pEBG empty vector. Cells were treated with 50 nM 17β-estradiol or vehicle for 10 min at 37 °C and were lysed for 1 h on ice in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-NaOH, pH 7.5, 0.53% (v/v) Triton including the protease inhibitors leupeptin (0.01 mg/ml), pepstatin (0.05 mg/ml), and phenylmethylsulfonyl fluoride (1 mM)). After centrifugation, supernatants of the lysates were incubated over-night with glutathione-Sepharose 4B beads at 4 °C. After extensive washing in lysis buffer, the bound proteins were eluted and separated by SDS-PAGE. The co-precipitation was analyzed using the anti-REA antibody (Abcam, Cambridge, UK).

**Cell Surface Labeling with Biotin**—HEK293 cells were transiently transfected with 15 μg HA-TRPM6 in poly-L-lysine (Sigma)-coated 10-cm dishes. 72 h after transfection, cells were treated with 50 nM 17β-estradiol or vehicle for 10 min at 37 °C. Cell surface labeling with NHS-LC-LC-biotin (Pierce, Etten-Leur, The Netherlands) was performed as previously described (32). 1 h after homogenizing, biotinylated proteins were precipitated using neutravidin-agarose beads (Pierce, Etten-Leur, The Netherlands).
TRPM6 expression was analyzed by immunoblot for the precipitates (plasma membrane fraction) and for the total cell lysates using the anti-HA antibody (Sigma).

**Immunoblotting**—Protein samples were denatured by incubation for 30 min at 37 °C in Laemmli buffer and then subjected to SDS-PAGE. Immunoblots were incubated with either mouse anti-HA (Sigma) or rabbit anti-REA (Abcam, Cambridge, UK) antibody. Subsequently, blots were incubated with sheep horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma) and then visualized using the enhanced chemiluminescence system.

**Immunohistochemistry**—Immunohistochemistry was performed as previously described (33). Briefly, mouse kidney serial sections were incubated for 16 h at 4 °C with rabbit anti-REA (Abcam, Cambridge, UK) and guinea pig anti-TRPM6 (8). To visualize TRPM6, tyramide signal amplification kit (NEN Life Science Products, Zaventem, Belgium) was used after incubation with biotin-coated goat anti-mouse secondary antibody. Images were taken with a Bio-Rad MRC 100 confocal laser scanning microscope.

**In Vitro Phosphorylation Assays**—HA-TRPM6 was precipitated by using the anti-HA antibody. The precipitates were incubated in a total volume of 30 μl of kinase reaction buffer (50 mM HEPES-KOH, pH 7.4, 4 mM MnCl₂, 0.5 mM CaCl₂, 100 μM ATP) and 2 μCi of [γ³²P]ATP for 30 min at 30 °C. The reaction was terminated by three washing steps with phosphorylation washing buffer (50 mM HEPES-KOH, pH 7.4, 4 mM MnCl₂, 0.5 mM CaCl₂). Phosphorylation was analyzed after gel electrophoresis by autoradiography.

**Statistical Analysis**—Values are expressed as mean ± S.E. Statistical significance between groups was determined by analysis of variance (ANOVA). In the case of significance, differences between the means of two groups were analyzed by unpaired Student's t test. p < 0.05 was considered statistically significant.

**RESULTS**

**REA Associates with TRPM6**—To identify proteins potentially interacting with the TRPM6 α-kinase domain, the combination of a GST pull-down with the α-kinase domain of TRPM6 in mouse kidney lysate followed by Fourier Transform Mass Spectrometry (FTMS), was performed. REA (34), was identified as an interacting protein of the TRPM6 α-kinase domain. Next, GST pull-down binding assays were used to confirm the interaction between REA and TRPM6 α-kinase domain. [³⁵S]methionine-labeled REA protein was incubated with GST and GST-α-kinase immobilized on glutathione-Sepharose 4B beads. REA interacted specifically with the GST-α-kinase, but not with GST alone (Fig. 1A). Next, the association between TRPM6 and REA was further substantiated in mammalian cells by co-pre-

