The Epithelial Mg^{2+} Channel Transient Receptor Potential Melastatin 6 Is Regulated by Dietary Mg^{2+} Content and Estrogens

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The kidney is the principal organ responsible for the regulation of the body Mg^{2+} balance. Identification of the gene defect in hypomagnesemia with secondary hypocalcemia recently elucidated transient receptor potential melastatin 6 (TRPM6) as the gatekeeper in transepithelial Mg^{2+} transport, whereas its homolog, TRPM7, is implicated in cellular Mg^{2+} homeostasis. The aim of this study was to determine the tissue distribution in mouse and regulation of TRPM6 and TRPM7 by dietary Mg^{2+} and hormones. This study demonstrates that TRPM6 is expressed predominantly in kidney, lung, cecum, and colon, whereas TRPM7 is distributed ubiquitously. Dietary Mg^{2+} restriction in mice resulted in hypomagnesemia and renal Mg^{2+} and Ca^{2+} conservation, whereas a Mg^{2+}-enriched diet led to increased urinary Mg^{2+} and Ca^{2+} excretion. Conversely, Mg^{2+} restriction significantly upregulated renal TRPM6 mRNA levels, whereas a Mg^{2+} enriched diet increased TRPM6 mRNA expression in colon. Dietary Mg^{2+} did not alter TRPM7 mRNA expression in mouse kidney and colon. In addition, it was demonstrated that 17β-estradiol but not 1,25-dihydroxyvitamin D_3 or parathyroid hormone regulates TRPM6 renal mRNA levels. Renal TRPM7 mRNA abundance remained unaltered under these conditions. The renal TRPM6 mRNA level in ovariectomized rats was significantly reduced, whereas 17β-estradiol treatment normalized TRPM6 mRNA levels. In conclusion, kidney, lung, cecum, and colon likely constitute the main sites of active Mg^{2+} (re)absorption in the mouse. In addition, Mg^{2+} restriction and 17β-estradiol upregulated renal TRPM6 mRNA levels, whereas a Mg^{2+}-enriched diet stimulated TRPM6 mRNA expression in colon, supporting the gatekeeper function of TRPM6 in transepithelial Mg^{2+} transport.

J Am Soc Nephrol 17: 1035–1043, 2006. doi: 10.1681/ASN.2005070700

Mg^{2+} is the second most abundant intracellular cation and plays an essential role as co-factor in many enzymatic reactions (1). Mg^{2+} homeostasis depends on the balance among intestinal absorption, renal excretion, and exchange with bone (2). Regulation of the total body Mg^{2+} balance principally resides within the kidney that tightly matches the intestinal absorption of Mg^{2+}. Approximately 80% of the total plasma Mg^{2+} is filtered in the glomeruli (3,4), the majority of which subsequently is reabsorbed along the nephron (5). Eighty-five percent of the filtered Mg^{2+} is reabsorbed passively in the proximal tubule and the thick ascending limb of Henle (TAL). The distal convoluted tubule (DCT) reabsorbs 5 to 10% of the filtered Mg^{2+}, and the reabsorption rate in this segment defines the final urinary Mg^{2+} concentration. Mg^{2+} transport in DCT is transcellular in nature and influenced by dietary Mg^{2+} restriction and a number of hormones (6,7). However, the molecular details and regulation of this pathway remain largely unknown (5,6,8,9).

Received July 10, 2005. Accepted January 20, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

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Hereditary disorders with primary hypomagnesemia have greatly facilitated the identification of epithelial ion transporters in the kidney. For instance, the elucidation of the genetic basis of isolated dominant hypomagnesemia and hypomagnesemia with secondary hypocalcemia resulted in the identification of the γ-subunit of the Na^+,K^+-ATPase and the epithelial Mg^{2+} transient receptor potential melastatin 6 (TRPM6), respectively (10–12). TRPM6, which is a member of the TRP superfamily, is localized along the apical membrane of the DCT. Heterologous expression in human embryonic kidney 293 cells of TRPM6 but not TRPM6 mutants identified in patients with hypomagnesemia with secondary hypocalcemia induces a Mg^{2+}-permeable cation channel that is tightly regulated by the intracellular Mg^{2+} concentration (13). TRPM6 shows the highest homology with TRPM7, which has been identified as a Mg^{2+}-permeable ion channel that primarily is required for cellular Mg^{2+} homeostasis (13–15).

In analogy with active Ca^{2+} (re)absorption in the distal part of the nephron and the small intestine through the epithelial Ca^{2+} channels (transient receptor potential vanilloid 5 [TRPV5] and TRPV6) (16), the process of transcellular Mg^{2+} transport is envisaged by the following sequential steps. Driven by a favorable transmembrane potential, Mg^{2+} enters the epithelial cell through the apical epithelial Mg^{2+} channel TRPM6. Next, Mg^{2+} will diffuse through the cytosol to be extruded actively

 ISSN: 1046-6673/1704-1035

across the basolateral membrane (13). The molecular identity of these latter Mg^{2+} transporters is not known. Most physiologic studies favor a Na^+-dependent exchange mechanism (17). The Mg^{2+} entry seems to be the rate-limiting step and the site of regulation.

The aim of our study was to determine the sites of active Mg^{2+} absorption in mice by investigating the expression profile of TRPM6. In addition, we established whether TRPM6 is regulated by dietary Mg^{2+} content and the calciotropic hormones 17β-estradiol (17β-E_2), 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), and parathyroid hormone (PTH). The effect of dietary Mg^{2+} content was studied by analyzing TRPM6 regulation at the mRNA and protein levels and Mg^{2+} excretion and serum levels in C57BL6 mice fed Mg^{2+}-deficient, -normal, and -enriched diets.

