

# Epithelial $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ Channels in Health and Disease

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A near constancy of the extracellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentration is required for numerous physiologic functions at the organ, tissue, and cellular levels. This suggests that minor changes in the extracellular concentration of these divalents must be detected to allow the appropriate correction by the homeostatic systems. The maintenance of the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  balance is controlled by the concerted action of intestinal absorption, renal excretion, and exchange with bone. After years of research, rapid progress was made recently in identification and characterization of the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  transport proteins that contribute to the delicate balance of divalent cations. Expression-cloning approaches in combination with knockout mice models and genetic studies in families with a disturbed  $\text{Mg}^{2+}$  balance revealed novel  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  gatekeeper proteins that belong to the super family of the transient receptor potential (TRP) channels. These epithelial  $\text{Ca}^{2+}$  (TRPV5 and TRPV6) and  $\text{Mg}^{2+}$  channels (TRPM6 and TRPM7) form prime targets for hormonal control of the active  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  flux from the urine space or intestinal lumen to the blood compartment. This review describes the characteristics of epithelial  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  transport in general and highlights in particular the distinctive features and the physiologic relevance of these new epithelial  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  channels in (patho)physiologic situations.

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**C**  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  are of great physiologic importance by their intervention in many enzymatic systems and their function in neural excitability, muscle contraction, blood coagulation, bone formation, hormone secretion, and cell adhesion. The human body is equipped with an efficient negative feedback system that counteracts variations of the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  balance. This system encompasses parathyroid glands, bone, intestine, and kidneys. These divalents are maintained within a narrow range by the small intestine and kidney, which both increase their fractional (re)absorption under conditions of deprivation (1,2). If depletion continues, then the bone store assists to maintain appropriate serum concentrations by exchanging part of its content with the extracellular fluid. The  $\text{Ca}^{2+}$ -sensing receptor (CaSR) represents the molecular mechanism by which parathyroid cells detect changes in the ionized  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  concentration and modulate parathyroid hormone (PTH) secretion (3,4). In addition to the effects of these divalents on PTH secretion, this hormone in turn regulates directly the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  balance by modulating bone resorption, renal reabsorption, and indirectly intestinal absorption by stimulating  $1\alpha$ -hydroxylase activity and consequently  $1,25$ -dihydroxyvitamin  $\text{D}_3$  ( $1,25$ - $(\text{OH})_2\text{D}_3$ ) synthesis to maintain serum  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  levels within a narrow physiologic range.

## $\text{Ca}^{2+}$ (Re)absorption

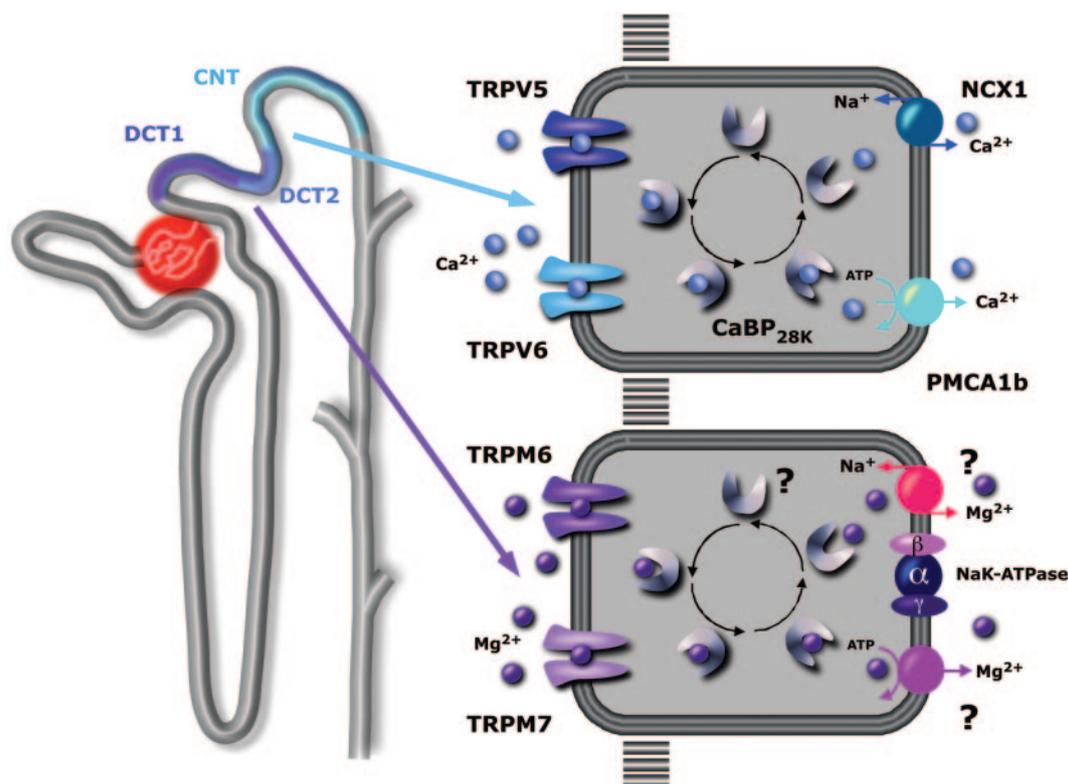
The major part of the  $\text{Ca}^{2+}$  reabsorption takes place along the proximal tubule (PT) and thick ascending limb of Henle's loop (TAL) through a paracellular and, therefore, passive pathway (5). Fine-tuning of the  $\text{Ca}^{2+}$  excretion occurs in the distal part of the nephron, where approximately 15% of the filtered load of  $\text{Ca}^{2+}$  is reabsorbed. This section consists of the distal convoluted tubule (DCT), the connecting tubule (CNT), and the initial portion of the cortical collecting duct (CCD; Figure 1). In these latter nephron segments (DCT, CNT, CCD),  $\text{Ca}^{2+}$  reabsorption is active and occurs against the existing electrochemical gradient. Together with the fact that here the tight junctions are impermeable for  $\text{Ca}^{2+}$  ions, this substantiates that  $\text{Ca}^{2+}$  is reabsorbed through an active transcellular pathway. Active  $\text{Ca}^{2+}$  reabsorption is generally envisaged as a multistep process that consists of passive entry of  $\text{Ca}^{2+}$  across the luminal or apical membrane, cytosolic diffusion of  $\text{Ca}^{2+}$  bound to vitamin  $\text{D}_3$ -sensitive calcium-binding proteins (calbindin- $\text{D}_{28\text{K}}$  and/or calbindin- $\text{D}_{9\text{K}}$ ), and active extrusion of  $\text{Ca}^{2+}$  across the opposite basolateral membrane by a  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchanger (NCX1) and/or  $\text{Ca}^{2+}$ -ATPase (PMCA1b) (6) (Figure 1, top). This active transcellular  $\text{Ca}^{2+}$  transport is under hormonal control of PTH (7,8),  $1,25$ - $(\text{OH})_2\text{D}_3$  (7,9–12,103), and calcitonin (13) but also estrogen (14,15), androgen (16), and dietary  $\text{Ca}^{2+}$  (10) are primary regulators.