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**FIGURE 1.** REA interacts with the α-kinase domain in TRPM6. A, GST pull-down assay between [³⁵S]methionine-labeled REA protein and GST or GST fused to the α-kinase domain. B, co-precipitation studies of GST and GST-α-kinase in REA-expressing HEK293 cells (top panel). REA input (1%) expression was analyzed by immunoblotting (bottom panel). C, mapping of the REA binding site within the α-kinase domain. Predicted three-dimensional structure model of the α-kinase domain with different truncations of the α-kinase. D, Coomassie Blue staining of the SDS-PAGE gel. E, co-precipitation studies of GST and GST-α-kinase in REA-expressing HEK293 cells in the presence of different amounts of RACK1 (dilution 1:10 (0.1), 1:2 (0.5), and non-diluted (1.0)) (bottom panel). REA and RACK1 input (1%) expression was analyzed by immunoblotting (bottom panels).
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**REA Binds to the 6th, 7th, and 8th $\beta$-Sheets in the TRPM6 $\alpha$-Kinase Domain**—To determine the REA binding site within TRPM6, a series of deletion mutants in the $\alpha$-kinase domain was constructed. Truncated TRPM6 $\alpha$-kinase mutants were expressed as GST-fused proteins and evaluated for their interaction with [$^{35}$S]methionine-labeled REA protein (Fig. 1C). Truncation at position 1857 abolished the interaction between the two proteins, whereas truncation at position 1885 had no effect on the interaction with REA. Moreover, a GST fusion protein containing only the short peptide between the amino acids 1857 and 1885 of the TRPM6 $\alpha$-kinase domain bound to REA (Fig. 1C). Therefore, the REA 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 Blue staining of SDS-PAGE gel (Fig. 1D). The lower abundant product bands of different sizes might be due to binding of so far unknown proteins to the bigger truncation mutants or degradation products.

**REA Co-expresses with TRPM6 in Kidney**—To address the tissue distribution of REA, reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed on mouse tissues. The expected DNA fragment of 916 bp for REA was detected in most tested tissues as indicated in Fig. 2A. The $\beta$-actin fragment was present in all the tissues evaluated. Of note, REA is present in kidney where TRPM6 is predominantly expressed (8). To further study the co-expression between REA and TRPM6 in kidney, immunohistochemistry was performed on serial mouse kidney sections. This analysis indicated immunopositive staining for REA in the TRPM6-expressing DCT cells that have been implicated in active Mg$^{2+}$ reabsorption (8) (Fig. 2B).

**REA Inhibits TRPM6 Channel Activity in a Phosphorylation-dependent Manner**—The functional role of REA on TRPM6 channel activity was investigated by whole-cell patch clamp recordings in HEK293 cells. Steeply outward rectifying currents carried by Na$^+$ were measured in HEK293 cells co-expressing TRPM6 and empty vector (mock) (Fig. 3, A and B). These currents were significantly inhibited in cells co-expressing TRPM6 and REA (Fig. 3, A and B). Of note, the inhibitory effect of REA was specific for TRPM6, because REA did not affect the current in TRPM7-expressing HEK293 cells (Fig. 3, C and D). To further analyze the inhibitory effect of REA on TRPM6, GST and GST-REA were expressed in HEK293 cells and purified using glutathione-Sepharose 4B beads (Fig. 4A). These purified GST and GST-REA proteins were subsequently perfused into TRPM6-expressing HEK293 cells via the patch pipette before whole-cell recording. As shown in Fig. 4, B and C, REA significantly inhibited the TRPM6-mediated current, but the associated protein did not change the electrophysiological characteristics of the recorded currents. GST alone did not affect the...
TRPM6-mediated current. Next, the phosphotransferase-deficient mutant (K1804R) was used to investigate the role of TRPM6 phosphorylation activity in this process. REA was unable to inhibit the K1804R mutant-mediated current, similar as GST alone (Fig. 4D and E). Importantly, the co-precipitation assay showed that the K1804R mutation did not influence the binding between TRPM6 and REA (Fig. 4F). Importantly, our in vitro phosphorylation assay demonstrated equal autophosphorylation of TRPM6 with or without REA co-expression (Fig. 4G). Next, we investigated the effect of PKC activation by phorbol 12-myristate 13-acetate-PMA (PMA) on the regulatory effect of REA, since it has been shown that RACK1 inhibition can be prevented by 5 min of preincubation of 100 nM PMA (15). Pretreatment of cells co-expressing TRPM6 and REA significantly decreased TRPM6 channel activity. Of note, the current amplitude in the presence of PMA was not altered in cells expressing TRPM6 alone (Fig. 4H). Finally, we investigated whether REA is a phosphorylation target of TRPM6. The in vitro phosphorylation assay showed that REA is not phosphorylated by TRPM6 (Fig. 4G).