Materials and Methods

Animal Studies

To evaluate TRPM6, TRPM7, and TRPV6 mRNA expression in various tissues, we constructed a cDNA panel. To this end, four C57BL6 mice fed a complete diet that contained 0.2% Mg^{2+} (wt/wt; SSNIFF spezialdiäten GmbH, Soest, Germany) were killed; kidney, spleen, brain, heart, skeletal muscle, liver, lung, stomach, bone, duodenum, jejunum, ileum, cecum, and colon were collected; and total RNA was isolated. To study the effect of dietary Mg^{2+} content on TRPM6 and TRPM7 expression in kidney and colon, we fed C57BL6 mice (12 wk of age) for 10 d a Mg^{2+}-deficient diet (0.005% wt/wt Mg), a Mg^{2+}-normal diet (0.19% wt/wt Mg), or a Mg^{2+}-enriched diet (0.48% wt/wt Mg; SSNIFF spezialdiäten GmbH). During the last 24 h of the dietary treatment, animals were housed in metabolic cages and 24-h urine was collected. At the end of the dietary treatment, blood samples were taken and the animals were killed. Kidney and colon tissues were sampled and frozen immediately in liquid nitrogen.

The effect of 17β-E_2 on the renal TRPM6 mRNA expression level was evaluated by sham-operated, bilateral ovariectomized (OVX), and OVX rats that received 2×500 μg 17β-E_2/d as described previously (18). The effect of PTH was studied by sham-operated, parathyroidectomized (PTX), and PTX rats that received 6 units/d bovine PTH as described previously (19). In addition, the effect of 1,25$(OH)_2D_3$ was studied by 1α-hydroxylase knockout (1α-OHase$^{-/-}$) mice, heterozygous (1α-OHase$^{+/-}$) mice that are phenotypically identical to wild-type mice, and 1α-OHase$^{-/-}$ mice supplemented intraperitoneally with 1,25$(OH)_2D_3$ as described previously (20). The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from kidney, complete segments of the intestine, and the other tissues using TriZol Total RNA Isolation Reagent (Life Technologies BRL, Breda, The Netherlands) according to the manufacturer's protocol. The obtained RNA was subjected to DNase treatment (Promega, Madison, WI) to prevent genomic DNA contamination. Thereafter, 2 μg of RNA was reverse transcribed by Molony-Murine Leukemia Virus-Reverse Transcriptase (Invitrogen) as described previously (21). The cDNA was used to determine TRPM6, TRPM7, and TRPV6 mRNA expression levels, as well as mRNA levels of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) as an endogenous control. The mRNA expression levels were quantified by real-time PCR on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). Primers and probes that target the genes of interest were designed using the com-

Table 1. Sequences of primers and Taqman probes for real-time quantitative PCR

Gene	Forward Primer	Reverse Primer	Probe
HPRT			
R	5′-TATCAGACTGAAGAGCTACTGTAATGACC-3′	5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′	5′-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3′
M	5′-TTATCAGACTGAAGAGCTACTGTAATGATC-3′	5′-TTACCAGTGTCAATTATATCTTCAACAATC-3′	5′-TGAGAGATCATCTCCACCAATAACTTTTATGTCCC-3′
TRPM6			
R	5′-AAAGCCATGCGAGTTATCAGC-3′	5′-CTTCACAATGAAAACCTGCCC-3′	5′-CCTGGTCTGAGGATGATGTTCTCAAGCC-3′
M	5′-AAAGCCATGCGAGTTATCAGC-3′	5′-CTTCACAATGAAAACCTGCCC-3′	5′-CCTGGTCTGAGGATGATGTTCTCAAGCC-3′
TRPM7			
R	5′-GGTTCCTCCTGTGGTGCCTT-3′	5′-CCCCATGTCGTCTCTGTCGT-3′	5′-TTCCCAAGTGCTGTTTCTCCCCCA-3′
M	5′-GGTTCCTCCTGTGGTGCCTT-3′	5′-CCCCATGTCGTCTCTGTCGT-3′	5′-TTCCCAAGTGCTGTTTCTCCCCCA-3′
TRPV6			
M	5′-ATCCGCCGCTATGCACA-3′	5′ AGTTTTTCTCCTGAATCTTTTTCCAA-3′	5′-TTCCAGCAACAAGATGGCCTCTACTCTGA-3′

PCR primers and fluorescent probes (5′FAM–3′TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Biolegio (Malden, The Netherlands). HPRT, hypoxanthine-guanine phosphoribosyl transferase; R, rat; M, mouse.

puter program Primer Express (Applied Biosystems, Foster City, CA) and are listed in Table 1.

In Vivo $^{45}Ca^{2+}$ *Absorption Assay*

Intestinal Ca^{2+} absorption was assessed in two groups of C57BL6 mice by measuring the amount of $^{45}Ca^{2+}$ in serum at early time points after oral gavage (15 μl/g body wt). Mice fasted for 12 h before the test. Animals were hemodynamically stable under anesthesia during the experiment. The solution that was used to measure Ca^{2+} absorption contained 0.1 mM $CaCl_2$, 125 mM NaCl, 17 mM Tris (pH 7.4), and 1.8 g/L fructose and was enriched with 20 μCi $^{45}CaCl_2$/ml (18 Ci/g; New England Nuclear, Newton, MA). One group received the $^{45}Ca^{2+}$ solution supplemented with $MgCl_2$ to a final concentration of 10 mM, whereas the $^{45}Ca^{2+}$ solution of group 2 was not supplemented with $MgCl_2$. Blood samples were obtained at different time intervals (2, 4, 8, and 12 min). Radioactive $^{45}Ca^{2+}$ was analyzed in serum (10 μl) by liquid scintillation counting. The change in the serum Ca^{2+} concentration was calculated from the $^{45}Ca^{2+}$ content of the serum samples and the specific activity of the administrated Ca^{2+}.