## $\text{Mg}^{2+}$ (Re)absorption

Regulation of the total body  $\text{Mg}^{2+}$  balance principally resides within the kidney that tightly matches the intestinal absorption of  $\text{Mg}^{2+}$ . In the kidney, approximately 80% of the total plasma  $\text{Mg}^{2+}$  is ultrafiltrated across the glomerular membrane and subsequently reabsorbed in consecutive segments of the

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**Figure 1.** Transcellular  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  transport. Active  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  transport is carried out as a three-step process in the distal part of the nephron. (Top) After entry of  $\text{Ca}^{2+}$  in distal convoluted tubule (DCT2) and connecting tubule (CNT) through the epithelial  $\text{Ca}^{2+}$  channels TRPV5 and TRPV6,  $\text{Ca}^{2+}$  bound to calbindin diffuses to the basolateral membrane. At the basolateral membrane,  $\text{Ca}^{2+}$  is extruded via an ATP-dependent  $\text{Ca}^{2+}$ -ATPase (PMCA1b) and an  $\text{Na}^{+}$ - $\text{Ca}^{2+}$ -exchanger (NCX1). (Bottom) Apical  $\text{Mg}^{2+}$  entry in DCT via TRPM6 (and TRPM7). As extrusion mechanisms are postulated a basolateral  $\text{Na}^{+}/\text{Mg}^{2+}$  exchanger and/or ATP-dependent  $\text{Mg}^{2+}$  pump. The  $\text{Na}^{+},\text{K}^{+}$ -ATPase complex including the  $\gamma$ -subunit controls this transepithelial  $\text{Mg}^{2+}$  transport. In this way, there is net  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  reabsorption from the luminal space to the extracellular compartment.

nephron (1). Approximately 10 to 20% of  $\text{Mg}^{2+}$  is reabsorbed by the PT. However, the bulk amount of  $\text{Mg}^{2+}$  (50 to 70%) is reabsorbed by the TAL, which likely mediates  $\text{Mg}^{2+}$  reabsorption via paracellular transport. The final urinary excretion of  $\text{Mg}^{2+}$  is mainly determined by active reabsorption of  $\text{Mg}^{2+}$  in DCT, because virtually no reabsorption takes places beyond this segment (Figure 1) (1). Microperfusion studies have shown that  $\text{Mg}^{2+}$  is reabsorbed in the superficial DCT, but little knowledge has been gained concerning the cellular mechanisms of transcellular  $\text{Mg}^{2+}$  reabsorption (1,17,18). Speculatively,  $\text{Mg}^{2+}$  can passively enter the DCT cell across the luminal membrane driven by a favorable plasma membrane voltage (Figure 1, bottom). The molecular identity of the responsible influx protein was unknown, however, and previous studies hypothesized that a  $\text{Mg}^{2+}$ -specific ion channel is a possible candidate (1). Subsequently,  $\text{Mg}^{2+}$  will be transported through the cytosol and extruded at the opposing basolateral membrane by an active mechanism given the existing electrochemical gradient. Again, the identity of responsible transport proteins remains to be defined, and candidate mechanisms are  $\text{Mg}^{2+}$ -binding proteins,  $\text{Na}^{+}/\text{Mg}^{2+}$  exchanger and/or an ATP-dependent  $\text{Mg}^{2+}$  pump (Figure 1, bottom).

### Search for Epithelial $\text{Ca}^{2+}$ Channels

Several genes involved in the process of transepithelial  $\text{Ca}^{2+}$  transport have now been identified, but the  $\text{Ca}^{2+}$  influx mechanism remained unknown for a long time. An expression-cloning approach using *Xenopus laevis* oocytes revealed the molecular identity of the  $\text{Ca}^{2+}$  influx systems (19,20). The first member, named TRPV5, was cloned from primary cultures of rabbit renal distal tubules that are primarily involved in active transcellular  $\text{Ca}^{2+}$  transport and encodes a  $\text{Ca}^{2+}$  channel that belongs to the TRP family (19). Likewise, a homologous member of this family, known as TRPV6, was successfully cloned from rat duodenum (20).

### Search for Genes Involved in $\text{Mg}^{2+}$ Homeostasis

During the past few years, several genes that encode proteins that are either directly or indirectly involved in renal  $\text{Mg}^{2+}$  handling have been identified following a positional cloning strategy in families with hereditary hypomagnesemia. The first gene involved, *PCLN-1* (or *CLDN16*), encodes the protein paracellin-1 (or claudin-16) (21). This protein is specifically expressed in the TAL and shows sequence and structural homol-

ogy to the claudin family of tight junction proteins. Paracellin-1 is mutated in patients who have hypomagnesemia, hypercalciuria, and nephrocalcinosis (HHN; MIM 248250). In this autosomal recessive disorder, there is profound renal Mg<sup>2+</sup> and Ca<sup>2+</sup> wasting. The hypercalciuria often leads to nephrocalcinosis, resulting in progressive renal failure (22,23). Other symptoms that have been reported in patients with HHN include urinary tract infections, nephrolithiasis, incomplete distal tubular acidosis, and ocular abnormalities (22,24). Immunohistologic studies have shown that claudin-16 co-localizes with occludin in intercellular junctions of human kidney sections, indicating that it is a tight junction protein (21). The second gene, *FXRD2*, encodes the  $\gamma$ -subunit of the Na<sup>+</sup>,K<sup>+</sup>-ATPase pump, which is predominantly expressed in the kidney, with the highest expression levels in DCT and medullary TAL (25). *FXRD2* is mutated in patients with autosomal dominant renal hypomagnesemia associated with hypocalciuria (IDH; MIM 154020). Hypomagnesemia in these patients can be severe (<0.40 mM) and cause convulsions. Remarkably, in some affected individuals, there are no symptoms of Mg<sup>2+</sup> deficiency except for chondrocalcinosis at adult age. The molecular mechanism for renal Mg<sup>2+</sup> loss in this autosomal dominant type of primary hypomagnesemia remains to be elucidated. The third gene involved, *SLC12A3*, encodes the thiazide-sensitive sodium chloride co-transporter (NCC) in DCT and is mutated in patients with Gitelman syndrome (MIM 263800) (26). This autosomal recessive disorder is a frequent hereditary tubular disorder that affects renal Mg<sup>2+</sup> handling, which is characterized by hypokalemia, hypomagnesemia, and hypocalciuria. Hypomagnesemia is found in most patients with Gitelman syndrome and is assumed to be secondary to the primary defect in NCC, but the mechanisms underlying hypomagnesemia are poorly understood.

