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New insights in the regulation of the epithelial magnesium channel TRPM6 in kidney and intestine

Wouter Tiel Groenestege
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General introduction

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**Mg²⁺ homeostasis**

Magnesium (Mg²⁺) is the second most abundant intracellular cation and the fourth most abundant cation in the body. Approximately 50% of the total body Mg²⁺ is mineralized in bone, whereas almost the other half is localized intracellularly and only 1% of the total body Mg²⁺ is present extracellularly (1). Of the intracellular Mg²⁺, merely up to 10% exists as the metabolically active ionized form which plays an essential role as a co-factor in many biochemical and physiological processes such as activation of ATPases, regulation of many plasma membrane ion channel activities, and translational processes (2-4). In serum, 60% of the Mg²⁺ exists as the physiologically active ionized form, 30% is protein bound, mainly to albumin (5), and the remaining 10% forms complexes with serum anions such as phosphates and citrates (6). Disturbances of serum Mg²⁺ levels can result in serious symptoms such as seizures and coma (1, 7). Therefore, an adequate homeostasis of Mg²⁺ resulting in tightly regulated serum Mg²⁺ levels, within a narrow range of 0.7 to 1.1 mM, is essential (8). The extracellular Mg²⁺ concentration is controlled by the concerted action of renal Mg²⁺ reabsorption, intestinal absorption, and exchange of Mg²⁺ from bone.

**Hypomagnesemia**

Hypomagnesemia (serum Mg²⁺ < 0.7 mM), is a common phenomenon occurring up to 15% in the general population (9), 12% in hospitalized patients (10) and as high as 65% in intensive care patients (11-13). This is of special interest in view of the association between hypomagnesemia and common chronic diseases such as diabetes mellitus type II, hypertension, coronary heart disease and asthma bronchiale (14-18). Importantly, aggravation of these disorders is observed with further Mg²⁺ depletion. Mg²⁺ deficiency has an effect on multiple body functions. Symptoms of Mg²⁺ deficiency are mostly related to muscle dysfunctioning, such as muscle weakness, tetany, prolonged QT interval and cardiac arrhythmias. Failure of early diagnosis or noncompliance with treatment can be fatal or result in permanent neurological damage. A long-term complication seen in many adult patients with chronic hypomagnesemia is chondrocalcinosis, which can lead to impairment of joint function (19). At present hypomagnesemia is treated with oral Mg²⁺ supplements (20). However, normalization of serum Mg²⁺ in patients with chronic hypomagnesemia is difficult to achieve because high doses of Mg²⁺ cause severe diarrhea. In general,
reduced dietary intake, intestinal malabsorption or renal Mg\(^{2+}\) loss can result in secondary or primary hypomagnesemia. This can be the result of for instance the use of osmotic agents, diuretics, alcohol, and drugs such as foscarnet and cisplatin (1). Furthermore, primary and secondary Mg\(^{2+}\) deficiency is observed in several monogenic disorders, which will be discussed in more detail further on.

**Hypermagnesemia**

Compared to hypomagnesemia, hypermagnesemia (serum Mg\(^{2+}\) > 1.1 mM) is rare, but frequently fatal. Symptoms of hypermagnesemia include lethargy, nausea, loss of deep tendon reflexes, paralysis and coma (1). The most common cause of hypermagnesemia is impaired renal excretion of Mg\(^{2+}\) which can be caused by progressive renal insufficiency, lithium therapy or hypocalciuric hypercalcemia (1). Dialysis is a rapid way to lower Mg\(^{2+}\) levels in patients with renal insufficiency.

**Intestinal Mg\(^{2+}\) absorption**

Normal dietary intake of Mg\(^{2+}\) is 300 to 360 mg/d (12.5-15 mmol/d) of which 40-50% is absorbed. Dietary Mg\(^{2+}\) intake is the only source by which the body can replete Mg\(^{2+}\) stores and a Mg\(^{2+}\) intake of approximately 4.0 mg/kg/d is necessary to maintain body Mg\(^{2+}\) homeostasis (21, 22). The net intestinal Mg\(^{2+}\) absorption is dependent on the fractional Mg\(^{2+}\) absorption within a specific segment of the intestine, the length of that specific segment and the transit time of the food bolus. Considering these parameters, the ileum and colon absorb most of the ingested Mg\(^{2+}\) (23, 24). In the intestine Mg\(^{2+}\) is reabsorbed via an unsaturable passive paracellular pathway which is dependent on the electrochemical gradient and a saturable active transcellular pathway (24, 25). The total intestinal Mg\(^{2+}\) absorption can be described by a curvilinear function which is proposed to be the sum of passive and transcellular transport mechanisms. The paracellular passive transport mechanism linearly rises with elevated luminal Mg\(^{2+}\) concentrations whereas the active transcellular transport mechanism saturates at high luminal Mg\(^{2+}\) concentrations and is of functional importance at low luminal Mg\(^{2+}\) concentrations (24, 25).

**Renal Mg\(^{2+}\) reabsorption**

The principal organ responsible for the regulation of the body Mg\(^{2+}\) balance is the kidney, which tightly matches the intestinal absorption of Mg\(^{2+}\) (figure 1). About 80% of the total plasma Mg\(^{2+}\) is filtered in the glomeruli (26, 27). With a glomerular filtration rate of 125 ml/min, approximately 140 mmol/d Mg\(^{2+}\) is filtered, of which the majority is subsequently reabsorbed along the nephron (4, 28). Approximately 20% of Mg\(^{2+}\) is reabsorbed by the proximal tubule (PCT). However, the bulk amount of Mg\(^{2+}\) (70%) is reabsorbed by the thick ascending limb of Henle’s loop (TAL), which mediates Mg\(^{2+}\) reabsorption via passive paracellular transport. The distal convoluted tubule (DCT) actively reabsorbs 5 to 10% of the filtered Mg\(^{2+}\) and the reabsorption rate in this segment defines the final urinary Mg\(^{2+}\) concentration, as no reabsorption takes place beyond this segment (4). Finally, 3 to 5% of the filtered Mg\(^{2+}\) is excreted in the urine. In the presence of hypermagnesemia the fractional excretion of Mg\(^{2+}\) can raise to nearly 100%. In contrast, in the occurrence of hypomagnesemia or reduced Mg\(^{2+}\) intake, the kidney minimizes urinary losses by increased reabsorption and can lower the fractional excretion to less than 1% (1, 29).

**Figure 1. Mg\(^{2+}\) reabsorption along the nephron**

The percentages of the filtered Mg\(^{2+}\) that are being reabsorbed by the various nephron segments and the final urinary excretion of Mg\(^{2+}\) is indicated. PCT, proximal convoluted tubule; TAL, thick ascending limb of Henle’s loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct.
Paracellular Mg$^{2+}$ transport

Passive paracellular Mg$^{2+}$ reabsorption takes place in PCT and TAL. In PCT approximately 20% of the filtered Mg$^{2+}$ is reabsorbed. Although Mg$^{2+}$ reabsorption in this segment is not well understood it appears to be paracellular and dependent on the filtered load, and net salt and water reabsorption (30). The major site of paracellular Mg$^{2+}$ transport is the TAL, where Mg$^{2+}$ transport is driven by a favorable lumen-positive electrochemical gradient, which is generated by the transcellular reabsorption of NaCl. Transcellular NaCl reabsorption is dependent on the concerted activity of Na$^+$, K$^+$, 2Cl$^-$ cotransporter (NKCC2), renal outer medullary K$^+$ channel (ROMK), Na$^+$,K$^+$-ATPase and the renal Cl$^-$ channel CLC-Kb (figure 2A) (31). The lumen-positive electrochemical gradient allows Mg$^{2+}$ to be transported into the blood compartment via the tight junction proteins claudin-16 and -19 (figure 2A) (32, 33). Furthermore, plasma Mg$^{2+}$ and Ca$^{2+}$ levels are monitored by the Ca$^{2+}$ sensing receptor (CaSR) which can also influence Mg$^{2+}$ reabsorption in TAL (figure 2A) (34).

Transcellular Mg$^{2+}$ transport

Hypothetically, the process of transcellular Mg$^{2+}$ transport in kidney and intestine is envisaged by the following sequential steps (figure 2B). Driven by a favorable transmembrane potential, Mg$^{2+}$ enters the epithelial cell through the apical epithelial Mg$^{2+}$ channel transient receptor potential subfamily M, member 6 (TRPM6). Next, Mg$^{2+}$ will diffuse through the cytosol to be extruded actively against an electrochemical negative gradient across the basolateral membrane (35). The key molecules that represent the cytosolic Mg$^{2+}$-binding proteins (if present) and the basolateral Mg$^{2+}$ extrusion mechanism in the process of transcellular Mg$^{2+}$ transport are still elusive. Until now, specific Mg$^{2+}$-binding proteins have not been identified, but it is interesting to mention that the Ca$^{2+}$-binding proteins parvalbumin and calbindins, which are present in DCT, also bind Mg$^{2+}$ (35, 36). For the basolateral Mg$^{2+}$ extrusion mechanism most physiological studies favor a Na$^+$-dependent exchange mechanism (3). Other candidate mechanisms include an ATP-dependent Mg$^{2+}$ pump. Importantly, Mg$^{2+}$ entry into the cells appears to be the rate-limiting step and the site of regulation. In kidney, the function of DCT in active ion transport is underlined by the fact that cells of this nephron segment have the highest energy consumption of the nephron (37). In addition to the proteins mentioned, the functioning of the Na$^+$, Cl$^-$ cotransporter (NCC), which is exclusively

Figure 2. Paracellular and transcellular Mg$^{2+}$ reabsorption in kidney

(A) Mg$^{2+}$ reabsorption in TAL is driven by a favorable electrochemical gradient generated by the transcellular reabsorption of NaCl. Mg$^{2+}$ is transported down this gradient through the tight junction proteins claudin-16 and claudin-19 to the blood compartment. (B) Active Mg$^{2+}$ reabsorption is presumably carried out as a 3-step-process in DCT. Following entry of Mg$^{2+}$ in DCT through the epithelial Mg$^{2+}$ channel, TRPM6, Mg$^{2+}$ diffuses possibly bound by cytosolic proteins to the basolateral membrane. As the extrusion mechanisms, a basolateral Na$^+$/Mg$^{2+}$ exchanger and/or ATP-dependent Mg$^{2+}$ pump are postulated. The Na$^+$,K$^+$-ATPase complex including its γ-subunit control this transepithelial Mg$^{2+}$ transport. In this way, net Mg$^{2+}$ reabsorption from the luminal space to the extracellular compartment takes place. See text for details and abbreviations.
present in DCT, the CaSR and CLC-Kb are also important for active Mg^{2+} reabsorption in DCT (34, 38-40) (figure 2B). The role of NCC, CLC-Kb and CaSR in Mg^{2+} homeostasis will be discussed in more detail in the following paragraphs.

Hormonal regulation of Mg^{2+} reabsorption
Several hormones including, parathyroid hormone (PTH), calcitonin, 1,25-dihydroxy-vitamin-D_{3} (1,25(OH)_{2}D_{3}), insulin, glucagon, vasopressin, aldosterone, and sex steroids (estrogens) have been reported to influence Mg^{2+} transport in vitro (41-43). Moreover, it was demonstrated that Mg^{2+} transport in DCT is influenced by dietary Mg^{2+} restriction and various hormones and that PTH in vivo stimulates Mg^{2+} reabsorption in TAL and DCT (44-46). In addition, it was shown that Mg^{2+} transport in rat colon was not responsive to 1,25(OH)_{2}D_{3} (47, 48). In spite of the reports described above, hormonal regulation of active transcellular Mg^{2+} transport remains largely unknown (4, 30, 45, 49). Moreover, it has been suggested that the hormones influencing the Mg^{2+} balance 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^{+} (42). Thus far, there is no evidence of specific physiological hormonal control of Mg^{2+} (re)absorption. Apical Mg^{2+} influx is the initial step of transcellular Mg^{2+} transport. Therefore, this step is an ideal target for hormonal regulation of active Mg^{2+} (re)absorption. The following paragraphs will highlight the distinctive features and the physiologic relevance of the proteins that represent the gatekeepers of epithelial Mg^{2+} transport.

The gatekeepers of epithelial Mg^{2+} transport
The transient receptor potential (TRP) superfamily consists of many cation channels of which most permeate both monovalent and divalent cations. Thus far, 28 mammalian TRP channels have been identified, which can be subdivided into six main subfamilies: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and the TRPA (ankyrin) groups (50). TRP channels are expressed in almost every tissue and cell type, and play an important role in the regulation of various cell functions. Each of the proteins is a cation channel composed of six transmembrane-spanning domains and a conserved pore-forming region that assemble in a tetrameric configuration (figure 3) (51-53). Two members of this superfamily, TRPM6 and TRPM7 have been identified as key regulators of total body and cellular Mg^{2+} homeostasis, respectively. TRPM6 and TRPM7 are unique bifunctional proteins combining Mg^{2+}-permeable cation channel properties with protein kinase activity (51, 54) and are therefore referred to as chanzymes (55).

**TRPM6**
Investigations of families suffering from autosomal-recessive hypomagnesemia with secondary hypocalcemia (HSH), which will be described in the following paragraphs, identified mutations in TRPM6 (20, 56). Through these and other studies, TRPM6 was discovered as an essential molecular determinant in transcellular Mg^{2+} transport in renal and intestinal epithelia (20, 35, 56). TRPM6 is one of the eight members of the identified TRPM cation channel subfamily and is composed of 2022 amino acids encoded by a large gene that contains 39 exons (20, 51, 56). The TRPM6 protein shows 52% homology with its closest homologue TRPM7. TRPM6 displays a restricted expression pattern and is predominantly present in (re)absorbing epithelia (20, 35, 56). Interestingly, three short alternative 5' exons, called
Mg2+ leak in patients with HSH, emphasizes the important role of TRPM6 in renal cells (20, 35). The renal and intestinal expression of TRPM6, in addition to the renal TRPM6 is detected along the brush-border membrane of the absorptive epithelial cells (20, 35). The renal and intestinal expression of TRPM6, in addition to the renal Mg2+ leak in patients with HSH, emphasizes the important role of TRPM6 in renal and intestinal Mg2+ (re)absorption. Moreover, the role of TRPM6 in total body Mg2+ homeostasis is underlined by the fact that TRPM6 and TRPM7 are not functionally redundant in humans, since the ubiquitously expressed TRPM7 can not rescue the severe phenotype of HSH patients (58).

TRPM7

Prior to the discovery of TRPM6, the Mg2+ channel TRPM7 was cloned from mouse brain cDNA (54). TRPM7 is ubiquitously expressed and is essential for cell growth and viability rather than for total body Mg2+ balance (58-60). The ubiquitous expression of TRPM7 supports its role in cellular viability and cellular Mg2+ homeostasis. It was demonstrated that Mg2+ supplementation of cells that lack TRPM7 expression rescued growth arrest and cell lethality that was caused by TRPM7 inactivation (60). Although TRPM7 is permeable for Ca2+, as well as trace divalents such as Zn2+, Ni2+, Ba2+, and Co2+, supplementation with these cations was ineffective, indicating the specific effect of Mg2+ on these cellular processes. TRPM7 has also been implicated to play a role in anoxic neuronal death (59, 61, 62), cell adhesion and actomyosin contractility (63, 64), and bone generation (65). In addition, a TRPM7 gene variant has been implicated in the neurodegenerative disorder amyotrophic lateral sclerosis-parkinsonism/dementia complex of Guam (66).

Mg2+ permeability of TRPM6

Electrophysiological characterization of TRPM6 demonstrated that TRPM6-transfected HEK293 cells exhibited outwardly rectifying currents upon establishment of the whole-cell configuration as demonstrated for TRPM7 (35, 54, 59). It is intriguing that at physiologic membrane potentials of the DCT cell (-80 mV), small but significant inward currents were observed in TRPM6-expressing HEK293 cells with all tested divalent cations as the sole charge carrier (35). However, mutations in TRPM6 are linked directly to HSH, emphasizing that this channel is an essential component of the epithelial Mg2+ uptake machinery. Interestingly, HEK293 cells transfected with the TRPM6 mutants identified in HSH patients (TRPM6-Ser90X and TRPM6-Arg736fsX737) displayed currents with similar amplitude and activation kinetics as nontransfected HEK293 cells, indicating that these mutant proteins are nonfunctional. This is in line with the postulated function of TRPM6 being the Mg2+ influx step in epithelial Mg2+ transport (35). The unique permeation rank order determined from the inward current amplitude at -80 mV was comparable to TRPM7 (Ba2+ ≥ Ni2+ > Mg2+ > Ca2+) (35, 54). Micropuncture studies have demonstrated that the luminal concentration of Mg2+ in DCT ranges from 0.2 to 0.7 mM (4). Because the luminal Ca2+ concentration is also in the millimolar range, the apical Mg2+ influx pathway should exhibit a higher affinity for Mg2+ than for Ca2+. It is interesting that dose-response curves for the Na+ current block at -80 mV indicated a four times higher partition coefficient value for Ca2+ compared with Mg2+ (35). These data suggest that the pore of TRPM6 has a higher affinity for Mg2+ than for Ca2+. In this way, TRPM6 comprises a unique channel because all known Ca2+-permeable channels, including members of the TRP superfamily, generally display a 10 to 1000 times lower affinity for Mg2+ than for Ca2+ (35).

TRPM6 regulation

Chronic metabolic acidosis results in renal Mg2+ wasting, whereas chronic metabolic alkalosis is known to exert the reverse effect. It was hypothesized that these adaptations are mediated, at least in part, by the renal Mg2+ transport protein TRPM6. It was demonstrated that chronic metabolic acidosis decreased renal TRPM6 expression, increased Mg2+ excretion, and decreased serum Mg2+ concentration, whereas chronic metabolic alkalosis resulted in the exact opposite effects (67). Thus, these data suggest that regulation of TRPM6 significantly contributes to the effect of the acid-base status on renal Mg2+ handling. Interestingly, extracellular protons enhance TRPM6 and TRPM7 inward currents by decreasing the divalent affinity to the channels (68, 69). Recently, the amino acids determining the pH sensitivity of TRPM6 and TRPM7 have been identified (70). Moreover, it was shown that TRPM7, in response to the extracellular pH, may contribute to the regulation of neurotransmitter release (71). The sensitivity of TRPM6 and TRPM7 to pH represents a novel feature and implies that both channels...
may play a role under acidic pathological conditions (68). In addition to the external pH, TRPM6 and TRPM7 are also regulated by intracellular Mg2+ levels. It was demonstrated that elevation of the intracellular Mg2+ concentration reduced the TRPM6-induced current, indicating that the channel is regulated by intracellular Mg2+ or Mg2+ bound to ATP (Mg2+·ATP) (35). These processes are called the Mg2+-inhibited cation current (MIC) or Mg2+/ATP-sensitive cation current (MgNuM), respectively (59, 72). Previous studies reported that TRPM7 channel activity is strongly suppressed by Mg2+ inhibition, TRPM7 is also inactivated by hydrolysis of the phosphoinositide lipid PIP2 (74). Interestingly, TRPM7 was discovered on the basis of binding to phospholipase C (PLC), which cleaves PIP2 (54). This raises the question whether there is a link between the two apparently dissimilar modes of channel inhibition: an increase in Mg2+ and hydrolysis of PIP2. It was postulated that Mg2+ could possibly activate PLC that associates with TRPM7 (55). This possibility is strengthened by a study in B lymphocytes that demonstrated the presence of phosphoinositide-specific PLC which is activated when the Mg2+ concentration is raised from 30 to 1000 µM (76). The role of PIP2 and PLC in the regulation of TRPM6 channel activity needs to be established.

TRPM6 and TRPM7 assembly

It was postulated that TRPM6 requires assembly with TRPM7 to form functional channel complexes in the plasma membrane and that disruption of multimer formation by a mutated TRPM6 variant, TRPM6-S141L, results in HSH (57). It was shown that, TRPM6-S141L was not directed to the cell surface by TRPM7 and failed to interact with the co-expressed TRPM7. Remarkably, in contrast to TRPM7, Gudermann and co-workers found that TRPM6 expression in *Xenopus laevis* oocytes and HEK293 cells did not entail significant ion currents (57). In addition, Schmitz et al. also supported the idea that TRPM6 and TRPM7 associate and that trafficking of TRPM6 to the cell surface is strongly dependent on TRPM7 co-expression (58). In variance, Voets et al. measured significantly larger currents in TRPM6-transfected HEK293 cells compared to mock-transfected cells (35). Accordingly, Li and colleagues indicated that TRPM6, TRPM7 and TRPM6/TRPM7 channels represent three different channels with unique functional characteristics (69). A plausible explanation for these discrepant findings is still elusive.

The α-kinase domain

Unlike other members of the TRP channel family, TRPM6 and TRPM7 contain long carboxy-terminal domains including an active threonine/serine kinase, which belongs to the atypical family of eukaryotic α-kinases (54). Genomic studies identified six α-kinases in mammals, including the ones fused to TRPM6 and TRPM7. The α-kinases share no sequence homology with the conventional kinases (77). A key question concerns the nature of the mechanisms underlying the activation and regulation of TRPM6 and TRPM7. In particular, what is the function of the atypical protein α-kinase domain located in the carboxyl terminus? The TRPM7 kinase is able to undergo autophosphorylation and to phosphorylate substrates such as myelin basic protein and histone H3 on serine and threonine residues (78). The kinase is specific for ATP and Mg2+ or Mn2+ is required for optimal activity. It was demonstrated that TRPM6 can phosphorylate TRPM7, but not vice versa (58). Recently, annexin 1 and myosin II A have been identified as endogenous substrates of the TRPM7 kinase (63, 79). Although, the biological role of annexin 1 phosphorylation via TRPM7 is currently unknown, both proteins have been linked to processes of cell survival and growth (62, 80). Future studies should elucidate whether the kinase domains of TRPM6 and TRPM7 have specific cellular phosphorylation targets that modulate ion channel activity and, therefore, the Mg2+ balance. It has been suggested that TRPM7 could potentially serve both as a Mg2+ uptake mechanism and a Mg2+ sensor when the ion channel domain could modulate the phosphotransferase activity by increasing intracellular Mg2+ or directly via conformational changes induced by gating of the channel (81). This modification could result in the phosphorylation of yet unidentified substrates providing real time information on channel activity or cellular Mg2+ status. While there is general consensus that TRPM6 and TRPM7 are inhibited by free intracellular Mg2+, the functional role of intracellular levels of Mg2+·ATP and the kinase domain in regulating channel activity remain controversial. Several groups suggested that the kinase domain is essential for channel activity (54, 82), whereas others indicated that it is not involved (60, 83). Furthermore, the phosphotransferase activity of the TRPM7 kinase domain affected channel activity by regulating the sensitivity of the channel to inhibition by Mg2+ and Mg2+·ATP (60, 84, 85), but this finding was not generally confirmed (74, 82). Further experiments are needed to elucidate these inconsistent results and firmly establish the functional role of the kinase domain and intracellular levels of Mg2+·ATP in regulating TRPM6 and TRPM7 channel activity.
Inherited disorders with primary hypomagnesemia

The investigation of families with primary hereditary hypomagnesemia has greatly facilitated the identification of genes and proteins directly involved in renal Mg\textsuperscript{2+} handling. Our knowledge concerning the epithelial Mg\textsuperscript{2+} transport pathways has evolved from physiological studies concerning these diseases described below. In humans, several inherited renal tubular disorders are known in which disturbances of serum Mg\textsuperscript{2+} levels are a primary or a secondary phenomenon (table 1 and 2). So far, seven disorders have been described in which hypomagnesemia is a primary defect. Two disorders are inherited in the autosomal dominant form, four in an autosomal recessive form and one is maternally inherited via mitochondrial DNA (table 1). The gene defects of isolated recessive renal hypomagnesemia (IRH) and isolated dominant hypomagnesemia (IDH) remain to be identified (table 1) (86, 87).

Isolated recessive renal hypomagnesemia (IRH)

In the literature a single family has been described thus far, which displays IRH (88). IRH is characterized by hypomagnesemia due to renal Mg\textsuperscript{2+} wasting, convulsions in the first year of life, psychomotor retardation during childhood and moderate mental retardation. Interestingly, in all disorders known with primary hypomagnesemia, a mutual disturbance of the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance is observed (table 2). However, IRH distinguishes itself by the fact that despite a disturbed Mg\textsuperscript{2+} balance, the Ca\textsuperscript{2+} urinary excretion and Ca\textsuperscript{2+} serum levels are unaffected. In addition, no abnormalities were observed for serum levels of Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{−}, HCO\textsubscript{3}−, PTH, calcitonin, renin activity, aldosterone and blood pH. The genetic analysis of this family with IRH is discussed in chapter 5 of this thesis.

Isolated dominant hypomagnesemia associated with hypocalciuria (IDHH)

IDHH has been described in two Dutch families with a common ancestor (88, 89). The gene FXYD2, encoding the $\gamma$-subunit of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase pump, is mutated in patients with IDHH, which display hypomagnesemia and lowered renal excretion of Ca\textsuperscript{2+}. The Na\textsuperscript{+},K\textsuperscript{+}-ATPase $\gamma$-subunit is predominantly expressed in the kidney and shows the highest expression levels in DCT and medullary TAL (90). The $\gamma$-subunit is a small (7 kDa) type I membrane protein, which specifically modulates the activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase (91-93). The identified glycine to arginine mutation at position 41 (G41R), is located in the single transmembrane domain of the $\gamma$-subunit and results in misrouting of this protein (90). Although the Na\textsuperscript{+},K\textsuperscript{+}-ATPase $\gamma$-subunit is critical for active transcellular Mg\textsuperscript{2+} reabsorption in DCT, the molecular mechanism for renal Mg\textsuperscript{2+} loss in this autosomal dominant type of primary hypomagnesemia remains to be elucidated (90). Interestingly, individuals with an 11q23.3 deletion, including FXYD2, showed normal serum Mg\textsuperscript{2+} levels (90). Furthermore, ablation of the FXYD2 gene in mouse did not result in significantly lowered serum Mg\textsuperscript{2+} levels compared to wild-type littermates (94). Based on the fact that haploinsufficiency in humans and loss of Na\textsuperscript{+},K\textsuperscript{+}-ATPase $\gamma$-subunit expression in mouse are not determining factors in the development of IDHH, it is assumed that the G41R mutation has a dominant negative effect resulting in this disease.

Isolated dominant hypomagnesemia (IDH)

Kantorovich et al. described another family with an autosomal dominant form of primary hypomagnesemia with an almost similar phenotype as patients with the mutation in FXYD2 (87). Patients displayed hypomagnesemia, lowered serum immunoreactive PTH levels and lowered bone mineral density at the lumbar spine and proximal femur. Although a trend was observed of decreased 24 hour urinary Ca\textsuperscript{2+} excretion in the affected individuals, no difference was detected when the urinary Ca\textsuperscript{2+} excretion was expressed as mg/100ml/GFR/1.73m\textsuperscript{2}. Serum Ca\textsuperscript{2+}, 25-hydroxyvitamin D and 1,25(OH)\textsubscript{2}D\textsubscript{3} were normal. The serum and urine values for Cl\textsuperscript{−}, HCO\textsubscript{3}−, K\textsuperscript{+} and amino acids were all within the normal range. Although the phenotype is very similar to IDHH caused by a mutation in FXYD2, no linkage was found to the 11q23 locus where FXYD2 is situated indicating that another unknown autosomal gene causes this disease (87). However, inheritance via the mitochondrial genome can not be excluded in this family with IDH.

Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC)

Patients with FHHNC suffer from severe hypomagnesemia due to profound renal Mg\textsuperscript{2+} wasting, which cannot be corrected by oral or intravenous Mg\textsuperscript{2+} supplementation (32). In addition, patients with FHHNC display renal failure and renal Ca\textsuperscript{2+} wasting resulting in parenchymal calcification. Other symptoms include urinary tract infections, kidney stones, hyperuricemia and ocular findings. Renal salt wasting is not observed in these patients. A positional cloning strategy identified the gene CLDN16 (or PCLN-1) encoding the protein claudin-16 (or paracellin-1) that was found to be mutated in these patients. The tight junction protein claudin-16, related
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<td>154020</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>(87)</td>
</tr>
<tr>
<td>Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC)</td>
<td>AR</td>
<td>248250</td>
<td>3q28</td>
<td>CLDN16 (PCLN-1)</td>
<td>claudin-16</td>
<td>TAL</td>
<td>(32)</td>
</tr>
<tr>
<td>Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis with severe ocular involvement (FHHNC)</td>
<td>AR</td>
<td>248190</td>
<td>1p34.2</td>
<td>CLDN19</td>
<td>claudin-19</td>
<td>TAL</td>
<td>(33)</td>
</tr>
<tr>
<td>Hypomagnesemia, Hypertension and Hypercholesterolemia, Mitochondrial (HHHM)</td>
<td>M</td>
<td>500005</td>
<td>mitochondrial</td>
<td>MTTL</td>
<td>-</td>
<td>DCT</td>
<td>(95)</td>
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<td>602014</td>
<td>9q21.13</td>
<td>TRPM6</td>
<td>TRPM6</td>
<td>DCT</td>
<td>(20, 56, 104)</td>
</tr>
<tr>
<td><strong>Secondary hypomagnesemia</strong></td>
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<tr>
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<td>601678</td>
<td>15q21</td>
<td>SLC12A1</td>
<td>NKCC2</td>
<td>TAL</td>
<td>(105)</td>
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<tr>
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<td>11q24</td>
<td>KCNJ1</td>
<td>ROMK</td>
<td>TAL</td>
<td>(106, 107)</td>
</tr>
<tr>
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<td>602023</td>
<td>1p36</td>
<td>CLCNKB</td>
<td>CLC-Kb</td>
<td>TAL/DCT</td>
<td>(108, 109)</td>
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<tr>
<td>Antenatal Bartter syndrome with sensorineural deafness (aBS with sensorineural deafness) Type IV</td>
<td>AR</td>
<td>602522</td>
<td>1p31</td>
<td>BSND</td>
<td>Barttin, B subunit of CLC-Ka and CLC-Kb</td>
<td>TAL</td>
<td>(110, 111)</td>
</tr>
<tr>
<td>Gitelman syndrome (GS)</td>
<td>AR</td>
<td>263800</td>
<td>16q13</td>
<td>SLC12A3</td>
<td>NCC</td>
<td>DCT</td>
<td>(38)</td>
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<tr>
<td>Autosomal Dominant Hypoparathyroidism (ADH) (activating mutations)</td>
<td>AD</td>
<td>601198</td>
<td>3q21</td>
<td>CASR</td>
<td>CaSR</td>
<td>TAL</td>
<td>(112)</td>
</tr>
<tr>
<td>Neocrotal Severe Hyperparathyroidism (NSHPT) (inactivating mutations)</td>
<td>AR</td>
<td>239300</td>
<td>3q21</td>
<td>CASR</td>
<td>CaSR</td>
<td>TAL</td>
<td>(113)</td>
</tr>
<tr>
<td>Familial Hypocalciuric Hypercalcemia (FHH or HHC1) (inactivating mutations)</td>
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<td>145980</td>
<td>3q21</td>
<td>CASR</td>
<td>CaSR</td>
<td>TAL</td>
<td>(113)</td>
</tr>
</tbody>
</table>

Genetic information about inherited disorders of Mg\(^{2+}\) homeostasis. AR, autosomal recessive; AD, autosomal dominant; M, mitochondrially inherited; ?, unknown; OMIM, online mendelian inheritance in man; Ref., reference; TAL, thick ascending limb of Henle’s loop; DCT, distal convoluted tubule; CLCN9, gene encoding claudin-16; CLCNKB, gene encoding claudin-19; FXYD2, gene encoding the γ-subunit of the Na+, K+-ATPase; TRPM6, gene encoding the epithelial Mg\(^{2+}\) channel TRPM6; MTTL, gene encoding mitochondrial tRNAile; KCNJ1, gene encoding the renal outer medullary K+ channel (ROMK); CLCNKB, gene encoding the Cl- channel Kb (KLC-Kb); BSND, gene encoding Barttin.
### Table 2. Clinical and biochemical characteristics

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Age at onset</th>
<th>Serum Mg²⁺</th>
<th>Serum Ca²⁺</th>
<th>Serum K⁺</th>
<th>Urine Mg²⁺ excretion</th>
<th>Urine Ca²⁺ excretion</th>
<th>Nephrocalcinosis</th>
<th>Ref.</th>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated Recessive Renal Hypomagnesemia (IRH)</td>
<td>childhood</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>↑</td>
<td>N</td>
<td>no</td>
<td>(86)</td>
</tr>
<tr>
<td>Isolated Dominant Hypomagnesemia with Hypocalciuria (IDHH)</td>
<td>childhood</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>↑</td>
<td>↓</td>
<td>no</td>
<td>(90)</td>
</tr>
<tr>
<td>Isolated Dominant Hypomagnesemia (IDH)</td>
<td>childhood</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>↑</td>
<td>↓</td>
<td>no</td>
<td>(87)</td>
</tr>
<tr>
<td>Familial Hypomagnesemia with Hypocalciuria and Nephrocalcinosis (FHINC)</td>
<td>childhood</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>↑↑</td>
<td>↑↑</td>
<td>yes</td>
<td>(32)</td>
</tr>
<tr>
<td>Familial Hypomagnesemia with Nephrocalcinosis with severe ocular involvement (FHINC)</td>
<td>childhood</td>
<td>↓</td>
<td>N</td>
<td>N</td>
<td>↑↑</td>
<td>↑↑</td>
<td>yes</td>
<td>(33)</td>
</tr>
<tr>
<td>Hypomagnesemia, Hypertension and Hypercholesterolemia, Mitochondrial (HHHM)</td>
<td>?</td>
<td>N ↓</td>
<td>N</td>
<td>N ↓</td>
<td>↑</td>
<td>N ↓</td>
<td>no</td>
<td>(95)</td>
</tr>
<tr>
<td>Familial Hypomagnesemia with Secondary Hypocalciuria (HSIH)</td>
<td>infancy</td>
<td>↓↓</td>
<td>↓</td>
<td>N</td>
<td>↑</td>
<td>N</td>
<td>no</td>
<td>(20, 56, 104)</td>
</tr>
<tr>
<td><strong>Secondary hypomagnesemia</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antenatal Bartter syndrome (aBS) Type I (NKCC2), Type II (ROMK)</td>
<td>antenatal</td>
<td>N</td>
<td>N</td>
<td>↓ - ↓</td>
<td>N</td>
<td>↑↑</td>
<td>yes</td>
<td>(106, 107)</td>
</tr>
<tr>
<td>Antenatal Bartter syndrome (aBS) Type III</td>
<td>childhood</td>
<td>N ↓</td>
<td>N</td>
<td>↓↓</td>
<td>N ↓</td>
<td>N ↑</td>
<td>variable</td>
<td>rare (108, 109)</td>
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<td>Gilfman syndrome (GS)</td>
<td>variable</td>
<td>↓</td>
<td>N</td>
<td>↓↓</td>
<td>↑</td>
<td>↓</td>
<td>no</td>
<td>(105)</td>
</tr>
<tr>
<td>Autosomal Dominant Hypoparathyroidism (ADH)</td>
<td>infancy/adulthood</td>
<td>N ↓</td>
<td>↓</td>
<td>N ↑</td>
<td>↑</td>
<td>↑↑</td>
<td>yes</td>
<td>(112)</td>
</tr>
<tr>
<td><strong>Secondary hypermagnesemia</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neonatal Severe Hyperparathyroidism (NSHPT)</td>
<td>infancy</td>
<td>N ↑</td>
<td>↑</td>
<td>N</td>
<td>N ↓</td>
<td>?</td>
<td>?</td>
<td>(113)</td>
</tr>
<tr>
<td>Familial Hypocalciuric Hypercalcemia (FH or HHC1)</td>
<td>infancy</td>
<td>N ↑</td>
<td>↑</td>
<td>N</td>
<td>N ↓</td>
<td>↓</td>
<td>no</td>
<td>(113)</td>
</tr>
</tbody>
</table>

The biochemical characteristics of serum and urine are indicated for the disorders described in the text. In addition the age of onset and the occurrence of nephrocalcinosis is indicated. N, normal; ↑, increased; ↓, decreased; ?, unknown. Ref., reference.
to the claudin family of tight junction proteins, is specifically expressed in TAL and is important for paracellular Mg\(^{2+}\) and Ca\(^{2+}\) reabsorption in this segment (32). Moreover, mutations in a second member of the claudin family, CLDN19 encoding claudin-19 have been identified. Patients with a CLDN19 mutation show an identical phenotype compared to patients affected with CLDN16 mutations, but display an additional severe ocular aberration (33).

### Hypomagnesemia, hypertension and hypercholesterolemia, mitochondrial (HHHM)

In a large kindred with patients displaying HHHHM, were shown to carry a mutation in the mitochondrial tRNA\(_{ile}\) gene (MTTI) (95). The patients displayed a fractional excretion of Mg\(^{2+}\) above 2%, which is inappropriately high in the presence of hypomagnesemia, indicating renal Mg\(^{2+}\) wasting (7). Furthermore, the affected individuals demonstrated a reduced urinary Ca\(^{2+}\) excretion (95). Electron microscopy of a skeletal muscle biopsy from a patient and in vivo nuclear magnetic resonance spectroscopy of the same skeletal muscle revealed morphological signs of mitochondrial dysfunction and reduced adenosine tri-phosphate production, respectively. It was hypothesized that the renal Mg\(^{2+}\) wasting resulting in hypomagnesemia is caused by mitochondrial dysfunctioning in DCT cells. As described before, DCT cells have the highest energy consumption of the nephron (37) and active transcellular Mg\(^{2+}\) transport in DCT requires ATP-dependent Na\(^+\) reabsorption (38). Consequently, the impaired basolateral extrusion of Mg\(^{2+}\) into the blood might result in renal Mg\(^{2+}\) wasting.

### Hypomagnesemia with secondary hypocalcemia (HSH)

HSH is an autosomal recessive disease of which the gene locus was mapped on chromosome 9q22 (96). Affected individuals showed neurological symptoms of hypomagnesemic hypocalcemia, including seizures and muscle spasms during infancy (20, 56, 97). Although in HSH the defect was originally established at the level of the intestine and physiological studies indicated that its pathophysiology is principally caused by a primary defect in intestinal Mg\(^{2+}\) transport, there is also evidence for impaired Mg\(^{2+}\) reabsorption (20, 98-101). Although most patients displayed normal renal Mg\(^{2+}\) conservation, for some patients inappropriately high fractional Mg\(^{2+}\) excretion rates with respect to their low serum Mg\(^{2+}\) levels were observed (20, 98). This indicates an additional role of impaired renal Mg\(^{2+}\) reabsorption in HSH (56, 98, 99). HSH can be treated by high dietary Mg\(^{2+}\) intake because passive Mg\(^{2+}\) absorption is not affected (102). When untreated, the disease may be fatal or could lead to severe neurological damage. The observed hypocalcemia in HSH is a secondary effect possibly caused by parathyroid failure resulting from Mg\(^{2+}\) deficiency (103). Using a positional candidate gene-cloning approach, mutations in the TRPM6 gene were found to be the cause of autosomal recessive HSH (20, 56, 104). This discovery identified TRPM6 as the molecular identity facilitating Mg\(^{2+}\) entry in transepithelial Mg\(^{2+}\) (re)absorption. The functional and physiological characteristics of TRPM6 have been highlighted in the paragraphs described previously.

### Inherited disorders with secondary hypo and hypermagnesemia

Several inherited renal tubular disorders are known in which the disturbed serum Mg\(^{2+}\) balance is likely a secondary phenomenon (table 1 and 2). These monogenic disorders do not directly involve Mg\(^{2+}\) transporting proteins and include the Bartter-like syndromes and the Ca\(^{2+} / \text{Mg}^{2+}\)-sensing receptor (CaSR) disorders.

#### Bartter-like syndromes

The Bartter-like syndromes (comprised of Gitelman syndrome, Bartter syndrome type I, II, III, and IV) are all characterized by renal salt wasting, hypokalemic metabolic alkalosis, elevated serum renin and aldosterone levels with normal blood pressure (114). Although, the underlying gene defects of the CaSR disorders and Bartter-like syndromes have been elucidated, the exact cause of the observed hypomagnesemia remains to be explained.

#### Bartter syndrome type I and II: antenatal Bartter syndrome (aBS)

aBS manifests in utero and is characterized by severe polyuria and polyhydramnios resulting in premature birth in most cases. Postnatally, affected individuals develop salt wasting, hypokalemic metabolic alkalosis, hypercalciumia, and nephrocalcinosis and only in exceptional cases hypomagnesemia (115). aBS is caused by mutations in either the SLC12A1 gene encoding the the furosemide-sensitive Na\(^+\),K\(^+\),2Cl\(^-\) co-transporter NKCC2 (Bartter type I) (105) or KCNJ1 encoding the K\(^+\) channel ROMK (Bartter type II) (106, 107). The hypokalemia in the ROMK patients is less...
severe compared with the NKCC2 patients (105). Mutations in NKCC2 or ROMK are believed to reduce salt reabsorption in the TAL and should thereby not only impair paracellular Ca2+ but also Mg2+ transport. However, Mg2+ wasting is not a common finding in patients with aBS which is consistent with the observation that NKCC2 knockout mice also display no disturbances in the Mg2+ balance (116). In addition, chronic furosemide treatment is in general not associated with hypomagnesemia. The mechanism explaining the absence of Mg2+ wasting in most aBS patients is still elusive. Possibly, increased renal prostaglandin synthesis, may contribute to increased Mg2+ reabsorption in DCT as has been shown in vitro (117). Alternatively, passive Mg2+ reabsorption in the proximal tubule and TAL could be stimulated by chronic volume depletion.

**Bartter syndrome type III:** Classic Bartter syndrome (cBS)

The majority of patients with cBS display hypokalemia, hypochloremia, and failure to thrive during the first two years of life (118). However, the clinical phenotype of cBS (Bartter type III) is broad ranging from a phenotype similar to aBS to a phenotype highly comparable with that of Gitelman syndrome (see below) (119-121). Hypomagnesemia is observed in almost 50% of the cBS patients and although the excretion of Ca2+ is variable, hypocalciuria is commonly detected (108, 120). cBS is caused by mutations in the CLCNKB gene encoding the renal Cl- channel CLC-Kb (108, 109). CLC-Kb is basolaterally expressed in TAL and DCT where it mediates Cl- efflux from the tubular epithelial cell into the blood (figure 2) (108, 109). Some cBS patients display hypomagnesemia combined with hypocalciuria, a characteristic phenotype also found in GS and IDHH, both caused by defects in DCT. Therefore, it can be hypothesized that disturbed reabsorption of Na+ and Cl- in DCT impairs active reabsorption of Mg2+.

**Bartter syndrome type IV:** antenatal Bartter syndrome with sensorineural deafness (aBS with sensorineural deafness)

Patients with aBS with sensorineural deafness (Bartter type IV) form a variant of aBS. The clinical phenotype of these patients is different from aBS since hypercalciuria and nephrocalcinosis are uncommon and patients often show progression to renal failure of unknown origin. In addition, renal water and salt losses can be even more severe than in type I and II aBS patients. aBS with sensorineural deafness is caused by mutations in the BSND gene which encodes Barttin, an activating β-subunit of the renal Cl- channels CLC-Ka and CLC-Kb (110, 111, 122). Barttin is expressed in the TAL, thin ascending limb of Henle’s loop and in the stria vascularis of the inner ear (111). In contrast to cBS, where mutations in CLC-Kb result in hypomagnesemia in approximately 50% of the patients, hypomagnesemia in patients with type IV aBS with sensorineural deafness is uncommon.

**Gitelman variant of Bartter syndrome (GS)**

GS forms the mild form of the salt-wasting renal disorders since GS patients have a higher urine concentrating ability than patients with aBS, aBS with sensorineural deafness or cBS, resulting in less water and salt loss. GS usually manifests during childhood or adolescence. Patients with GS display hypokalemia, metabolic alkalosis, hypomagnesemia and hypocalciuria. Furthermore, they show muscle weakness or tetanic episodes that are related to profound hypomagnesemia. GS is caused by mutations in SLC12A3, which encodes the thiazide-sensitive Na+,Cl- cotransporter NCC. NCC is expressed exclusively at the apical membrane of the DCT (38). Ablation of SLC12A3 in mice resulted in a phenotype that closely resembles GS including hypomagnesemia and hypocalciuria. However, hypokalemia was not detected indicating that the hypomagnesemia observed is not secondary to this characteristic in GS (39). Chronic thiazide treatment, which is a model for GS, does not always result in Mg2+ wasting and hypomagnesemia. Loffing and colleagues showed that chronic thiazide treatment in rats, which enhances renal Na+ excretion, resulted in an increased rate of apoptosis in DCT cells (40). This is in line with the observation that the number and height of DCT cells is decreased in NCC knockout mice (39). It could be hypothesized that a reduction in reabsorptive surface area results in diminished Mg2+ reabsorption leading to Mg2+ wasting. In addition, in SLC12A3 knockout mice, an animal model of GS, and during chronic hydrochlorothiazide administration, the hypomagnesemia is accompanied by decreased expression levels of TRPM6 (123). Thus, TRPM6 downregulation may represent a general mechanism involved in the pathogenesis of hypomagnesemia accompanying NCC inhibition or inactivation.

**CaSR disorders**

The CaSR is a large glycoprotein belonging to the superfamily of G protein-coupled receptors (124). In kidney, the CaSR is expressed in various nephron segments including the proximal convoluted and straight tubule (125), the TAL, DCT and in
some cells of the collecting duct (126-128). It is generally accepted that the CaSR can sense both Ca\(^{2+}\) and Mg\(^{2+}\) levels and regulates the reabsorption and thereby the total body balance of these ions (34). As described previously, the kidney plays an important role in the Ca\(^{2+}\) and Mg\(^{2+}\) homeostasis by adjusting the tubular reabsorption of these cations. Frequently a disturbed Mg\(^{2+}\) balance is observed in patients with mutations in the CASR gene. The mutations that have been described are either activating or inactivating. Activating CASR mutations cause autosomal-dominant hypoparathyroidism (ADH) (112), which is associated with hypocalcemia, hypercalciuria and frequently with hypomagnesemia. This phenotype is explained by the enhanced sensitivity of the CaSR for both Ca\(^{2+}\) and Mg\(^{2+}\), resulting in decreased PTH secretion and thereby reduced reabsorption of Ca\(^{2+}\) and Mg\(^{2+}\) in the cortical TAL and DCT (124). The inactivating CASR mutations can be divided into heterozygous mutations that have been identified in patients with familial hypocalciuric hypercalcemia (FHH or HHC1) and homozygous or compound heterozygous mutations causative for neonatal severe hyperparathyroidism (NSHPT). In contrast to ADH, patients with FHH are characterized by hypercalcemia, hypocalciuria and display a tendency towards hypermagnesemia (129). Patients with NSHPT exhibit hypercalcemia and elevated PTH levels from birth (113). In addition, several NSHPT patients display hypermagnesemia (129).

In conclusion, investigations of families with primary and secondary hereditary hypomagnesemia have uniquely resulted in the identification of several genes and proteins that can be either directly or indirectly linked to renal and/or intestinal Mg\(^{2+}\) handling. Interestingly, all identified gene products involved in Mg\(^{2+}\) homeostasis are expressed in kidney (figure 4). The importance of studying families with hereditary hypomagnesemia is stressed by the fact that no suitable radioactive Mg\(^{2+}\) isotope is available, which would greatly facilitate the electrophysiological characterization of Mg\(^{2+}\) pathways.

**Outline of this thesis**

Mg\(^{2+}\) is an abundant cation in the human body and is required for a wide variety of cellular processes. Serum Mg\(^{2+}\) concentration is maintained within a narrow range (0.7-1.1 mM) (24) by changes in urinary Mg\(^{2+}\) excretion in response to altered uptake by the intestine. Thus, the kidney plays a key role in Mg\(^{2+}\) homeostasis. The majority
Mg²⁺-normal and a Mg²⁺-enriched diet. Furthermore, this chapter discusses the regulation of TRPM6 in kidney by hormones including PTH, 1,25(OH)₂D₃ and 17β-estradiol. Chapter 4 illustrates how the Mg²⁺ balance is influenced in TRPV5 knockout mice (TRPV5⁻/⁻), which display robust hypercalciuria and hypervitaminosis D. In addition, the analysis of TRPM6 expression levels and the Mg²⁺ and Ca²⁺ balance in TRPV5⁻/⁻ mice fed various Mg²⁺ diets is described. The aim of the study described in chapter 5 was to identify one or more genes involved in renal Mg²⁺ handling and systemic Mg²⁺ homeostasis. To this end, we performed a positional candidate cloning strategy on a family previously described by Geven et al. (86) with isolated recessive renal Mg²⁺ wasting and normocalciuria. This study, presents the search for the gene defect causing the aforementioned disease and the mechanism underlying the pathophysiology of the observed hypomagnesemia. Furthermore, this study provides for the mechanism explaining hypomagnesemia induced by cetuximab, which is a monoclonal antibody directed against the epidermal growth factor receptor (EGFR). The relation of specific EGFR signaling cascades, elicited after EGFR activation, with respect to TRPM6 channel activity is described in chapter 6. To this end, various inhibitors, blocking specific EGFR signaling pathways and patch clamp analysis were used. Finally, the results of this thesis are summarized and discussed in chapter 7.

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Chapter 1

General introduction
Molecular determinants of permeation through the cation channel TRPM6

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Abstract

TRPM6 and its closest relative TRPM7 are members of the Transient Receptor Potential Melastatin (TRPM) subfamily of cation channels and are known to be Mg²⁺ permeable. By aligning the sequence of the putative TRPM6 pore with the pore sequences of the other subfamily members, we located in the loop between the 5th and the 6th transmembrane domain, a stretch of amino acids residues, 1028GEIDVC1033, as the potential selectivity filter. Two negatively charged residues, E1024 (conserved in TRPM6, TRPM7, TRPM1 and TRPM3) and D1031 (conserved along the entire TRPM subfamily), were identified as important determinants of cation permeation through TRPM6, because neutralization of both residues into an alanine resulted in non-functional channels. Neutralization of E1029 (conserved in TRPM6, TRPM7, TRPM4 and TRPM5) resulted in channels with increased conductance for Ba²⁺ and Zn²⁺, decreased ruthenium red sensitivity and larger pore diameter compared to wild-type TRPM6. Changing the residue I1030 into methionine, resulted in channels with lower conductance for Ni²⁺, decreased sensitivity to ruthenium red block and reduced pore diameter. Thus, these data demonstrate that amino acid residues E1024, I1030 and D1031 are important for channel function and that subtle amino acid variation in the pore region accounts for TRPM6 permeation properties.

Introduction

Being the most abundant intracellular cation after K⁺, Mg²⁺ is an important cofactor for many biological processes, such as protein synthesis, nucleic acid stability and neuromuscular excitability (1, 2). Mg²⁺ homeostasis involves the kidney as the primary regulatory site, the intestine as the absorption place, while the bone is the storage location (3). Transcellular Mg²⁺ transport in renal and intestinal epithelia is of vital importance for overall Mg²⁺ homeostasis. There is both a passive (paracellular) mechanism and an active (transcellular) transport for Mg²⁺ (re)absorption in renal and intestine epithelia (2). However, the molecular mechanism of transcellular Mg²⁺ transport is not fully understood. Recently, investigations in consanguineous families suffering from hypomagnesaemia with secondary hypocalcaemia (HSH) have identified mutations in the gene encoding the Transient Receptor Potential Melastatin 6 (TRPM6) protein as the cause of the disease (4, 5).
It was demonstrated that TRPM6 functions as a molecular determinant of transcellular Mg\(^{2+}\) transport in renal and intestinal epithelia (6).

TRPM6 shares with its closest relative TRPM7 approximately 50% sequence homology at the amino acid level (6). TRPM7 appears to be ubiquitously expressed, whereas TRPM6 expression is restricted to kidney and intestine (6), where it plays an important role in Mg\(^{2+}\) (re)absorption (6). Consistent with a role in Mg\(^{2+}\) homeostasis, we demonstrated recently that dietary Mg\(^{2+}\) restriction upregulates TRPM6 expression in mice kidney, while a Mg\(^{2+}\)-enriched diet increases TRPM6 expression in colon (7). These diets did not affect TRPM7 expression levels in kidney and colon. Atypically for other ion channels, TRPM6 and TRPM7 contain a serine/threonine protein kinase domain at the C-terminus resembling that of elongation factor 2 (eEF-2) kinase and other α-kinases. The kinase function in channel regulation remains elusive (4, 8-12).

Recently, we have functionally characterized TRPM6 using electrophysiological analysis (6). This protein forms constitutively active divalent selective cation channels, with a higher affinity for Mg\(^{2+}\) than for Ca\(^{2+}\) at physiological membrane potentials and divalent concentrations. TRPM6 becomes permeable to monovalent cations when all divalent cations are omitted from the extracellular solution. Despite this initial characterization of TRPM6, the molecular determinants of its Mg\(^{2+}\) permeability remain unknown. Moreover, in a recent paper, Li et al. described in detail the functional differences in divalent ion permeability between homomeric TRPM6, homomeric TRPM7 and heteromeric TRPM6/TRPM7 channel complexes demonstrating unequivocally that TRPM6 can form by itself functional channels without TRPM7 co-expression (13). The study also demonstrated that TRPM6 displays single channel conductance that is 2- and 1.5-fold bigger than TRPM7 and TRPM6/TRPM7 complexes.

To date there is limited knowledge about the structures of TRP channels pores and even less is known about TRPM channels pores. In a recent study Owsianik et al. summarized the current knowledge about permeation and selectivity of TRP channels (14). They propose and describe a general approach as a toolkit for characterizing the permeation and selectivity of ion channels. Therefore, the aim of the present study was to identify the amino acid stretch responsible for Mg\(^{2+}\) permeation of TRPM6. To this end, a combined approach consisting in site-directed mutagenesis and electrophysiological measurements was used to investigate the role of amino acid residues from the putative selectivity filter in the permeation properties of TRPM6. Our data indicate that a short amino acid stretch of the pore region, 1028GEIDVC1033, determines the TRPM6 conductance properties and its sensitivity to the channel pore blocker, the hexavalent cation ruthenium red.

Materials and methods

Molecular biology

The full-length open reading frame from N-terminally HA-tagged human (h)TRPM6 was cloned as a BclI - BspEI fragment in the pCINeo/IRES-GFP vector (6). This bicistronic expression vector, pCINeo/IRES-GFP/HA-hTRPM6, was used to co-express hTRPM6 and enhanced GFP in Human Embryonic Kidney cells (HEK293). Mutagenesis of the amino acids E1024, E1029, I1030, D1031, V1032 and of the TRPM6-TRPM4 and TRPM6-TRPV6 chimeric constructs was performed using QuickChange™ site-directed mutagenesis (Stratagene, La Jolla, USA). The sequence of each mutant was verified by sequence analysis of the corresponding cDNA.

Cell culture and transfection

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine and 10 µg/ml ciproxin at 37°C in a humidity-controlled incubator with 5% (v/v) CO\(_2\). The cells were transiently transfected with the respective constructs using Lipofectamine 2000 (Invitrogen-Life Technologies, Breda, the Netherlands), as described previously (15), and electrophysiological recordings were performed 16 to 36 hours post-transfection. Transfected cells were identified by their green fluorescence when illuminated at 480 nm. Non-transfected (GFP-negative) cells from the same batch were used as controls.

Electrophysiology

Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature (20-25°C) using an EPC-9 patch clamp amplifier computer.
Statistical analysis
Data analysis and graphs were prepared using IgorPro software (WaveMetrics, Lake Oswego, USA). Data are expressed as mean ± s.e.m. Overall statistical significance was determined by analysis of variance. In case of significance (p < 0.05), individual groups were compared using Student’s t test.

Results

Sequence analysis of the putative pore-forming region of TRPM6
Figure 1 shows a sequence alignment of the putative pore region of the TRPM subfamily. The structure of the loop between the 5th and the 6th transmembrane domains (TM5 and TM6) was modeled based on the K⁺ channel KcsA crystal structure (19). In this region, TRPM6 shares the highest sequence homology with TRPM7, also known to conduct Mg²⁺ when heterologously expressed in HEK293 cells (8, 9, 20). TRPM6 and TRPM7 contain two glutamate residues, E1024 and E1029 (horizontal pattern in figure 1). The E1024 is conserved in TRPM1 and TRPM3. In addition, TRPM6 shares an aspartate residue D1031 (vertical pattern in figure 1) with all the other members of the TRPM subfamily. To assess the contribution of these amino acid residues in the permeation properties of TRPM6, these residues were neutralized by alanine substitution (mutants E1024A, E1029A, and D1031A). To delineate the role of the conserved aspartate residue (D1031), adjacent amino acids were mutated. I1030 was substituted by a methionine in order to mimic the pore properties of the monovalent selective channel TRPM4 (mutant I1030M), and the V1032 was substituted by an alanine (mutant V1032A). The positions for the amino acid residues mutated for this study are marked with asterisks in figure 1.

Functional characterization of TRPM6 pore mutants
To determine which amino acids in the pore region account for the permeation properties of TRPM6, the activation kinetics of wild-type channels were compared with those of the mutant proteins. The currents recorded from TRPM6-transfected cells reached a plateau level of 295 ± 40 pA/pF (n = 30 cells) within 100-200 s, while non-transfected (NT) cells (n = 10 cells) displayed only background current, probably endogenous TRPM7 (75 ± 27 pA/pF) that is activated over a longer period of time, as depicted in figure 2A. When expressed in HEK293 cells, the TRPM6

Solutions
The standard pipette solution contained (in mM): 150 NaCl, 10 EDTA, and 10 HEPES (pH 7.2 adjusted with NaOH). The extracellular solution contained (in mM): 150 NaCl and 10 HEPES (pH 7.4 adjusted with NaOH), supplemented with either 1 mM CaCl₂ (to assess the time-course for development of currents), 10 mM EDTA (divalent-free solutions, DVF), or the same concentration of divalent cations (for the permeation profile determinations). The relative permeabilities (Pₓ/PNa) of mono-, di-, tri-, and tetramethyl ammonium substituents and of N-methyl-D-glucamine (NMDG) were measured using solutions in which all Na⁺ was substituted by the respective cations and calculated from the biionic reversal potentials (16). All potentials were corrected for possible liquid junction potentials, which were calculated according to Barry (17). In these permeation experiments, the standard pipette solution was used as intracellular solution. For the ammonium derivatives, the following compounds diameters were used (in nm): 0.36, 0.46, 0.52, 0.58, and 0.68 for monomethylammonium (MA⁺), dimethylammonium (DMA⁺), trimethylammonium (TriMA⁺), tetramethylammonium (TetMA⁺), and for NMDG⁺ respectively. To fit the points from the graph plotting permeation ratios of the different organic cations (C) versus their estimated diameters, the excluded volume considering friction of the permeating ion Equation 1 (18) was used:

\[ \frac{P_x}{P_{Na}} = \frac{k(1-a/d)^2}{a} \]

where a is the organic cation diameter, k is a constant factor and d the minimal pore diameter. The ammonium derivates were purchased from Sigma.

Chapter 2
Permeation through TRPM6

Statistical analysis
Data analysis and graphs were prepared using IgorPro software (WaveMetrics, Lake Oswego, USA). Data are expressed as mean ± s.e.m. Overall statistical significance was determined by analysis of variance. In case of significance (p < 0.05), individual groups were compared using Student’s t test.

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pore mutants showed three different phenotypes: $E_{1029}A$ displayed a time-course of activation and a current amplitude similar to wild-type TRPM6 ($n = 10$ cells) (figure 2B), $I_{1030}M$ and $V_{1032}A$ showed activation kinetics similar with TRPM6, while their current amplitudes were significantly reduced ($178 \pm 40$ and $150 \pm 45$ pA/pF respectively versus $295 \pm 40$ pA/pF, $p < 0.05$, $n = 10$ cells for each mutant) as depicted in figure 2C and D, and $E_{1024}A$ and $D_{1031}A$ showed no current ($n = 5$ cells) (figure 2E).