Analytical Procedures

Serum Mg^{2+} and Ca^{2+} were measured using a colorimetric assay kit according the manufacturer's protocol (Roche Diagnostics, Woerden, The Netherlands). Urinary Mg^{2+} and Ca^{2+} excretion was determined by atomic absorption spectrophotometry on a Perkin Elmer AAnalyst 300 (Perkin Elmer, Milano, Italy).

Immunohistochemistry

Immunohistochemical staining was performed on 7-μm cryosections of periodate-lysine-paraformaldehyde–fixed kidney samples. Sections were stained with affinity-purified guinea pig anti-TRPM6, as described previously (13). Photographs of the entire cortex were taken with a Zeiss fluorescence microscope (Sliedrecht, The Netherlands) equipped with a digital photo camera (Nikon DMX1200). For semi-quantitative determination of protein levels, images were analyzed with the Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD), resulting in quantification of the protein levels as the mean of integrated optical density (22).

Statistical Analyses

Values are expressed as mean ± SEM. Differences between groups were tested by one-way ANOVA and further evaluated using Fisher multiple comparison procedure. Differences in means with $P < 0.05$ were considered statistically significant. All analyses were performed using the Statview Statistical Package (Power PC version 4.51; Berkeley, CA) on a Macintosh computer.

Results

Tissue Distribution of TRPM6 and TRPM7 in Mouse

To study the quantitative expression levels of TRPM6 and TRPM7 in various tissues, we constructed a mouse cDNA panel. Subsequently, we quantified TRPM6 and TRPM7 mRNA levels by real-time PCR analysis and normalized for HPRT expression. The highest level of TRPM6 expression was measured in kidney, intestine, and lung (Figure 1A), whereas TRPM7 showed a ubiquitous expression pattern (Figure 1B). To determine in more detail the intestinal site of active Mg^{2+} absorption in relation to transcellular Ca^{2+} absorption, we quantified mRNA expression levels of TRPM6, TRPM7, and TRPV6 in different segments of the mouse intestinal tract by

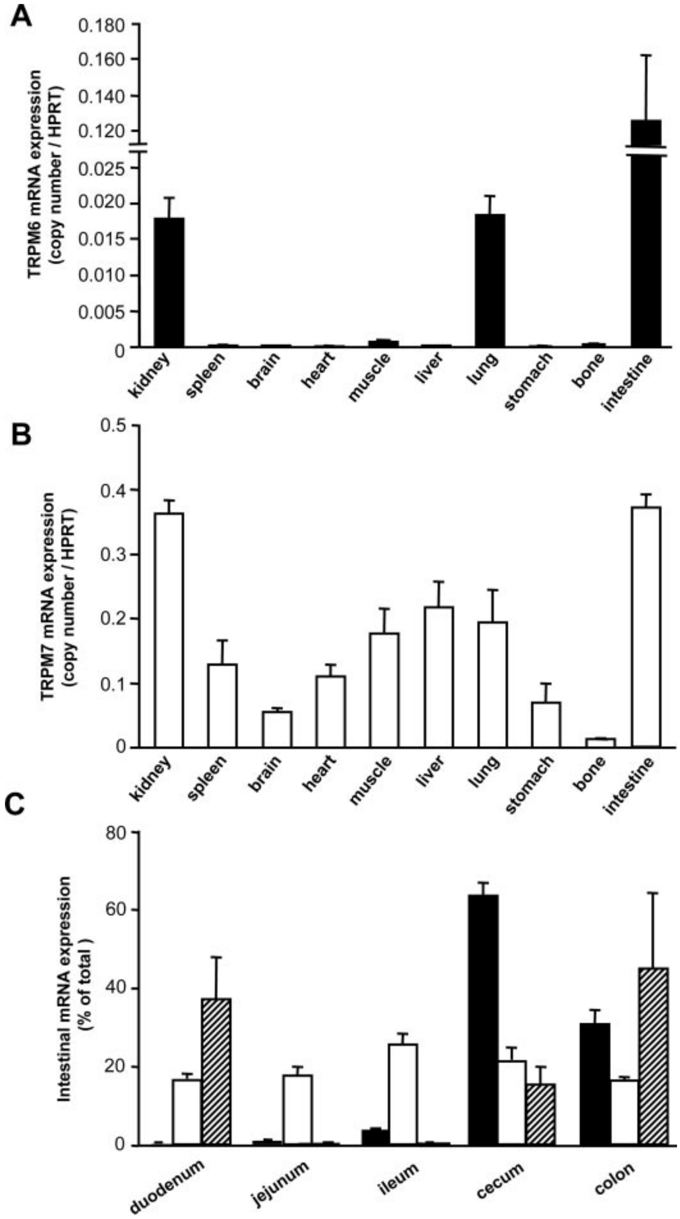

Figure 1. Expression profile of transient receptor potential melastatin 6 (TRPM6) and TRPM7 channels in various mouse tissues. (A and B) The mRNA expression levels of TRPM6 and TRPM7 in a panel of mouse tissues were measured by using quantitative real-time PCR. (C) Quantification of mRNA expression levels of TRPM6 (■), TRPM7 (□), and transient receptor potential vanilloid 6 (TRPV6) (▨) along the intestinal tract are presented as percentage of total intestinal mRNA expression. mRNA quantified by real-time PCR analysis is calculated as ratio to the hypoxanthine-guanine phosphoribosyl transferase (HPRT) RNA level. Data are presented as means ± SEM ($n = 4$).

real-time PCR analysis and presented them relative to their total intestinal expression. The highly Ca^{2+} selective channel TRPV6 forms the apical entry mechanism in active Ca^{2+} absorption in the small intestine. TRPM6 was expressed predominantly in cecum and colon, whereas no expression was detectable in duodenum and jejunum (Figure 1C). TRPM7 was equally expressed in the different segments of the intestinal tract. The epithelial Ca^{2+} channel TRPV6 was expressed primarily in duodenum, cecum, and colon but was not detectable in jejunum and ileum (Figure 1C).