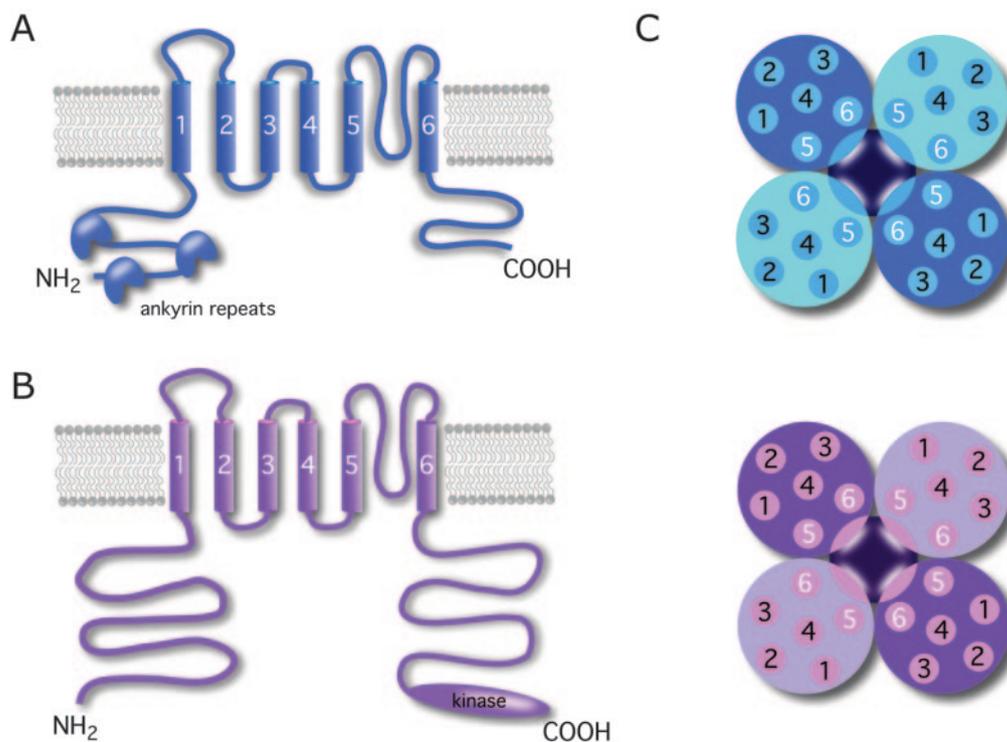
Although these linkage analyses revealed the identification of genes involved in Mg<sup>2+</sup> homeostasis, the key molecules that represent the mechanisms for luminal Mg<sup>2+</sup> influx and basolateral Mg<sup>2+</sup> extrusion in the process of transcellular Mg<sup>2+</sup> transport are still elusive. Importantly, Walder *et al.* (27) reported that hypomagnesemia associated with secondary hypocalciuria (HSH; MIM 602014) is an autosomal recessive disease that is genetically linked to chromosome 9p22. This disease is primarily due to defective intestinal Mg<sup>2+</sup> absorption, and affected individuals show neurologic symptoms of hypomagnesemic hypocalcemia, including seizures and muscle spasms during infancy (28–30). Because passive Mg<sup>2+</sup> absorption is not affected, the disease can be treated by high dietary Mg<sup>2+</sup> intake (31). Renal Mg<sup>2+</sup> conservation has been reported to be normal in most patients. In some cases, however, a renal leak has been reported, suggesting impaired renal Mg<sup>2+</sup> reabsorption. Patients who were studied by Konrad *et al.* and others (28,32) showed inappropriately high fractional Mg<sup>2+</sup> excretion rates with respect to their low serum Mg<sup>2+</sup> levels. When untreated, the disease may be fatal or may lead to severe neurologic damage. Hypocalcemia is secondary to parathyroid failure resulting from Mg<sup>2+</sup> deficiency. Using a positional candidate gene-cloning approach, two groups headed by Konrad and Sheffield (28,29) independently identified mutations in TRPM6

in autosomal recessive HSH, previously mapped to chromosome 9q22. The TRPM6 protein is a new member of the long TRP channel (TRPM) family and is similar to TRPM7 (also known as TRP-PLIK), a unique bifunctional protein known as a Mg<sup>2+</sup>-permeable cation channel properties with protein kinase activity (33–35). TRPM6 and TRPM7 are distinct from all other ion channels in that they are composed of a channel linked to a protein kinase domain recently abbreviated as chanzymes (36). These chanzymes are essential for Mg<sup>2+</sup> homeostasis, which is critical for human health and cell viability (37,38).

In summary, a variety of approaches, including a genetic screen in patients with primary hypomagnesemia and expression cloning in Ca<sup>2+</sup>-transporting epithelial cells, revealed the identification of TRP cation channels as potential gatekeepers in the maintenance of the Ca<sup>2+</sup> and Mg<sup>2+</sup> balance. The TRP superfamily is a newly discovered family of cation-permeable ion channels (33). There are at least three previously recognized subfamilies of proteins—TRPC (conical), TRPV (vanilloid), and TRPM (metastatin)—that are expressed throughout the animal kingdom (<http://clapham.tch.harvard.edu/trps/>). Recently, the polycystins were also included in the TRP superfamily abbreviated as TRPP (polycystin) (39). Each of the proteins seems to be a cation channel composed of six transmembrane-spanning domains and a conserved pore-forming region (Figure 2) (6,33,40). Most members of the TRPC have been characterized as Ca<sup>2+</sup>-permeable cation channels playing a role in Ca<sup>2+</sup> signaling (41). The functional characterization of other TRP members, including TRPV5 and TRPV6, and TRPM6 and TRPM7, has recently been started.

#### TRPV5 and TRPV6

TRPV5 and TRPV6 belong to the TRPV subfamily. These homologous channel proteins are composed of approximately 730 amino acids, whereas the corresponding genes consist of 15 exons juxtaposed on chromosome 7q35 (42–44). In human embryonic kidney 293 (HEK293) cells heterogeneously expressing TRPV5 or TRPV6, currents can be activated under conditions of high intracellular buffering of Ca<sup>2+</sup>. In addition, the current is increased by hyperpolarizing voltage steps, which enhances the driving force for Ca<sup>2+</sup> (45,46). Outward currents are extremely small, indicating that these channels are inwardly rectifying (Table 1, Figure 3). TRPV5 and TRPV6 are subject to Ca<sup>2+</sup>-dependent feedback inhibition (46,47). Both channels rapidly inactivate during hyperpolarizing voltage steps, and this inactivation is reduced when Ba<sup>2+</sup> or Sr<sup>2+</sup> is used as a charge carrier, confirming the Ca<sup>2+</sup> dependence (47). Currents also diminish during repetitive activation by short hyperpolarizing pulses (46). TRPV5 and TRPV6 are so far the only known highly Ca<sup>2+</sup>-selective channels in the TRP superfamily. It has been demonstrated that the molecular determinants of the Ca<sup>2+</sup> selectivity and permeation of TRPV5 and TRPV6 reside at a single aspartate residue (TRPV5<sup>D542</sup> [48,49] and TRPV6<sup>D541</sup> [50], respectively) present in the predicted pore-forming region. Neutralization of these negatively charged residues affects the high Ca<sup>2+</sup> selectivity of these channels. Therefore, it was suggested that Ca<sup>2+</sup> selectivity in TRPV5 and TRPV6 depends on a ring of four aspartate residues in the channel pore, similar to