17β-Estradiol Dissociates the REA-TRPM6 Interaction and Stimulates Channel Activity—Given the role of REA in the estrogen signaling pathway (34–36), the influence of estrogen on the TRPM6 and REA interaction was investigated by co-precipitation experiments. To this end, GST-α-kinase and GST were co-expressed with REA in HEK293 cells, subjected to 17β-estradiol (50 nM) or vehicle for 10 min at 37°C, and then precipitated using glutathione-Sepharose 4B beads. The interaction between REA and TRPM6 α-kinase domain was significantly reduced by 17β-estradiol treatment (Fig. 5A, upper panel), whereas REA was equally expressed in all situations (Fig. 5A, lower panel). Next, the influence of estrogen on TRPM6 channel activity was investigated. To this end, HEK293 cells expressing TRPM6 were preincubated for 10 min with 17β-estradiol (50 nM) at 37°C and analyzed by whole-cell patch clamp recordings. As shown in Fig. 5, B and C, the TRPM6 current was significantly increased when treated with 17β-estradiol compared with control. Subsequently, the influence of 17β-estradiol treatment on the amount of TRPM6 channels expressed at the plasma membrane was investigated by cell surface biotinylation experiments using HEK293 cells. As shown in Fig. 5D (upper panel), treatment with 17β-estradiol does 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 (Fig. 5D, bottom panel).
DISCUSSION

In the present study, we identified REA as a TRPM6-associated protein and demonstrated that this estrogen-responsive protein inhibits the channel activity in a phosphorylation activity-dependent manner. First, REA binds to the TRPM6 α-kinase domain and co-expresses with the Mg$^{2+}$ channel in the renal DCT. Second, REA specifically inhibits channel activity of TRPM6, but not TRPM7. Third, this inhibition depends on TRPM6 phosphorylation activity, because REA has no effect on the TRPM6 phosphotransferase-deficient K1804R mutant. Fourth, the association between REA and TRPM6 is a dynamic process, which is regulated by 17β-estradiol treatment. Finally, 17β-estradiol treatment stimulates TRPM6 activity via a rapid non-transcriptional pathway.

TRPM6 functions as the gatekeeper of active transepithelial Mg$^{2+}$ transport (6–8). Here, REA was identified as a dynamic regulator of TRPM6 channel activity and, therefore, a molecular component in Mg$^{2+}$ homeostasis. REA binds to the α-kinase domain of TRPM6, which was initially identified by the combination of a GST-pull down with the α-kinase domain of TRPM6 in mouse kidney lysate followed by FTMS analysis. This interaction was confirmed by co-precipitation between REA and TRPM6 α-kinase domain in HEK293 cells. Interestingly, our GST pull-down experiment and structure modeling analysis suggest that REA specifically binds to the 6th, 7th, and 8th β-sheets in the α-kinase domain, which has been demonstrated as the binding region for RACK1 as well (15). This might be due to the fact that most amino acids in this area are located on the peripheral region of the tertiary structure of the TRPM6 α-kinase domain, thereby facilitating the interaction with its regulatory proteins. Importantly, we demonstrated that RACK1 has no effect on the binding between REA and the α-kinase domain. This could be explained either by different binding sites within this region or by a higher binding affinity for REA than RACK1.

REA was first identified as an interacting protein of the estrogen receptor (ER), where it functions as a selective co-regulator repressing the transcriptional activity of ERs (34). Genetic deletion of REA significantly enhances estrogen responsiveness in vivo (35). Recently, He et al. (36) showed that REA forms a

FIGURE 5. Effect of 17β-estradiol on the binding between REA on TRPM6 and on TRPM6 channel activity. A, GST-fused TRPM6 α-kinase domain, GST (control) and REA were co-expressed in HEK293 cells. After treatment with 50 nm 17β-estradiol (17β-E2) or vehicle, REA was co-precipitated with GST-fused α-kinase domain and visualized using the anti-REA antibody (top panel). Immunoblotting (bottom panel) and densitometry quantification (right panel) of binding between TRPM6 α-kinase domain and REA. * indicates p < 0.05 compared with vehicle treatment.

B, time course of the current density (pA/pF) at +80 mV of TRPM6 transfected HEK293 cells in control condition (■) and treated with 50 nm 17β-estradiol (E2) for 10 min at 37 °C (▲). C, averaged values of the current density at +80 mV after 200 s of TRPM6 (n = 37) and TRPM6 pretreated with 50 nm of 17β-estradiol (E2) (n = 37). * indicates p < 0.05 compared with control condition. D, TRPM6 expressing HEK293 cells were treated with 50 nm 17β-estradiol (E2) or vehicle and subsequently subjected to cell surface biotinylation assay. TRPM6 expression was analyzed by immunoblot for the plasma membrane fraction (top panel) and for the total cell lysates (bottom panel). Surface expression of TRPM6 was quantified by densitometry (right panel). As negative controls, mock cells were used.