Urine and Serum Analysis of Mice Fed Various Mg^{2+} Diets

Mice were fed a Mg^{2+}-deficient (0.005% wt/wt), -normal (0.19% wt/wt), or -enriched (0.48% wt/wt) diet for 10 d. At the end of this period, mice were housed for 24 h in metabolic cages, and urine samples were collected for investigation of the electrolyte metabolism of Mg^{2+} and Ca^{2+}. The serum Mg^{2+} and Ca^{2+} concentrations and total urinary Mg^{2+} and Ca^{2+} excretion are shown in Figures 2 and 3, respectively. The Mg^{2+}-deficient diet resulted in significant hypomagnesemia (Figure 2B), whereas serum Ca^{2+} values were not significantly altered in mice fed the various Mg^{2+} diets (Figure 2A). Dietary Mg^{2+} restriction significantly reduced the urinary Mg^{2+} and Ca^{2+} excretion compared with mice fed a Mg^{2+}-normal diet (Figure 3). The Mg^{2+}-enriched diet significantly increased the urinary Mg^{2+} and Ca^{2+} excretion, compared with mice fed the normal diet.

Effect of Dietary Mg^{2+} on $^{45}Ca^{2+}$ Absorption

To investigate whether dietary Mg^{2+} competes with the rate of Ca^{2+} absorption, two groups of C57BL6 mice were orally administered a $^{45}Ca^{2+}$ solution that contained 0.1 mM Ca^{2+} with or without 10 mM $MgCl_2$. Changes in the serum $^{45}Ca^{2+}$ concentration ($\Delta\mu M$) were measured within 10 min after administration of the $^{45}Ca^{2+}$ solutions. No significant differences in the rate of $^{45}Ca^{2+}$ absorption were observed between the group that was administered the $^{45}Ca^{2+}$ solution that contained an excess of Mg^{2+} and the group that received the $^{45}Ca^{2+}$ solution only (Figure 4).

Effect of Dietary Mg^{2+} Content on TRPM6 and TRPM7 Expression

The effect of dietary Mg^{2+} content on renal and intestinal expression of TRPM6 and TRPM7 mRNA was studied. The Mg^{2+}-deficient diet resulted in a significant upregulation of the

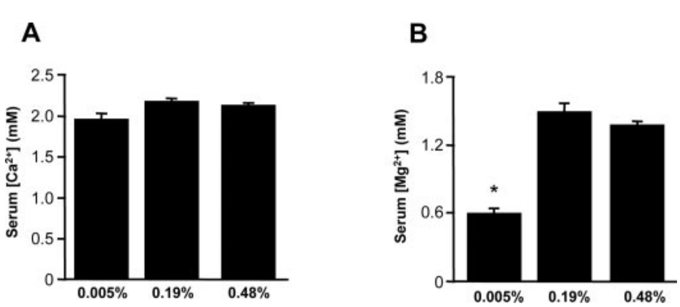

Figure 2. Effect of dietary Mg^{2+} content on serum Mg^{2+} and Ca^{2+} concentrations. The effect of a Mg^{2+}-deficient diet (0.005% wt/wt), Mg^{2+}-normal diet (0.19% wt/wt), and Mg^{2+}-enriched diet (0.48% wt/wt) on serum Mg^{2+} and Ca^{2+} concentration in mouse. Values are presented as means ± SEM ($n = 6$). $^*P < 0.05$ *versus* all groups.

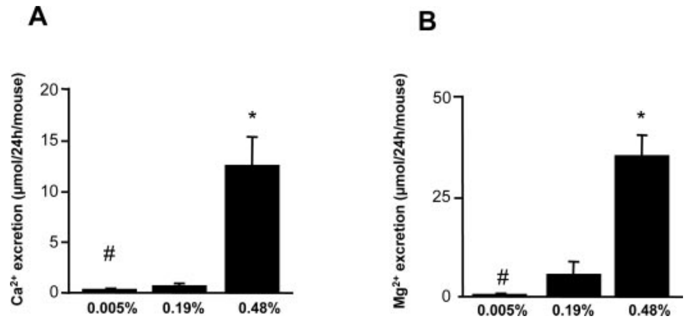

Figure 3. Effect of dietary Mg^{2+} content on the total urinary excretion of Mg^{2+} and Ca^{2+}. Net urinary excretion of Mg^{2+} (A) and Ca^{2+} (B) of mice on a Mg^{2+}-deficient diet (0.005% wt/wt), Mg^{2+}-normal diet (0.19% wt/wt), and Mg^{2+}-enriched diet (0.48% wt/wt). Values are presented as means ± SEM ($n = 6$). $^*P < 0.05$ *versus* all groups; $^\#P < 0.05$ *versus* all groups.