**Figure 2.** Structural organization of TRPV5/6 and TRPM6/7. TRPV5 and TRPV6 contain a cytosolic amino- and carboxyl-terminus containing ankyrin (ANK) repeats (A). TRPM6 and TRPM7 belong to the largest TRP channels consisting of approximately 2000 amino acids, including very large cytosolic amino- and carboxyl-termini including an atypical protein kinase domain (B). The six-transmembrane unit is one of four identical or homologous subunits presumed to surround the central pore (C). The gate and selectivity filter are formed by the four two-transmembrane domains (TM5–pore loop–TM6) facing the center of the channel. Cations are selected for permeation by the extracellular-facing pore loop, held in place by the TM5 and TM6  $\alpha$ -helices.

the ring of four negative residues (aspartates and/or glutamates) in the pore of voltage-gated  $\text{Ca}^{2+}$  channels (48,50). Recently, the substituted cysteine accessibility method was used to map the pore region of TRPV5 (51) and TRPV6 (50). On the basis of the permeability of the TRPV6 channel to organic cations, a pore diameter of 5.4 Å was estimated (50). Mutating TRPV6<sup>D541</sup>, a residue involved in high-affinity  $\text{Ca}^{2+}$  binding, altered the apparent pore diameter, indicating that this residue indeed lines the narrowest part of the pore (50).

The renal expression profile of TRPV5 has been studied in great detail (Table 1). In different species, it was demonstrated that TRPV5 co-localizes in the kidney with the other  $\text{Ca}^{2+}$  transport proteins, including calbindin- $\text{D}_{28\text{K}}$  and the extrusion proteins PMCA1b and NCX1 in DCT2 and CNT, with the highest immunochemical abundance in DCT2, and a gradual decrease along CNT (52,53). A minority of cells along CNT lacked immunopositive staining for TRPV5 and the other  $\text{Ca}^{2+}$ -transporting proteins. These negative cells were identified as intercalated cells (52). Taken together, these findings suggest that the major sites of transcellular  $\text{Ca}^{2+}$  transport are DCT2 and, probably to a lesser extent, CNT. Recently, Hoenderop *et al.* (54) generated TRPV5 null (TRPV5<sup>-/-</sup>) mice by genetic ablation of *TRPV5* to investigate the function of TRPV5 in renal and intestinal  $\text{Ca}^{2+}$  (re)absorption. It is interesting that metabolic studies demonstrated that TRPV5<sup>-/-</sup> mice exhibit a ro-

bust calciuresis compared with wild-type (TRPV5<sup>+/+</sup>) littermates. Serum analysis showed that TRPV5<sup>-/-</sup> mice have normal plasma  $\text{Ca}^{2+}$  concentrations but significantly elevated 1,25-(OH) $_2\text{D}_3$  levels (54). For locating the defective site of the  $\text{Ca}^{2+}$  reabsorption along the nephron, *in vivo* micropuncture studies were performed. Collections of tubular fluid revealed unaffected  $\text{Ca}^{2+}$  reabsorption in TRPV5<sup>-/-</sup> mice up to the last surface loop of the late proximal tubule. In contrast,  $\text{Ca}^{2+}$  delivery to puncturing sites within DCT and CNT were significantly enhanced in TRPV5<sup>-/-</sup> mice. It is interesting that polyuria and polydipsia were consistently observed in TRPV5<sup>-/-</sup> mice compared with control littermates. Polyuria facilitates the excretion of large quantities of  $\text{Ca}^{2+}$  by reducing the potential risk of  $\text{Ca}^{2+}$  precipitations. This hypercalciuria-induced polyuria has been observed in humans and animal models (55,56). Furthermore, TRPV5<sup>-/-</sup> mice produced urine that was significantly more acidic compared with TRPV5<sup>+/+</sup> littermates. Acidification of the urine is known to prevent renal stone formation in hypercalciuria, because the formation of  $\text{Ca}^{2+}$  precipitates is less likely at an acidic pH (57). A significant increase in the rate of  $\text{Ca}^{2+}$  absorption was observed in TRPV5<sup>-/-</sup> mice compared with wild-type littermates, indicating a compensatory role of the small intestine. Expression studies using quantitative real-time PCR in TRPV5<sup>-/-</sup> mice dem-

Table 1. Characteristics of TRPV5/6 and TRPM6/7<sup>a</sup>

	TRPV5	TRPV6	TRPM6	TRPM7	references
Expression	Kidney (DCT2,CNT), bone, intestine	Widely	Kidney (DCT), intestine	Widely	(28,29,38,52, 59,61,103)
Localization	Luminal membrane	(Brushborder) membrane	Luminal membrane	Membrane	(38,52,59,61)
Structure	Homo/heterotetramer	Homo/heterotetramer	Homo/heteromultimer	Homo/heteromultimer	(66,70,83)
Splice variants	Not detected	2kb-cDNA in testis	TRPM6a,b,c	Not detected	(63,83)
Channel properties	Inward rectifier	Inward rectifier	Outward rectifier	Outward rectifier	(35,37,38,45,46, 79,82)
Selectivity	Ca <sup>2+</sup> >>> Mg <sup>2+</sup>	Ca <sup>2+</sup> >>> Mg <sup>2+</sup>	Mg <sup>2+</sup> > Ca <sup>2+</sup>	Mg <sup>2+</sup> > Ca <sup>2+</sup>	(38,45,46,79,82)
Hormonal regulation	1,25-(OH) <sub>2</sub> D <sub>3</sub> , PTH estradiol dietary Ca <sup>2+</sup>	1,25-(OH) <sub>2</sub> D <sub>3</sub> estradiol dietary Ca <sup>2+</sup> androgen	ND	ND	(9-11,14-16,72, 103,105)
Associated proteins	S100A10, 80KH	S100A10, calmodulin	ND	ND	(58,75-78)
Function	Ca <sup>2+</sup> reabsorption	Ca <sup>2+</sup> absorption	Mg <sup>2+</sup> (re)absorption	Cellular Mg <sup>2+</sup> homeostasis	(38,54,60,82,83)
Malfunction	Knockout mice: hypercalciuria, Ca <sup>2+</sup> hyperabsorption, low bone mineral density	Knockout mice: Ca <sup>2+</sup> malabsorption expression correlates with prostate carcinoma tumor grade	Patient: hypomagnesemia with secondary hypocalciuria	Knockout cells: growth arrest of cells, lethality	(28,29,54,60, 62,63,82,83)

<sup>a</sup>TRPV5/6 and TRPM6/7 share several features, including expression profiling, transport function of divalent ions, and structural organization. It is interesting that one of the ion channel pairs is widely expressed (TRPV6 and TRPM7), whereas the homologous member shows a more restricted expression pattern in epithelial tissues (TRPV5 and TRPM6). Elusive information, such as the hormonal regulation of TRPM6/7; mouse knockout models of TRPV6, TRPM6, and TRPM7; and clinical consequences of malfunction needs to be addressed in the future. DCT, distal convoluted tubule; CNT, connecting tubule; ND, not determined. For explanation, see text.