Mutations in the pore region of TRPM6 did not change the current-voltage properties of the currents as summarized in figure 2F.

Interestingly, when co-expressed with wild-type TRPM6 in a 1:1 cDNA ratio, $D_{1031}A$ showed a dominant negative effect since the currents recorded from these cells ($n = 8$ cells) were identical in the time-course of development, current-voltage relation and amplitude with the currents from NT cells as shown in figure 3. The dominant negative effect of $D_{1031}A$ suggests that assembly with wild-type TRPM6 is not disturbed. Channel complexes containing wild-type and mutant

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**Figure 1.** The putative pore-forming region of TRPM6

Alignment of the putative pore sequences of the TRPM subfamily on the rows, which represent the amino acid sequences from the respective human TRPM subfamily channels. The GenBank accession numbers are: NP_002411, AAY22174, NP_066003, NP_060106, NP_055370, NP_060132, NP_060142, and NP_076985 starting from TRPM1 to TRPM8. The highly conserved hydrophobic residues are highlighted in diagonal pattern, the conserved aspartate is shown in vertical pattern, and the conserved residues with polar or charged groups are shown in horizontal pattern (adapted from (24, 35)). Asterisks denote the amino acid residues mutated in this study.

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**Figure 2.** Functional expression of wild-type TRPM6 and pore mutant proteins

(A) Average time-course of inward (at $-80$ mV) and outward currents (at $+80$ mV) development from cells expressing wild-type TRPM6 ($\bullet$), and non-transfected cells (NT $\bigtriangleup$). (B-E) Average time course of inward and outward currents development from cells expressing the mutant $E_{1029}A$ (B), $I_{1030}M$ (C), $V_{1032}A$ (D), $E_{1024}A$ (●) and $D_{1031}A$ (i) (E); the dotted line indicates the averaged time course for wild-type TRPM6. (F) Current-voltage relations from cells transfected with either TRPM6, or mutant proteins as indicated, obtained under experimental conditions as in other panels, measured 200 s after whole-cell establishment.
proteins could be retained at the endoplasmic reticulum or reach the plasma membrane showing defective pore architecture. Likewise, expression of D1031A alone could have the same outcome as the co-expression with wild-type TRPM6. Despite repetitive attempts, the low abundance of TRPM6 proteins at the plasma membrane did not allow us to determine whether these proteins are expressed at the cell surface.

Conductance profile of TRPM6 pore mutants

To establish the conductance properties of TRPM6, we determined the permeation profile of the wild-type channel and the functional pore mutants. The permeation rank order of divalent cations compared to Ca\(^{2+}\) was assessed by the ratio of inward current at -80 mV, when Ca\(^{2+}\) ions are equimolar substituted by other divalent cations. When normalized to the inward current amplitude in the presence of 10 mM Ca\(^{2+}\), the following conductance profile for TRPM6 was obtained: Ba\(^{2+}\) (1.66) > Ni\(^{2+}\) (1.16) > Mg\(^{2+}\) (1.08) > Zn\(^{2+}\) (1.01) ≥ Ca\(^{2+}\) (1.00) (\(n\) = 5-8 cells) (figure 4A). The permeation rank order obtained for the mutant E1029A was: Ba\(^{2+}\) (2.34) > Zn\(^{2+}\) (1.29) > Ni\(^{2+}\) (1.14) > Mg\(^{2+}\) (1.08) > Ca\(^{2+}\) (1.00) (figure 4B). The E1029A displayed an increased conductance to Ba\(^{2+}\) and Zn\(^{2+}\) compared to TRPM6. For the permeation rank order of the mutant I1030M, the following values were obtained: Ba\(^{2+}\) (1.74) > Mg\(^{2+}\) (1.01) ≥ Ca\(^{2+}\) (1.00) ≥ Zn\(^{2+}\) (0.99) > Ni\(^{2+}\) (0.92), (\(n\) = 5 cells) (figure 4C). The I1030M mutant had a lower conductance for Ni\(^{2+}\) compared to TRPM6. These data are summarized in figure 4D. To further characterize the TRPM6 pore permeability, its permeation profile was compared with the homologous TRPM7 channel as previously described by Monteilh-Zoller et al. (20). When normalized to the inward current amplitude in 10 mM Ca\(^{2+}\), the following relative values were obtained for TRPM7: Ba\(^{2+}\) (4.62) > Ni\(^{2+}\) (3.69) > Zn\(^{2+}\) (2.11) > Mg\(^{2+}\) (1.24) > Ca\(^{2+}\) (1.00) (\(n\) = 8 cells). This comparison, summarized in figure 4E, revealed that TRPM6 and TRPM7 display different permeation rank orders in the way that TRPM6 has a higher conductance for Mg\(^{2+}\) than TRPM7.

Next, the effect of micromolar concentrations of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)) present in the extracellular solutions on inward monovalent (Na\(^{+}\)) current was investigated. As shown in figure 5A and B, both Mg\(^{2+}\) and Ca\(^{2+}\) inhibited the TRPM6 current in the micromolar range. The concentration for half-maximal TRPM6 current inhibition (IC\(_{50}\)) was calculated from the dose-response curve for Mg\(^{2+}\).
Figure 4. Divalent permeation through wild-type TRPM6 and pore mutant proteins

(A) Magnified views of the inward currents recorded with extracellular solutions containing 10 mM of the indicated divalent cations, from HEK293 cells expressing TRPM6 (A), E1029A (B), and I1030M (C). (D) Histogram summarizing relative permeation rank order determined from the ratio of inward current for the indicated divalent cation, for TRPM6 (black bars) and pore mutants E1029A (white bars) and I1030M (grey bars), (asterisks represent p < 0.05 versus wild-type TRPM6). (E) Histogram summarizing the relative permeation profile for TRPM6 (black columns) and TRPM7 (white columns) determined from the ratio of inward current amplitude in the presence of the indicated divalent cation, and the inward current amplitude with Ca2+ containing extracellular solution.

Figure 5. Mg2+ and Ca2+ inhibit TRPM6-mediated inward monovalent currents

(A) and (B) Current-voltage relations obtained from HEK293 cells expressing TRPM6 in divalent-free solution (DVF) and in the presence of 0.1 and 1 µM Mg2+, or 1 and 5 µM Ca2+. 
(C) Dose-response curve for the inhibition of inward monovalent current at -80 mV by Mg2+. 
(D) Dose-response curve for the inhibition of inward monovalent current at -80 mV by Ca2+. 
(E) and (F) Dose-response curves for the inhibition of inward monovalent current by Mg2+ (E) and Ca2+ (F) for the pore mutant proteins, E1029A (●), I1030M (■) and V1032A (▼).
Effect of pore mutations on TRPM6 ruthenium red sensitivity

As previously demonstrated, TRPM6 can be blocked by the hexavalent cation ruthenium red (RR) in a voltage-dependent manner (6). As depicted in figure 6 A-D, RR blocked specifically the inward monovalent currents carried by wild-type TRPM6 or the pore mutants. To determine which amino acid residues in the TRPM6 pore are involved in the inhibitory effect of RR, a dose-response curve was established for RR block of the inward TRPM6 and pore-mutant’s monovalent currents (n = 5 cells for each point) (figure 6E). The following IC50 for RR sensitivity were obtained (in µM): 15.3 for E1029 A with the Hill coefficient of 0.92, 11.9 for I1030 M with Hill coefficient of 0.77 and 4.8 for V1032 A with a Hill coefficient of 0.84 compared to 5.3 for TRPM6 with a Hill coefficient of 0.90. E1029 A and I1030 M mutants showed a significantly (p < 0.05) reduced sensitivity to RR compared to wild-type channel.

Measurement of the pore diameter of TRPM6

To estimate the TRPM6 pore diameter, the permeability ratios of currents carried by organic monovalent cations of increasing size relative to Na+ current were measured. When Na+ was used as the sole charge carrier, the current reverted close to 0 mV and had a slightly inward-rectifying shape (figure 7A). All Na+ ions from the extracellular solution were substituted by methyl-ammonium or its di-, tri- and tetra methyl derivatives (MA+, DMA+, TriMA+, TetMA+), or by the larger organic cation N-methyl-D-glucamine (NMDG+). All the tested cations were able to permeate wild-type TRPM6, E1029A and I1030M channels (figure 7A-C). The permeability ratios relative to Na+ (P×PNa) were calculated from the bionic reversal potentials (16): 0.827 ± 0.004, 0.483 ± 0.007, 0.360 ± 0.015, 0.262 ± 0.016, and 0.230 ± 0.015 for MA+, DMA+, TriMA+, TetMA+, and NMDG+ respectively. Figure 7D depicts the permeability ratios of the different cations versus their

\( \text{IC}_{50} \) for Mg2+ sensitivity was (in µM): 1.1, 1.0, and 0.8, for E1029A, I1030M and V1032A, respectively (figure 5E). The following IC50 for Ca2+ sensitivity for the pore mutants was (in µM): 3.4, 3.6 and 3.4 for E1029A, I1030M and V1032A, respectively (figure 5F). No significant differences were observed between the pore mutants and wild-type TRPM6.
Both constructs resulted in non-functional channels when expressed in HEK293 cells (data not shown).

Discussion

The Mg$^{2+}$-selective cation channel TRPM6 represents the first molecular key player of transcellular Mg$^{2+}$ (re)absorption in renal and intestinal epithelia. Mutations in TRPM6 described for HSH patients imply that TRPM6 indeed plays a crucial role as the gatekeeper of Mg$^{2+}$ (re)absorption. The present study indicates that a short amino acid stretch of the pore region, 1028GEIDVC1033, determines the TRPM6 permeability properties and its sensitivity to the channel pore blocker, RR. Our conclusions are based on the following experimental observations. First, the mutant E1029A shows different permeation properties compared to wild-type TRPM6. Second, the estimated pore diameter of TRPM6 increases when E1029 is neutralized into an alanine and decreases when I1030 is changed into a methionine. Third, the sensitivity of TRPM6 to RR block depends on the E1029 and I1030 residues in the pore forming region of the channel.

TRPM6 pore localization

Mutations that affect basic pore properties have been mainly described for the TRPV (vanilloid) subfamily, namely TRPV1, TRPV4, TRPV5 and TRPV6 (21-23), while the only characterized member of TRPM subfamily is TRPM4 (24). For TRPV5 and TRPV6, it has been shown that neutralization of an aspartate residue, D542 in TRPV5, which corresponds to D541 in TRPV6, abolishes the Ca$^{2+}$ permeation, Ca$^{2+}$-dependent current decay and block by extracellular Mg$^{2+}$, whereas permeation of monovalent cations remains basically intact (25). Although the molecular determinants of TRPM6 Mg$^{2+}$ selectivity were not fully revealed with this study, we obtained essential evidences that the region between the residues 1028GEIDVC1033 forms a part of the TRPM6 pore based on the fact that neutralizing the negatively charged amino acid residues in this area has dramatic consequences on TRPM6 functionality. To further delineate the selectivity filter of TRPM6, two chimeras were prepared in which the identified selectivity filters of TRPM4 or TRPV6 were inserted. To this respect an amino acid stretch from the TRPM6 pore was replaced by the sequence 981EDMDVA986 to construct the TRPM6-TRPM4 selectivity filter chimera and by the sequence 539TIIDGP544 to create the TRPM6-TRPV6 chimera.
As demonstrated for TRPM4 (24), when mutating D^{1030} into alanine, which is conserved throughout the entire TRPM subfamily, we obtained non-functional channels. This non-functional phenotype could be explained by a crucial role of D^{1030} for the integrity of TRPM6 pore. In contrast to TRPM4, at positions 1028 and 1036, TRPM6 contains non-charged residues while TRPM4 possesses glutamates at these positions and therefore, the delineation of the TRPM6 selectivity filter sequence appears different. However, we showed by alteration of the pore diameter due to mutation of E^{1029} and I^{1030} that the region from 1029 to 1031 determines the narrowest area of the channel pore.

Traditionally, the minimal pore size of an ion channel can be estimated by the largest permeant ion that carries a measurable current (16, 18). Recently, using this approach, the pore diameter of TRPV6 was estimated (26). Surprisingly, TRPM6-associated inward currents could be measured with all organic cations including NMDG^+ whereas for TRPV6, DMA^+ was the largest cation that could permeate the channel pore (26). The remarkable inward current measured with NMDG^+ as the sole charge carrier could be blocked in the presence of RR (data not shown), proving clearly that NMDG^+ is able to permeate TRPM6 pore. NMDG^+ permeation has been already described for TRPV1 and purinergic receptors P2X (27, 28). For estimation of the pore diameter of TRPM6, fitting of the relative permeation versus the cation diameter had to be extrapolated because we did not find any organic cation that does not permeate through TRPM6. Interestingly, the estimated diameter of the TRPM6 pore (1.15 nm) is significantly larger compared to other channels like TRPV6 (0.54 nm), voltage-gated Ca^{2+} channels and estimations for Ca^{2+} release activated currents (0.38 nm in Jurkat cells and 0.32-0.55 nm in rat basophilic leukemia cells (29-31). However, further studies are required to debate if molecular sieving is the main mechanism underlying the selectivity of Mg^{2+} channels. Recently, the selectivity filter of TRPM4 was revealed by Nilius et al. (24). They demonstrated that the pore properties of TRPM4 are determined by a subset of amino acid residues, in contrast to members of the TRPV subfamily in which a single amino acid residue accounts for the permeability properties of the pore. Our results support their observations and prove that members of the TRPM subfamily have a more complex pore structure compared to their close relatives of the TRPV subfamily.

**TRPM6 pore properties**

To investigate the key determinants of TRPM6 pore properties, two main directions were followed: first, the negatively charged residues of the putative pore region were neutralized, and second, the role of the conserved D^{1031} and other amino acid residues in its close vicinity were investigated. Mutation of the three negatively charged residues in the putative TRPM6 selectivity filter (E^{1024}, E^{1029} and D^{1031}) or in the conserved pore helix (E^{1012}, E^{1016}, data not shown) had important functional consequences since neutralization of these residues resulted in non-functional channels, except for E^{1029}. These data suggest that E^{1012}, E^{1016}, E^{1024} and D^{1031} play an important role in determining the pore properties of the channel. Neutralization of E^{1029} did not alter the current amplitude, but significantly reduced the sensitivity to RR, and Zn^{2+} and Ba^{2+} conductance. Most likely, negative charge at the inner mouth of the channel pore provides a binding site for positively charged cations, inducing open channel block (32). These data are in line with the fact that the pore diameter of this mutant is increased. We suggest that E^{1029} plays an important role in the TRPM6 pore architecture and takes part in the RR binding site of TRPM6.

Second, alteration of the amino acid residues in close vicinity of the conserved D^{1031} had relevant consequences as the mutation of I^{1030} and V^{1032} resulted in functional channels, but with different characteristics compared to wild-type TRPM6. Mutation of I^{1030} resulted in a channel with a smaller pore size. For this latter mutant additional properties have been affected: I^{1030}M is less permeable to Ni^{2+}, but displays the same conductance for Zn^{2+} and Ca^{2+} with wild-type TRPM6 and is more sensitive to RR. Despite the fact that Ni^{2+} has a smaller diameter than Ca^{2+} and Zn^{2+} and that I^{1030}M forms channels with a reduced pore diameter, the altered conductance could be explained by differences in ion’s hydration energy with water molecules and by structural changes in the selectivity filter encompassing the permeation of ions (33). Next, the V^{1032}A mutant showed no change in RR block and pore diameter, but had decreased current amplitudes indicating that the molecular determinants of the TRPM6 gating parameters may lay up-stream in the TRPM6 sequence. None of these mutations (E^{1029}, I^{1030}, V^{1032}) affected the Ca^{2+} and Mg^{2+} sensitivity which is in line with the hypothesis that pore mutations are not expected to influence Ca^{2+} binding to the activation site(s) (34).

We and other groups (6, 13) are able to record TRPM6-associated currents in HEK293 cells without co-expressing TRPM7, whereas Gudermann and co-workers...
suggested that TRPM6 requires co-expression with TRPM7 to form functional channels at the plasma membrane (11). Moreover, Li et al. could demonstrate functional differences at the divalent ions permeation and single channel conductance between homomeric TRPM6 and TRPM7 and heteromeric TRPM6/TRPM7 channels (13). These apparent contradictions could be reconciled by the fact that we can detect low TRPM7-like endogenous currents in non-transfected HEK293 cells.

In conclusion, the molecular determinants of Mg\(^{2+}\) selectivity and permeation of TRPM6 appear to be determined by changes in a stretch of amino acid residues from the pore region, \texttt{1028GEIDVC1033}, rather than by a single residue as demonstrated for the Ca\(^{2+}\) selective TRPV5 and TRPV6 channels. Subsequent studies like crystallographic assays will be needed to investigate in more detail the pore structure of TRPM6.

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The epithelial Mg$^{2+}$ channel TRPM6 is regulated by dietary Mg$^{2+}$ content and estrogens

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Abstract

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 (HSH) has recently elucidated TRPM6 as the gatekeeper in transepithelial Mg\(^{2+}\) transport, whereas its homolog TRPM7 is implicated in cellular Mg\(^{2+}\) homeostasis. The aim of the present study was to determine the tissue distribution in mouse and regulation of TRPM6 and TRPM7 by dietary Mg\(^{2+}\) and hormones. The present study demonstrates that TRPM6 is predominantly expressed in kidney, lung, cecum and colon, whereas TRPM7 is ubiquitously distributed. 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, we demonstrated that 17ß-estradiol, but not 1,25-dihydroxyvitamin D\(_3\) and parathyroid hormone, regulate 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 17ß-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 17ß-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.

Introduction

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 between 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\(^{2+}\). About 80% of the total plasma Mg\(^{2+}\) is filtered in the glomeruli (3, 4), of which the majority is subsequently
hormone (PTH). The effect of dietary Mg^{2+} content was studied by analyzing TRPM6 regulation at the mRNA and protein level and Mg^{2+} excretion and serum levels in C57BL6 mice fed a Mg^{2+} deficient, normal and enriched diet.

Materials and Methods

Animal studies

To evaluate TRPM6, TRPM7 and TRPV6 mRNA expression in various tissues, a cDNA panel was constructed. To this end, four C57BL6 mice, fed a complete diet containing 0.2% Mg^{2+} (w/w) (SSNIFF spezialdiäten GmbH, Soest, Germany), were sacrificed and 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 TRPM 7 expression in kidney and colon, C57BL6 mice (12 weeks of age), were fed for 10 days either a Mg^{2+} deficient diet (0.005% w/w Mg), normal Mg^{2+} diet (0.19% w/w Mg), or a Mg^{2+} enriched diet (0.48% w/w Mg) (SSNIFF spezialdiäten GmbH, Soest, Germany). The last 24 hours of the dietary treatment, animals were housed in metabolic cages and 24-hours urine was collected. At the end of the dietary treatment, blood samples were taken and the animals were sacrificed. Kidney and colon tissues were sampled and immediately frozen in liquid nitrogen.

The effect of 17ß-estradiol (17ß-E_2) on the renal TRPM6 mRNA expression level was evaluated by sham operated, bilateral ovariectomized (OVX) and OVX rats that received 2 x 500 µg 17ß-E_2/day as described previously (18). The effect of PTH was studied by sham operated, parathyroidectomized (PTX) rats and PTX rats that received 6 units/day bovine PTH as described previously (19). In addition, the effect of 1,25(OH)_{2}D_3 was studied by 1α-hydroxylase knockout (1α-OHase-/-) mice, heterozygous (1α-OHase +/-) mice phenotypically identical to wild type mice, and 1α-OHase-/- mice intraperitoneally supplemented with 1,25(OH)_{2}D_3 as described previously (20). The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from kidney, complete segments of the intestine and the other tissues using Trizol Total RNA Isolation Reagent (Gibco BRL, Breda, the...
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 (Gibco BRL) 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 targeting the genes of interest were designed using the computer program Primer Express (Applied Biosystems, Foster City, CA) and are listed in Table 1.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5'-TCGCAAGCTGATTTGTTATCATG-3'</td>
<td>5'-ATCCGCCGCTATGCACA-3'</td>
<td>5'-GAGCATGACGGAGGCTGCAC-3'</td>
</tr>
<tr>
<td>TRPM6</td>
<td>5'-ACAGGCTGGAGGAGCTGGGA-3'</td>
<td>5'-CCCTGCTGAACTCTCTTCCTG-3'</td>
<td>5'-AGTCTGGTCTGCTGCAGAGAG-3'</td>
</tr>
<tr>
<td>TRPM7</td>
<td>5'-CCCTGCTGAACTCTCTTCCTG-3'</td>
<td>5'-AGTCTGGTCTGCTGCAGAGAG-3'</td>
<td></td>
</tr>
<tr>
<td>TRPV6</td>
<td>5'-TACGTGGAGGAGCTGGGA-3'</td>
<td>5'-CCCTGCTGAACTCTCTTCCTG-3'</td>
<td>5'-AGTCTGGTCTGCTGCAGAGAG-3'</td>
</tr>
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PCR primers and fluorescent probes 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

**In vivo 45Ca2+ absorption assay**

Intestinal Ca2+ absorption was assessed in 2 groups of C57BL6 mice by measuring the amount of 45Ca2+ in serum at early time points after oral gavage (15 µl/g body weight). Mice were fasted 12 hours before the test. Animals were hemodynamically stable under anesthesia during the experiment. The solution used to measure Ca2+ absorption contained 0.1 mM CaCl2, 125 mM NaCl, 17 mM Tris pH 7.4, and 1.8 g/l fructose and was enriched with 20 µCi 45CaCl2/ml (18 Ci/g; New England Nuclear, Newton, Massachusetts, USA). One group received the 45Ca2+ solution supplemented with MgCl2 to a final concentration of 10 mM, whereas the 45Ca2+ solution of group 2 was not supplemented with MgCl2. Blood samples were obtained at different time intervals (2, 4, 8, 12 minutes). Radioactive 45Ca2+ was analyzed in serum (10 µl) by liquid scintillation counting. The change in the serum Ca2+ concentration was calculated from the 45Ca2+ content of the serum samples and the specific activity of the administrated Ca2+.

**Analytical procedures**

Serum Mg2+ and Ca2+ were measured using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, the Netherlands). Urinary Mg2+ and Ca2+ 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 (Gliedrecht, the Netherlands) equipped with a digital photo camera (Nikon DMX1200). For semiquantitative 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 analysis

Values are expressed as mean ± SEM. Differences between groups were tested by one-way analysis of variance (ANOVA) and further evaluated using Fisher's 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, California, USA) on a Macintosh computer.

Results

Tissue distribution of TRPM6 and TRPM7 in mouse

In order to study the quantitative expression levels of TRPM6 and TRPM7 in various tissues, a mouse cDNA panel was constructed. Subsequently, TRPM6 and TRPM7 mRNA levels were quantified 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), while 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, mRNA expression levels of TRPM6, TRPM7 and TRPV6 were quantified in different segments of the mouse intestinal tract by real-time PCR analysis and presented 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 predominantly expressed 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 primarily expressed in duodenum, cecum and colon, but was not detectable in jejunum and ileum (figure 1C).

Figure 1.

Expression profile of TRPM6 and TRPM7 in various mouse tissues

The mRNA expression levels of TRPM6 (A) and TRPM7 (B) in a panel of mouse tissues were measured by using quantitative real-time PCR. (C) Quantification of mRNA expression levels of TRPM6 (black), TRPM7 (white) and TRPV6 (grey) 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 HPRT RNA level. Data are presented as means ± SEM ($n = 4$).
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 figure 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 different Mg$^{2+}$ diets (figure 2A). Dietary Mg$^{2+}$ restriction significantly reduced the urinary Mg$^{2+}$ and Ca$^{2+}$ excretion compared to mice fed the normal Mg$^{2+}$ diet (figure 3). The Mg$^{2+}$ enriched diet significantly increased the urinary Mg$^{2+}$ and Ca$^{2+}$ excretion, compared to mice fed the normal diet.

Effect of dietary Mg$^{2+}$ content on 45Ca$^{2+}$ absorption
To investigate whether dietary Mg$^{2+}$ competes with the rate of Ca$^{2+}$ absorption, 2 groups of C57BL6 mice were orally administered a 45Ca$^{2+}$ solution containing 0.1 mM Ca$^{2+}$ with or without 10 mM MgCl$_2$. Changes in the serum 45Ca$^{2+}$ concentration ($\Delta$µM) were measured within 10 minutes after administration of 45Ca$^{2+}$ by oral gavage to C57BL6 mice. Data are averaged values ± SEM (n = 5) from mice of 12 weeks old. ■ 0.1 mM Ca$^{2+}$ and ▲ 0.1 mM Ca$^{2+}$ + 10 mM Mg$^{2+}$.

Effect of dietary Mg$^{2+}$ content on TRPM6 and TRPM7 expression
The effect of dietary Mg$^{2+}$ content on TRPM6 and TRPM7 expression

Urine and serum analysis of mice fed various Mg$^{2+}$ diets
Mice were fed a Mg$^{2+}$ deficient (0.005% w/w), normal (0.19% w/w) and enriched diet (0.48% w/w) for 10 days. At the end of this period, mice were housed for 24 hours in metabolic cages and urine samples were collected to investigate 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 figure 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 different Mg$^{2+}$ diets (figure 2A). Dietary Mg$^{2+}$ restriction significantly reduced the urinary Mg$^{2+}$ and Ca$^{2+}$ excretion compared to mice fed a normal Mg$^{2+}$ diet (figure 3). The Mg$^{2+}$ enriched diet significantly increased the urinary Mg$^{2+}$ and Ca$^{2+}$ excretion, compared to mice fed the normal diet.

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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
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 Mg²⁺ deficient (0.005% w/w), were significantly higher than those in the other groups. The regulation of TRPM6 and TRPM7 mRNA levels in colon was studied. The Mg²⁺ enriched diet resulted in an upregulation of TRPM6 mRNA expression level in colon compared to mice on a Mg²⁺ deficient diet (figure 6A). Like in kidney, variation in dietary Mg²⁺ content did not alter TRPM7 mRNA expression levels in complete colon 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 calcitropic hormones including 1,25(OH)₂D₃, PTH and 17β-E₂, on renal mRNA expression levels of TRPM6 and TRPM7, different animal models were used. 1α-OHase⁻/⁻ mice represent a unique animal model to...
expression levels remained unaffected in 1α-OHase<sup>−/−</sup> compared to 1α-OHase<sup>+/−</sup> mice and 1α-OHase<sup>−/−</sup> mice supplemented with 1,25(OH)<sub>2</sub>D<sub>3</sub> (figure 7A). Similarly, no effect was observed of PTH (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 17ß-E<sub>2</sub> normalized the TRPM6 expression levels in kidney of OVX rats. No differences in renal expression level of TRPM7 mRNA were observed in 1α-OHase<sup>−/−</sup>, PTX and OVX animals (figure 7B, D, F).

**Discussion**

The present study shows novel regulatory hallmarks of TRPM6 further supporting a gatekeeper function in the process of transepithelial Mg<sup>2+</sup> transport. First, this epithelial Mg<sup>2+</sup> channel was predominantly expressed in mouse epithelia including kidney, cecum, colon and lung. Second, 17ß-E<sub>2</sub> specifically upregulated TRPM6 mRNA expression in kidney pointing to the first magnesiotropic hormone in the maintenance of the Mg<sup>2+</sup> balance. Third, Mg<sup>2+</sup> depletion increased TRPM6 mRNA expression in kidney, whereas a Mg<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> reabsorption (13). Here, we now show that Mg<sup>2+</sup> excretion and TRPM6 expression in kidney is strongly regulated by the dietary Mg<sup>2+</sup> content. Dietary Mg<sup>2+</sup> restriction resulted in Mg<sup>2+</sup> conservation, whereas a Mg<sup>2+</sup> enriched diet increased urinary Mg<sup>2+</sup> excretion. The Mg<sup>2+</sup> deficient diet resulted in a significant upregulation of renal TRPM6 mRNA and protein levels, while the enriched diet tended to reduce TRPM6 abundance. TRPM7 expression was not influenced by the dietary Mg<sup>2+</sup> content supporting a general role in cellular Mg<sup>2+</sup> homeostasis (14, 15). Interestingly, dietary Mg<sup>2+</sup> restriction in humans also leads to renal Mg<sup>2+</sup> conservation (24-26), while high

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**Figure 7.** Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub>, PTH and 17ß-E<sub>2</sub> on the expression of TRPM6 and TRPM7 mRNA in kidney

The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub>, PTH and 17ß-E<sub>2</sub> on renal TRPM6 (A, C, E) and TRPM7 (B, D, F) mRNA expression levels was quantified by real-time PCR analysis and normalized for HPRT expression. Data are presented as means ± SEM (n = 4 - 5). * p < 0.05 versus all groups.

study the effect of the hormone 1,25(OH)<sub>2</sub>D<sub>3</sub> (23). To investigate the effect of PTH and 17ß-E<sub>2</sub>, parathyroidectomized and ovariectomized rats were used, respectively (18, 19). Quantitative real-time PCR demonstrated that renal TRPM6 mRNA
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). Thus, upregulation of TRPM6 in kidney supports a critical role of this channel facilitating maximal Mg\(^{2+}\) reabsorption during Mg\(^{2+}\) deficiency.