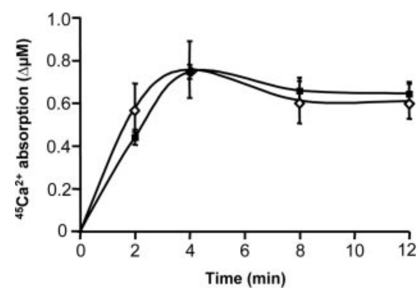

Figure 4. Effect of dietary Mg^{2+} content on Ca^{2+} absorption. Changes in the serum $^{45}Ca^{2+}$ concentration ($\Delta\mu M$) within 10 min after administration of $^{45}Ca^{2+}$ by oral gavage to C57BL6 mice. Data are averaged values ± SEM ($n = 5$) from 12-wk-old mice. ◇, 0.1 mM Ca^{2+}; ■, 0.1 mM Ca^{2+} + 10 mM Mg^{2+}.

renal TRPM6 mRNA level (Figure 5A). In addition, differences in dietary Mg^{2+} content did not influence renal TRPM7 mRNA expression (Figure 5B). Next, the abundance of TRPM6 protein in kidneys was examined. Figure 5C presents representative immunofluorescence labeling of distal tubules in sections of kidney from mice fed the Mg^{2+}-deficient (0.005% wt/wt), -normal (0.19% wt/wt), and -enriched (0.48% wt/wt) diets. More TRPM6 protein was detected in tissue from mice fed the Mg^{2+}-deficient (0.005% wt/wt) diet, as indicated by the increased staining in the kidney cortex. In these immunopositive tubules, TRPM6 was localized to the apical membrane of DCT. For semiquantitative assessment of TRPM6 protein expression, the relative amounts of immunopositive tubules in the complete kidney cortex were counted for each kidney section. Figure 5D presents the average values for each experimental group. The levels of TRPM6 protein expression in mice fed the Mg^{2+}-deficient (0.005% wt/wt) diet were significantly higher than those in the other groups. The regulation of TRPM6 and TRPM7 mRNA levels in colon was studied. The Mg^{2+}-enriched diet resulted in an upregulation of TRPM6 mRNA expression level in colon compared with mice fed the Mg^{2+}-deficient diet (Figure 6A). Like in kidney, variation in dietary Mg^{2+} content did not alter TRPM7 mRNA expression levels in complete colon

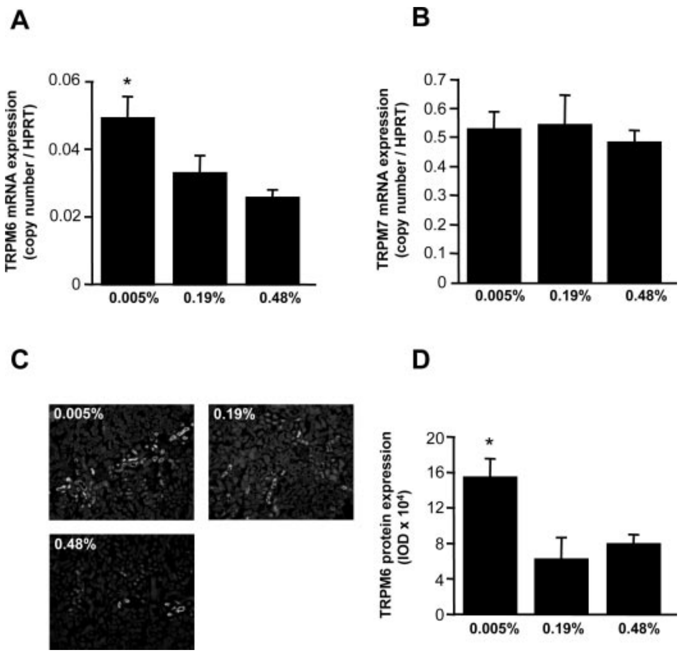

Figure 5. Effect of dietary Mg^{2+} content on renal expression levels of TRPM6 and TRPM7. (A and B) Real-time quantitative PCR was used to determine TRPM6 and TRPM7 mRNA expression levels in kidney of mice fed the Mg^{2+}-deficient diet (0.005% wt/wt), Mg^{2+}-normal diet (0.19% wt/wt), and Mg^{2+}-enriched diet (0.48% wt/wt). (C and D) TRPM6 protein abundance was determined by computerized analysis of immunohistochemical images and is presented as integrated optical density (IOD). Data are presented as means ± SEM (n = 6). *$P < 0.05$ *versus* all groups.

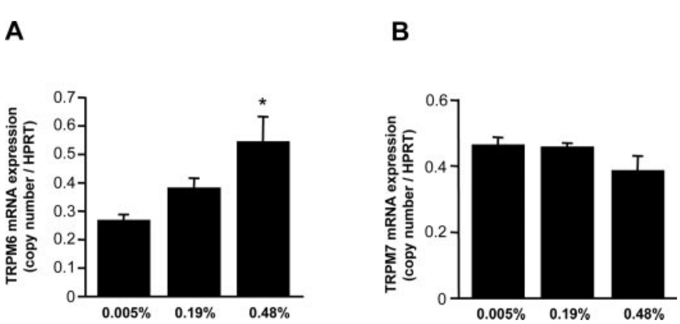

Figure 6. The effect of dietary Mg^{+} content on TRPM6 and TRPM7 mRNA expression in mouse colon. mRNA expression levels of TRPM6 (A) and TRPM7 (B) mRNA in colon of mice fed the Mg^{2+}-deficient diet (0.005% wt/wt), Mg^{2+}-normal diet (0.19% wt/wt), and Mg^{2+}-enriched diet (0.48% wt/wt) were assessed by quantitative real-time PCR analysis and calculated as ratio to HPRT RNA. Data are presented as means ± SEM (n = 6). *$P < 0.05$ *versus* 0.005% (wt/wt) Mg^{2+} diet.

sections (Figure 6B) and in the mucosa of the colon (data not shown).