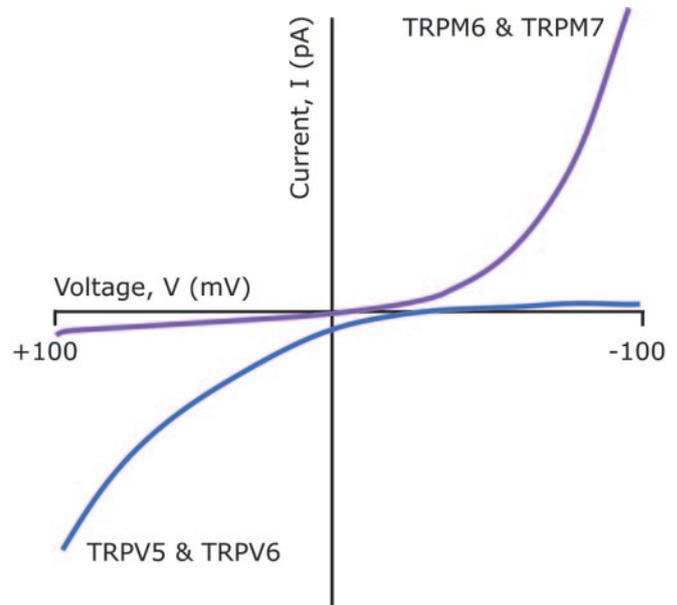


Figure 3. Current-voltage relationship of TRP channels. Representative transmembrane currents in response to a voltage ramp (I–V relation) of TRPV5/6 and TRPM6/7 channels.

onstrated increased TRPV6 and calbindin-D<sub>9K</sub> levels in duodenum consistent with Ca<sup>2+</sup> hyperabsorption.

Immunohistochemical studies indicated that TRPV6, originally cloned from duodenum, is localized to the brush-border membrane of the small intestine. In enterocytes, TRPV6 is co-expressed with calbindin-D<sub>9K</sub> and PMCA1b (58,59). It is interesting that Hediger and co-workers (60) studied the functional role of TRPV6 in Ca<sup>2+</sup> absorption by inactivation of the mouse *TRPV6* gene. These TRPV6 null (*TRPV6*<sup>-/-</sup>) mice were placed on a Ca<sup>2+</sup>-deficient diet and subsequently challenged in a <sup>45</sup>Ca<sup>2+</sup> absorption assay. *TRPV6*<sup>-/-</sup> mice showed a consistent decrease in Ca<sup>2+</sup> absorption over time. From these initial data, it was concluded that *TRPV6*<sup>-/-</sup> mice show a significant Ca<sup>2+</sup> malabsorption, suggesting that TRPV6 is indeed the rate-limiting step in 1,25-OH<sub>2</sub>D<sub>3</sub>-dependent Ca<sup>2+</sup> absorption (60). Recently, it was found that TRPV6 is expressed in the mouse kidney along the apical domain of the late portion of the DCT (DCT2) through inner medullary collecting duct (61). TRPV6 co-localizes with TRPV5 and the other Ca<sup>2+</sup> transport proteins in DCT2, suggesting a role in Ca<sup>2+</sup> reabsorption. In addition, the protein is detected in the intercalated cells and the inner medullary collecting duct that are not involved in transepithelial Ca<sup>2+</sup> transport, pointing to additional functions of TRPV6. Thus, the precise role of this epithelial Ca<sup>2+</sup> channel in kidney remains to be established, but given the widespread distribution of TRPV6 throughout the nephron segments, functions of TRPV6 could involve Ca<sup>2+</sup> reabsorption, Ca<sup>2+</sup> signaling, and others. Detailed characterization of the *TRPV6*<sup>-/-</sup> mice will address these important questions shortly. It is interesting that quantitative PCR measurements indicated that the mouse prostate contains high expression levels of TRPV6 (61). Although the exact function in this organ remains to be elucidated, previous reports have suggested that TRPV6 expression correlates

with prostate carcinoma tumor grade (16,62,63). Together, these findings indicate that TRPV6 expression is associated with prostate cancer progression and, therefore, represents a prognostic marker and a promising target for new therapeutic strategies to treat advanced prostate cancer (63). TRPV5 and TRPV6 share several functional properties, including the permeation profile for monovalent and divalent cations, anomalous mole fraction behavior, and  $\text{Ca}^{2+}$ -dependent inactivation (47,64). However, detailed comparison of the amino- and carboxyl-termini of the TRPV5 and TRPV6 channels illustrates significant differences, which may account for the unique electrophysiologic properties of these homologous channels (65). The initial inactivation is faster in TRPV6 compared with TRPV5, and the kinetic differences between  $\text{Ca}^{2+}$  and  $\text{Ba}^{2+}$  currents are more pronounced for TRPV6 than for TRPV5 (66,67). It is interesting that structural determinants of these functional dissimilarities are not located in either the amino- or carboxyl-terminus but in the TM2-TM3 linker (67). It is intriguing that TRPV5 has a 100-fold higher affinity for the potent channel blocker ruthenium red than TRPV6 (65). Physiologic consequences of these functional differences remain to be established and are of interest with respect to the structural organization of these channels (66). Cross-linking studies, co-immunoprecipitations, and molecular mass determination of TRPV5/6 complexes using sucrose gradient sedimentation showed that TRPV5 and TRPV6 form homo- and heterotetrameric channel complexes (Figure 2C). Hetero-oligomerization of TRPV5 and TRPV6 might influence the functional properties of the formed  $\text{Ca}^{2+}$  channel complex. As TRPV5 and TRPV6 exhibit different channel kinetics with respect to  $\text{Ca}^{2+}$ -dependent inactivation and  $\text{Ba}^{2+}$  selectivity and sensitivity for the inhibitor ruthenium red, the influence of the heterotetramer composition on these channel properties was investigated. Concatemers that consisted of four TRPV5 and/or TRPV6 subunits that were configured in a head-to-tail manner were constructed. A different ratio of TRPV5 and TRPV6 subunits in these concatemers showed that the phenotype resembles the mixed properties of TRPV5 and TRPV6. An increased number of TRPV5 subunits in such a concatemer displayed more TRPV5-like properties, indicating that the stoichiometry of TRPV5/6 heterotetramers influences the channel properties (66). Consequently, regulation of the relative expression levels of TRPV5 and TRPV6 may be a mechanism to fine-tune the  $\text{Ca}^{2+}$  transport kinetics in TRPV5/6-co-expressing tissues (68). It is interesting that Niemeyer and colleagues (69) identified the third ankyrin (ANK) repeat as being a stringent requirement for physical assembly of TRPV6 subunits. Deletion of this repeat or mutation of critical residues within this repeat renders nonfunctional channels that do not co-immunoprecipitate or form tetramers. It was proposed that the third ANK repeat initiates a molecular zippering process that proceeds past the fifth ANK repeat and creates an intracellular anchor that is necessary for functional subunit assembly (69).