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heteromeric with prohibitin and acts as a transcriptional co-repressor for ERα. Our study demonstrated a novel role of REA in TRPM6 regulation by showing that the inhibitory effect of REA on TRPM6 channel activity is not via the long-term transcriptional pathway. This notion is supported by the fact that rapid perfusion of purified GST-REA through the patch pipette into TRPM6-expressing cells induced a significant inhibition of channel activity. Moreover, this inhibitory effect is dependent on the kinase activity of the TRPM6 α-kinase domain since the TRPM6 phosphotransferase-deficient K1804R mutant is insensitive to REA. Of note, REA can still bind to the TRPM6 K1804R mutant. Next, we demonstrated that PKC activation by PMA potentiated the inhibitory effect of REA. Taken together, these data suggest that the phosphorylation activity of TRPM6 α-kinase domain plays a role in the REA effect.

Although the phosphorylation activity of the TRPM6 and TRPM7 α-kinase domains has been well established (15, 37–42), the regulatory role of this catalytic domain on channel activity remains elusive. While it has previously been shown that these domains are not essential for TRPM6/7 channel activation (15, 39, 42), several studies have demonstrated an indirect regulatory role in modulation of channel activity (15, 43). In this line, we previously demonstrated that ATP can regulate TRPM6 channel activity through the ATP binding motif within the α-kinase domain, independent of the TRPM6 α-kinase activity (43). Moreover, we showed earlier that the inhibitory effect of RACK1 on TRPM6 is dependent on the TRPM6 phosphorylation activity (15). It has also been illustrated that the phosphorylation-impaired T1851A mutant is less sensitive to [Mg$^{2+}$], in agreement with previous studies demonstrating that TRPM7 α-kinase activity influences the Mg$^{2+}$-dependent inhibition of channel activity (42, 44). Remarkably, while REA inhibits TRPM6 channel activity, it has no effect on TRPM7, the closest homologue of TRPM6. Based on the structural analysis, the REA binding site in TRPM6 α-kinase domain is highly conserved in TRPM7, therefore REA most likely binds to TRPM7 α-kinase domain as well. However, it has been reported that the α-kinase of TRPM6 is capable of cross-phosphorylation of TRPM7, but not vice versa, suggesting the functional non-redundancy of these two α-kinase domains (40). One hypothesis is that REA is a specific phosphorylation target of TRPM6, but not TRPM7, and this phosphorylation may function as a switch of the REA regulatory activity on TRPM6. However, we demonstrated that REA could not be phosphorylated by TRPM6. Furthermore, TRPM6 autophosphorylation is not affected in the presence of REA. So, the present study revealed another indirect regulation of TRPM6 channel activity, free from TRPM6 autophosphorylation, but dependent on its α-kinase activity.

REA has been well documented as a regulator in the estrogen signaling pathway (34–36). Interestingly, our data showed that the interaction between REA and TRPM6 is a dynamic process that is regulated by estrogen. Treatment with 17β-estradiol disassociated the interaction between TRPM6 and REA, and attenuated its inhibitory effect on TRPM6 channel activity. According, our electrophysiological data demonstrated that preincubation of 17β-estradiol indeed induced a significant increase of TRPM6-mediated current. It is well known that estrogen can enhance Mg$^{2+}$ utilization and uptake (41, 45–47). In this line, Groenestege et al. (9) demonstrated that the renal TRPM6 mRNA level in ovariectomized rats was significantly reduced, whereas 17β-estradiol treatment normalized TRPM6 mRNA level, suggesting that estrogen can regulate Mg$^{2+}$ homeostasis via a transcriptional pathway. Accumulating evidence showed that, in addition to the classical transcriptional pathway, estrogen has the ability to facilitate rapid, membrane-initiated, fast signaling cascades via plasma membrane-associated receptors (19–22). Our current data pointed out a rapid regulatory pathway for estrogen, since the TRPM6-expressing cells were only treated with 17β-estradiol for 10 min prior to path clamp analysis. Interestingly, we demonstrated that 17β-estradiol significantly disassociated the interaction between TRPM6 α-kinase domain and REA. In reminiscence of REA inhibition on TRPM6 channel activity, the molecular mechanism of estrogen stimulation might be due to disassociation of the TRPM6 and REA interaction and subsequent diminishment of the REA inhibition. It would, therefore, be of interest to further elucidate the detailed signaling pathway involved in estrogen stimulation leading to attenuation of the TRPM6 and REA interaction. It is conceivable that estrogen activates an estrogen receptor and its downstream signaling pathway, which might couple to TRPM6 or REA and influence their structure.

Taken together, we presented REA as a novel negative modulator involved in active transepithelial Mg$^{2+}$ transport via regulating TRPM6 channel activity. Furthermore, estrogen disassociated the interaction between TRPM6 and REA and thereby increases TRPM6-mediated Mg$^{2+}$ influx, which may function as a rapid pathway for estrogen to regulate the Mg$^{2+}$ balance. These findings shed new light on the molecular regulation of TRPM6 and contribute to a further understanding of the molecular basis of transepithelial Mg$^{2+}$ (re)absorption.

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