Hormonal Regulation of Renal TRPM6 and TRPM7 Expression

To determine the effect of the calciotropic hormones, including $1,25(OH)_2D_3$, PTH, and 17β-E_2, on renal mRNA expression levels of TRPM6 and TRPM7, different animal models were used. 1α-OHase$^{-/-}$ mice represent a unique animal model to study the effect of the hormone $1,25(OH)_2D_3$ (23). To investigate the effect of PTH and 17β-E_2, we used PTX and OVX rats, respectively (18,19). Quantitative real-time PCR demonstrated that renal TRPM6 mRNA expression levels remained unaffected in 1α-OHase$^{-/-}$ compared with 1α-OHase$^{+/-}$ mice and 1α-OHase$^{-/-}$ mice supplemented with $1,25(OH)_2D_3$ (Figure 7A). Similarly, no effect of PTH was observed (Figure 7C). However, a two-fold decrease in TRPM6 mRNA expression levels was measured in kidneys of ovariectomized (OVX) rats (Figure 7E). Importantly, administration of 17β-E_2 normalized the TRPM6 expression levels in kidney of OVX rats. No differences in renal expression level of TRPM7 mRNA were observed in 1α-OHase$^{-/-}$, PTX, and OVX animals (Figure 7, B, D, and F).

Discussion

Our study shows novel regulatory hallmarks of TRPM6, further supporting a gatekeeper function in the process of transepithelial Mg^{2+} transport. First, this epithelial Mg^{2+} channel was expressed predominantly in mouse epithelia, including kidney, cecum, colon, and lung. Second, 17β-E_2 specifically upregulated TRPM6 mRNA expression in kidney, pointing to the first magnesiotropic hormone in the maintenance of the Mg^{2+} balance. Third, Mg^{2+} depletion increased TRPM6 mRNA expression in kidney, whereas a Mg^{2+}-enriched diet increased TRPM6 mRNA levels in colon. Fourth, the high abundant expression of TRPM6 in cecum and colon, together with the low expression level in duodenum and jejunum, suggests that active Mg^{2+} absorption takes place primarily in the distal part of the intestine.

Our study showed that the expression of TRPM6 is restricted to epithelial tissues, including kidney, whereas TRPM7 can be found in all tested tissues. Previous immunohistochemical studies in mouse kidney indeed indicated that TRPM6 is localized exclusively along the apical domain in DCT, which is in line with the postulated gatekeeper function in the process of active Mg^{2+} reabsorption (13). Here, we show that Mg^{2+} excretion and TRPM6 expression in kidney is strongly regulated by the dietary Mg^{2+} content. Dietary Mg^{2+} restriction resulted in Mg^{2+} conservation, whereas a Mg^{2+}-enriched diet increased urinary Mg^{2+} excretion. The Mg^{2+}-deficient diet resulted in a significant upregulation of renal TRPM6 mRNA and protein levels, whereas the enriched diet tended to reduce TRPM6 abundance. TRPM7 expression was not influenced by the dietary Mg^{2+} content, supporting a general role in cellular Mg^{2+} homeostasis (14,15). It is interesting that dietary Mg^{2+} restriction in humans also leads to renal Mg^{2+} conservation (24–26), whereas high dietary Mg^{2+} intake markedly stimulates Mg^{2+} excretion without significant increases in plasma Mg^{2+}, which is in accordance with the results of our study in mouse (27). Therefore, upregulation of TRPM6 in kidney supports a critical role of this channel in facilitating maximal Mg^{2+} reabsorption during Mg^{2+} deficiency.

It is interesting that dietary Mg^{2+} content also influenced the Ca^{2+} excretion in our study, because Mg^{2+} restriction resulted in Ca^{2+} conservation, whereas Mg^{2+} supplementation led to an