Previous studies in animal and cell models demonstrated that TRPV5 and TRPV6 channels are tightly regulated at the transcriptional level by various hormones, including  $1,25\text{-(OH)}_2\text{D}_3$ , estradiol, androgen, and also dietary  $\text{Ca}^{2+}$  intake,

which are described in detail (6,70). Recently, the effect of PTH was studied on the renal expression of TRPV5 (71). The parathyroid glands play a key role in maintaining the extracellular  $\text{Ca}^{2+}$  concentration through their capacity to sense minute changes in the level of blood  $\text{Ca}^{2+}$  (4). Early studies using micropuncture and cell preparations demonstrated that PTH stimulates active  $\text{Ca}^{2+}$  reabsorption in the distal part of the nephron via a dual signaling mechanism involving protein kinase A- and protein kinase C-dependent processes (7,8,72,73). Preliminary studies demonstrated that parathyroidectomy in rats resulted in decreased serum PTH levels and hypocalcemia, which were accompanied by decreased levels of TRPV5, calbindin- $\text{D}_{28\text{K}}$ , and NCX1. Supplementation with PTH restored serum  $\text{Ca}^{2+}$  concentrations and abundance of these  $\text{Ca}^{2+}$  transporters in kidney. These data suggest that long-term treatment with PTH affects renal  $\text{Ca}^{2+}$  handling through the regulation of the expression of the  $\text{Ca}^{2+}$  transport proteins, including TRPV5 (71). Promoter analysis should reveal the molecular mechanism of this PTH-mediated increase in TRPV5 expression. In addition, several regulatory proteins that interact with TRPV5 and/or TRPV6 have been identified, including calmodulin (74–76), S100A10-annexin 2 (58), and 80K-H (77) (Table 1). These newly identified associated proteins have facilitated the elucidation of important molecular pathways modulating transport activity. Calmodulin and 80K-H both have been implicated as  $\text{Ca}^{2+}$  sensors. Disturbance of the EF-hand structures in these proteins directly affects TRPV5/6 channel activity. Interaction of TRPV5/6 with the S100A10-annexin 2 complex is critical for trafficking of these epithelial  $\text{Ca}^{2+}$  channels toward the plasma membrane.

#### *TRPM6 and TRPM7*

TRPM6 is a protein of approximately 2000 amino acids encoded by a large gene that contains 39 exons (28,29,33). TRPM6 shows approximately 50% sequence homology with TRPM7, which forms a  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -permeable cation channel (38). Unlike other members of the TRP family, TRPM6 and TRPM7 contain long carboxyl-terminal domains with similarity to the  $\alpha$ -kinases (Figure 2B) (35). The combination of channel and enzyme domains in TRPM6 and TRPM7 is unique among known ion channels and raises intriguing questions concerning the function of the enzymatic domains and physiologic role of these channels. The identification of TRPM6 as the gene mutated in HSH represents the first case in which a human disorder has been attributed to a channel kinase. However, the precise function of this kinase domain remains to be established. To date, TRPM7 regulation has received most of the attention. TRPM7 is ubiquitously expressed and implicated in cellular  $\text{Mg}^{2+}$  homeostasis, whereas TRPM6 has a more restricted expression pattern predominantly present in absorbing epithelia (28,29,38). Although in HSH the defect at the level of the intestine is established, there is also evidence for impaired renal  $\text{Mg}^{2+}$  reabsorption (28,32). The renal expression of TRPM6, in addition to the renal leak in patients with HSH, stresses the potential important role of TRPM6 in renal  $\text{Mg}^{2+}$  reabsorption. In kidney, TRPM6 is expressed in DCT, known as the main site of active transcellular  $\text{Mg}^{2+}$  reabsorption along

the nephron (38). In line with the expected function of being the gatekeeper of Mg<sup>2+</sup> influx, TRPM6 was predominantly localized along the apical membrane of these immunopositive tubules. Immunohistochemical studies of TRPM6 and NCC, which were used as specific markers for DCT, indicated a complete co-localization of these transport proteins in the kidney (38). Until now, specific Mg<sup>2+</sup>-binding proteins have not been identified, but it is interesting to mention that the Ca<sup>2+</sup>-binding proteins parvalbumin and calbindins also bind Mg<sup>2+</sup> (78). Importantly, TRPM6 co-localized with parvalbumin in DCT1 and with calbindin-D<sub>28K</sub> in DCT2 (38). In addition to the DCT segment, Schlingmann *et al.* (28,38) reported the presence of TRPM6 mRNA by nephron segment-specific PCR analysis in the proximal tubule, which was not confirmed by immunohistochemistry. In small intestine, absorptive epithelial cells stained positively for TRPM6 detected by *in situ* hybridization and immunohistochemistry (28,38). In these cells, TRPM6 was localized along the brush-border membrane (38).

To functionally characterize TRPM6, the protein was heterogeneously expressed in HEK293 cells. TRPM6-transfected HEK293 cells perfused with an extracellular solution that contained 1 mM Mg<sup>2+</sup> or Ca<sup>2+</sup> exhibited characteristic outwardly rectifying currents upon establishment of the whole-cell configuration as was demonstrated for TRPM7 (Figure 3) (37,38,79). 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. However, mutations in TRPM6 are linked directly to HSH, emphasizing that this channel is an essential component of the epithelial Mg<sup>2+</sup> uptake machinery. It is possible that the TRPM6-mediated Mg<sup>2+</sup> inward current is more pronounced in native DCT and intestinal cells as a result of specific co-factors, such as intracellular Mg<sup>2+</sup> buffers, that are missing in overexpression systems such as HEK293 cells.

The unique permeation rank order determined from the inward current amplitude at −80 mV was comparable to TRPM7 (Ba<sup>2+</sup> ≥ Ni<sup>2+</sup> > Mg<sup>2+</sup> > Ca<sup>2+</sup>) (35,38). Experiments using the Mg<sup>2+</sup>-sensitive radiometric fluorescent dye Magfura-2 demonstrated a coherent relationship between the applied extracellular Mg<sup>2+</sup> concentration and the measured intracellular Mg<sup>2+</sup> level in TRPM6-expressing cells. Intracellular Mg<sup>2+</sup> was elevated further when the plasma membrane was hyperpolarized to the physiologic level of −80 mV, consistent with influx through the TRPM6 channel. For evaluating the effect of intracellular Mg<sup>2+</sup> on TRPM6 activity, the Mg<sup>2+</sup> concentration was altered directly in a spatially uniform manner using flash photolysis of the photolabile Mg<sup>2+</sup> chelator DM-nitrophen. Elevation of intracellular Mg<sup>2+</sup> by a flash of ultraviolet light reduced the TRPM6-induced current, indicating that the channel is regulated by the intracellular concentration of this ion. Likewise, TRPM7 channel activity is strongly suppressed by Mg<sup>2+</sup>-ATP concentrations in the millimolar range (37,80). Kozak and Cahalan (81) demonstrated that internal Mg<sup>2+</sup> rather than ATP inhibits channel activity.