Interestingly, dietary Mg\(^{2+}\) content also influenced the Ca\(^{2+}\) excretion in our study since Mg\(^{2+}\) restriction resulted in Ca\(^{2+}\) conservation, whereas Mg\(^{2+}\) supplementation led to an increased urinary Ca\(^{2+}\) excretion. In accordance with this finding, Shafik and Quamme have shown that urinary Ca\(^{2+}\) excretion decreases in rats maintained on a low Mg\(^{2+}\) diet (7). This coupling between the Mg\(^{2+}\) and Ca\(^{2+}\) excretion observed under various dietary Mg\(^{2+}\) regimes could occur at the level of intestinal absorption and/or renal excretion. Since dietary Mg\(^{2+}\) contents did not affect the intestinal Ca\(^{2+}\) absorption rate, the coupling mechanism presumably resides within the kidney. This implies the existence of a common pathway or regulatory mechanism 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 can thus be envisaged that Mg\(^{2+}\) and Ca\(^{2+}\) are competitively transported by paracellin-1. This competition would imply 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 due to a Mg\(^{2+}\)-enriched diet. Indeed, Ikari et al. demonstrated that 45Ca\(^{2+}\) transport across monolayers of cells expressing paracellin-1 is inhibited by increased Mg\(^{2+}\) concentration (31). In addition, in patients with Gitelman syndrome or IDHH the observed hypomagnesemia is accompanied by a seriously diminished urinary Ca\(^{2+}\) excretion (32). Thus, 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 (CaSR) could play a role in the coupling between the Mg\(^{2+}\) and Ca\(^{2+}\) excretion under various dietary Mg\(^{2+}\) conditions, because the CaSR 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 that existing for Ca\(^{2+}\), Na\(^{+}\) and K\(^{+}\) (34). Interestingly, 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. Interestingly, 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 pre-eclampsia. However, different studies measuring estrogen levels in plasma from pre-eclamptic women have been inconsistent (38-41). Interestingly, 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 pre-eclampsia.

Previous reports demonstrated that PTH stimulates Mg\(^{2+}\) reabsorption in TAL and DCT (43, 44). In addition, 1,25(OH)\(_2\)D\(_3\) has also been shown to enhance the influx of Mg\(^{2+}\) in a mouse distal convoluted tubule (mDCT) cell line (5). Our study suggests that PTH and 1,25(OH)\(_2\)D\(_3\) are not involved in the stimulation of Mg\(^{2+}\) reabsorption via upregulation of renal TRPM6 expression levels since 1,25(OH)\(_2\)D\(_3\) and PTH did not change the TRPM6 expression in kidney. In addition, Karbach et al. demonstrated that cellular Mg\(^{2+}\) transport in rat colon is not responsive to 1,25(OH)\(_2\)D\(_3\) (45, 46).
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, however, not established \((11, 13)\). The highly abundant expression of TRPM6 in colon is further supported by the fact that colectomy in rats 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 implies that active intestinal Mg\(^{2+}\) absorption only partly overlaps with the process of active Ca\(^{2+}\) absorption. The transepithelial absorption of Ca\(^{2+}\) takes predominantly place 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 \((48, 49)\). Thus, active Mg\(^{2+}\) and Ca\(^{2+}\) absorption simultaneously take place in the distal part of the intestine, whereas in duodenum solely 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 transepidermal Mg\(^{2+}\) absorption capacity when fed a Mg\(^{2+}\) enriched diet. In comparison with Ca\(^{2+}\) absorption and TRPV6 expression, Brown et al. demonstrated that in rats fed a low Ca\(^{2+}\) diet, TRPV6 mRNA levels in duodenum were significantly increased \((50)\). In contrast, Van Abel et al. showed that supplementation of 1α-OHase knockout mice with a high dietary Ca\(^{2+}\) intake resulted in an increase of duodenal TRPV6 mRNA levels \((51)\). Thus, if the calcitropic hormone 1,25(OH)\(_2\)D\(_3\) is not present, 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 there is not a magnesiotropic hormone identified, which regulates intestinal TRPM6 expression and thereby Mg\(^{2+}\) absorption. We suggest that physiologically, an excess of Mg\(^{2+}\) absorption due to high dietary Mg\(^{2+}\) intake together with TRPM6 upregulation in colon, can be totally corrected by the kidney. Normally the kidney excretes only 2-4% of the filtered Mg\(^{2+}\) but is capable of increasing fractional excretion to nearly 100% in the face of increased serum Mg\(^{2+}\) levels \((52)\). Thus, the absence of a magnesiotropic hormone regulating 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 transepidermal 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 primarily involved in cellular Mg\(^{2+}\) homeostasis \((14, 15)\).

Interestingly, besides kidney and the intestine, TRPM6 is also 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 directly related to lung function, airway reactivity and respiratory symptoms in the general population \((53)\). Moreover, treatment of subjects with 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 is certainly needed to determine the precise function of TRPM6 in lung (patho)physiology.

In conclusion, TRPM6 is predominantly expressed in kidney, cecum, colon and lung suggesting that these organs are primarily involved in Mg\(^{2+}\) (re)absorption. Furthermore, we provide evidence that the intestinal site of active Mg\(^{2+}\) absorption is primarily located 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**

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Downregulation of renal TRPM6 leads to hypermagnesuria in TRPV5 knockout mice

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Manuscript in preparation
Abstract

The Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance is controlled by the concerted action of intestinal absorption, renal excretion, and exchange with bone of these ions. Primary disturbances of the Ca\textsuperscript{2+} balance often display a concomitant disturbance of Mg\textsuperscript{2+} homeostasis and vice versa. The kidney tightly regulates the amount of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} that is excreted in the urine. The epithelial Mg\textsuperscript{2+} channel transient receptor potential melastatin member 6 (TRPM6) is predominantly expressed in kidney and colon and constitutes the gatekeeper and postulated rate-limiting entry step in active Mg\textsuperscript{2+} (re)absorption. The epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6 have a similar function in active Ca\textsuperscript{2+} (re)absorption in kidney and small intestine, respectively. The aim of this study was to study the Mg\textsuperscript{2+} balance and regulation of TRPM6 and TRPM7 by dietary Mg\textsuperscript{2+} content in TRPV5 knockout (TRPV5\textsuperscript{-/-}) mice, which display severe hypercalcuria and hypervitaminosis D-mediated Ca\textsuperscript{2+} hyperabsorption. First, ablation of the TRPV5 gene in mice resulted in hypermagnesuria and a significant downregulation of the renal TRPM6 mRNA and protein expression levels. Second, a Mg\textsuperscript{2+}-deficient diet upregulated renal TRPM6 mRNA and protein expression levels in wild-type (TRPV5\textsuperscript{+/+}) and TRPV5\textsuperscript{-/-} mice and resulted in concomitant renal Mg\textsuperscript{2+} and Ca\textsuperscript{2+} conservation. Third, dietary Mg\textsuperscript{2+} supplementation resulted in increased expression levels of TRPM6 mRNA in colon and increased the urinary excretion of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}. Fourth, this study provided evidence that hypervitaminosis D, which is present in TRPV5\textsuperscript{-/-} mice, and not the lack of TRPV5 caused the hypermagnesuria. The results of this study showed that the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance in TRPV5\textsuperscript{-/-} mice is tightly coupled and provided new insight about the regulation of TRPM6 in the absence of the epithelial Ca\textsuperscript{2+} channel TRPV5.

Introduction

Numerous enzymatic reactions are dependent on the presence of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}. Moreover, maintenance of the body Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance is of crucial importance for many physiological functions including neural excitability, muscle contraction, and cell adhesion (1-5). The Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance is controlled by the concerted action of intestinal absorption, renal excretion, and exchange with bone. In kidney, the majority of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} reabsorption occurs via passive paracellular...
transport in the proximal tubule and the thick ascending loop of Henle (1, 6, 7). The distal convoluted tubule (DCT) reabsorbs 5-10% of the filtered Mg^{2+} and beyond this segment virtually no Mg^{2+} reabsorption takes place. Reabsorption of Mg^{2+} in DCT is active and transcellular in nature and determines the final amount of Mg^{2+} excreted in the urine. Of the filtered Ca^{2+}, approximately 10-15% is actively reabsorbed in DCT and the connecting tubule (CNT). Thus, the DCT fine-tunes the urinary Mg^{2+} concentration and DCT and CNT together do the same for the urinary Ca^{2+} concentration. Similarly, intestinal Ca^{2+} and Mg^{2+} absorption consists of a passive paracellular and an active transcellular pathway (8-10).

Transcellular transport of Mg^{2+} and Ca^{2+} through the renal and intestinal epithelia can be envisaged by three sequential steps. First, driven by a favorable transmembrane potential, Mg^{2+} enters the cell through the luminal Mg^{2+} channel TRPM6, whereas Ca^{2+} enters through TRPV5 and TRPV6. The second step is the intracellular diffusion to the basolateral side of the cell. During this process Ca^{2+} is bound to the Ca^{2+}-binding proteins calbindinD_{28K} and/or calbindin-D_{32K}. The presence and identity of the cytosolic Mg^{2+} binding proteins facilitating intracellular diffusion of Mg^{2+} are completely unknown. Finally, Mg^{2+} and Ca^{2+} are extruded at the basolateral side of the cell. Ca^{2+} is extruded basolaterally by two pathways: the plasma membrane Ca^{2+}-ATPase and the Na^{+}/Ca^{2+} exchanger. The protein(s) involved in the extrusion of Mg^{2+} remain(s) to be identified. Since the luminal Mg^{2+} and Ca^{2+} influx forms the initial step of transcellular transport, the transporters involved in this process provide efficient targets for the regulation of Mg^{2+} and Ca^{2+} (re)absorption.

A positional candidate gene-cloning approach identified mutations of TRPM6 in patients with hypomagnesemia with secondary hypocalcemia (HSH) (8, 11). The TRPM6 protein is a member of the TRP superfamily and is homologous to the more ubiquitously expressed Mg^{2+} channel TRPM7. The epithelial Mg^{2+} channel TRPM6 facilitates Mg^{2+} influx in the process of active Mg^{2+} (re)absorption and is localized along the luminal membrane of the DCT and in the brush border membrane of the small intestine (12). TRPM7 has been identified as a Mg^{2+}-permeable ion channel primarily required for cellular Mg^{2+} homeostasis (13-15). TRPM6 and TRPM7 are cation channels with permeability for Mg^{2+}, which play a pivotal role in Mg^{2+} homeostasis in vertebrates at the body and cellular level, respectively (8, 11, 15-17).

Both TRPM6 and TRPM7 are unique bifunctional proteins as they combine channel function with protein kinase activity (13, 18, 19). The analogous epithelial influx pathway for Ca^{2+} is constituted by the epithelial Ca^{2+} channels TRPV5 and TRPV6 (1). These channels are by far the most Ca^{2+} selective channels of the TRP superfamily and constitute the rate-limiting influx step in active Ca^{2+} (re)absorption that takes place in kidney, small intestine, placenta and bone (1, 20).

Many diseases have been reported in which patients show mutual disturbances of the Mg^{2+} and Ca^{2+} balance and a tight coupling of the Mg^{2+} and Ca^{2+} homeostasis is frequently observed in animal models (6, 21). For most of these diseases, the cascades of cellular and molecular events that lead to this combined disturbance are largely unknown. Previous studies showed that TRPV5 knockout (TRPV5-/-) mice displayed renal Ca^{2+} wasting, hypervitaminosis D, compensatory Ca^{2+} hyperabsorption and bone abnormalities (22). Since mutual disturbances of the Mg^{2+} and Ca^{2+} balance are common and TRPV5-/- mice display renal Ca^{2+} wasting, the aim of the present study was to reveal the effect of TRPV5 ablation and the effect of dietary Mg^{2+} content on the Mg^{2+} and Ca^{2+} balance in TRPV5-/- mice. To this end, the current study investigated the systemic Mg^{2+} balance and the expression levels of the Mg^{2+} transport proteins TRPM6 and TRPM7 in kidney and colon of TRPV5-/- mice fed a Mg^{2+}-deficient, Mg^{2+}-normal or a Mg^{2+}-enriched diet. In addition, the effect of 1,25-dihydroxyvitamin D_{3} (1,25(OH)_{2}D_{3}) on the urinary excretion of Mg^{2+} was studied in 25-hydroxyvitamin D_{3}-1α-hydroxylase knockout (1α-OHase –/–) and TRPV5/1α-hydroxylase double knockout (TRPV5 –/–/1α-OHase –/–) mice, which show undetectable serum 1,25(OH)_{2}D_{3} levels because the ablation of the cytochrome P450 enzyme 25-hydroxyvitamin D_{3}-1α-hydroxylase prevents the synthesis of 1,25(OH)_{2}D_{3} (22-24).

Materials and methods

Animal experiments
To study the effect of dietary Mg^{2+} content on TRPM6 and TRPM7 expression in kidney and colon, wild-type (TRPV5+/+), and TRPV5 knockout (TRPV5-/-) mice (12 weeks of age) were fed for 10 days a Mg^{2+}-deficient diet (0.005% w/w Mg^{2+}), a Mg^{2+}-normal diet (0.19% w/w Mg^{2+}), or a Mg^{2+}-enriched diet (0.48% w/w Mg^{2+}; SSNIFF spezialdiäten GmbH, Germany).
TRPV5−/− mice were generated as described previously (23). During the last 24 hours of the dietary treatment, animals were housed in metabolic cages and urine was collected. Subsequently, blood samples were taken and the animals were sacrificed. Kidney and colon tissues were sampled and frozen immediately in liquid nitrogen. In order to evaluate the effect of the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) on the Mg2+ balance, the 24-hour urinary Mg2+ excretion was analyzed of TRPV5+/+, TRPV5−/−, 25-hydroxyvitamin D3-1α-hydroxylase knockout (1α-OHase −/−) (25) and TRPV5/1α-hydroxylase double knockout (TRPV5−/−/1α-OHase−/−) mice. Cross-breeding 1α-OHase−/− and TRPV5−/− mice resulted in offspring heterozygous for both TRPV5 and 1α-OHase (TRPV5+/−/1α-OHase+/−). This offspring was subsequently intercrossed to obtain TRPV5−/−/1α-OHase−/− mice (24). Genotypes were determined by PCR analysis, using specific primers as described previously (22, 25). In order to obtain 24-hour urine samples of all mouse genotypes, 12 week old mice were kept in a light- and temperature-controlled room in metabolic cages that enabled 24-h urine collection. Standard pelleted chow (1.1% w/w Ca2+, 0.19% w/w Mg2+, SSNIFF spezialdiäten GmbH, Soest, Germany) and drinking water were available *ad libitum*. The animal ethics board of the Radboud University Nijmegen Medical Centre approved all experimental procedures.

**Quantitative real-time PCR analysis**

Total RNA was extracted from kidney and colon 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, USA) to prevent genomic DNA contamination. Thereafter, 2 µg of RNA was reverse transcribed by Molony-Murine Leukemia Virus-Reverse Transcriptase (Invitrogen, Breda, the Netherlands) as described previously (26). The cDNA was used to determine TRPM6 and TRPM7 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 TRPM6, TRPM7 and HPRT cDNA were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA) and are listed in table 1.
Mg²⁺-deficient diet resulted in a significant hypomagnesemia in TRPV5 +/+ mice (0.59 ± 0.05 mM vs. 1.49 ± 0.08 mM, p < 0.05) as well as in TRPV5 -/- mice (0.67 ± 0.05 mM vs. 1.46 ± 0.02 mM) (figure 1). In contrast, no significant differences were observed in serum Mg ²⁺ and Ca²⁺ concentrations when mice were fed the Mg²⁺-enriched diet. To examine the effect of dietary Mg²⁺ content on the urinary Mg²⁺ and Ca²⁺ excretion in TRPV5 -/- mice, both TRPV5 +/+ and TRPV5 -/- mice were placed in metabolic cages to enable 24 hours urine sample collection. TRPV5 -/- mice fed a Mg²⁺-normal (0.19% w/w) diet displayed robust hypercalciuria compared to TRPV5 +/+ mice (9.9 ± 0.7 µmol/24h/mouse vs. 0.6 mM ± 0.3 µmol/24h/mouse, respectively, p < 0.05). Hypercalciuria in TRPV5 -/- mice was observed in all three dietary Mg²⁺ groups. However, the total amount of urinary Ca²⁺ excreted in 24 hours by TRPV5 -/- mice varied among these groups. TRPV5 -/- mice on a Mg²⁺-deficient diet excreted significantly less Ca²⁺ than TRPV5 -/- mice on a Mg²⁺-normal diet (1.9 µmol/24h/mouse ± 0.8 vs. 9.9 µmol/24h/mouse ± 0.7, respectively, p < 0.05). In contrast, TRPV5 -/- mice on a Mg²⁺-enriched diet excreted significantly more Ca²⁺ than TRPV5 -/- mice fed a Mg²⁺-normal diet (25.9 ± 3.5 µmol/24h/mouse vs. 9.9 ± 0.7 µmol/24h/mouse, respectively, p < 0.05) (figure 2A). Interestingly, besides hypercalciuria, TRPV5 -/- mice display robust hypermagnesuria compared to TRPV5 +/+ mice (32.6 ± 6.6 µmol/24h/mouse vs. 5.6 ± 3.0 µmol/24h/mouse, respectively, p < 0.05) (figure 2B). Similarly with hypercalciuria in TRPV5 -/- mice, the presence of hypermagnesuria in these mice is independent of dietary Mg²⁺ content but the magnitude of the hypermagnesuria decreases significantly when fed a Mg²⁺-deficient diet compared to a Mg²⁺-normal diet (0.2 ± 0.1 µmol/24h/mouse vs. 5.6 ± 3.0 µmol/24h/mouse, respectively, p < 0.05). In addition, increased Mg²⁺ excretion was observed when TRPV5 -/- mice were fed the Mg²⁺-enriched diet compared to TRPV5 -/- mice fed the Mg²⁺-normal diet (92.6 ± 7.7 µmol/24h/mouse vs. 5.6 ± 3.0 µmol/24h/mouse, respectively, p < 0.05) (figure 2B).

Effect of dietary Mg²⁺ content on the renal TRPM6 and TRPM7 expression levels
To determine the molecular mechanism responsible for the hypermagnesuria presented by TRPV5 -/- mice, renal TRPM6 and TRPM7 mRNA expression levels were determined by quantitative real-time PCR. A significant decrease in renal

Analytical procedures
Serum Mg²⁺ and Ca²⁺ concentrations were measured using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Almere, the Netherlands). Urinary Mg²⁺ and Ca²⁺ concentration was determined by atomic absorption spectrophotometry on a Perkin Elmer Analyst 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 an affinity-purified guinea pig anti-TRPM6 antibody, as described previously (12). Pictures covering the entire kidney 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, USA), resulting in semi-quantification of the protein levels as the mean of integrated optical density (27).

Statistical analysis
Values are expressed as means ± SEM. Differences between groups were tested by one-way ANOVA and evaluated using Fisher’s 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, USA) on a Macintosh computer.

Results
Effect of dietary Mg²⁺ content on the Mg²⁺ and Ca²⁺ balance
TRPV5 -/- mice and their TRPV5 +/+ (wild-type) littermates were fed a Mg²⁺-deficient (0.005% w/w), Mg²⁺-normal (0.19% w/w) and a Mg²⁺-enriched diet (0.48% w/w) for 10 days. Directly after this period blood was sampled for Mg²⁺ and Ca²⁺ analysis. The serum Mg²⁺ and Ca²⁺ concentrations are depicted in figure 1. As previously shown, inactivation of both alleles of the TRPV5 gene did not result in altered serum Mg²⁺ and Ca²⁺ concentrations (22). Moreover, dietary Mg²⁺ content did not influence serum Ca²⁺ levels in TRPV5 +/+ and TRPV5 -/- mice. Interestingly, the Mg²⁺-deficient diet resulted in a significant hypomagnesemia in TRPV5 +/+ mice (0.59 ± 0.05 mM vs. 1.49 ± 0.08 mM, p < 0.05) as well as in TRPV5 -/- mice (0.67 ± 0.05 mM vs. 1.46 ± 0.02 mM) (figure 1).
expression, the relative amounts of immunopositive tubules in the total kidney cortex were counted for each section. The averaged values for each experimental group are presented in figure 4B. Within all dietary groups, significantly reduced TRPM6 protein abundance was detected in TRPV5 -/- mice compared to TRPV5 +/+ mice (All values TRPV5 +/+ vs. TRPV5 -/-, respectively: Mg 2+-deficient diet; 251 ± 61 vs. 102 ± 46, Mg 2+-normal diet; 100 ± 41 vs. 19 ± 4, Mg 2+-enriched diet; 129 ± 19 vs. 42 ± 15, \( p < 0.05 \)), whereas an increase in renal TRPM6 protein expression was observed in TRPV5 +/+ and TRPV5 -/- mice fed the Mg 2+-deficient diet compared to those mice fed the Mg 2+-normal diet (All values Mg 2+-deficient diet vs. Mg 2+-normal diet, respectively: TRPV5 +/+; 251 ± 61 vs. 100 ± 41 and TRPV5 -/-; 102 ± 46 vs. 19 ± 4, \( p < 0.05 \), (figure 4)).

Effect of dietary Mg 2+ content on TRPM6 and TRPM7 expression in colon

The effect of dietary Mg 2+ content on intestinal TRPM6 and TRPM7 mRNA expression levels was studied by quantitative real-time PCR. The abundance of TRPM6 mRNA in colon of TRPV5 +/+ and TRPV5 -/- mice was not significantly

TRPM6 mRNA expression was observed in TRPV5 +/+ mice fed a Mg 2+-normal and Mg 2+-deficient diet compared to TRPV5 -/- mice on a Mg 2+-normal and Mg 2+-enriched diet, respectively (0.019 ± 0.004 and 0.015 ± 0.001 vs. 0.033 ± 0.005 0.026 ± 0.002, respectively, \( p < 0.05 \)) (figure 3A). Interestingly, increased renal TRPM6 mRNA expression levels were observed in TRPV5 +/+ and TRPV5 -/- mice fed a Mg 2+-deficient diet (0.049 ± 0.006 and 0.047 ± 0.009, respectively) compared to TRPV5 +/+ and TRPV5 -/- mice, respectively, fed a Mg 2+-normal (0.034 ± 0.005 and 0.019 ± 0.004, respectively, \( p < 0.05 \)) or a Mg 2+-enriched diet (0.026 ± 0.002 and 0.015 ± 0.001, respectively, \( p < 0.05 \)) (figure 3A). The Mg 2+-enriched diet did not result in altered renal TRPM6 mRNA levels in TRPV5 +/+ and TRPV5 -/- mice compared to both mice fed the Mg 2+-normal diet. No difference in TRPM6 mRNA expression was observed between TRPV5 +/+ and TRPV5 -/- mice fed a Mg 2+-deficient diet. In contrast to TRPM6, TRPM7 mRNA expression levels were unaffected in TRPV5 -/- mice. Furthermore, renal TRPM7 mRNA expression levels were not altered by dietary Mg 2+ content in these mice. Next, the abundance of TRPM6 protein in kidneys was examined by immunohistochemistry. Figure 4A presents representative immunofluorescence labeling of DCT in renal sections of mice fed the various Mg 2+ diets. In order to semi-quantify TRPM6 protein

![Figure 3A](image1.png)

**Figure 3A.** Effect of dietary Mg 2+ content on renal TRPM6 mRNA expression levels in TRPV5 +/+ and TRPV5 -/- mice. TRPM6 mRNA expression was observed in TRPV5 -/- mice fed a Mg 2+-normal and Mg 2+-enriched diet compared to TRPV5 +/+ mice on a Mg 2+-normal and Mg 2+-enriched diet, respectively (0.019 ± 0.004 and 0.015 ± 0.001 vs. 0.033 ± 0.005 0.026 ± 0.002, respectively, \( p < 0.05 \)) (figure 3A). Interestingly, increased renal TRPM6 mRNA expression levels were observed in TRPV5 +/+ and TRPV5 -/- mice fed a Mg 2+-deficient diet (0.049 ± 0.006 and 0.047 ± 0.009, respectively) compared to TRPV5 +/+ and TRPV5 -/- mice, respectively, fed a Mg 2+-normal (0.034 ± 0.005 and 0.019 ± 0.004, respectively, \( p < 0.05 \)) or a Mg 2+-enriched diet (0.026 ± 0.002 and 0.015 ± 0.001, respectively, \( p < 0.05 \)) (figure 3A). The Mg 2+-enriched diet did not result in altered renal TRPM6 mRNA levels in TRPV5 +/+ and TRPV5 -/- mice compared to both mice fed the Mg 2+-normal diet. No difference in TRPM6 mRNA expression was observed between TRPV5 +/+ and TRPV5 -/- mice fed a Mg 2+-deficient diet. In contrast to TRPM6, TRPM7 mRNA expression levels were unaffected in TRPV5 -/- mice. Furthermore, renal TRPM7 mRNA expression levels were not altered by dietary Mg 2+ content in these mice. Next, the abundance of TRPM6 protein in kidneys was examined by immunohistochemistry. Figure 4A presents representative immunofluorescence labeling of DCT in renal sections of mice fed the various Mg 2+ diets. In order to semi-quantify TRPM6 protein

expression, the relative amounts of immunopositive tubules in the total kidney cortex were counted for each section. The averaged values for each experimental group are presented in figure 4B. Within all dietary groups, significantly reduced TRPM6 protein abundance was detected in TRPV5 -/- mice compared to TRPV5 +/+ mice (All values TRPV5 +/+ vs. TRPV5 -/-, respectively: Mg 2+-deficient diet; 251 ± 61 vs. 102 ± 46, Mg 2+-normal diet; 100 ± 41 vs. 19 ± 4, Mg 2+-enriched diet; 129 ± 19 vs. 42 ± 15, \( p < 0.05 \)), whereas an increase in renal TRPM6 protein expression was observed in TRPV5 +/+ and TRPV5 -/- mice fed the Mg 2+-deficient diet compared to those mice fed the Mg 2+-normal diet (All values Mg 2+-deficient diet vs. Mg 2+-normal diet, respectively: TRPV5 +/+; 251 ± 61 vs. 100 ± 41 and TRPV5 -/-; 102 ± 46 vs. 19 ± 4, \( p < 0.05 \), (figure 4)).
different when fed the same diet (figure 4). However, the Mg$_{2+}$-enriched diet resulted in a significant upregulation of TRPM6 mRNA expression levels in colon of TRPV5$^+$/+ and TRPV5$^-$/- mice compared to both groups, respectively, fed the Mg$_{2+}$-deficient diet (0.54 ± 0.09 and 0.49 ± 0.08, respectively, $p < 0.05$) (figure 4A). In addition, no differences in TRPM7 mRNA expression levels in colon were observed between TRPV5$^+$/+ and TRPV5$^-$/- mice. Moreover, differences in dietary Mg$_{2+}$ content did not influence TRPM7 mRNA expression levels in colon of both TRPV5$^+$/+ and TRPV5$^-$/- mice (figure 4B).

**Effect of 1,25(OH)$_{2}$D$_{3}$ on urinary excretion of Mg$_{2+}$**

To assess whether hypervitaminosis D in TRPV5$^-$/- mice has an effect on the amount of urinary Mg$_{2+}$ excreted, TRPV5$^+$/+, TRPV5$^-$/-, 1α-OHase$^-$/- and TRPV5$^-$/-/1α-OHase$^-$/- mice were placed in metabolic cages to collect urine over a 24 hour period, enabling the subsequent analysis of the urinary Mg$_{2+}$ excretion. Hypermagnesuria was observed for TRPV5$^-$/- mice compared to wild-type mice (47.3 ± 4.7 µmol/24h/mouse vs. 23.7 ± 3.9 µmol/24h/mouse, respectively, $p < 0.05$).
whereas the 1α-OHase−/− and TRPV5−/-/1α-OHase−/− mice, which show undetectable levels of serum 1,25(OH)_{2}D_{3}, show significantly lowered 24 hour urinary Mg^{2+} excretion compared to TRPV5+/+ mice (6.5 ± 3.8 µmol/24h/mouse and 5.1 ± 2.9 µmol/24h/mouse, respectively, vs. 23.7 ± 3.9 µmol/24h/mouse, p < 0.05) (figure 5).

**Discussion**

This present study showed that the Mg^{2+} and Ca^{2+} balance in TRPV5−/− mice is tightly coupled and how TRPM6 is regulated in the absence of the epithelial Ca^{2+} channel TRPV5. First, ablation of the TRPV5 gene in mice resulted in robust hypermagnesuria and a significant reduction in renal TRPM6 mRNA and protein abundance. TRPM7 mRNA expression levels in kidney and colon were unaltered in TRPV5−/− mice. Second, dietary Mg^{2+} restriction resulted in upregulation of renal TRPM6 expression and simultaneous renal Mg^{2+} and Ca^{2+} conservation in TRPV5+/+ and TRPV5−/− mice. Third, dietary Mg^{2+} supplementation resulted in an upregulation of TRPM6 expression levels in colon of both TRPV5+/+ and TRPV5−/− mice and increased the urinary excretion of Mg^{2+} and Ca^{2+}. Fourth, absence of 1,25(OH)_{2}D_{3} results in hypomagnesuria even in mice in which TRPV5 is knocked out.

Our study demonstrated that TRPV5−/− mice, which display robust renal Ca^{2+} wasting and compensatory intestinal Ca^{2+} hyperabsorption (22), also present hypermagnesuria. Interestingly, serum Mg^{2+} levels and TRPM6 mRNA expression levels in colon were unaffected, whereas renal TRPM6 expression levels were downregulated in TRPV5−/− mice. Although reduced renal TRPM6 expression levels can explain increased urinary Mg^{2+} excretion, the mechanism underlying diminished TRPM6 expression is elusive.

In addition, Mg^{2+} excretion and TRPM6 expression in kidney and colon are strongly regulated by dietary Mg^{2+} content in TRPV5−/− mice. Dietary Mg^{2+} restriction resulted in Mg^{2+} conservation, whereas a Mg^{2+}-enriched diet increased urinary Mg^{2+} excretion. Interestingly, the dietary Mg^{2+} content also influenced the Ca^{2+} excretion in TRPV5−/− mice. Mg^{2+} restriction resulted in Ca^{2+} conservation, whereas Mg^{2+} supplementation stimulated urinary Ca^{2+} excretion. Similarly, Shafik and Quamme reported that rats fed a low Mg^{2+}-diet displayed decreased urinary Ca^{2+}

**Figure 5.** The effect of 1,25(OH)_{2}D_{3} on urinary Mg^{2+} excretion

Urinary Mg^{2+} excretion was determined in wild-type (TRPV5+/+), TRPV5 knockout (TRPV5−/−), 1α-hydroxylase knockout (1α-OHase−/−) and TRPV5/1α-hydroxylase double knockout mice (TRPV5−/−/1α-OHase−/−). Data are presented as means ± SEM (n = 5). *p < 0.05 versus TRPV5+/+ mice.