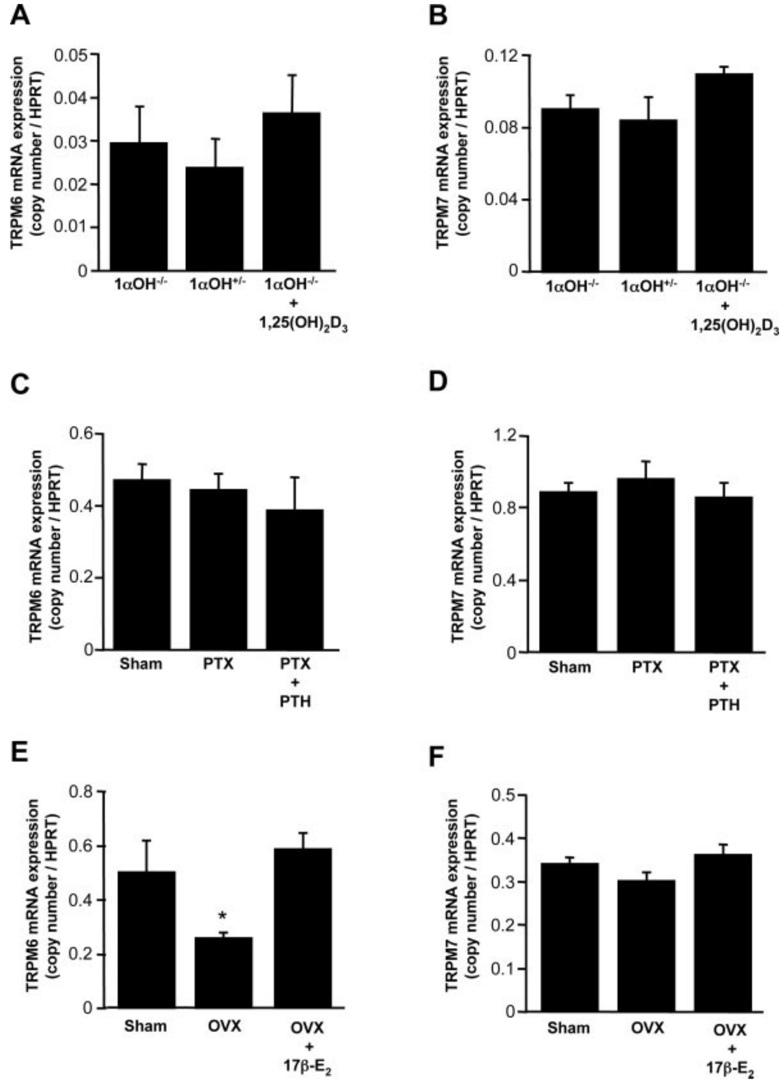

Figure 7. Effect of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), parathyroid hormone (PTH), and 17β-estradiol (17β-E_2) on expression of TRPM6 and TRPM7 channels mRNA in kidney. Effect of $1,25(OH)_2D_3$, PTH, and 17β-E_2 in renal TRPM6 (A, C, and E) and TRPM7 (B, D, and F) mRNA expression levels was quantified by real-time PCR analysis and normalized for HPRT expression. Data are presented as means ± SEM (n = 4 to 5). *$P < 0.05$ *versus* all groups.

increased urinary Ca^{2+} excretion. In accordance with this finding, Shafik and Quamme (7) showed that urinary Ca^{2+} excretion decreases in rats that are maintained on a low-Mg^{2+} diet. This coupling between the Mg^{2+} and Ca^{2+} excretion observed under various dietary Mg^{2+} regimens could occur at the level of intestinal absorption and/or renal excretion. Because dietary Mg^{2+} contents did not affect the intestinal Ca^{2+} absorption rate, the coupling mechanism presumably resides within the kidney. This suggests the existence of a common pathway or regulatory mechanism in facilitating urinary Mg^{2+} and Ca^{2+} excretion. In this respect, it is interesting to notice that paracellin-1 has been shown to be instrumental for the paracellular reabsorption of both divalent cations in TAL (28,29). Importantly, the majority of the renal Mg^{2+} reabsorption takes place in this particular segment, where the lumen positive transepithelial potential difference drives paracellular transport of cations (30). It therefore can be envisaged that Mg^{2+} and Ca^{2+} are competitively transported by paracellin-1. This competition suggests that paracellular Ca^{2+} reabsorption in TAL is favored in the presence of a low luminal Mg^{2+} concentration resulting from Mg^{2+} deficiency. Conversely, renal Ca^{2+} excretion will be increased by a high Mg^{2+} load as a result of a Mg^{2+}-enriched diet. Indeed, Ikari *et al.* (31) demonstrated that $^{45}Ca^{2+}$ transport across monolayers of cells that express paracellin-1 is inhibited by increased Mg^{2+} concentration. In addition, in patients with Gitelman syndrome or isolated dominant hypomagnesemia, the observed hypomagnesemia is accompanied by a seriously diminished urinary Ca^{2+} excretion (32). Therefore, competition between Mg^{2+} and Ca^{2+} for a common paracellular route could explain the observed coupling between the urinary Mg^{2+} and Ca^{2+} excretion in response to different Mg^{2+} diets. In addition, the extracellular Ca^{2+} sensing receptor could play a role in the coupling between the Mg^{2+} and Ca^{2+} excretion under various dietary Mg^{2+} conditions, because the Ca^{2+} sensing receptor is believed to sense Ca^{2+} and Mg^{2+} levels and to regulate the reabsorption of these ions (33).

Despite early proposals for the existence of a specific hormonal control of the Mg^{2+} balance, our understanding of the endocrine factors that regulate circulating or urinary Mg^{2+} is incomplete. Several hormones, including PTH, calcitonin, vitamin D, insulin, glucagons, antidiuretic hormone, aldosterone, and sex steroids, have been reported to influence the Mg^{2+} balance (2,34,35). It was suggested that these hormones are only indirect regulators of Mg^{2+} homeostasis, because Mg^{2+} lacks a specific endocrine control similar to what exists for Ca^{2+}, Na^{+}, and K^{+} (34). It is interesting that our study indicates a magnesiotropic role for estrogens in Mg^{2+} homeostasis *via* the regulation of the Mg^{2+} channel TRPM6. In OVX rats, the renal TRPM6 mRNA level was significantly reduced and subsequently normalized by 17β-E_2 supplementation. It has been demonstrated that postmenopausal hypermagnesuria significantly decreased after estrogen substitution therapy (36,37). This finding is in line with our results and suggests that 17β-E_2 increases Mg^{2+} reabsorption *via* an enhanced renal TRPM6 expression. This stimulatory effect of 17β-E_2 could be attributable to enhanced transcriptional activity or mRNA stabilization. Thus far, detailed analysis did not result in 17β-E_2–responsive elements in the putative promoter sequence of human and mouse TRPM6. The magnesiotropic action of estrogens in Mg^{2+} homeostasis *via* regulation of TRPM6 could be of importance during the menstrual cycle, pregnancy, and preeclampsia. However, different studies that have measured estrogen levels in plasma from preeclamptic women have been inconsistent (38–41). It is interesting that menstrual migraine is preceded by a decline in the plasma estrogen level and shows a high incidence of free ionized Mg^{2+} deficiency (42). Further studies should elucidate the possible interrelationship of estrogens and TRPM6 in Mg^{2+} homeostasis during the menstrual cycle, pregnancy, and preeclampsia.