Micropuncture studies have demonstrated that the luminal concentration of free Mg<sup>2+</sup> in DCT ranges from 0.2 to 0.7 mM

(1). Because the Ca<sup>2+</sup> concentration is in the millimolar range, the apical Mg<sup>2+</sup> influx pathway should exhibit a higher affinity for Mg<sup>2+</sup> than for Ca<sup>2+</sup>. It is interesting that dose-response curves for the Na<sup>+</sup> current block at −80 mV indicated four times higher K<sub>D</sub> values for Ca<sup>2+</sup> compared with Mg<sup>2+</sup> (38). These data suggest that the pore of the TRPM6 has a higher affinity for Mg<sup>2+</sup> than for Ca<sup>2+</sup>. In this way, TRPM6 comprises a unique channel because all known Ca<sup>2+</sup>-permeable channels, including members of the TRP superfamily, generally display a 10 to 1000 times lower affinity for Mg<sup>2+</sup> than for Ca<sup>2+</sup>. It is interesting that HEK293 cells transfected with the TRPM6 mutants identified in HSH patients (TRPM6<sup>Ser590X</sup> and TRPM6<sup>Arg736fsX737</sup>) displayed currents with similar amplitude and activation kinetics as nontransfected HEK293 cells, indicating that these mutant proteins are nonfunctional, in line with the postulated function of TRPM6 being Mg<sup>2+</sup> influx step in epithelial Mg<sup>2+</sup> transport (38). The observation that TRPM7 conducts Mg<sup>2+</sup> and is required for cell viability suggested that the TRPM7-mediated Mg<sup>2+</sup> influx is essential for cellular Mg<sup>2+</sup> homeostasis rather than the extracellular Mg<sup>2+</sup> homeostasis (37). It is interesting that Schmitz *et al.* (82) demonstrated that Mg<sup>2+</sup> supplementation of cells that lack TRPM7 expression rescued growth arrest and cell lethality that was caused by TRPM7 inactivation (Table 1). Although TRPM7 is permeable for Ca<sup>2+</sup>, as well as trace divalents such as Zn<sup>2+</sup>, Ni<sup>2+</sup>, Ba<sup>2+</sup>, and Co<sup>2+</sup>, supplementation with these cations was ineffective, indicating the specific effect of Mg<sup>2+</sup> on these cellular processes.

Recently, 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<sup>S141L</sup>, results in HSH (83). In this study, TRPM6<sup>S141L</sup> was not directed to the cell surface by TRPM7 and failed to interact with the coexpressed TRPM7. Remarkably, in contrast to TRPM7, Gudermann and co-workers (83) found that TRPM6 expression in *Xenopus* oocytes and HEK293 cells did not entail significant ion currents. In contrast, Voets *et al.* (38) measured significantly larger currents in TRPM6-transfected HEK293 cells compared with control cells. An explanation for this discrepancy might be the existence of specific TRPM6 splice variants with different functional properties. Chubanov *et al.* (83) demonstrated that 5' rapid amplification of cDNA ends revealed three short alternative 5' exons, called 1A, 1B, and 1C, that were found to be individually spliced onto exon 2, suggesting that the TRPM6 gene harbors a promoter with alternative transcription start sites. These cDNA have been named accordingly TRPM6a, TRPM6b, and TRPM6c, and additional functional measurements are needed to explain possible biophysical differences.

A key question concerns the nature of mechanisms underlying the activation and regulation of TRPM6 and TRPM7. In particular, what is the function of the atypical protein  $\alpha$ -kinase domain located in the carboxyl terminus?  $\alpha$ -Kinases are a recently discovered family of proteins that have no detectable sequence homology to conventional protein kinases (84). To characterize the TRPM7 kinase activity *in vitro*, we performed studies in which the catalytic domain was expressed in bacteria

(85). This kinase is able to undergo autophosphorylation and to phosphorylate substrates such as myelin basic protein and histone H3 on serine and threonine residues. The kinase is specific for ATP and requires Mg<sup>2+</sup> or Mn<sup>2+</sup> for optimal activity. Clapham and co-workers (35) found that kinase activity is necessary for TRPM7 channel function. Although kinases have long been known to modulate ion channels, TRPM7 is unusual in that the channel has its own kinase. Future studies will address the question of whether the kinase, present in TRPM6 and TRPM7, has specific cellular targets that might modulate ion channel activity and, therefore, the Mg<sup>2+</sup> balance.

### Mutual Disturbance of the Ca<sup>2+</sup> and Mg<sup>2+</sup> Balance

A tight coupling of the Ca<sup>2+</sup> and Mg<sup>2+</sup> balance is frequently observed in patients and animal models (86,87). In hypomagnesemia with secondary hypocalcemia, Simon *et al.* (21) proposed that paracellin-1 is involved in controlling both the Ca<sup>2+</sup> and Mg<sup>2+</sup> permeability of the paracellular pathway in TAL. Immunolocalization studies demonstrated that this tight junction protein is expressed in TAL. Defective paracellular Ca<sup>2+</sup> and Mg<sup>2+</sup> absorption by inactive paracellin-1 explains the observed hypomagnesemia and hypercalciuria in patients with HHN (Table 2).

Mutations in the Ca<sup>2+</sup>-sensing receptor (CaSR) are also associated with disturbed Mg<sup>2+</sup> handling (Table 2). Mutations in this receptor are identified in autosomal dominant hypoparathyroidism, which is characterized by hypercalcemia and hypocalciuria (88). Hypomagnesemia is observed in up to half of the patients (89). Mutations in the parathyroid and kidney CaSR result in a lower set point for plasma Ca<sup>2+</sup> and Mg<sup>2+</sup> on PTH secretion (86). Consequently, renal Ca<sup>2+</sup> and Mg<sup>2+</sup> reabsorption is suppressed, and the disease is characterized by inappropriately low serum PTH and increased Ca<sup>2+</sup> and Mg<sup>2+</sup> excretion. Furthermore, mutations in CaSR were identified in patients with hypercalcemic disorders of familial benign (hypocalciuric) hypercalcemia and neonatal severe primary hyperparathyroidism (90). Inactivation of the CaSR likely leads to inappropriate reabsorption of Ca<sup>2+</sup> and Mg<sup>2+</sup> in the TAL (91) and Mg<sup>2+</sup> transport in DCT (92). Therefore, renal excretion of Ca<sup>2+</sup> and Mg<sup>2+</sup> is reduced, which leads to hypercalcemia and in some cases hypermagnesemia (93). It should be noted that the CaSR plays a role in controlling renal Ca<sup>2+</sup> and Mg<sup>2+</sup> secretion independent of its role in regulating PTH release. Recent studies using double knockout mice for CaSR and PTH showed a much wider range of values for serum Ca<sup>2+</sup> and renal excretion of Ca<sup>2+</sup> than those in control (PTH<sup>-/-</sup>) littermates, despite the absence of any circulating PTH (94,95).