Excretion (28). The mechanism responsible for the concomitant excretion of Ca^{2+} in response to altered dietary Mg^{2+} intake remains elusive. Possibly, Mg^{2+} and Ca^{2+} are competitively (re)absorbed in intestine and kidney via a paracellular pathway.

Thus, in the presence of low luminal Mg^{2+} concentrations when fed the Mg^{2+}-deficient diet, Ca^{2+} (re)absorption is favoured, which could explain the renal conservation of Ca^{2+}. Conversely, the Mg^{2+}-enriched diet could result in a high luminal Mg^{2+}-load in intestine and kidney resulting in decreased (re)absorption of Ca^{2+}. 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 (re)absorption of these ions (29).

The Mg^{2+}-deficient diet significantly upregulated renal TRPM6 mRNA and protein levels, possibly maximizing Mg^{2+} reabsorption in the presence of a reduced filtered Mg^{2+} load. Although TRPV5−/− mice fed the Mg^{2+}-deficient diet were hypomagnesemic, these mice still displayed hypermagnesuria compared to TRPV5+/+ mice fed the
same diet, indicating the presence of renal Mg\(^{2+}\)-loss. However, the renal upregulation of TRPM6 protein in TRPV5\(^{-/-}\) mice fed the Mg\(^{2+}\)-deficient diet was significantly less compared to TRPV5\(^{+/+}\) mice fed the same diet. After 10 days of dietary Mg\(^{2+}\) restriction, the TRPV5\(^{-/-}\) mice still displayed hypermagnesuria. Thus, the upregulation of renal TRPM6 protein abundance after this dietary period was insufficient to normalize the Mg\(^{2+}\) reabsorption for the TRPV5\(^{-/-}\) mice. In contrast, renal TRPM6 mRNA abundance of wild-type and TRPV5\(^{-/-}\) mice fed a Mg\(^{2+}\)-deficient diet was similar, suggesting equal amounts of TRPM6 protein expression. Interestingly, in both TRPV5\(^{+/+}\) and TRPV5\(^{-/-}\) mice, TRPM6 mRNA levels in colon are upregulated when fed a Mg\(^{2+}\)-enriched diet but not during dietary Mg\(^{2+}\)-restriction. Previously, we suggested that this apparent discrepancy in the regulation of TRPM6 expression upon dietary Mg\(^{2+}\) content is caused by the absence of a hormone regulating intestinal Mg\(^{2+}\) absorption (30). In analogy with Ca\(^{2+}\), feeding a Ca\(^{2+}\)-enriched diet to 1α-OHase\(^{-/-}\) mice, in which the synthesis of 1,25(OH)\(_2\)D\(_3\) is prevented, resulted in increased duodenal mRNA expression levels of the epithelial Ca\(^{2+}\)-channel TRPV6 (31). The molecular mechanisms resulting in the opposite regulation of TRPM6 expression in kidney and colon in response to Mg\(^{2+}\) enrichment and a Mg\(^{2+}\)-enriched diet, need additional investigation. Possibly, the Ca\(^{2+}\) sensing receptor, which is present in kidney and colon (32-34), exerts an opposite function in the regulation of renal and intestinal TRPM6 expression levels upon Mg\(^{2+}\) restriction and supplementation. Dietary Mg\(^{2+}\) content did not influence TRPM7 expression levels in kidney and colon, excluding a major role for TRPM6 closest homologue in systemic Mg\(^{2+}\) handling (15, 17).

Previously, it was reported that hypervitaminosis D mediates compensatory Ca\(^{2+}\) hyperabsorption in TRPV5\(^{-/-}\) mice, in order to maintain normocalcemia (24). Here, we show that 1α-OHase\(^{-/-}\) and TRPV5\(^{-/-}/1α-OHase^{-/-}\) mice, which both have undetectable levels of 1,25(OH)\(_2\)D\(_3\), display hypomagnesuria. Thus, the lack of TRPV5 does not directly result in hypomagnesuria and renal TRPM6 downregulation. Interestingly, 1α-OHase\(^{-/-}\) mice display hypomagnesuria and unaltered renal TRPM6 mRNA expression levels compared to wild-type mice (30). This suggests that the disturbance of the Mg\(^{2+}\) balance in these mice originates from the intestine possibly due to disturbed Mg\(^{2+}\) absorption in the absence of 1,25(OH)\(_2\)D\(_3\). Thus, the normal renal TRPM6 expression levels in 1α-OHase\(^{-/-}\) mice are relatively high in the presence of the suggested reduction of Mg\(^{2+}\) absorption. To delineate the role of 1,25(OH)\(_2\)D\(_3\) in the Mg\(^{2+}\) homeostasis of these mice, the Mg\(^{2+}\) balance and TRPM6 expression levels in kidney and colon of 1α-OHase\(^{-/-}\) mice should be studied after different dietary Mg\(^{2+}\) regimes and during 1,25(OH)\(_2\)D\(_3\) supplementation. Moreover, the observed hypomagnesuria in TRPV5\(^{-/-}/1α-OHase^{-/-}\) mice strongly suggest the involvement of hypervitaminosis D as a cause for the hypomagnesuria in TRPV5\(^{-/-}\) mice. Therefore, we hypothesize that hypervitaminosis D in TRPV5\(^{-/-}\) mice, which stimulates the compensatory hyperabsorption of Ca\(^{2+}\), results in parallel hyperabsorption of Mg\(^{2+}\). Consequently, to maintain a normal Mg\(^{2+}\) balance the surplus of Mg\(^{2+}\) absorbed needs to be corrected by the kidney, which responds by downregulation of TRPM6 resulting in hypermagnesuria. Although there is evidence that increased paracellular Mg\(^{2+}\) transport can be stimulated by 1,25(OH)\(_2\)D\(_3\), the presence of simultaneous hyperabsorption of Mg\(^{2+}\) and Ca\(^{2+}\) in TRPV5\(^{-/-}\) mice needs further research (35). Karbach demonstrated that cellular Mg\(^{2+}\) transport in rat colon is not responsive to 1,25(OH)\(_2\)D\(_3\) and paracellular transport of Ca\(^{2+}\) has been suggested to be positively influenced by 1,25(OH)\(_2\)D\(_3\) via an yet unknown mechanism (1, 36, 37). In the intestinal tract, the duodenum constitutes the site which absorbs most of the ingested Ca\(^{2+}\) and would therefore be the obvious segment to study simultaneous 1,25(OH)\(_2\)D\(_3\)-induced hyperabsorption of Mg\(^{2+}\) and Ca\(^{2+}\) (1, 36, 37). Possibly hypervitaminosis D stimulates paracellular Mg\(^{2+}\) and Ca\(^{2+}\) transport or increases transcellular Mg\(^{2+}\) absorption via TRPM6 in colon. Whether hypervitaminosis D results in hypermagnesuria, the Mg\(^{2+}\) balance of TRPV5\(^{-/-}\) mice with induced hypervitaminosis D needs to be investigated. Although it is tempting to speculate that hypermagnesuria and renal TRPM6 downregulation in TRPV5\(^{-/-}\) mice is caused by hypervitaminosis D, the Mg\(^{2+}\) balance and TRPM6 expression in kidney and intestine of 1α-OHase\(^{-/-}\) and TRPV5\(^{-/-}/1α-OHase^{-/-}\) mice could provide additional evidence to delineate the role and mechanism of the coupled Mg\(^{2+}\) and Ca\(^{2+}\) balance with respect to 1,25(OH)\(_2\)D\(_3\).

In conclusion, TRPV5\(^{-/-}\) mice, which display hypercalcemia, hypervitaminosis D and compensatory Ca\(^{2+}\) hyperabsorption showed hypermagnesuria and renal TRPM6 downregulation. Furthermore, dietary Mg\(^{2+}\) restriction and supplementation are positively involved in the regulation of TRPM6 in kidney and colon of TRPV5\(^{-/-}\) mice, respectively. In addition, we demonstrated that not the functional loss of TRPV5 is primarily responsible for the hypomagnesuria in TRPV5\(^{-/-}\) mice and that disruption of 1,25(OH)\(_2\)D\(_3\) synthesis in TRPV5\(^{-/-}\) mice results in hypomagnesuria, indicating an
important role of 1,25(OH)2D3 in Mg2+ homeostasis. Future research should aim to further unravel the mechanism controlling the coupled regulation of the Mg2+ and Ca2+ balance in TRPV5−/− mice, and in particular the role of 1,25(OH)2D3 in the Mg2+ balance, which may lead to new insights regarding Mg2+ and Ca2+ homeostasis-related disorders.

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Chapter 4

Hypermagnesuria in TRPV5 knockout mice


Impaired basolateral sorting of pro-EGF causes isolated recessive renal hypomagnesemia
Abstract

Primary hypomagnesemia constitutes a rare heterogeneous group of disorders characterized by renal or intestinal magnesium (Mg²⁺) wasting resulting in generally shared symptoms of Mg²⁺ depletion, such as tetany and generalized convulsions, and often including associated disturbances in calcium excretion. However, most of the genes involved in the physiology of Mg²⁺ handling are unknown. Through the discovery of a mutation in the EGF gene in isolated autosomal recessive renal hypomagnesemia, we have, for what we believe is the first time, identified a magnesiotropic hormone crucial for total body Mg²⁺ balance. The mutation leads to impaired basolateral sorting of pro-EGF. As a consequence, the renal EGFR is inadequately stimulated, resulting in insufficient activation of the epithelial Mg²⁺ channel TRPM6 (transient receptor potential cation channel, subfamily M, member 6) and thereby Mg²⁺ loss. Furthermore, we show that colorectal cancer patients treated with cetuximab, an antagonist of the EGFR, develop hypomagnesemia, emphasizing the significance of EGF in maintaining Mg²⁺ balance.

Introduction

Mg²⁺ is established as a central electrolyte in a large number of cellular metabolic reactions, including DNA and protein synthesis, neurotransmission, and hormone-receptor binding. It is a component of GTPase and a cofactor for Na⁺,K⁺-ATPase, adenylate cyclase, phospho-inositide kinases and phosphofructokinase (1). Mg²⁺ is also important for the regulation of parathyroid hormone release (2, 3). Accordingly, Mg²⁺ deficiency (plasma Mg²⁺ concentrations below 0.70 mM) has an effect on multiple body functions. Symptoms of Mg²⁺ deficiency are mostly related to muscle dysfunctioning, such as tetany, prolonged QT interval and cardiac arrhythmias (4). Children with hypomagnesemia often present with tetany and/or convulsions. Hypomagnesemia is a problem frequently observed in more than 10% of the hospitalized patients and can be as high as 65% in intensive care patients (5). A long-term complication seen in many adult patients with chronic hypomagnesemia is chondrocalcinosis, which can lead to impairment of joint function (4). Mg²⁺ deficiency can be secondary to systemic diseases (for instance diabetes mellitus and Crohn’s disease) or to the use of osmotic agents, diuretics and drugs such as...
cyclosporin and cysplatin (6). In addition, primary Mg^{2+} deficiency is observed in several monogenetic disorders. Failure of early diagnosis or noncompliance with treatment can be fatal or result in permanent neurological damage.

The plasma Mg^{2+} concentration is regulated within a narrow range by changes in urinary Mg^{2+} excretion in response to altered intake by the intestine. Thus, the kidney plays a key role in the Mg^{2+} homeostasis (4, 7). Most renal reabsorption of Mg^{2+} occurs in the proximal tubule and the thick ascending limb of the loop of Henle via a passive paracellular transport process, but the fine-tuning of the Mg^{2+} excretion takes place in the distal convoluted tubule (DCT), where Mg^{2+} is reabsorbed via an active transcellular transport process (6, 7). Apical entry into DCT cells is mediated by the Mg^{2+} permeable channel; transient receptor potential channel subfamily M, member 6 (TRPM6) driven by a favourable transmembrane voltage (8). The mechanism of basolateral Mg^{2+} transport into the interstitium is unknown. Mg^{2+} has to be extruded against an unfavourable electrochemical gradient, most likely by a Na^{+}/Mg^{2+} dependent exchange mechanism and/or a Mg^{2+}-ATPase. Finally, 3-5% of the filtered Mg^{2+} is excreted in the urine.

Despite the critical role in Mg^{2+} handling, the exact mechanisms of transepithelial Mg^{2+} transport remain obscure. Studies of disorders with primary hypomagnesemia are very important to gain more insight into the molecular and cellular mechanisms that underlie Mg^{2+} (re)absorption. Genetic studies in families with hereditary renal Mg^{2+} wasting syndromes have identified several genes that are either directly or indirectly involved in active Mg^{2+} handling. In the past few years, genetic studies of inborn errors of the Mg^{2+} balance revealed several new proteins unexpectedly involved in transepithelial Mg^{2+} transport in the DCT, e.g., thiazide-sensitive sodium chloride cotransporter (NCC), the γ subunit of the Na^{+},K^{+}-ATPase, and the previously mentioned epithelial Mg^{2+} channel, TRPM6 (9-12).

The aim of the present study was, therefore, to elucidate the gene defect and molecular mechanism underlying Isolated Recessive Hypomagnesemia (IRH) that is characterized by renal Mg^{2+} loss. To this end, a homozygosity-based mapping strategy and mutation detection was performed. In addition, the molecular mechanism of IRH was studied in vitro using patch clamp analysis and in vivo by clinical studies in humans.

## Materials and methods

### Statistical analysis

In all experiments, data are expressed as mean ± SEM. Overall statistical significance was determined by ANOVA. Where differences between the means of 2 groups were significant, they were analyzed by pairwise comparison using Scheffe’s method. For the statistical analysis in figure 4B, an unpaired t-test was performed. $p < 0.05$ was considered significant. The statistical analysis was performed using the SPSS software 12.0 (SPSS).

### Subjects

Informed consent was obtained from the parents and unaffected daughters of the family in this study. The parents gave proxy consent for the 2 affected daughters in this study. In this family, 2 Dutch sisters suffered from primary renal Mg^{2+} loss associated with normocalciuria. These women are the offspring of a consanguineous mating, and since both parents did not display this disorder, the inheritance pattern was likely to be autosomal recessive. The clinical data of this family have previously been described in detail by Geven et al. (13). In short, 2 patients of this family, V3 and V4, had serum Mg^{2+} levels of 0.53 mM and 0.56 mM and urinary Mg^{2+} values of 3.9 mmol/24 h and 3.7 mmol/24 h, respectively. Given the low serum Mg^{2+} levels, urinary Mg^{2+} excretion was relatively high, indicating a renal Mg^{2+} reabsorption defect. Both patients suffer from epileptic seizures that started in their first year of life and are controlled by conventional antiepileptic drugs. During childhood, they showed psychomotor retardation, and they are presently moderately mentally retarded women. Serum and urinary Ca^{2+} were in the normal range, as were serum Na^{+}, K^{+}, Cl^{-}, HCO_{3}^{-}, and blood pH values. In both patients, plasma renin activity, plasma aldosterone, and parathyroid hormone concentrations were in the normal range. Blood was collected for genotyping from the parents, both affected and both unaffected daughters, the sister of the father, and the sister’s 2 unaffected children.

### Mutation analysis

We extracted DNA using standard protocols. The exons of the EGF gene were amplified separately from genomic DNA, using the primers listed in table 1. PCR products were purified with QIAGEN PCR Purification Kit according to the manufacturer’s protocol, and products were sequenced using the same primers. Mutation analysis of
Calculation of FE Mγ

The FE Mγ was calculated using the following formula:

$$\text{FE Mγ} = \frac{\text{U Mγ} \times \text{P Cr}}{(0.7 \times \text{P Mγ}) \times \text{U Cr}} \times 100$$

U and P refer to the urine and serum concentrations of Mg²⁺ (Mγ) and creatinine (Cr). Serum Mg²⁺ concentration was multiplied by 0.7, since only approximately 70% of the circulating Mg²⁺ was unbound by albumin and therefore able to be filtered across the glomerulus.

Expression profiling

PCRs were performed in 50 ml reaction volumes containing 1 µl of cDNA, 10 pmol of each primer (table 2), 2.5 mM MgCl₂, 200 µM of each deoxyribonucleotide triphosphate (dATP, dTTP, dCTP, and dGTP), and 0.5 U of Taq polymerase. PCRs had an initial denaturation stage of 1 minute at 95°C, followed by 39 cycles of 30 seconds at 95°C, 1 minute at 55°C, 1 minute at 72°C, and a final extension step at 72°C for 10 minutes.

Immunohistochemistry

Staining of rat kidney sections for NCC and EGF was performed on 7-µm cryosections of periodate-lysine paraformaldehyde-fixed kidney samples. Sections were stained with affinity-purified anti-NCC (1:200) and affinity-purified rabbit anti-EGF (1:100) (Calbiochem; EMD Biosciences). Images were made with a Bio-Rad MRC 1000 laser scanning confocal imaging system using a ×60 oil-immersion objective.
Linkage analysis

Samples of peripheral EDTA blood were collected from each person, and genomic DNA was isolated using standard procedures. Homozygosity mapping was performed with a genome-wide set of 400 evenly distributed microsatellite markers (LMS-MD10 2.5; Applied Biosystems), with an average intermarker distance of 10 cM, on the basis of Marshfield genetic maps (http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp).

The average heterozygosity of these markers was 0.76. Five additional markers, D4S1570, D4S2623, D4S2392, D4S1615, and D4S422, were selected for fine mapping purposes from the public databases on chromosome 4 (http://research.marshfieldclinic.org/genetics/GeneticResearch/data/Maps/Map4.txt and http://genome.ucsc.edu/cgi-bin/hgGateway). The analysis of the markers was according to the protocol provided for the LMS-MD10 version 2.5 with some small modifications. PCRs were performed in 10 µl reaction volumes containing 40 ng of genomic DNA, 5 pmol of each primer, 2.5 mM MgCl₂, 250 µM dATP, dTTP, dCTP, and dGTP each, 10 mM Tris/Cl (pH 8.3), 50 mM KCl, and 0.4 U of Taq polymerase (AmpliTaq Gold; Applied Biosystems). PCRs had an initial denaturation stage of 12 minutes at 95°C, followed by 10 cycles of 15 seconds at 94°C, 15 seconds at 55°C, and 30 seconds at 72°C, then 22 cycles of 15 seconds at 89°C, 15 seconds at 55°C, and 30 seconds at 72°C, and a final extension step at 72°C for 10 minutes. Reactions for each marker were performed separately, with products being pooled into size-specific sets before typing. Markers were typed on an ABI 3100 sequencer (Applied Biosystems) using GeneMapper 4.0 software (Applied Biosystems). Allele binning was performed with the Excel 2000 (Microsoft) macro linkage designer developed by van Camp and coworkers (14), and we checked Mendelian inheritance of alleles with PedCheck 1.0 software (http://watson.hgen.pitt.edu). Multipoint LOD scores were calculated with the GeneHunter, version 2.1 release 5, program in the easyLINKAGE software package.

Electrophysiology

The bicistronic expression vector pCINeo-IRES-GFP containing the full-length open reading frames of N terminally HA-tagged human TRPM6 (GenBank accession number NM_017662) was used to coexpress human TRPM6 and enhanced GFP in HEK293 cells. HEK293 cells were grown in DMEM (BioWhittaker) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine, and 10 µg/ml Ciproxin at 37°C in a humidity-controlled incubator with 5% (v/v) CO2. Cells were transiently transfected with the respective constructs using Lipofectamine 2000 (Invitrogen), as described previously (16), and electrophysiological recordings were performed 48 hours after transfection. Transfected cells were identified by their green fluorescence when illuminated at 480 nm. Nontransfected (GFP-negative) cells from the same batch were used as controls. Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature (20-25°C) using an EPC-10 patch clamp amplifier computer controlled by PatchMaster Classic 1.20 software (HEKA Elektronik). Currents were digitized at 10 kHz and digitally filtered at 2.9 kHz. Patch pipettes had resistances between 2 and 5 MΩ after filling with the standard intracellular solution. Cells were held at 0 mV, and voltage ramps of 200 ms duration ranging from -100 to +100 mV were applied every 2 seconds. Extracting the current amplitudes at +80 mV from individual ramp current records provided an assessment of the temporal development of membrane currents. 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 mV. Step protocols were applied from holding potentials of 0 mV and consisted of 400-ms steps to -100 to +100 mV (increment of 20 mV). The standard pipette solution contained 150 mM NaCl, 10 mM EDTA, and 10 mM HEPES/NaOH (pH 7.2). Extracellular solutions contained 150 mM NaCl and 10 mM HEPES/NaOH.
Transfection of HEK293 cells
HEK293 cells were transiently transfected with pSAD-EGF, pSAD-EGF-P1070L, or empty-vector pCINeo-IRES-GFP cDNA using polyethylenimine (Polysciences Inc.). Six million cells were seeded on a petri dish (57 cm²) (10 plates per vector) in a total volume of 5 ml DMEM. After 3 hours, the cells were transfected. The transfection mix for each plate consisted of 15 µg cDNA and 90 µl polyethylenimine (1 µg/µl) in a total volume of 500 µl Opti-MEM (Invitrogen). The transfection mix was incubated for 20 minutes at room temperature. Subsequently, the transfection mix was added to the cells and incubated overnight. After 1 day of transfection, the culture media was replaced by 8 ml DMEM medium as described above but without FCS.

Collection and concentration of conditioned culture media
Two days after replacement of the medium of HEK293 and MDCK cells, the FCS-free culture media was collected and proteinase inhibitors were added (1 mM PMSF, 1 µg/ml pepstatin A, and 10 ng/ml leupeptin). Subsequently, the collected FCS-free culture media of HEK293 cells with added proteinase inhibitors was concentrated 400 times using Centriprep YM-3 followed by Microcon YM-3 (Millipore). Prior to concentration of the collected HEK293 medium, 2 ml of the collected FCS-free culture medium was kept separately and used for patch clamp analysis.

Human EGF ELISA
EGF concentrations were determined in 400 times concentrated culture supernatant by using Centriprep columns and a human EGF ELISA kit according the manufacturer’s protocol (RayBiotech Inc.).

Results and Discussion
IRH is characterized by low serum Mg²⁺ levels and mental retardation. Two affected sisters V3 and V4 (figure 1A) displayed low serum Mg²⁺ levels (0.53-0.66 mM) in combination with urinary fractional excretion (FE) values of Mg²⁺ of 4.8% and 3.6%, respectively. These values are well above a FE of 2% indicating renal Mg²⁺ wasting as previously described (5, 20). Thus, the fact that the urinary excretion of Mg²⁺ is in the normal range, while their serum Mg²⁺ values are hypomagnesemic, points to
childhood, and presently they are moderately mentally retarded women who suffer from epileptic seizures. The previously identified genes involved in renal Mg\textsuperscript{2+} handling encoding the thiazide-sensitive sodium chloride cotransporter NCC (SLC12A3), paracellin-1 (CLDN16), the γ subunit of the Na\textsuperscript{+},K\textsuperscript{+}-ATPasae (FXYD2), and the epithelial Mg\textsuperscript{2+} channel (TRPM6) have been excluded for these 2 affected sisters (figure 1A) (8, 11, 12, 21). To determine the disease locus of this consanguineous family, a homozygosity-based mapping strategy with a set of highly polymorphic microsatellite markers spread over the genome was performed and followed by fine-mapping. This resulted in the identification of a critical linkage interval with LOD score 2.66 ($p < 0.004$) on chromosome 4 of 18.4 cM between markers D4S2623 and D4S1575 (figure 1B). A search for candidate genes within this region revealed the EGF gene that was considered a highly relevant positional candidate since it had previously been linked to electrolyte homeostasis (22).

We sequenced EGF in the affected individuals (figure 1A) and identified the homozygous mutation C3209T in exon 22, which cosegregated with the disorder and was absent in 126 ethnically matched control chromosomes (figure 1C). The mutation causes the substitution of the highly conserved proline 1070 within the cytoplasmic tail of pro-EGF by leucine (P1070L) (figure 1D and E). We found that both parents (figure 1A) and the two unaffected sisters (figure 1A) of this Dutch family were heterozygous for the mutation. Furthermore, the paternal aunt was heterozygous for the mutation, whereas her 2 children (figure 1A) exhibited the homozygous wild-type genotype (figure 1A and C).

The EGF gene consists of 24 exons encoding a large type I membrane-anchored precursor protein of 1,207 amino acid residues that exists as a membrane-bound molecule, which is proteolytically cleaved into pro-EGF to finally generate the 53-amino acid peptide hormone EGF (15). EGF has a profound effect on cell differentiation in vivo and is a potent mitogenic factor for a variety of cultured cells of both ectodermal and mesodermal origin (23). EGF belongs to the EGF-like family of growth factors that bind with high-affinity to the EGFR. Other members of this family are TGF-α, amphiregulin, heparin-binding EGF-like growth factor, betacellulin and epiregulin (24-26). These membrane-anchored growth factor precursors are characterized by the fact that they are biologically active at the cell surface, although they can be proteolytically cleaved to release soluble, diffusible factors (24-26).
expression was detected in the adrenal gland, liver, cerebellum and placenta. The EGFR showed a ubiquitous expression pattern since all tissues tested were positive by RT-PCR analysis (figure 2A). Immunohistochemistry on rat kidney sections showed that EGF is consistently coexpressed with NCC, a marker of the DCT (27) (figure 2B). Interestingly, the immunopositive staining is mainly localized along the apical domain in DCT. Coffey and coworkers demonstrated that human pro-EGF overexpressed in Madin-Darby Canine Kidney (MDCK) cells is delivered equally to the apical and basolateral membrane but is found predominantly at the apical membrane domain (28). They proposed that preferential ectodomain cleavage at the basolateral surface explains the apparent apical localization of pro-EGF.

Sack and Talor provided evidence for the existence of specific binding sites in tubular basolateral membranes suggesting a physiologic role of EGF in the kidney (29). In kidney, the EGFR was detected in glomerular endothelial cells, peritubular capillaries and arteriole walls, as well as along the thick ascending limb of Henle’s loop and DCT (30, 31). Immunohistochemical analysis revealed that the EGFR was located particularly along the basolateral membrane of the tubular cells. The DCT reabsorbs approximately 10% of the filtered Mg2+ load and the reabsorption rate in this segment defines the final urinary Mg2+ excretion, because virtually no reabsorption takes place beyond this section (1). In kidney, the epithelial Mg2+ channel TRPM6 is specifically expressed in DCT, where it forms the rate-limiting step of epithelial Mg2+ transport (8). Thus, pro-EGF and TRPM6 are both predominantly expressed in DCT, the main site of active renal Mg2+ reabsorption (1, 15, 22, 32). To determine whether EGF can regulate the activity of TRPM6, Human Embryonic Kidney 293 (HEK293) cells were transiently transfected with TRPM6 and treated for 30 minutes with EGF (10 nM), resulting in increased channel activity (figure 3A). Western blot analysis demonstrated endogenous expression of EGFR in these HEK293 cells (data not shown). EGF dose-dependently stimulated TRPM6 activity with half maximal effective concentration of 1.7 nM, an apparent affinity that is in the physiological range as reported previously (figure 3B) (29).

Previous in vivo studies showed that the predominant form of EGF released from epithelial cells is the high-molecular mass 160-170 kDa EGF, as found at high concentrations in serum, urine and milk (33, 34). To investigate the functional effect of the identified mutation, HEK293 cells were transfected with wild-type or mutant

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To study the tissue distribution of pro-EGF and EGFR, we performed RT-PCR on various human tissues (figure 2A). For EGF, PCR amplification products were detected in, e.g., kidney, salivary gland, cerebrum, and prostate, whereas no

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Figure 2. Expression profiling of EGF and EGFR

(A) The expression of EGF and EGFR mRNA was determined by RT-PCR on various human tissues. Signals for EGF were detected in, e.g., kidney, salivary gland, prostate, and cerebrum whereas no signals were detected in the adrenal gland, cerebellum, liver, lung, and placenta. The EGFR showed a ubiquitous expression pattern since PCR amplification products were obtained in all tissues tested.

(B) Immunohistochemical analysis of EGF (green) and thiazide-sensitive sodium chloride cotransporter (NCC, red) in rat kidney sections (upper panel, overview of a cortical region; lower panel, magnified image of an immunopositive tubule). EGF colocalized with NCC, a marker for the DCT. Original magnification, ×180 (B, top panels); ×360 (B, bottom panels).
EGF maintains the total body Mg²⁺ balance

Chapter 5

Figure 3. Electrophysiological analysis of pro-EGF and pro-EGF-P1070L on TRPM6 channel activity

(A) Average time course of outward (at +80 mV) current densities from HEK293 cells transfected with TRPM6 in control conditions (open circles) and after EGF treatment (30 minutes, 10 nM, filled circles) in comparison with mock-transfected cells without EGF treatment (triangles) and after EGF stimulation (filled triangles). n = 17-20. (B) Dose-response curve of EGF-induced current in TRPM6-transfected HEK293 cells indicating half maximal effective concentration of 1.7 nM. n = 11-14. (C) Histogram presenting the current densities at +80 mV of TRPM6-transfected cells (200 seconds after establishment of the whole-cell configuration) treated with mock (mock), pro-EGF (EGF WT) or pro-EGF-P1070L (EGF-P1070L) supernatant. All treatments were performed for 30 minutes at 37°C. White bars indicate experimental conditions in which HEK293 cells were transfected with mock DNA whereas black bars indicate HEK293 cells transfected with TRPM6. Asterisk indicates significance in comparison with nontreated TRPM6-transfected cells. p = 0.024; n = 6. (D) Supernatants of mock-, pro-EGF-, or pro-EGF-P1070L-transfected HEK293 cells were analyzed by ELISA. Asterisk indicates mock and pro-EGF-P1070L supernatant were significantly different from EGF supernatant. p = 0.006; n = 4. (E) Histogram summarizing the current density (pA/pF) at +80 mV (200 seconds after break-in) of HEK293 cells treated with the apical or basolateral supernatant of MDCK cells stably transfected with either wild-type pro-EGF, pro-EGF-P1070L, or mock DNA (30 minutes, 37°C). White bars indicate experimental conditions in which HEK293 cells were transfected with mock DNA whereas black bars indicate HEK293 cells transfected with TRPM6. Crosses indicate significance in comparison with the mock treatment at the apical side (wild-type pro-EGF apical, p = 0.0001; n = 6; pro-EGF-P1070L apical, p = 0.029, n = 6), and pound symbol represents significance in comparison with the mock treatment at the basolateral side (wild-type pro-EGF basolateral p = 0.031; n = 6). (F) Schematic model illustrating how pro-EGF-P1070L mutation results in IRH. The pro-EGF-P1070L mutation leads to impaired basolateral sorting of pro-EGF, resulting in abrogated stimulation of the EGFR localized at the basolateral membrane. Activation of the EGFR in DCT by EGF is necessary to prevent renal Mg²⁺ wasting by stimulation of the epithelial Mg²⁺ channel TRPM6.