Previous reports demonstrated that PTH stimulates Mg^{2+} reabsorption in TAL and DCT (43,44). In addition, $1,25(OH)_2D_3$ has been shown to enhance the influx of Mg^{2+} in a mouse DCT cell line (5). Our study suggests that PTH and $1,25(OH)_2D_3$ are not involved in the stimulation of Mg^{2+} reabsorption *via* upregulation of renal TRPM6 expression levels because $1,25(OH)_2D_3$ and PTH did not change the TRPM6 expression in kidney. In addition, Karbach (45,46) demonstrated that cellular Mg^{2+} transport in rat colon is not responsive to 1,25-dihydroxyvitamin D_3.

The predominant expression of TRPM6 in cecum and colon together with the low expression level in duodenum and jejunum suggests that active Mg^{2+} absorption takes place exclusively in the distal part of the intestine. Although initial studies already detected TRPM6 in small intestine, a quantitative comparison in the expression along the gastrointestinal tract was not established (11,13). The highly abundant expression of TRPM6 in colon is further supported by the fact that colectomy in rat results in a syndrome of Mg^{2+} deficiency with decreased urinary Mg^{2+} excretion, normal serum Mg^{2+} levels, and, interestingly, decreased bone Mg^{2+} content (47). Remarkably, our data further suggest that active intestinal Mg^{2+} absorption only partly overlaps with the process of active Ca^{2+} absorption. The transepithelial absorption of Ca^{2+} takes place predominantly in duodenum and possibly colon as illustrated by the robust expression of the epithelial Ca^{2+} channel TRPV6 in these particular intestinal segments. TRPV6 constitutes the luminal Ca^{2+} entry mechanism in active Ca^{2+} absorption (48,49). Therefore, active Mg^{2+} and Ca^{2+} absorptions simultaneously take place in the distal part of the intestine, whereas in duodenum, only active Ca^{2+} absorption occurs. Expression levels of TRPM6 mRNA in colon were upregulated by the Mg^{2+}-enriched diet, whereas Mg^{2+} restriction did not significantly affect TRPM6 mRNA expression levels. This suggests that mice can increase their transcellular Mg^{2+} absorption capacity when fed a Mg^{2+}-enriched diet. In comparison with Ca^{2+} absorption and TRPV6 expression, Brown *et al.* (50) demonstrated that in rats fed a low-Ca^{2+} diet, TRPV6 mRNA levels in duodenum were significantly increased. In contrast, van Abel *et al.* (51) showed that supplementation of 1α-OHase$^{-/-}$ mice with a high dietary Ca^{2+} intake resulted in an increase of duodenal TRPV6 mRNA levels. Therefore, if the calciotropic hormone 1,25-dihydroxyvitamin D_3 is not present, then duodenal TRPV6 mRNA expression is upregulated in response to high dietary Ca^{2+} intake. The important difference between intestinal regulation of TRPM6 and TRPV6 is that no magnesiotropic hormone has been identified to regulate intestinal TRPM6 expression and thereby Mg^{2+} absorption. We suggest that, physiologically, an excess of Mg^{2+} absorption as a result of high dietary Mg^{2+} intake together with TRPM6 upregulation in colon can be totally corrected by the kidney. Normally, the kidney excretes only 2 to 4% of the filtered Mg^{2+} but is capable of increasing fractional excretion to nearly 100% in the face of increased serum Mg^{2+} levels (52). Therefore, the absence of a magnesiotropic hormone that regulates intestinal TRPM6 expression and Mg^{2+} absorption could explain why TRPM6 mRNA levels in colon are upregulated in response to high dietary Mg^{2+} content.

The unaltered expression levels of TRPM6 mRNA in colon during Mg^{2+} restriction suggests that the Mg^{2+} absorptive capacity is sufficient to obtain maximal transcellular Mg^{2+} transport. The ubiquitous and diet-unresponsive expression of TRPM7 suggests that this particular Mg^{2+} channel does not participate in the extracellular Mg^{2+} homeostasis. In line with previous studies, this indicates that TRPM7 is involved primarily in cellular Mg^{2+} homeostasis (14,15).

It is interesting that besides kidney and the intestine, TRPM6 is highly abundant in lung tissue. The exact function of TRPM6 in this organ, however, remains to be elucidated. The importance of Mg^{2+} in lung is supported by the fact that dietary Mg^{2+} intake is related directly to lung function, airway reactivity, and respiratory symptoms in the general population (53). Moreover, treatment of patients who have chronic asthma is currently receiving attention because of a role for Mg^{2+} in relaxation of arterial and bronchial smooth muscle cells (54–56). Further research certainly is needed to determine the precise function of TRPM6 in lung (patho)physiology.

Conclusion

TRPM6 is expressed predominantly in kidney, cecum, colon, and lung, suggesting that these organs are involved primarily in Mg^{2+} (re)absorption. Furthermore, we provide evidence that the intestinal site of active Mg^{2+} absorption is located primarily in the distal part of the intestine. In addition, 17β-E_2 and dietary Mg^{2+} are positively involved in the regulation of TRPM6, underlining the gatekeeper function of this epithelial Mg^{2+} channel.

Acknowledgments

This study was supported by the Dutch Kidney Foundation (C02.2030, C03.6017) and the Dutch Organization of Scientific Research (Zon-Mw 016.006.001).

We thank NV Organon Nederland for kindly providing kidney tissue from the OVX rat model study. In addition, we thank Dr. René St. Arnaud for kindly providing kidney tissue from the 1α-OHase$^{-/-}$ mouse model and Dr. Marlies Cornelissen for stimulating discussions.

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