Patients with mutations in TRPM6, the  $\gamma$ -subunit of the Na<sup>+</sup>-K<sup>+</sup>-ATPase, or NCC exhibit besides hypomagnesemia also hypocalciuria (Table 2). Expression of these affected genes is restricted to the DCT. However, an interaction between transcellular Ca<sup>2+</sup> and Mg<sup>2+</sup> pathways in the distal part of the nephron is still unclear. There is limited overlap in expression between the Ca<sup>2+</sup> transport proteins and TRPM6,  $\gamma$ -subunit, or NCC (38,53,96). It is interesting that hypocalcemia in HSH patients can be corrected only by administration of high dietary Mg<sup>2+</sup> content. Several studies reported that normalization of

Table 2. Inherited disorders with mutual disturbance in Ca<sup>2+</sup> and Mg<sup>2+</sup> balance<sup>a</sup>

	Location Defect	Affected Gene/Transporter	Ca <sup>2+</sup> and Mg <sup>2+</sup> Homeostasis	
			Ca <sup>2+</sup> Disturbance	Mg <sup>2+</sup> Disturbance
Hypomagnesemia with hypercalciuria and nephrocalcinosis	TAL	CLDN16, paracellin 1	Hypercalciuria	Hypomagnesemia
Autosomal dominant hypoparathyroidism	TAL, DCT	CASR, CaSR	Hypocalciuria hypercalcemia	Hypermagnesemia
Hypomagnesemia with secondary hypocalciuria	DCT	TRPM6, TRPM6	Hypocalciuria	Hypomagnesemia
Gitelman syndrome	DCT	SLC12A3, NCC	Hypocalciuria	Hypomagnesemia
Isolated dominant hypomagnesemia	DCT	$\gamma$ -subunit Na <sup>+</sup> -K <sup>+</sup> -ATPase	Hypocalciuria	Hypomagnesemia

<sup>a</sup>TAL, thick ascending loop; NCC, sodium chloride co-transporter.

the hypomagnesemia by dietary supplementation resulted in a prompt release of PTH and subsequent correction of the hypocalcemia (97–99). These findings suggest that hypocalcemia in HSH is caused by a disturbance in PTH-mediated Ca<sup>2+</sup> reabsorption. The factors that determine whether Mg<sup>2+</sup> deficiency will result in inhibition of PTH release, a lack of response of the bone to PTH, or both remain to be clarified.

Gitelman syndrome in adults is characterized by consistent hypomagnesemia, hypocalciuria, and hypokalemic metabolic alkalosis (Table 2). The affected NCC gene results in loss of normal thiazide function, and the phenotype is identical to patients with chronic use of thiazide diuretics. Likewise, hypocalciuria is observed when animals receive long-term treatment with thiazides (100). Recently, it was postulated that thiazides induce hypovolemia, which stimulates proximal electrolyte reabsorption, explaining the observed hypocalciuria (100). This finding is in line with the observation that thiazide exposure leads to structural degeneration of DCT, resulting in downregulation of Ca<sup>2+</sup> transport proteins, arguing an increased transcellular Ca<sup>2+</sup> transport in this segment (100,101). This increased rate of apoptosis might reduce the absorptive surface area of the DCT in general and, therefore, could explain the observed hypomagnesemia.

Furthermore, mutations in the  $\gamma$ -subunit of Na<sup>+</sup>-K<sup>+</sup>-ATPase are the cause of IDH (Table 2). A cell model that hypothesized that the mutated  $\gamma$ -subunit manipulates the activity of the Na<sup>+</sup>,K<sup>+</sup>-ATPase by disturbing routing of the total protein complex to the plasma membrane has been proposed (25). As a consequence, the reduced intracellular K<sup>+</sup> concentration, increased intracellular Na<sup>+</sup> concentration, or depolarization of the membrane may subsequently lead to reduced Mg<sup>2+</sup> influx through the apical TRPM6 channel, resulting in Mg<sup>2+</sup> wasting (25). However, the exact molecular mechanism of decreased Mg<sup>2+</sup> reabsorption and the associated hypocalciuria remains to be elucidated.

Taken together, many diseases in which Ca<sup>2+</sup> and Mg<sup>2+</sup> disturbances are linked have been reported (Table 2). In some cases, there is an explanation for the mutual disorder in Ca<sup>2+</sup> and Mg<sup>2+</sup> handling, but in the majority of the diseases, the origin of this coupling is still unclear. Particularly, the limited overlap between the Ca<sup>2+</sup> transport (DCT2-CNT) and Mg<sup>2+</sup> transport (DCT1-DCT2) machinery indicated that additional mechanisms might be involved.

## Research Directions

This review has focused on the identification, function, and regulation of the Ca<sup>2+</sup> and Mg<sup>2+</sup> transport proteins. In the past few years, significant advances were achieved in our knowledge about the maintenance of the Ca<sup>2+</sup> and Mg<sup>2+</sup> balance. The identification of the epithelial Ca<sup>2+</sup> (TRPV5 and TRPV6) and Mg<sup>2+</sup> (TRPM6 and TRPM7) channels provided insight in a new molecular concept of Ca<sup>2+</sup> and Mg<sup>2+</sup> influx in specialized epithelia and other cell systems in which these channels facilitate Ca<sup>2+</sup> and Mg<sup>2+</sup> transport. There are striking similarities between the characteristics of the TRPV5/6 and TRPM6/7 channel pairs, such as expression profiling, structural organization, and function with respect to the maintenance the Ca<sup>2+</sup>

and Mg<sup>2+</sup> balance (Table 1). To date, several studies have focused on the regulation of TRPV5 and TRPV6, whereas many questions remain to be investigated for TRPM6 and TRPM7. For instance, the hormonal regulation of these Mg<sup>2+</sup> channels has not been studied yet. The next step is to clarify the cellular events in epithelial Ca<sup>2+</sup> and Mg<sup>2+</sup> transport. For instance, the mechanisms by the DCT cells to sense the extracellular Ca<sup>2+</sup> and Mg<sup>2+</sup> concentration and appropriately adapt the transport rates are fertile areas for future research. The continued use of molecular and cell physiologic techniques to probe the constitutive and congenital disturbances of Ca<sup>2+</sup> and Mg<sup>2+</sup> metabolism will increase further our understanding of renal electrolyte transport and provide new insights into the way in which renal diseases are diagnosed and managed.

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