To demonstrate that the observed stimulatory effects are due to EGFR activation, a preincubation with cetuximab was performed. This IgG human/mouse chimeric monoclonal antibody competitively inhibits the activation of the EGFR. It binds externally to the EGFR to block binding of the ligand and subsequent signal transduction mediated via the receptor-associated tyrosine kinase and prevents phosphorylation of the EGFR and other downstream proteins in the signal transduction cascade (35). The stimulatory effect of the supernatant collected from pro-EGF-expressing HEK293 cells on TRPM6 was significantly inhibited by preincubation for 1 hour with cetuximab (191 ± 22 pA/pF, 491 ± 62 pA/pF, and 286 ± 48 pA/pF for TRPM6-expressing HEK293 cells measured at +80 mV and treated with mock supernatant, EGF supernatant, or EGF supernatant plus cetuximab, respectively; p < 0.05, n = 6). Thus, the stimulatory effect of the supernatant on TRPM6 activity is due to direct activation of the EGFR.

As outlined above the localization of EGFR is restricted to the basolateral membrane, whereas pro-EGF is present at both the apical and basolateral membranes (28). This prompted us to investigate in detail the cellular metabolism of pro-EGF. Recently, several basolateral sorting motifs were identified including the sequence PXXP (with X being an arbitrary amino acid), determining the targeting of proteins to the basolateral membrane in MDCK cells (36). Our mutation substitutes the second highly conserved proline in the cytoplasmic 1067PKNP1070 motif into a leucine (figure 1D). We hypothesized that this mutation results in improper trafficking of pro-EGF thereby preventing an adequate secretion of the hormone into the circulation. Since, EGFR is only localized basolaterally, the mutation should prevent the ligand from reaching its receptor. Interestingly, He and coworkers have shown previously that a 22-amino acid sequence in the EGFR juxtamembrane
mutations at P667 and P670 were associated with impaired basolateral delivery of the EGFR.

To determine the effect of the mutation on the sorting of pro-EGF in polarized epithelial cells, MDCK cells stably expressing either wild-type or mutant pro-EGF were grown on semipermeable filter supports until they reached confluency. After 2 days, media of the basolateral and apical compartment were collected individually. Next, HEK293 cells expressing TRPM6 were treated with the collected preconditioned media of wild-type pro-EGF, mutant pro-EGF, or mock-expressing cells. Equal stimulation of TRPM6 channel activity was observed when the cells were incubated with the apically collected media of wild-type or mutant pro-EGF-expressing MDCK cells (figure 3E). However, stimulation of TRPM6 channel activity was only observed with basolaterally collected wild-type pro-EGF medium, not with the basolateral mutant pro-EGF media (figure 3E). Of note, the apically and basolaterally collected mock medium did not affect TRPM6 activity. These observations suggest that the basolateral release of mutant pro-EGF is diminished, seriously hampering the EGF-dependent activation of the basolaterally localized EGFR (figure 3F).

Clinical trials directed to the treatment of patients with colorectal cancer have demonstrated that cetuximab is synergistic with chemotherapy for these patients (37). We observed that serum Mg²⁺ levels gradually decreased in all studied colorectal cancer patients on cetuximab treatment (figure 4A). This is in line with a recent report showing that a significant number of similarly treated patients develop hypomagnesemia (35). Our patients treated with cetuximab displayed an FE of Mg²⁺ of 5.1%, which is inappropriately high given their hypomagnesemia (35). As previously established by Agus (5), an FE of Mg²⁺ above 2% in the presence of hypomagnesemia indicates renal Mg²⁺ loss.

The affected sisters with IRH exhibited a similar relatively high Mg²⁺ excretion profile (figure 4B). The urinary Mg²⁺ patterns of the patients treated with EGFR antibodies, the patients described in our Dutch family and patients with Hypomagnesemia with Secondary Hypocalcemia (HSH; OMIM 602014) (11, 12) were identical and suggest mutual defects in renal TRPM6 activity. Indeed, preincubation with cetuximab abolished the stimulatory effect of EGF on TRPM6 activity as shown by patch clamp analysis of TRPM6-expressing HEK293 cells (figure 4C). Taken together, these data strongly suggest a pivotal role for EGFR.

**domain contains autonomous sorting information necessary for basolateral localization of the receptor in MDCK cells (36). This sorting motif contains a polyproline core comprising residues P667 and P670 (667PXXP670). Site-directed
signaling in the maintenance of normal Mg\textsuperscript{2+} balance and indicate TRPM6 as a critical link between EGFR inhibition and IRH.

Through the discovery of an EGF mutation in IRH, we have for the first time, to our knowledge, identified a magnesiotropic hormone crucial for total body Mg\textsuperscript{2+} balance that directly stimulates renal tubular Mg\textsuperscript{2+} reabsorption via activation of the epithelial Mg\textsuperscript{2+} channel TRPM6. Moreover, it raises the question of whether EGF plays a role in mental development since our IRH patients (figure 1A) are mentally retarded. EGF is present in cerebrospinal fluid, and EGF mRNA has been detected in the developing brain of various species. Furthermore, EGF is a well-known neurotrophic factor regulating the development of various neuronal cells (38). Fatamura et al. reported that serum EGF levels were markedly decreased in schizophrenic patients and suggested that EGF might provide a link between impaired EGF signaling and the pathology/etiology of schizophrenia (39). An association between an EGF polymorphism and schizophrenia has been suggested although data from several studies are conflicting (40, 41). Interestingly, in our Dutch family with IRH, 1 unaffected, EGF P1070L-carrier sister (figure 1A) suffers from schizophrenia, and 2 brothers and 1 sister of the carrier mother (figure 1A) are known with cluster A (Diagnostic and statistical manual of mental disorders, fourth edition, text revision) personality disorders. In addition, EGF could be involved in Mg\textsuperscript{2+} absorption in the intestine, a site that also expresses TRPM6. Future therapeutic intervention might be able to treat hypo- and hypermagnesemia by regulating EGFR activity.

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References


Chapter 5


Chapter 5

EGF maintains the total body Mg²⁺ balance
EGF activates the distal tubular magnesium channel, TRPM6, via a Src kinase and Rac1 mediated increase in plasma membrane expression
Abstract

A mutation in the epidermal growth factor (EGF) gene, causing isolated recessive hypomagnesemia, was recently identified. EGF was shown to increase the activity of the epithelial Mg$^{2+}$ channel TRPM6. The aim of this study is to elucidate the molecular mechanism mediating this effect. To this end, whole-cell patch clamp recordings of TRPM6 expressed in Human Embryonic Kidney 293 (HEK293) cells were employed. EGF receptor stimulation increased TRPM6, but not TRPM7, current independently of its unique carboxy-terminal α-kinase domain. This activation relied on both the Src family of tyrosine kinases and the downstream effector Rac1. Activation of Rac1 increased the mobility of TRPM6, as assessed by fluorescent recovery after photobleaching. Moreover, dominant negative Rac1 decreased TRPM6 mobility, abrogated current development and prevented the EGF-mediated increase in activity. In contrast, a constitutively active mutant of Rac1 mimicked the stimulatory effect of EGF on TRPM6 mobility and activity. TRPM6 activation was ultimately the result of increased cell surface abundance. In summary, EGF-mediated stimulation of TRPM6 activity occurs via signaling through Src kinases and Rac1, thereby redistributing endomembrane TRPM6 to the plasma membrane.

Introduction

Recently we described a mutation in the EGF gene, encoding pro-EGF, which is responsible for a rare form of renal magnesium (Mg$^{2+}$) wasting, Isolated Recessive Hypomagnesemia (IRH) (1). A link between a defect in EGF and renal Mg$^{2+}$ wasting was made by demonstrating that pro-EGF is expressed in the distal convoluted tubule (DCT), where regulated transcellular Mg$^{2+}$ reabsorption occurs via transient receptor potential M6 (TRPM6). Further, supernatant from the basolateral compartment of polarized epithelial cells expressing pro-EGF was able to activate TRPM6, while supernatant from cells expressing the mutant pro-EGF was unable to. The EGF receptor (EGFR/ErbB1) is expressed in the basolateral membrane of DCT, suggesting an autocrine/paracrine activation of TRPM6 by EGF through its receptor. This has not however been confirmed, nor have the molecular details of this activation been elucidated.

EGFR activation mediates its downstream effects via numerous signaling cascades,
including the extracellular signal-regulated kinase (ERK) limb of the mitogen-activated protein kinase (MAPK) superfamily, protein kinase A (PKA), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and the phospholipases C and D pathways (2). Activation of the EGFR in DCT could therefore result in phosphorylation and consequently activation of the previously described target proteins. That EGFR signaling is implicit to Mg\(^{2+}\) homeostasis is further supported by the observation that patients treated with cetuximab, a monoclonal antibody directed against the EGFR, develop hypomagnesemia (1, 3, 4). However, the signaling pathway(s) downstream of EGFR activation, which stimulate TRPM6-mediated Mg\(^{2+}\) influx, remain to be determined.

The aim of the present study was to ascertain the downstream signaling events, after EGFR engagement, and ultimately the mechanism responsible for increased TRPM6 activity. Elucidation of the molecular details responsible for EGFR-mediated stimulation of TRPM6 will facilitate the development of treatments for hypomagnesemia in general and during cetuximab treatment. Using electrophysiological measurements in combination with biochemical and live cell imaging techniques we provide evidence that EGF stimulates TRPM6 through the specific activation of the EGFR. This activates the Src family of tyrosine kinases and the small Rho-GTPase, Rac1, which results in a redistribution of vesicular TRPM6 to the plasma membrane.

Materials and methods

Reagents

EGF, AG1478/tyrphostin, forskolin, U73122, U17343, LY492005, cytochalasin D and wortmannin were from Sigma-Aldrich (Steinheim, Germany). UO126, PP2 (4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), PP3 (4-Amino-7-phenylpyrazolo[3,4-d]pyrimidine), PD98059 (2’-Amino-3’-methoxyflavone) and RG13022 were obtained from Calbiochem Inc. (La Jolla, CA, USA). The EZ-link Sulfo-NHS-LC-LC-Biotin was purchased from Pierce (Rockford, IL, USA) and Neutravidin agarose resin from Thermo Scientific (Rockford, IL, USA).

DNA constructs

Wild-type TRPM6 in the pCINeo/IRES-GFP vector was HA-tagged at the N-terminal tail as described previously (7). GFP-fused-TRPM6 in pCINeo vector was constructed by site-directed mutagenesis and Polymerase Chain Reaction (PCR) based on the TRPM6 cDNA in pCINeo/RES-GFP. The α-kinase (L1749X) mutant was created using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s protocol (5). All constructs were verified by sequence analysis. Rac1-T17N and Rac1-G12V plasmids in the pcDNA3.1+ vector were purchased from UMR cDNA resource center (Rolla, MO, USA). Mouse TRPM7 in pTracer-CMV2 was a kind gift from David Clapham (Harvard, Boston, MA, USA). A HA-tag has been inserted at the C-terminus of TRPM7.

Immunoblotting

Protein samples were denatured by incubation for 30 min at 37°C in Laemmli buffer, and then subjected to SDS-PAGE. Blots were incubated with either a goat anti-EGFR polyclonal antibody (1:500, Santa-Cruz, CA, USA) or a rabbit anti-GFP polyclonal antibody (1:5,000, Transduction Laboratories), at 4ºC for 16 h. Subsequently, blots were incubated with a goat anti-rabbit or donkey anti-goat, peroxidase-conjugated secondary antibody (both 1:10,000, Sigma, St Louis, MO, USA) and then visualized using the enhanced chemiluminescence method (Pierce, Rockford, IL, USA) as described previously.

Electrophysiology

A ramp protocol, consisting of linear voltage ramp from -100 to +100 mV (within 450 ms) was applied every 5 s from a holding potential of +0 mV. Extracting the current amplitudes at +80 and -80 mV from individual ramp current records assessed the temporal development of membrane currents. Current densities, expressed in units of membrane capacitance, were calculated from the current at +80 and -80 mV from individual ramp current records assessed the temporal development of membrane currents. Current densities, expressed in units of membrane capacitance, were calculated from the current at +80 and -80 mV during the ramp protocols. The step protocol consisted of a series of 100 ms long voltage steps applied from the resting membrane potential to voltages between -100 and +100 mV with an increment of 20 mV. The standard pipette solution (150 mM NaCl, 10 mM EDTA and 10 mM HEPES / NaOH, pH 7.2) was used. The extracellular solution (150 mM NaCl, 10 mM HEPES / NaOH, pH 7.4) was supplemented with 1 mM CaCl\(_2\). All experiments were performed at room temperature. Transfected cells were identified by their green appearance and GFP-negative cells from the same batch of cells were used as negative controls.

Fluorescence recovery after photobleaching

HEK293 cells were transfected with GFP-TRPM6 and empty vector or the Rac1
EGF activates TRPM6 current in HEK293

The presence of the EGFR was first confirmed in HEK293 cells by immunoblotting, where it was detected as a single immunopositive band with the expected molecular size of 170 kD (Figure 1A). Thus, HEK293 cells provide an excellent model to study the effect of EGF on TRPM6 activation. TRPM6 has been expressed and characterized in this model system where it is detectable at the appropriate molecular weight of 230 kD (Figure 1B) (8). The effect of EGF on TRPM6 current activity was then assessed using whole-cell recordings. All time courses were analyzed at +80 and -80 mV for a 200s period. EGF pre-incubation stimulated TRPM6 current by 34 ± 6 % and 65 ± 8 % at +80 and -80 mV respectively, compared to TRPM6 current in the absence of EGF (Figure 1C and D). TRPM6 displayed an outwardly rectifying current-voltage relation with a reversal potential close to 0 mV. This was unaltered by EGF pre-treatment (Figure 1E).

EGF-stimulated TRPM6 current is specifically mediated by the EGFR

To address whether the stimulatory action of EGF was specific to activation of the EGFR, the receptor antagonist, AG1478 (also named tyrphostin) was added prior to treatment with EGF. Incubation of TRPM6-expressing HEK293 cells with AG1478 prevented the EGF stimulatory effect (Figure 2A). To confirm that EGFR stimulation mediated the observed increase in TRPM6 current, a specific tyrosine kinase receptor inhibitor, RG13022, was used to block EGFR activation (9). Following a 10 min exposure, EGF failed to elicit an increase in TRPM6 activity (Figure 2B). In fact, incubation with either of these compounds reduced baseline TRPM6 activity (Figure 2A and B). TRPM7, the closest homologue of TRPM6, was also examined for an EGF-mediated increase in activity. Consistent with the notion that EGFR engagement by EGF is specific for TRPM6 activation, treatment with EGF did not affect TRPM7 activity (Figure 2C).

Mutants. The samples were plated onto glass bottom Petri dishes (0.17 mm thick Wilco Wells, USA) chambers and then mounted on the stage of a confocal laser scanning microscope (Zeiss LSM 510) and bathed in a standard solution (130 mM NaCl, 20 mM HEPES / Tris, pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂ and 5 mM KCl) with or without EGF. A confocal plane through the center of the cell was brought into focus, and two equal areas (2 μm in diameter) were defined. After acquiring two base-line fluorescence measurements, one of the selected areas was irreversibly photobleached, and then the fluorescence of both areas was measured over time. The fractional fluorescence recovery of the bleached area was determined relative to the average of the two measurements acquired before photobleaching. The unbleached area was used to estimate possible bleaching incurred during image acquisition. FRAP data were fitted by nonlinear regression analysis.

Cell culture and transfection

HEK293 cells were cultured in (DMEM) medium with 10% FCS and transfected as described previously (5). Electrophysiological recordings, live cell imaging and biochemical assays were performed 48 h post-transfection. MDCK cells were maintained in DMEM medium with 10% FCS. MDCK cells were stably transfected with GFP-TRPM6, generated by sub-cloning TRPM6 into the GFP- vector as outlined below. To select a stable line (MDCK-GFP-TRPM6), the cells were cloned by limiting dilution in the presence of 500 μg/ml G418 and screened by immunofluorescence and immunoblotting for expression of GFP-tagged TRPM6. The experiments were performed at least 72 h after the monolayers had reached confluence.

Cell surface biotinylation

MDCK-GFP-TRPM6 cells were incubated at 37°C in the presence or absence of EGF (10 nM, 30 min) before cells were placed on ice to inhibit endo- and exocytosis. Biotinylation was performed on ice, and cells were lysed as described previously (7). Subsequently, cell surface proteins were precipitated with neutravidin-agarose beads. The purified biotinylated surface proteins were probed for the presence of TRPM6 by immunoblotting with an anti-GFP antibody (Sigma-Aldrich, St. Louis, USA).

Statistical analysis

Values are expressed as mean ± SE. Statistical significance between groups was determined by analysis of variance (ANOVA) followed by pair-wise comparison using the method of Scheffé. Differences in means with \( p < 0.05 \) were considered statistically significant. The statistical analysis was performed using the SPSS software (SPSS Inc, Chicago, Illinois, USA).
Stimulation of TRPM6 by EGF does not require the α-kinase domain

TRPM6 uniquely encompasses an α-kinase domain with a channel. To determine whether the α-kinase domain is necessary for EGFR-mediated stimulation of TRPM6 activity, HEK293 cells transiently expressing an α-kinase-truncated TRPM6 mutant (TRPM6 α-kinase) (7) were treated with EGF and subjected to whole-cell recordings. EGFR stimulation by EGF was able to increase TRPM6 channel activity, even in the absence of the α-kinase domain. EGF increased the current in TRPM6 α-kinase transfected cells by 75 ± 11% and 73 ± 8% at +80 and -80 mV, respectively (figure 3A).

Stimulation of TRPM6 by EGF involves both Src-family kinases and MAPK

Src family tyrosine kinases, including Fyn and Lyn, are important downstream signaling intermediates of the EGFR. They are also known to regulate ion channel activity (10). We therefore examined whether Src family tyrosine kinases were necessary for TRPM6 activation by EGF. Pre-incubation with PP2, a compound that selectively inhibits Src kinases (11), prevented the stimulatory effect of EGF while its inactive analog PP3, failed to prevent EGF from stimulating TRPM6 activity (figure 3B).
To confirm that ERK1/2 activation was necessary for the EGF-mediated increase in TRPM6 activity we pre-treated TRPM6-transfected HEK293 cells with forskolin, thereby activating PKA. Application of forskolin had no effect on TRPM6 current, it did however prevent EGF-mediated activation (figure 3B). These findings support the conclusion that TRPM6 activation following EGFR engagement is mediated via activation of the MAPK pathway.

**Stimulation of TRPM6 by EGF depends on Phosphoinositide Kinase-3 (PI3K) and Rac1**

A downstream target of Src family kinases is the PI3K/Akt pathway. To address whether PI3K is involved in the EGF-mediated stimulation of TRPM6, cells were preincubated with the PI3K inhibitors LY294005 or wortmannin. Preincubation with either compound prevented the EGF-induced activation of TRPM6 (figure 4A). Neither compound altered the TRPM6 current when used in isolation (data not shown). A common PI3K effector, implicated in cytoskeletal remodeling and membrane traffic is Rac1 (13-15). We therefore sought to ascertain whether modulation of Rac1 activity would alter baseline or the EGF-mediated increase in TRPM6 current. HEK293 cells were transiently co-transfected with either a dominant negative mutant of Rac1 (Rac1-T17N) and TRPM6, or a constitutively active mutant of Rac1 (Rac1-G12V) and TRPM6, and then subjected to patch clamp analysis. Control cells were transiently co-transfected with empty vector and TRPM6. Cells co-expressing Rac1-T17N and TRPM6 displayed significantly decreased TRPM6 currents compared to TRPM6-transfected cells (figure 4B and C). In these cells, EGF pre-treatment did not restore TRPM6 current. In contrast, cells co-transfected with Rac1-G12V and TRPM6 exerted a significant increase in TRPM6 current (figure 4B and C). The addition of EGF to HEK293 cells co-expressing Rac1-G12V and TRPM6 did not further increase the TRPM6 current.

**EGF increases cell surface expression of TRPM6**

Activation of Rac1 results in actin cytoskeletal rearrangement. To assess whether this downstream effect of Rac1 was responsible for the EGF-mediated increase in TRPM6 activity we pre-treated TRPM6-transfected HEK293 cells with forskolin, thereby activating PKA. Application of forskolin had no effect on TRPM6 current, it did however prevent EGF-mediated activation (figure 3B). These findings support the conclusion that TRPM6 activation following EGFR engagement is mediated via activation of the MAPK pathway.

Next, a series of experiments were conducted to identify which signaling pathway(s), downstream of the Src family kinases, are necessary for TRPM6 activation. The involvement of phospholipase C-gamma (PLCγ) was excluded using the PLC inhibitor, U73122 and its inactive analog, U73343 (figure 3B). The putative role of the MEK1/2 and ERK1/2 limb of the MAPK superfamily was then evaluated. To this end, HEK293 cells were pre-incubated with selective inhibitors of MEK1/2 and ERK1/2 activation, UO126 and PD98059, respectively. Treatment with either UO126 or PD98059 prevented EGF from increasing TRPM6 activity, implicating the ERK1/2 pathway in this signaling cascade (figure 3B). None of these agents had an effect on TRPM6 in the absence of EGF. Stimulation of ERK1/2 by the EGFR is preceded by Raf-1 activation, an event that can be blocked by protein kinase A (PKA) (12).

To confirm that ERK1/2 activation was necessary for the EGF-mediated increase in TRPM6 activity we pre-treated TRPM6-transfected HEK293 cells with forskolin, thereby activating PKA. Application of forskolin had no effect on TRPM6 current, it did however prevent EGF-mediated activation (figure 3B). These findings support the conclusion that TRPM6 activation following EGFR engagement is mediated via activation of the MAPK pathway.

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Implicit to the observed prevention of TRPM6 activation by cytochalasin D is the notion that EGFR signaling promotes the mobility of endomembrane TRPM6, presumably by redistributing endomembrane channels into the plasma membrane. To this end, we measured the mobility of TRPM6 in HEK293 cells. This was accomplished with a GFP-fused TRPM6 construct and the imaging technique, fluorescence recovery after photobleaching (FRAP). GFP-TRPM6 can be expressed and detected in HEK293 cells by immunoblotting and fluorescent microscopy (figure 5A and B). The electrophysiological characteristics of GFP-TRPM6 were evaluated by patch clamp analysis and shown to display a similar baseline and EGF-stimulated current (data not shown). As is evident from figure 5C and E, EGF treatment increased both the rate and fraction of endomembrane TRPM6 that is mobile. That Rac1 alters the mobility of endomembrane TRPM6 was further supported by repeating these measurements after co-transfection with either Rac1-G12V, the constitutively active mutant, or Rac1-T17N, the dominant negative mutant (figure 5D and E). Consistent with the whole-cell patch clamp analysis (figure 4C), Rac1-G12V increased both the rate of recovery and the mobile fraction of TRPM6 (figure 5D and E). Moreover, EGF treatment failed to further increase the mobility or the mobile fraction. Conversely, a decreased mobile fraction is observed in cells co-transfected with Rac1-T17N. EGF treatment in the presence of Rac1-T17N failed to even return the mobile fraction of TRPM6 to that of the control (figure 5D and E). These observations correspond to decreased channel activity irrespective of EGF treatment (figure 4C). Taken together these findings support the concept that EGFR activation stimulates Rac1, alters cytoskeletal dynamics, and consequently increases the mobility of endomembrane TRPM6.

Given that TRPM6 current is enhanced by EGF treatment together with the above FRAP results, this implies that endomembrane TRPM6 is moving into the plasma membrane, thereby increasing the number of active channels there. To confirm this that was occurring cell surface biotinylation experiments were performed, utilizing the fused GFP for immuno-detection. We used a TRPM6 stably expressing renal epithelial cell line, Madin Darby Canine Kidney (MDCK), to perform these studies (referred to hereafter as MDCK-GFP-TRPM6). Quantification of cell surface expressed TRPM6 by this method revealed a significant increase in the cell surface expression of TRPM6 after incubation with EGF (figure 6).
elucidate the downstream signaling events. This revealed TRPM6 stimulation to be dependent on the activation of the Src family of tyrosine kinases, the ERK/MEK pathway and PI3K. Cytoskeleton remodeling was clearly implicated. This was evinced through a pharmacological inhibition of EGF-mediated activation by cytochalasin D, an observed parallel increase in TRPM6 current induced by constitutively active Rac1 and abrogation of this effect in the presence of dominant negative Rac1. FRAP was further employed to demonstrate that EGF increases the mobility of TRPM6, an effect that is also Rac1 dependent. Ultimately, it is shown that EGF activates TRPM6 by increasing the number of channels at the plasma membrane. Together these findings clearly outline the activation of TRPM6 by EGF (figure 6C), a process fundamental to Mg^{2+} homeostasis.

Discussion

The present study delineates the molecular details of TRPM6 stimulation by EGF, from receptor to channel. First, it was demonstrated that EGF specifically activates TRPM6 via engagement of its receptor, the EGFR. A combination of live cell imaging, biochemical and electrophysiological approaches were employed to elucidate the downstream signaling events. This revealed TRPM6 stimulation to be dependent on the activation of the Src family of tyrosine kinases, the ERK/MEK pathway and PI3K. Cytoskeleton remodeling was clearly implicated. This was evinced through a pharmacological inhibition of EGF-mediated activation by cytochalasin D, an observed parallel increase in TRPM6 current induced by constitutively active Rac1 and abrogation of this effect in the presence of dominant negative Rac1. FRAP was further employed to demonstrate that EGF increases the mobility of TRPM6, an effect that is also Rac1 dependent. Ultimately, it is shown that EGF activates TRPM6 by increasing the number of channels at the plasma membrane. Together these findings clearly outline the activation of TRPM6 by EGF (figure 6C), a process fundamental to Mg^{2+} homeostasis.

EGFR signaling pathway and TRP channel regulation

Our study is the first to demonstrate that EGF regulates the melastatin subfamily of TRP channels. Further, the effect is specific to member TRPM6 as no increase in TRPM7
current, the closest homologue, was observed upon EGF application. To confirm that EGF specifically activates the EGFR we demonstrated the presence of this receptor in our model system and then showed an inhibition of the observed effect when the receptor was blocked with selective antagonists. Further evidence that EGFR engagement is required for TRPM6 activation was provided by the demonstration that inhibition of the receptor’s tyrosine kinase activity, prevented the EGF-induced increase in TRPM6 activity. Thus, EGF-mediated activation of TRPM6 is novel within this subfamily of channels and requires the specific engagement of the EGFR.

Among EGFR effectors, PLCγ is a known regulator of TRPC channel activity (13, 16). Furthermore, TRPM7 has been shown to interact with PLCγ and β isoforms (17) and to be inactivated by PIP₂ hydrolysis (18). However, inhibition of PLCγ failed to prevent EGF-mediated TRPM6 regulation, suggesting that PLCγ is not necessary for the activation of TRPM6 by EGF. Thus, PLCγ is a potential discriminator between EGF regulation of TRPM6 and other TRP channels (16, 17). Further, because TRPM7 is activated by PLCγ and not EGF, this finding highlights the specificity of the EGF signaling cascade for TRPM6.

Inhibition of PI3K activity prevented EGF from activating TRPM6. The role of the small Rho GTPase Rac1, a common effector of PI3K was, therefore, investigated (19). Dominant negative Rac1 (T17N) abrogated TRPM6 current development and prevented the EGF-mediated increase in TRPM6 activity. In contrast, a constitutively active mutant of Rac1 (G12V) mimicked the EGF stimulation of TRPM6 activity. Both PI3K and Rac1 components have been implicated in TRPC5 and TRPV1 stimulation upon EGF and nerve growth factor application, respectively (13, 20). Therefore, in addition to the regulation of brain function such as neurite extension and pain perception through TRP channels, our findings implicate Rac1 in renal Mg²⁺ reabsorption via the regulation of TRPM6 channel activation, independently of the constitutively expressed TRPM7.

Regulation of TRPM6 at the plasma membrane

The requirement of PI3K, Rac1 and a dynamic actin cytoskeleton for EGF-mediated activation of TRPM6 implies that altered endomembrane traffic and potentially redistribution to the plasma membrane is responsible for the increased current observed (21, 22). To test this hypothesis we measured the mobility of TRPM6 before and after treatment with EGF. This increased both the rate that endomembrane TRPM6 was moving and the fraction that was mobile. Indeed the application of EGF appeared to mobilize an immobile subset of TRPM6 (figure 5, C and E). The co-expression of Rac1 mutants altered the mobile fraction of TRPM6, in parallel to the observed changes in activity. Moreover, co-expression of these constructs prevented the EGF-mediated alteration in TRPM6 mobility. That EGF activation ultimately leads to increased expression at the plasma membrane was substantiated by cell surface biotinylation analysis. Together, these observations provide strong evidence that EGF increases the abundance of TRPM6 at the plasma membrane.

What then explains the increased abundance of TRPM6 at the plasma membrane? We propose that TRPM6 activation subsequent to EGFR stimulation regulates the amount of recycling TRPM6 and/or the balance between constitutive exocytosis and endocytosis in favor of TRPM6 accumulation at the cell surface. This hypothesis is supported by our FRAP analysis which demonstrates that EGF activation increases the mobility of endomembrane TRPM6 and releases a pool of immobile, or poorly mobile, endomembrane channels. The total number of channels within the plasma membrane is determined by the combined effects of membrane insertion, retrieval and number of recycling channels. EGF activation likely increases the number of recycling channels and the balance between endocytosis and exocytosis, to favor increased plasma membrane expression. Consistent with an effect of EGF on the recycling kinetics of the TRP family of channels, EGF prevents the internalization of plasma membrane TRPC3 (23).

Potential role(s) of EGF-mediated regulation of TRPM6

Our findings implicate both Raf-1 and the ERK1/2 limb of the MAPK superfamily in EGF-mediated activation of TRPM6. EGF is known to alter ion homeostasis through inhibition of the amiloride-sensitive Na⁺ reabsorption from collecting duct. This also occurs downstream of ERK1/2 activation (24). In kidney, TRPM6 and EGF are predominantly expressed in DCT, the nephron segment responsible for regulated, transepithelial Ca²⁺ and Mg²⁺ reabsorption (25). TRPM6 mediates and regulates this latter process, a conclusion supported by its unique permeation characteristics, carrying almost exclusively divalent cations with a higher affinity for Mg²⁺ than Ca²⁺ (5, 8) and its specific localization to Mg²⁺-transporting epithelia. That this process is regulated by the MAP kinases is consistent with previous findings. However in DCT, EGF appears to uniquely activate TRPM6, to the exclusion of TRPM7, TRPV5.
and TRPV6 (figure 2C & data not shown). TRPV6 mediates active transcellular Ca\textsuperscript{2+} reabsorption from the distal part of the nephron, hence EGF signaling confers a precision to the control of Mg\textsuperscript{2+} homeostasis and can therefore be defined as a magnesiotropic hormone.

The specificity of this pathway is highlighted by clinical examples. The patient we originally described (1), displayed clinical symptoms secondary to hypomagnesemia, and lacked evidence of altered Ca\textsuperscript{2+} homeostasis. Further, treatment with the anti-EGFR antibody, cetuximab, specifically alters plasma Mg\textsuperscript{2+} levels (1, 3). The work described herein delineates the pathway between EGFR engagement and apical Mg\textsuperscript{2+} influx through TRPM6. A process whose specificity is mediated by the restricted location of TRPM6 and unique signaling within this location. Both the Src family of tyrosine kinases and the small Rho GTPase, Rac1 are central to the latter. The increased activity of TRPM6 is ultimately the result of increased cell surface expression. These findings provide molecular insight into a novel mechanism specifically regulating transepithelial Mg\textsuperscript{2+} transport and consequently whole body Mg\textsuperscript{2+} homeostasis.

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References


Chapter 6
EGF stimulates TRPM6
General discussion and summary
Introduction

Mg$^{2+}$ is an important, predominantly intracellular cation that is indispensable for a wide variety of biological processes (1). Although Mg$^{2+}$ is a dominant divalent intracellular cation and is required for the function of diverse types of enzymes that participate in virtually every cellular process, the molecular mechanisms that regulate its homeostasis are poorly understood. The plasma Mg$^{2+}$ concentration is regulated within a narrow range by changes in urinary Mg$^{2+}$ excretion in response to altered uptake by the intestine. Thus, the kidney plays a key role in Mg$^{2+}$ homeostasis (2). Most renal reabsorption of Mg$^{2+}$ occurs in the proximal tubule and the thick ascending limb of Henle’s loop (TAL) via a passive paracellular transport process, but the fine-tuning of the Mg$^{2+}$ excretion takes place in the distal convoluted tubule (DCT), where Mg$^{2+}$ is reabsorbed via an active transcellular transport process (2). The first step of transcellular transport is the influx of Mg$^{2+}$ across the apical plasma membrane of the epithelial cell. Subsequently Mg$^{2+}$ diffuses or is transported to the basolateral membrane where it is extruded into the bloodstream. Genetic studies of disorders with hereditary hypomagnesemia have proven to be very important in gaining more insight into the molecular and cellular mechanisms that underlie Mg$^{2+}$ (re)absorption. Through the discovery of the epithelial Mg$^{2+}$ channel TRPM6 by studying patients with hypomagnesemia with secondary hypocalcemia (HSH) and the identification of its closest homologue TRPM7, the knowledge of active Mg$^{2+}$ transport was greatly boosted. TRPM6 constitutes the gatekeeper of transcellular Mg$^{2+}$ transport in kidney and intestine and is essential for the maintenance of total body Mg$^{2+}$ homeostasis. In contrast, TRPM7 displays a ubiquitous expression pattern and is important for Mg$^{2+}$ homeostasis at the cellular level. Since TRPM6 forms the entry gate for Mg$^{2+}$ into the DCT cells, it is the ideal target for hormonal regulation of active Mg$^{2+}$ reabsorption. Although TRPM6 has been identified as the apical entry mechanism of transcellular Mg$^{2+}$ transport, the identity of proteins and (hormonal) factors that regulate and coordinate the essential steps in transcellular Mg$^{2+}$ transport, including cytosolic Mg$^{2+}$ diffusion and basolateral extrusion, are still elusive. Therefore, the aim of this thesis was to increase the knowledge of renal Mg$^{2+}$ handling at the molecular, cellular and body level in health and disease. This chapter discusses the research presented in this thesis, which significantly attributed to our understanding of active transcellular Mg$^{2+}$ reabsorption in general and in particular of TRPM6.
Structural analysis of the TRPM6 pore

At present, little is known about the structure and the specific amino acids that determine the specific conductive properties of TRP and more specifically TRPM pores. To identify the molecular pore determinants responsible for the Mg$^{2+}$ permeability of TRPM6, the putative pore-forming region of this channel was modeled based on the crystal structure of the K$^+$ channel KcsA (3). The putative pore-forming region of TRPM6 contains three negatively charged amino acid residues, i.e. E1024, E1029 and D1031, which are potential Mg$^{2+}$ binding sites determining the conductive properties of this channel (figure 1). The latter two are present in the putative selectivity filter which stretches from G1028 to C1033. Interestingly, E1024 is conserved in TRPM1, TRPM3 and TRPM7 and E1029 in TRPM5 and TRPM7. D1031 is fully conserved throughout all members of the TRPM subfamily. To evaluate the functional role of these negatively charged residues all were neutralized to alanine. This resulted in non-functional channels except for E1029A. Unfortunately, numerous attempts trying to detect the plasma membrane localization of HA- and VSV-tagged TRPM6 by biotinylation were unsuccessful. Therefore, detection of wild-type HA-tagged TRPM6 channels and the non-functional channels at the plasma membrane was unattainable. As a consequence, we were unable to determine whether the non-functional channels resulting from the mutations described above, are either inserted in the plasma membrane or are retained intracellularly. Future studies, using methods with improved TRPM6 plasma membrane detection by for example the use of other tags and antibodies should address this question. The mutant E1029A displayed dissimilar permeation properties as increased pore diameter and reduced ruthenium-red (RR) sensitivity compared to wild-type TRPM6. Substitution of I1030 by methionine to resemble the pore of TRPM4 and V1032 by alanine resulted in functional TRPM6 channels with distinctive pore properties. The I1030M mutant displayed a decreased pore diameter and reduced RR sensitivity compared to wild-type TRPM6. The V1032 mutant showed decreased current amplitudes, but no change in pore diameter or RR sensitivity.

Taken together, these data indicated that several amino acids together in the putative pore region 1028GEIDVC1033 determine the Mg$^{2+}$ selectivity of TRPM6 (figure 1), which is in accordance with a report of Li and colleagues who demonstrated that E1024 and E1029 residues are essential for the Mg$^{2+}$ permeability of TRPM6 (4). In addition, these results correspond with the pore properties of TRPM4, which also has a more complex pore structure, but are in contrast with the members of the TRPV family in which a single amino acid determines the pore permeation properties (5, 6). Interestingly, the permeation rank order of divalent cations for TRPM6 is: Ba$^{2+}$ > Ni$^{2+}$ > Mg$^{2+}$ > Zn$^{2+}$ ≥ Ca$^{2+}$ whereas for TRPM7 this is: Ba$^{2+}$ > Ni$^{2+}$ > Zn$^{2+}$ > Mg$^{2+}$ > Ca$^{2+}$. Compared to the permeation profile of TRPM7, the selectivity for Mg$^{2+}$ conductance is higher for TRPM6 (chapter 2). Although the putative pore-forming regions of both proteins are highly homologous, differences are found at positions Y1022, G1028, C1034, S1035 and Q1036 and P1037 for TRPM6 corresponding to F1045, Y1051, A1057, N1058, D1059, and S1060 in TRPM7, respectively (figure 1).

Further investigation is needed to unveil the role of these individual amino acids in constituting the difference in selectivity for Mg$^{2+}$ between TRPM6 and TRPM7. In addition to individual differences between TRPM6 and TRPM7, the identification of molecular pore determinants of TRPM6 and TRPM7 is even more complex of TRPM6 and TRPM7.
TRPM7 and trafficking was not affected, detailed patch clamp analysis is required to investigate the P1070R pore properties in detail. In addition to studying pore mutants by patch clamp analysis, future crystallographic structure assays of the TRPM6 pore allow a detailed study of the pore structure of TRPM6.

Tissue distribution of TRPM6

To characterize the distribution of the epithelial Mg\(^{2+}\) channels TRPM6 and TRPM7, we studied the expression profile of these channels in various mouse tissues. We demonstrated that TRPM6 mRNA is predominantly expressed in kidney, lung, cecum and colon, whereas TRPM7 showed a ubiquitous mRNA expression pattern (chapter 3). The omnipresent expression pattern of TRPM7 is in line with its essential role in cell viability and maintaining cellular Mg\(^{2+}\) homeostasis (10-12). The expression pattern of TRPM6 is in accordance with the observed disturbance of Mg\(^{2+}\) absorption in HSH patients and its physiological function to maintain a proper body Mg\(^{2+}\) balance (13, 14). Moreover, by Northern blot analysis of human tissues, Walder et al. demonstrated that TRPM6 mRNA was abundantly expressed in kidney and colon (13). In addition, Northern blot analysis of mouse tissues revealed that full-length TRPM6 is predominantly expressed in kidney and to a lesser extent in lung (13). Moreover, Fonfria and colleagues reported that TRPM6 mRNA in human is principally expressed in kidney, intestine, brain and pituitary gland (10). Although Fonfria et al. determined the TRPM6 mRNA expression levels by two different techniques (SYBR green and TaqMan), the values were not corrected for the mRNA expression levels of a housekeeping gene but for the amount of total mRNA used, rendering the results less reliable. Moreover, the expression of TRPM6 mRNA in human brain was not confirmed by the study of Walder and colleagues, who did not detect TRPM6 mRNA expression in both human and mouse brain (13). Thus, in human and mouse, TRPM6 is predominantly expressed in kidney and intestine.

In kidney, TRPM6 protein was demonstrated to be specifically expressed in DCT (15). In contrast, Schlingmann and colleagues showed by reverse transcriptase PCR (RT-PCR) that TRPM6 mRNA in rat kidney is not only expressed in DCT but also in the proximal convoluted tubule, connecting tubule, cortical collecting duct, outer medullary collecting duct, and inner medullary collecting duct. A possible explanation for this discrepancy could be the result of cross contamination of these

because different pore properties were described for TRPM6, TRPM7 and TRPM6/TRPM7 heteromeric channels (8). Moreover, TRPM7 is expressed in every cell type and therefore possibly contributes to TRPM6/TRPM7 tetramer formation and observed channel properties in cells transiently overexpressing TRPM6.

It can be speculated that the selectivity filter for Mg\(^{2+}\) in TRPM6 consists of a ring of 8 negatively charged residues in a tetrameric pore molecule (figure 2). Consequently, mutations in the selectivity filter of TRPM6 could result in defective channels and the identification of such mutations in hypomagnesemic patients can further unveil the molecular determinants of Mg\(^{2+}\) permeability. The mutation P1017R, which results in a dominant-negative inhibition of TRPM6 channel activity, was the first identification of a mutation in the putative pore-forming region upstream of the selectivity filter in a patient with hypomagnesemia with secondary hypocalcemia (HSH) (9). Although hetrotetrameric assembly of this mutant with
segments with DCT during isolation. In addition, Schlingmann et al. revealed with RT-PCR that TRPM6 mRNA is expressed in all intestinal segments of rat. However, RT-PCR is not quantitative and might not represent physiological expression levels (14). Therefore, based on our results of quantitative PCR in mouse, we suggest that cecum and colon, which comprise the distal part of the intestine, are the dominant intestinal segments important for transcellular Mg2+ absorption. By Northern blot analysis of human tissues Walder et al. also demonstrated TRPM6 mRNA expression in colon but not in the small intestine (13). Interestingly, over 60% of the total intestinal TRPM6 mRNA expression of mouse resides in cecum and suggests that this segment is most important for transcellular Mg2+ absorption (chapter 3). Although the explanation for the high cecum TRPM6 expression levels remains obscure, the fermentation processes and the breakdown of cellulose by bacteria in the cecum possibly generate a favorable environment for transcellular Mg2+ absorption. Detailed analysis of TRPM6 expression levels in the human intestinal tract is necessary to conclude which intestinal segments are (most) important for transcellular Mg2+ absorption. Preliminary results indicate that in human, TRPM6 is predominantly expressed in colon (data not shown).

The role of TRPM6 in lung remains elusive and further research, first determining in which cells and tissues the Mg2+ channel is specifically expressed, is needed to ascertain the function of TRPM6 in this organ. We and Walder et al. demonstrated that TRPM6 mRNA is expressed in lung tissue of mouse (13). However, expression of TRPM6 mRNA was not detected in human lung tissue (10, 13). It was suggested that low dietary Mg2+ intake may be involved in the etiology of asthma and chronic obstructive airways disease (16).

 Regulation of TRPM6 in kidney by 17ß-estradiol

In chapter 3 it was demonstrated that 17ß-estradiol (17ß-E2), which has been implicated in maintenance of the Ca2+ balance (17-19), positively regulates renal TRPM6 mRNA expression levels. In addition it was shown that 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) and parathyroid hormone (PTH) did not alter renal TRPM6 mRNA expression levels. Although the calcitropic hormones 1,25(OH)2D3 and PTH (20, 21) did not modify the renal expression levels of TRPM6 and TRPM7, these hormones have been suggested to influence Mg2+ homeostasis (2, 14, 22). However, the role of 1,25(OH)2D3 and PTH in regulation of the systemic Mg2+ balance needs further investigation. Since a simultaneous disturbance of the Mg2+ and Ca2+ balance is often observed in various electrolyte disorders (chapter 1), the interrelationship between 17ß-E2, Ca2+ and the Mg2+ balance requires further investigation to reveal whether TRPM6 expression is primarily regulated by 17ß-E2 or is a secondary effect of changes in the Ca2+ balance induced by 17ß-E2. Interestingly, Mg2+ homeostasis and 17ß-E2 have been linked to menstrual migraine, pregnancy, pre-eclampsia, and menopause (23-25). This raises the question whether in males, who express lower concentrations of 17ß-E2, the Mg2+ reabsorption is differentially regulated (26). Moreover, it would be interesting to study the role of androgens and estrogens in male and female Mg2+ homeostasis and in particular its effect on TRPM6 mediated transcellular Mg2+ transport in more detail.

Transepithelial transport of Mg2+ and Ca2+ in intestine

In chapter 3 was demonstrated that the epithelial Ca2+ channel TRPV6, which constitutes the luminal Ca2+ entry mechanism in active Ca2+ absorption (27, 28), is predominantly expressed in duodenum and colon. Since in mouse intestine, TRPM6 is predominantly expressed in cecum and colon with very low expression levels in duodenum and jejunum, it is likely that Mg2+ is exclusively absorbed in the distal part of the intestinal tract, including cecum and colon, whereas Ca2+ is transcellularly absorbed in both the proximal (duodenum) and distal (colon) part of the intestinal tract. This is supported by the fact that 45Ca2+ absorption studies, which measure the rate of Ca2+ absorption in the proximal intestine, showed that dietary Mg2+ content did not compete with the rate of Ca2+ absorption (chapter 3). Possibly, differences of the intestinal epithelium and the intestinal mucosa of different parts of the intestinal tract affect or create an environment favoring the absorption of Mg2+ or Ca2+ divalents. This could explain the partial overlap of active Mg2+ and Ca2+ absorption.

Regulation of TRPM6 by dietary Mg2+ and coupling of the Mg2+ and Ca2+ balance

Disturbances in the Mg2+ balance are often accompanied with disturbances in the Ca2+ balance (29). Mutual disturbances of the Mg2+ and Ca2+ balance are for example observed in HSH and Gitelman syndrome. However, the concurrence of these symptoms in these disorders is not well understood. As described in chapters 3 and 4, dietary Mg2+ restriction in mice resulted in hypomagnesemia, renal Mg2+ and Ca2+ conservation, and increased expression of TRPM6 in kidney. In contrast, the

General discussion and summary
Mg\textsuperscript{2+}-enriched diet increased urinary Mg\textsuperscript{2+} and Ca\textsuperscript{2+} excretion and TRPM6 expression levels in colon. These different dietary Mg\textsuperscript{2+} regimes did not alter TRPM7 mRNA expression levels in kidney and intestine supporting the role of TRPM7 in cellular Mg\textsuperscript{2+} homeostasis. Dietary Mg\textsuperscript{2+} content did not influence serum Ca\textsuperscript{2+} levels. Dietary Mg\textsuperscript{2+} content influences Ca\textsuperscript{2+} excretion at the level of the intestine or kidney possibly via hormones. In addition, the involvement of the Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-sensing receptor (CaSR) and transport of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} by claudin-16 and/or claudin-19 need further attention since all three proteins are involved in maintenance of both the total body Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance (30-34). The exact mechanism resulting in the altered Ca\textsuperscript{2+} balance in response to dietary Mg\textsuperscript{2+} restriction or supplementation, remains unresolved.

The upregulation of TRPM6 in kidney during dietary Mg\textsuperscript{2+} restriction supports a mechanism actively regulating the expression level of this channel to maximally facilitate Mg\textsuperscript{2+} reabsorption during Mg\textsuperscript{2+} deficiency. This emphasizes the gatekeeper function of TRPM6 in transepithelial Mg\textsuperscript{2+} transport. Remarkably, TRPM6 mRNA levels in colon are upregulated by the Mg\textsuperscript{2+}-enriched diet, but not after dietary Mg\textsuperscript{2+} restriction. The molecular details resulting in the differential upregulation of TRPM6 expression in kidney and intestine in response to Mg\textsuperscript{2+} restriction and a Mg\textsuperscript{2+}-enriched diet, respectively, need to be elucidated. It is possible that the CaSR, which is present in kidney and colon (35-37), fulfills an opposite role in the regulation of renal and intestinal TRPM6 expression levels upon alternating dietary Mg\textsuperscript{2+} content. In analogy, a Ca\textsuperscript{2+}-enriched diet increased duodenal mRNA expression levels of the epithelial Ca\textsuperscript{2+} channel TRPV6 of 25-hydroxyvitamin D\textsubscript{3}-1\alpha-hydroxylase (1\alpha-OHase) knockout mice, which have undetectable levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} (38). Thus, in the absence of 1,25(OH)\textsubscript{2}D\textsubscript{3} regulating intestinal Ca\textsuperscript{2+} absorption, TRPV6 mRNA expression levels increase during high dietary Ca\textsuperscript{2+} intake. Since a similar effect of TRPM6 expression was observed in response to high dietary Mg\textsuperscript{2+} content in wild-type mice, this suggests that no hormone, like 1,25(OH)\textsubscript{2}D\textsubscript{3} regulating Ca\textsuperscript{2+} absorption, exists for Mg\textsuperscript{2+}.

**Hypermagnesuria in TRPV5 knockout mice**

In chapter 4 the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance of TRPV5 knockout (TRPV5\textsuperscript{-/-}) mice was studied. Surprisingly, TRPV5\textsuperscript{-/-} mice displayed renal Mg\textsuperscript{2+} wasting, decreased renal TRPM6 mRNA and protein abundance together with normal serum Mg\textsuperscript{2+} levels. Although the observed reduced renal expression levels of TRPM6 in TRPV5\textsuperscript{-/-} mice could be an explanation for the renal Mg\textsuperscript{2+} wasting in these mice, the question remains what triggered the downregulation of TRPM6 protein expression. It was demonstrated that TRPV5\textsuperscript{-/-} mice show impaired Ca\textsuperscript{2+} reabsorption, hypercalciuria, hypervitaminosis D, and intestinal hyperabsorption of Ca\textsuperscript{2+} (39). Moreover, it was shown that the hypervitaminosis D mediates the compensatory Ca\textsuperscript{2+} hyperabsorption in TRPV5\textsuperscript{-/-} mice (40). Possibly, the high vitamin D levels present in TRPV5\textsuperscript{-/-} mice, which result in hyperabsorption of Ca\textsuperscript{2+}, also lead to hyperabsorption of Mg\textsuperscript{2+}. Consequently, downregulation of renal TRPM6 expression levels in TRPV5\textsuperscript{-/-} mice could be a secondary effect in response to an increased filtered load of Mg\textsuperscript{2+} resulting from the concomitant hyperabsorption of Mg\textsuperscript{2+} with Ca\textsuperscript{2+}. Thus, if hypervitaminosis D induces hyperabsorption of Mg\textsuperscript{2+}, the hypermagnesuria and renal downregulation of TRPM6 expression in TRPV5\textsuperscript{-/-} mice might be explained.

Interestingly, we showed that 1\alpha-hydroxylase knockout (1\alpha-OHase –/-) and TRPV5/1\alpha-hydroxylase double knockout (TRPV5\textsuperscript{-/-} /1\alpha-OHase –/-) mice (40, 41), which have undetectable levels of 1,25(OH)\textsubscript{2}D\textsubscript{3}, display hypomagnesurias. This indicated that the lack of TRPV5 does not primarily cause hypermagnesuria and renal downregulation of TRPM6 in TRPV5\textsuperscript{-/-} mice. In addition to wild-type mice, the Mg\textsuperscript{2+} and Ca\textsuperscript{2+} balance of TRPV5\textsuperscript{-/-} mice fed a Mg\textsuperscript{2+}-deficient, Mg\textsuperscript{2+}-normal and a Mg\textsuperscript{2+}-enriched diet was studied (chapter 4). TRPV5\textsuperscript{-/-} mice responded similarly as wild-type mice to the three different dietary Mg\textsuperscript{2+} regimes, as described previously. Dietary Mg\textsuperscript{2+} restriction resulted in hypomagnesemia, renal Mg\textsuperscript{2+} and Ca\textsuperscript{2+} conservation, and increased expression of TRPM6 in kidney. Moreover, the Mg\textsuperscript{2+}-enriched diet increased renal Mg\textsuperscript{2+} and Ca\textsuperscript{2+} excretion and TRPM6 expression levels in colon. In addition, dietary Mg\textsuperscript{2+} content did not alter TRPM7 mRNA expression levels in kidney and intestine. Moreover, dietary Mg\textsuperscript{2+} content did not influence serum Ca\textsuperscript{2+} levels.

**Elucidation of the gene defect of isolated recessive renal hypomagnesemia (IRH)**

The role of epidermal growth factor (EGF) as a magnesiotropic hormone was discovered by careful analysis of a Dutch family with two daughters suffering from IRH, which is characterized by hypomagnesemia, convulsions from the first year of life, and moderate mental retardation. Remarkably, other biochemical abnormalities were not identified, especially no disturbance in urinary Ca\textsuperscript{2+} excretion. Previously, the Mg\textsuperscript{2+} balance in these two affected daughters was extensively studied as reported in 1987 (42). Twenty years later, improvement of linkage and DNA analysis...
resulted in the discovery of the candidate gene EGF, encoding pro-EGF, and subsequent identification of a mutation in this gene. The identified mutation substitutes a proline at position 1070 for a leucine (P1070L) and is located in the intracellular domain of the protein. Through the elucidation of the gene defect in IRH we could demonstrate that EGF is a novel autocrine/paracrine magnesiotropic hormone, which regulates Mg\(^{2+}\) reabsorption by regulation of TRPM6 activity via EGFR binding (chapter 5). In addition, disruption of basolateral sorting and release of EGF by a point mutation or inhibition of the EGFR results in reduced activity of TRPM6 and renal Mg\(^{2+}\) wasting. Thus, stimulation of basolateral EGFR by EGF is required for baseline TRPM6 activity necessary to maintain Mg\(^{2+}\) homeostasis. This is supported by the fact that the hypomagnesemia observed in the two sisters with IRH (serum Mg\(^{2+}\) 0.53-0.66 mM) is less severe than the hypomagnesemia reported in patients with hypomagnesemia with secondary hypocalcemia (serum Mg\(^{2+}\) as low as 0.08 mM), in which TRPM6 itself is affected and non-functional (13-15, 42). The identification of autocrine/paracrine regulation of TRPM6 activity by EGF and thereby of active Mg\(^{2+}\) reabsorption could open new leads to develop therapeutic possibilities to control renal Mg\(^{2+}\) handling. In addition, this discovery might help to elucidate the mechanisms and epidemiological phenomena of hypomagnesemia in the general population and its correlation with diseases such as cardiovascular diseases, diabetes and hypertension.

**Impaired basolateral sorting of pro-EGF results in renal Mg\(^{2+}\) wasting**

Pro-EGF is a type I membrane protein, which in kidney is abundantly expressed at the apical and at a low level at the basolateral membrane of DCT cells (43, 44). Pro-EGF is cleaved by an unidentified protease to release the mature active 53 amino acid EGF in the luminal and basolateral compartments. A possible candidate for the EGF sheddase is ADAM17, which is a transmembrane protein and belongs to the superfamily of zinc proteases. ADAM17 can cleave peptides of many EGF family members including TGF-\(\alpha\), amphiregulin, betacellulin, epiregulin, and HB-EGF (45, 46). The function of apically expressed pro-EGF remains elusive since expression of the EGFR is restricted to the basolateral membrane. To date there is no evidence for a luminal effect of EGF, but it has been suggested that apically released EGF is involved in maintenance of epithelial surface integrity, because EGF constantly baths the epithelial surfaces of the urinary tract or that apically expressed pro-EGF might function as a receptor that is involved in ion transport (47, 48). The identified P1070L mutation resides in the intracellular tail of pro-EGF and results in aberrant secretion of EGF to the basolateral compartment, but not in disturbed secretion to the luminal space as studied in polarized MDCK cells. Based on the fact that pro-EGF-P1070L is efficiently secreted to the luminal space, we suggested that disturbed basolateral sorting of pro-EGF-P1070L results from missorting and is not due to affected pro-EGF processing or aberrant processing by proteases. In the literature, several basolateral sorting motifs have been described, including the PXXP motif, in which X is an arbitrary amino acid (49-51). In addition, pro-EGF contains a cytoplasmic arginine-arginine (RR) motif. Interestingly, both the PXXP and RR motifs have been identified as cellular kinase binding motifs and could be involved in the apical function of pro-EGF (52). We hypothesized that the P1070L mutation disrupts the basolateral sorting motif 1067PKNP1070 resulting in improper basolateral sorting of pro-EGF. Impaired basolateral sorting was demonstrated functionally by patch clamp analysis measuring the stimulatory effect on TRPM6 of the separately collected apical and basolateral pre-conditioned media of MDCK cells stably expressing either wild-type or mutant pro-EGF. Additional biochemical experiments are necessary to resolve the biochemical processing of mutant EGF in detail. In addition, experiments are required to elucidate all residues which constitute the basolateral sorting motif of pro-EGF. Interestingly, in the salivary gland, pro-EGF appears to be processed intracellularly to the mature 6 kDa EGF which is stored as a high molecular weight complex within secretory granules (53-55). Therefore, it is expected that patients with IRH display normal excretion of EGF in the saliva. Furthermore, the role of EGF in intestinal Mg\(^{2+}\) absorption needs to be investigated since TRPM6 is also abundantly expressed in human colon (unpublished observations).

**Additional role of EGF in ion reabsorption: EGF and transcellular phosphate transport**

In addition to the role of EGF as a magnesiotropic hormone, EGF has previously been reported to stimulate phosphate transport in the proximal convoluted tubule (PCT) via basolateral receptors (56). It is possible that the basolaterally released form of EGF, which enters the interstitium, can bind the basolaterally localized EGFRs of the PCT, because the PCT is in the immediate vicinity of the DCT. Therefore, phosphaturia in patients with IRH might be expected. However, the values of phosphate reabsorption of the patients with IRH were between 85 and
of the EGFR signaling pathway, which stimulates TRPM6 activity, could provide new clues for therapies to overcome the side effect of hypomagnesemia during cetuximab treatment. Surprisingly, thus far hypomagnesemia has not been reported for cancer patients treated with erlotinib (alias tarceva) or gefitinib (alias iressa), which are specific EGFR tyrosine kinase inhibitors (65). The mechanism by which these drugs circumvent the side effect hypomagnesemia is elusive and more detailed research is needed to investigate whether patients treated with these drugs have no or mild hypomagnesemia. The EGFR belongs to the erythroblastoma viral gene product, v-erbB (ErbB) family of proteins, which comprises four receptors (ErbB1-4, also known as HER1-4). After ligand binding, the EGFR (or ErbB1) forms homodimers, as well as three functional heterodimers (66). Possibly, cetuximab completely prevents homo- and heterodimerization completely abolishing EGFR activity, whereas erlotinib and gefitinib only prevent EGFR tyrosine kinase activity but not dimerization. Thus, in the presence of erlotinib and gefitinib the EGFR in kidney could still be phosphorylated by for instance its dimerization partner ErbB2 and subsequently recruit its unique set of signaling proteins, which stimulate TRPM6 activity thereby preventing hypomagnesemia. Because erlotinib and gefitinib are effectively used to treat cancer without the development of the side effect of hypomagnesemia, these drugs probably target mainly EGFR homodimers (66). Although ErbB2 is present in kidney, its basolateral localization in DCT and of the other ErbB members needs to be determined (67).

Molecular mechanism of TRPM6 activation by EGFR signaling

Studying the signaling pathways of the EGFR, which stimulate TRPM6 activity, and TRPM6 mRNA abundance, respectively. Interestingly, a coupling between estrogen and pro-EGF expression has been described (57-59). Mice treated for four weeks with 17β-E2 showed a 2-fold increase in pro-EGF mRNA expression in the uterus (60). It was speculated that estrogens stimulate processing of the pro-EGF precursor. Moreover, no EGF immunoreactivity was observed in the uterus of OVX mice compared to OVX mice supplemented with 17β-E2 (58). The administration of 17β-E2 to OVX mice was also shown to increase the level of uterine EGFR expression, increasing the number of EGF-binding sites (61). Furthermore, evidence was provided that exogenous EGF can mimic estrogen function in the stimulation of female genital-tract growth and differentiation and that EGF can interact with the estrogen receptor (57, 59). Taken together, these results suggest that estrogen is positively involved in the upregulation of EGF and EGFR in uterus and that EGF can mimic the effect of estrogen. However, the effect of 17β-E2 positively stimulating the expression of EGF and the EGFR reported for the uterus needs to be established for the kidney. Moreover, the relationship between EGF and 17β-E2 regarding Mg2+ homeostasis needs to be determined. Interestingly, it was demonstrated that the excretion of EGF by women taking oral contraceptives was significantly greater than in females who did not use contraceptives (62). The effect of gender differences with respect to levels of EGF and 17β-E2 in relation to regulation of the Mg2+ balance require additional research. Nevertheless, the relationship between EGF and estrogen is striking and should receive further attention with respect to their coupled interaction regarding TRPM6 regulation and Mg2+ homeostasis.

EGFR inhibition and hypomagnesemia

In chapter 5 we demonstrated that, in accordance with other studies, several colorectal cancer patients treated with cetuximab, which is an antagonist of the human EGFR, develop hypomagnesemia due to renal Mg2+ wasting (63, 64). These results emphasize the significance of EGF in maintenance of a proper Mg2+ balance. Importantly, cetuximab abolished EGF-stimulated TRPM6 activity underlining the involvement of the EGFR in hypomagnesemia. Detailed elucidation of the EGFR signaling pathway, which stimulates TRPM6 activity, could provide new clues for therapies to overcome the side effect of hypomagnesemia during cetuximab treatment. Surprisingly, thus far hypomagnesemia has not been reported for cancer patients treated with erlotinib (alias tarceva) or gefitinib (alias iressa), which are specific EGFR tyrosine kinase inhibitors (65). The mechanism by which these drugs circumvent the side effect hypomagnesemia is elusive and more detailed research is needed to investigate whether patients treated with these drugs have no or mild hypomagnesemia. The EGFR belongs to the erythroblastoma viral gene product, v-erbB (ErbB) family of proteins, which comprises four receptors (ErbB1-4, also known as HER1-4). After ligand binding, the EGFR (or ErbB1) forms homodimers, as well as three functional heterodimers (66). Possibly, cetuximab completely prevents homo- and heterodimerization completely abolishing EGFR activity, whereas erlotinib and gefitinib only prevent EGFR tyrosine kinase activity but not dimerization. Thus, in the presence of erlotinib and gefitinib the EGFR in kidney could still be phosphorylated by for instance its dimerization partner ErbB2 and subsequently recruit its unique set of signaling proteins, which stimulate TRPM6 activity thereby preventing hypomagnesemia. Because erlotinib and gefitinib are effectively used to treat cancer without the development of the side effect of hypomagnesemia, these drugs probably target mainly EGFR homodimers (66). Although ErbB2 is present in kidney, its basolateral localization in DCT and of the other ErbB members needs to be determined (67).

Molecular mechanism of TRPM6 activation by EGFR signaling

Studying the signaling pathways of the EGFR, which stimulate TRPM6 activity, the Src family of tyrosine kinases and the Rho GTPase Rac1 pathways have been identified as possible routes of TRPM6 activation (chapter 6). It was demonstrated that the EGF-mediated stimulation of TRPM6 occurs via signaling through Src kinases and Rac1, which increased the cell surface abundance of TRPM6. However, it is unknown whether TRPM6 activity is directly gated by Src. Moreover, the effect of both pathways simultaneously stimulating TRPM6 activity can not be excluded. Patch clamp analysis demonstrated that the stimulatory effect of EGF is specific for TRPM6 because EGF did not increase the activity of its closest homologue TRPM7. This supports the role of EGF in specifically maintaining total body Mg2+ homeostasis rather than the cellular Mg2+ balance. However, the effect of EGF on heterotetramers of TRPM6 and TRPM7 needs further investigation.
since TRPM6, TRPM7 and TRPM6/7 channels display different properties (8). Moreover, it is not clear how stimulation of the EGFR by EGF can result in specific stimulation of TRPM6 channel activity. Possibly, small differences between TRPM6 and TRPM7 allow specialized auxiliary proteins, which might be activated after stimulation of the EGFR, to specifically bind and regulate TRPM6 activity. To date, no regulatory proteins have been described that modify the biophysical, pharmacological, and expression properties of TRPM6 by direct interactions as has been described for the epithelial Ca2+ channels TRPV5 and TRPV6 (68). As described above, Src kinase is a downstream signaling molecule of the EGFR. Furthermore, this kinase is a non-receptor protein tyrosine kinase, which is known to be directly activated after a steroid stimulation, and is rapidly activated by co-stimulation of 17ß-E2 and EGF (69, 70). Subsequently, activated Src kinase phosphorylates a wide variety of substrate proteins, which finally induce gene transcription (71, 72). In contrast, androgens are able to activate Src without the EGF stimulation (73). It is tempting to speculate that estrogens, androgens and EGF cooperate together in a concerted action to tightly regulate active Mg2+ reabsorption by controlling EGFR signaling pathways and thereby regulate the activity and transcription/translation of TRPM6.

**EGF and schizophrenia**

The identification of a mutation in the EGF gene raised the question whether this mutation has an effect on mental development since the two previously described IRH patients are mentally retarded. Moreover, in our family with IRH, one unaffected, EGF P1070L-carrier sister suffers from schizophrenia and two brothers and one sister of the carrier mother are known with cluster A (diagnostic and statistical manual of mental disorders, fourth edition, text revision) personality disorders. Schizophrenia or cluster A personality disorders has not been diagnosed for the two affected sisters. EGF functions as a neurotrophic, survival and maintenance factor for selective neurons in the central nervous system (CNS). EGF is present in the developing nervous system and, based on the various studies reporting that EGF has neurotrophic and neuromodulatory effects on developing dopaminergic neurons both in vitro and in vivo, it is suggested that dopaminergic dysfunction might play a role in the etiopathology of schizophrenia (77-86). Moreover, EGF is expressed in brainstem, cerebral cortex, hippocampus, basal hypothalamus, striatum and thalamus which are all brain regions where changes have been demonstrated in schizophrenia (74-76). Interestingly, decreased levels of EGF were observed in serum, prefrontal cortex and striatum of schizophrenic patients (77). However, decreased of serum EGF levels in schizophrenia patients was not confirmed by another group (78). Two groups reported that the EGF nucleotide polymorphism, A61G, is associated with schizophrenia (79, 80), but this association was not confirmed by two other studies (81, 82). Recently, it was demonstrated that this polymorphism is not associated with the risk of schizophrenia but with the age of onset of the disease in male patients (82). In addition, a relationship between EGF and schizophrenia was found by studies performing genome-wide linkage analysis, which were used to map the complex genetic disorder schizophrenia in order to pinpoint susceptibility genes. Multiple linkage regions have been identified in schizophrenia and interestingly, linkage with a region on chromosome 4, which harbours the EGF gene and a single polymorphic marker pinpointing EGF have been reported (83, 84). Taken together, these data suggest that EGF might be a susceptibility gene for schizophrenia. Sequencing of this gene in families with schizophrenia, which show linkage to a region on chromosome 4 harbouring the EGF gene, should substantiate our hypothesis.

**Missing links in active transcellular Mg2+ transport**

The research presented in this thesis contributed to the understanding of molecular mechanisms that regulate active Mg2+ (re)absorption. However, a comprehensive model of active transepithelial Mg2+ transport is still lacking because the identity of proteins coordinating essential steps in transepithelial Mg2+ transport including cytosolic Mg2+ diffusion and basolateral extrusion are still unknown (figure 3). An interesting candidate for cytosolic Mg2+ transport is parvalbumin, a member of the family of cytoplasmic Ca2+-binding proteins, which has been suggested to function in renal Mg2+ handling and is specifically expressed in DCT (85). However, parvalbumin is not expressed in the entire DCT but only in the first segment DCT1, whereas TRPM6 is expressed in DCT1 and DCT2 (15). Possibly, parvalbumin mediates cytosolic transport for Mg2+, comparable to the function of calbindin-D28k, which transports cytosolic Ca2+ (21). Moreover, parvalbumin knockout mice display a disturbed contraction-relaxation cycle of fast twitch muscles, which supports a role of parvalbumin in renal Mg2+ handling (86). However, parvalbumin knockout mice did not show a disturbance of the Mg2+ balance (87). At present no candidate gene has been identified for the basolateral extrusion mechanism for Mg2+.
Our understanding of molecular mechanisms regulating the Mg\(^{2+}\) balance primarily arose from the elucidation of the genetic basis of rare monogenic Mg\(^{2+}\) human disorders (chapter 1 and 5). The identification of additional genes, by studying disorders with unexplained forms of hereditary Mg\(^{2+}\) disturbance, could reveal the missing links in the molecular mechanism of transcellular Mg\(^{2+}\) transport. Other approaches that were applied to identify mammalian Mg\(^{2+}\) transporters include homology searches with prokaryotic Mg\(^{2+}\) transport proteins and genomics to identify genes that are upregulated by low extracellular Mg\(^{2+}\) concentrations in cell lines or in mouse kidney. This resulted in the identification of hsaMrs2p, NIPA1 (alias SPG6), MagT1, SLC41A1, SLC41A2, and ACDP2 (89-94). Although it has been demonstrated that these newly identified proteins transport Mg\(^{2+}\), their physiologic function remains to be determined. However, the ubiquitously expression pattern of these proteins does not support a specialized role in epithelial Mg\(^{2+}\) transport in kidney and intestine. Irrespective of the approach used, the identification of new genes involved in body Mg\(^{2+}\) homeostasis will increase our knowledge about Mg\(^{2+}\) homeostasis and might explain other Mg\(^{2+}\) wasting disorders. In addition, increased insight into Mg\(^{2+}\) homeostasis could provide clues to make therapeutic intervention possible in the treatment of hypomagnesemia.

Conclusions

The overall aim of the research presented in this thesis was to elucidate the molecular mechanisms of active Mg\(^{2+}\) (re)absorption, and therefore the maintenance of the Mg\(^{2+}\) balance in health and disease. This thesis described the identification of factors regulating TRPM6 and the discovery of EGF as an autocrine/paracrine hormone essential for maintenance of the body Mg\(^{2+}\) balance by regulating renal Mg\(^{2+}\) handling. Herewith, a major step is taken towards elucidation of the mechanisms which regulate Mg\(^{2+}\) homeostasis. Future studies directed to disclose the molecular mechanisms and (hormonal) factors that regulate transepithelial Mg\(^{2+}\) transport will certainly contribute to a better understanding of Mg\(^{2+}\) homeostasis. Increased knowledge of molecular and cellular aspects of Mg\(^{2+}\) homeostasis may lead to a better treatment of patients with Mg\(^{2+}\) deficiencies that is directed towards correcting the underlying functional defect(s). This will be of great benefit to patients because of the adverse symptoms resulting from

**Figure 3.** Schematic representation of active transcellular Mg\(^{2+}\) transport in DCT

(A) Active and transcellular Mg\(^{2+}\) transport is carried out as a three-step process. Driven by the transmembrane voltage, Mg\(^{2+}\) leaves the pro-urine compartment and enters the cell through the epithelial Mg\(^{2+}\) channel TRPM6. Subsequently, Mg\(^{2+}\) diffuses, possibly bound to Mg\(^{2+}\)-binding proteins (?), to the basolateral plasma membrane. At the basolateral plasma membrane, Mg\(^{2+}\) is extruded into the interstitium by a hypothetical Na\(^{+}\)/Mg\(^{2+}\)-exchanger protein and/or a plasma membrane Mg\(^{2+}\)-ATPase (?) against both electrical and concentration gradients. Importantly, Mg\(^{2+}\) entry into the cells appears to be the rate-limiting step. TRPM6 is positively regulated by i) 17ß-estradiol (17ß-E\(_2\)), which binds the estrogen receptor (ER); ii) low Mg\(^{2+}\) levels in pro-urine and/or blood and iii) EGF, which binds the EGF receptor (EGFR). The functioning of the Na\(^{+}\)/Cl\(^{-}\)-cotransporter (NCC), the Ca\(^{2+}\) sensing receptor (CaSR), the \(\gamma\)-subunit of the Na\(^{+}\)/K\(^{+}\)-ATPase and the renal Cl\(^{-}\)-channel CLC-Kb are also important for active Mg\(^{2+}\) reabsorption.

Cell lines cultured on special media, e.g. with Mg\(^{2+}\) concentrations up to 100 mM can be used to identify responsive genes via a proteomics approach (88).
hypomagnesemia, such as tetany, prolonged QT interval and cardiac arrhythmias. In addition, it might help to explain and provide new treatment strategies for unknown Mg²⁺ wasting disorders and its association with common chronic diseases such as cardiovascular diseases and diabetes.

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Nederlandse samenvatting

Introductie
Magnesium (Mg$^{2+}$) is een belangrijk twee-vaardig positief geladen ion dat een cruciale rol speelt in een breed scala van verschillende biologische processen. Ondanks het feit dat Mg$^{2+}$ het op een na meest voorkomende intracellulaire kation is en een noodzakelijke rol vervult in het functioneren van meer dan driehonderd enzymen, zijn de moleculaire mechanismen die de Mg$^{2+}$ balans reguleren nog niet volledig opgehelderd. In het behoud van de Mg$^{2+}$ balans spelen de nieren een essentiële rol. De Mg$^{2+}$ concentratie in het bloed wordt nauwkeurig gereguleerd doordat de uitscheiding van Mg$^{2+}$ in de urine wordt aangepast aan de opname van Mg$^{2+}$ in het spijsverteringsstelsel. Het grootste deel van het door de nieren gefilterde Mg$^{2+}$ wordt geresorbeerd in de proximale tubulus en het dikke opstijgende been van de lis van Henle door middel van passief paracellulair transport. Het nauwkeurig aanpassen van de Mg$^{2+}$ excretie vindt plaats in het distaal convolut, een deel van de distale tubulus, waar Mg$^{2+}$ wordt geresorbeerd via een actief transcellulair transportproces. De eerste stap van het transcellulaire transport is de aanvoer van Mg$^{2+}$ vanuit de gevormde voorurine door het apicale plasmamembraan van de epitheelcel. Vervolgens bereikt het Mg$^{2+}$ door middel van diffusie de basolaterale plasmamembraan, waar het actief wordt getransporteerd naar het bloed. Genetisch onderzoek naar aandoeningen met erfelijk verlaagde bloed Mg$^{2+}$ concentraties is belangrijk in het verkrijgen van meer inzicht in de moleculaire en cellulaire mechanismen die ten grondslag liggen aan het Mg$^{2+}$ transport. De kennis over actief Mg$^{2+}$ transport is sterk toegenomen door de ontdekking van het epitheliale Mg$^{2+}$ kanaal TRPM6, na bestudering van patiënten met hypomagnesiëmie met secundaire hypocalcemie (HSH) en de ontdekking van een met TRPM6 overeenstemmend Mg$^{2+}$ kanaal, genaamd TRPM7. TRPM6 vormt de poortwachter van transcellulair Mg$^{2+}$ transport in nier en darm en is essentieel voor het behoud van de Mg$^{2+}$ balans in het lichaam. Het TRPM7 eiwit is in de meeste weefsels aanwezig en speelt een belangrijke rol in het constant houden van de cellulaire Mg$^{2+}$ concentratie. Omdat TRPM6 de toegangs-poort voor Mg$^{2+}$ in nier epitheliecellen, is het een ideaal doelwit voor de hormonale regulatie van het proces van actieve Mg$^{2+}$ resorptie. Ondanks dat TRPM6 is geïdentificeerd als het apicale instroommechanisme van Mg$^{2+}$, zijn de identiteit van de eiwitten en (hormonale) factoren die de vervolgstappen van het transcellulaire Mg$^{2+}$ transport
reguleren en coördineren, zoals intracellulaire Mg²⁺ diffusie en basolaterale uitscheiding, nog onbekend. Daarom was het doel van het onderzoek beschreven in dit proefschrift het vergroten van de kennis over de handhaving van de Mg²⁺ balans door de nier op het moleculaire-, cellulaire- en lichaamsniveau. Deze samenvatting geeft een bondig overzicht van dit onderzoek dat een significante bijdrage heeft geleverd aan het begrip van de regulatie van TRPM6 en daarmee aan het proces van actieve transcellulaire Mg²⁺ resorptie.

Structuur en functionele analyse van de porie-eigenschappen van TRPM6
Tussen het vijfde en zesde transmembraandomein van TRPM6 ligt de porie waardoor Mg²⁺ vanuit het lumen de cel in getransporteerd kan worden. Door de aminozuursequenties van deze porie te vergelijken met die van de overige leden van dezelfde TRPM subfamilie, is een potentieel selectiviteitsfilter voor Mg²⁺ geïdentificeerd zoals beschreven in hoofdstuk 2, bestaande uit de aminozuren 1028GEIDVC1033. De twee negatief geladen aminozuren E1024 en D1031, die geconserveerde zijn in de meeste leden van de TRPM subfamilie, zijn belangrijke determinanten voor kationpermeatie van TRPM6 omdat neutralisatie van beide aminozuren in een alanine residu resulteerde in niet-functionele ionkanalen. Neutralisatie van het geconserveerde aminozuur E1029 vormde kanalen met een verhoogde geleiding voor barium- en zink-ionen, een vermindere rutheniumrood gevoeligheid en een grotere poriediameter in vergelijking tot het ongemuteerde wild-type TRPM6. Bij vervanging van het aminozuur D1030 door methionine ontsloten kanalen met een vermindere permeabiliteit voor nikkel-ionen, een afgenomen gevoeligheid voor rutheniumrood blokkade en een kleinere poriediameter. Uit de hierboven beschreven resultaten werd geconcludeerd dat de aminozuren E1024, D1030 en D1031 belangrijk zijn voor het functioneren van TRPM6 en dat subtiele aminozuurvariaties in de porie resulteren in veranderingen van de permeatie-eigenschappen van het kanaal.

TRPM6 expressie wordt gereguleerd door Mg²⁺ in het dieet en oestrogenen
In hoofdstuk 3 werd aangetoond dat TRPM6 met name tot expressie komt in nier-, long-, caecum- en colonweefsel, terwijl TRPM7 in de meeste weefzels voorkomt. Deze analyse bewees bovendien dat de actieve Mg²⁺ resorptie plaatsvindt in het distale deel van de mizendarm. Bovendien toonden Mg²⁺ dieetstudies aan dat Mg²⁺ depraviait leidt tot hypomagnesiëmie en conservering van Mg²⁺ en Ca²⁺ door de nieren, terwijl een Mg²⁺ verrijkt dieet leidde tot een verhoogde uitscheiding van Mg²⁺ en Ca²⁺ in de urine. Onthouding van Mg²⁺ in het dieet zorgde voor een toename van het TRPM6 expressieniveau in de nier en een Mg²⁺ verrijkt dieet resulteerde in een toegenomen expressie van TRPM6 in colonweefsel. De expressieniveaus van TRPM7 in nier en colon bleven onveranderd na inname van de verschillende Mg²⁺ diëten. Bovendien werd in ratten aangetoond dat het steroidhormoon 17β-oestradiol het renale TRPM6 expressieniveau verhoogt. Daarentegen hadden de calciotrope hormonen 1,25-dihydroxyvitamine D₃ en het parathormoon geen invloed op het expressieniveau van TRPM6 in de nier.

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TRPM6 downregulatie en hypermagnesuria in TRPV5 knock-outmuisen
Uit voorgaande studies met TRPV5 knock-outmuisen bleek dat deze muizen een sterk verhoogde hoeveelheid Ca²⁺ uitscheiden via de urine. Ten gevolge van deze verhoogde Ca²⁺ uitscheiding is bij deze muizen het vitamine D gehalte verhoogd waardoor extra Ca²⁺ wordt geabsorbeerd in de darm om dit verlies aan te vullen. Een uitvoerige analyse van de Mg²⁺ balans in TRPV5 knock-outmuisen heeft aan het licht gebracht dat deze dieren ook een verhoogde hoeveelheid Mg²⁺ uitscheiden via de urine in combinatie met een verlaagde expressie van TRPM6 in de nier (hoofdstuk 4). Nadat TRPV5 knock-outmuisen op een Mg²⁺ arm dieet waren geplaatst was een verhoogde expressie van TRPM6 in de nier gevonden. Dit werd geëxpliekt door een verhoogde expressie van TRPM6 in de nier en een toename van de excretie van Ca²⁺ en Mg²⁺ in de urine. Bovendien toonden Mg²⁺ dieetstudies aan dat Mg²⁺ depraviait leidt tot hypomagnesiëmie en conservering van Mg²⁺ en Ca²⁺ door de nieren, terwijl een Mg²⁺ verrijkt dieet leidde tot een verhoogde uitscheiding van Mg²⁺ en Ca²⁺ in de urine. Onthouding van Mg²⁺ in het dieet zorgde voor een toename van het TRPM6 expressieniveau in de nier en een Mg²⁺ verrijkt dieet resulteerde in een toegenomen expressie van TRPM6 in colonweefsel. De expressieniveaus van TRPM7 in nier en colon bleven onveranderd na inname van de verschillende Mg²⁺ diëten. Bovendien werd in ratten aangetoond dat het steroidhormoon 17β-oestradiol het renale TRPM6 expressieniveau verhoogt. Daarentegen hadden de calciotrope hormonen 1,25-dihydroxyvitamine D₃ en het parathormoon geen invloed op het expressieniveau van TRPM6 in de nier.

Epidermale Groeifactor (EGF) is essentieel voor de Mg²⁺ homeostase
De ontdekking van een mutatie (C3209T) in het gen dat codeert voor epidermale groeifactor (EGF), in een familie met geïsoleerd recessief renaal Mg²⁺ verlies, heeft geleid tot de vondst dat het auto-en paracriene hormoon EGF een essentiële rol

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vervult in de Mg^{2+} homeostase (hoofdstuk 5). De bovengenoemde mutatie resulteert in de aminozuur substitutie P1070L in en in een verstoorde lokalisatie van het pro-EGF eiwit waardoor deze niet in de basolaterale plasmamembraan terechtkomt. Hierdoor kan de basolateraal gelokaliseerde epidermale groeffactorreceptor (EGFR) niet worden gestimuleerd, hetgeen uiteindelijk leidt tot een verminderde activiteit van het epitheliale Mg^{2+} kanaal TRPM6. De verminderde activiteit van TRPM6 zorgt voor een afname van Mg^{2+} resorptie en heeft zodoende Mg^{2+} verlies via de urine tot gevolg. Het belang van EGF in het constant houden van de Mg^{2+} balans wordt onderstreept door het feit dat patiënten met colorectaal kanker die behandeld werden met het medicijn cetuximab, een antagonisten van de EGFR, een verlaagde bloed Mg^{2+} concentratie ontwikkelden.

**Stimulatie van TRPM6 activiteit door EGFR activatie**

Stimulatie van de EGFR door EGF resulteerde in een toegenomen activiteit van TRPM6 via de stimulatie van zowel de Src familie van tyrosinekinases als het kleine Rho GTPase, Rac1 (hoofdstuk 6). Hierbij zorgde de activatie van de laatst genoemde effector voor een toegenomen mobiliteit van TRPM6 in de cel. Bovendien werd aangetoond dat een dominant negatieve vorm van Rac1 (T17N) niet alleen de basale activiteit van TRPM6 reduceerde, maar ook het stimulerende effect van EGF op de TRPM6 activiteit blokkeerde. Daarentegen bleek dat de constitutieve actieve vorm van Rac1 (G12V), de TRPM6 activiteit juist stimuleerde. De toegenomen activiteit van TRPM6 na stimulatie van de EGFR met EGF bleek het resultaat te zijn van een toegenomen expressie van TRPM6 op de plasmamembraan. Deze verhoogde activatie van TRPM6 door EGF is specifiek omdat EGF niet de activiteit van het homologe Mg^{2+} kanaal TRPM7 stimuleerde. Bovendien werd aangetoond dat het carboxy-uiteinde van TRPM6, waarin zich een ε-kinase domein bevindt, geen rol speelt in de stimulatie van TRPM6 door EGF.

**Conclusies en toekomstperspectieven**

Dit proefschrift beschrijft het onderzoek waarin werd getracht de moleculaire mechanismen en (hormonale) factoren die transepitheliaal Mg^{2+} transport reguleren op te helderen. Tijdens deze studie zijn verschillende factoren ontdekt die TRPM6 reguleren. Hierbij werd ontdekt dat EGF een essentiële functie vervult in de Mg^{2+} homeostase. Hiermee is verdere vooruitgang geboekt in de opheldering van de mechanismen die de Mg^{2+} balans reguleren. De toenemende kennis van de Mg^{2+} homeostase kan leiden tot een betere behandeling van patiënten met Mg^{2+} deficienties die erop gericht is de onderliggende functionele defecten te verhelpen. Aangezien hypomagnesiëmie gepaard gaat met ernstige symptomen zoals tetanus, spierzwakte en hartritmesstoornissen, zal een verbeterde behandeling deze patiënten ten goede komen. Bovendien kan deze kennis helpen om tot nu toe onopgehelderde aandoeningen met Mg^{2+} verlies en de associatie van hypomagnesiëmie met algemeen voorkomende chronische aandoeningen zoals hart- en vaatziekten en diabetes te verklaren en daarvoor nieuwe therapieën te ontwikkelen.
List of abbreviations

1,25(OH)2D3  1,25-dihydroxy-vitamin-D3 / calcitriol
1α-OHase  25-hydroxyvitamin-D3-1α-hydroxylase
45Ca2+  radioactive calcium isotope
ANOVA  analysis of variance
ADH  autosomal dominant hypoparathyroidism
ATP  adenosine 5’-triphosphate
ATPase  adenosine 5’-triphosphatase
bp  base pairs
BS  Bartter syndrome
BSND  gene encoding Barttin, the β subunit of CLC-Ka and CLC-Kb
Ca2+  calcium ion
CaSR  Ca2+, Mg2+-sensing receptor
cBS  classic Bartter syndrome
CCD  cortical collecting duct
CD  collecting duct
cDNA  copy DNA
Ci-  chloride ion
CLC-Kb  Cl- channel Kb
CLCNKB  gene encoding the Cl- channel Kb
CLDN16  gene encoding claudin-16 (= paracellin-1)
CLDN19  gene encoding claudin-19
CNT  connecting tubule
cM  centi-Morgan
cRNA  copy RNA
DCT  distal convoluted tubule
DMEM  dulbecco’s modified eagle medium
DMSO  dimethylsulphoxide
EGF  gene encoding pro-EGF
EGF  epidermal growth factor
EGFR  epidermal growth factor receptor
ELISA  enzyme-linked immunosorbent assay
ERBB  erythroblastoma viral gene product, v-erbB
FHH  familial hypocalciuric hypercalcaemia
GS  Gitelman syndrome
HCO3-  bicarbonate
HEK293 cells  human embryonic kidney cells
HHCI  hypocalciuric hypercalcaemia familial type 1
HHHM  hypomagnesemia, hypertension and hypercholesterolemia, mitochondrial
HHN  hypomagnesemia, hypercalciuria, and nephrocalcinosis
HPRT  hypoxanthine-guanine phosphoribosyl transfers
aBS  antenatal Bartter syndrome
HSH  hypomagnesemia with secondary hypocalcaemia
IC50  concentration required to induce 50% of the maximum effect
IDH  isolated dominant hypomagnesemia
IDHH  isolated dominant hypomagnesemia associated with hypocalciuria
IOD  integrated optical density
IRH  isolated recessive renal hypomagnesemia
K+  potassium ion
KCNJ1  gene encoding the renal outer medullary K+ channel
kDa  kilo dalton
M  mitochondrially inherited
MagNuM  Mg2+ nucleotide-regulated metal ion current
M16  gene encoding the mitochondrially encoded tRNA isoleucine
M2+  magnesium ion
Mg2+·ATP  Mg2+ bound to ATP
MiC  Mg2+-inhibited cation current
M-MLV-RT  moloney-murine leukaemia virus-reverse transcriptase
mRNA  messenger ribonucleic acid
mTAL  medullary thick ascending limb of Henle’s loop
MTTI  gene encoding the mitochondrially encoded tRNA isoleucine
Na+  sodium ion
NaCl  sodium chloride
NCC  Na+-Cl- cotransporter
NKCC2  Na+, K+-Cl- cotransporter 2
NSHPT  neonatal severe hyperparathyroidism
OMIM  online mendelian inheritance in man
PAGE  polyacrylamide gelelectrophoresis
PBS  phosphate-buffered saline

List of abbreviations
Curriculum Vitae

Dankwoord

Hier ligt het dan, het resultaat van de Nijmeegse aio vierjaarse! De route om dit doel te bereiken was buitengewoon uitdagend. Eerst terugkijkend was het een periode die ik voor geen goud heb willen missen en waarin ik zeer veel heb geleerd. Nimmer aflatende vastberadenheid, doelgerichtheid en interesse zijn onontbeerlijk bij het verrichten van wetenschappelijk onderzoek. Het vasthouden hiervan heeft niet alleen met mezelf te maken maar temeer met de steun en hulp die ik heb ontvangen van mijn collegaa’s, vrienden en familie.

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List of publications

Tiel Groenestege WM, Hoenderop JG, Knoers NV, Bindels RJ: Downregulation of renal TRPM6 leads to hypermagnesuria in TRPV5 knockout mice. Manuscript in preparation

Thébault S, Alexander RT, Tiel Groenestege WM, Hoenderop JG, Bindels RJ: EGF activates the distal tubular magnesium channel, TRPM6, via a Src kinase and Rac1 mediated increase in plasma membrane expression. Manuscript submitted


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