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2009 Homer W. Smith Award: Minerals in Motion: From New Ion Transporters to New Concepts

René J.M. Bindels

Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

ABSTRACT

The kidneys play a critical role in maintaining the systemic balance of Mg^{2+} and Ca^{2+} cations. The reabsorptive capacity of these divalent cations adapt to changes in their plasma concentrations. Active reabsorption of Mg^{2+} and Ca^{2+} takes place in the distal convoluted and connecting tubules, respectively, and is initiated by cellular transport through selective transient receptor potential (TRP) channels located along the luminal membrane and modulated by hormonal stimuli. Recent characterization of underlying molecular defects in renal Mg^{2+} handling illuminate complex transport processes in the kidney and their contribution to the overall mineral balance. Likewise, studies of Ca^{2+} transport proteins in null mice disclose molecular mechanisms maintaining normal plasma Ca^{2+} levels and the hypercalciuria-related adaptations important in the prevention of kidney stones. Current knowledge of Mg^{2+} and Ca^{2+} transport is summarized here as comprehensive cellular models of the distal nephron.

J Am Soc Nephrol 21: 1263–1269, 2010. doi: 10.1681/ASN.2010010001

Calcium (Ca^{2+}) and magnesium (Mg^{2+}) minerals are essential for many physiologic processes.[1] Ca^{2+} plays a pathologic role in osteoporosis, nephrolithiasis, vascular calcification, nephrocalcinosis, and chronic kidney disease, and disturbances in Mg^{2+} contribute to muscle cramps, paraesthesia, convulsions, arrhythmias, and cardiac arrest. Their overall mineral balance is regulated by the concerted actions of kidneys, intestine, and bone.[2] The kidneys determine the final excretion of these cations and fulfills, and therefore, an important step in homeostatic control. This role was recognized by Homer Smith, who wrote "…Little is known concerning Ca^{2+} excretion except that the total excretion can be increased or decreased by a variety of circumstances. Much that has been said about Ca^{2+} applies to Mg^{2+}. The mechanism by which renal excretion of Mg^{2+} is controlled is unknown…"[3]

In the last decade, considerable progress has been made in elucidating the molecular mechanisms underlying the reabsorption of these minerals by the kidney. Instrumental in this respect are studies of rare monogenic diseases related to defective renal Mg^{2+} handling and genetically modified mice with deleted Ca^{2+} transport proteins.[4,5] These studies identified new transport proteins and have led to the development of new concepts for the renal handling of minerals.

IMPORTANCE OF THE DISTAL PART OF THE NEPHRON

Active reabsorption of Mg^{2+} and Ca^{2+} takes place in the distal part of the nephron only. More precisely, this part of the nephron is comprised of the distal convoluted tubule (DCT) and the connecting tubule (CNT) leading to the collecting duct.[6] The former can be further subdivided into early (DCT1) and late (DCT2) segments. Based on micropuncture experiments and the conspicuous localization of transport proteins, active Mg^{2+} transport is confined to the DCT1 and DCT2 segments, whereas active Ca^{2+} reabsorption mainly occurs along the DCT2 and CNT segments (Figure 1).[1] Thus, DCT2 functions as a transition area between Mg^{2+} and Ca^{2+} reabsorption.

Mg^{2+} REABSORPTION IN DCT1 AND DCT2 SEGMENTS

The DCT is famous for the presence of the thiazide-sensitive NaCl co-transporter (NCC) along the luminal membrane, which is energized by a Na^+ gradient generated by the basolateral Na^+-K^+-ATPase.[7,8] Active transcellular Mg^{2+} transport along the DCT is envisaged by the following sequential steps (Figure 2).[9] Driven by a favorable membrane potential, Mg^{2+} enters the DCT cell through an apical epithelial Mg^{2+} channel. The chemical driving force for Mg^{2+} is limited because the extra- and intracellular Mg^{2+} concentrations are in the same millimolar range. Importantly, Mg^{2+} entry into the cells seems to be the rate-limiting

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

Correspondence: Dr. René J.M. Bindels, 286 Physiology, Radboud University Nijmegen Medical Centre, POB 9101, 6500 HB, Nijmegen, The Netherlands. Phone: 31-24-3614211; Fax: 31-24-3616413; E-mail: r.bindels@fysiol.umcn.nl

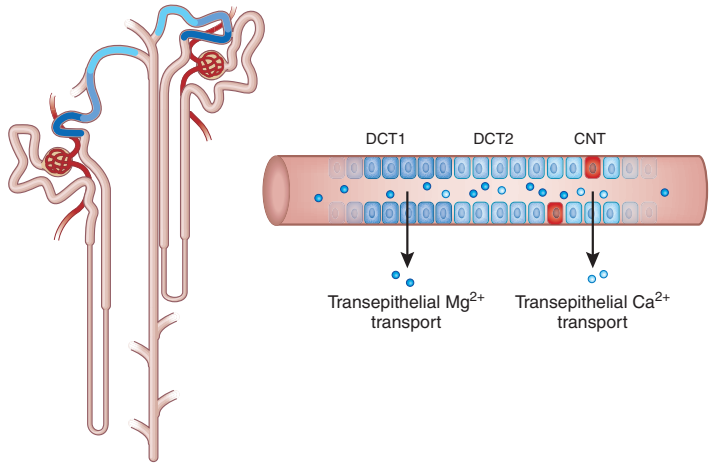

Figure 1. Overview of Mg^{2+} and Ca^{2+} handling in the distal nephron. The active reabsorption of the minerals Mg^{2+} and Ca^{2+} takes place in the distal part of the nephron only. More precisely, this part of the nephron is comprised of the DCT and the CNT to the collecting duct. The former can be further subdivided into an early (DCT1) and late (DCT2) portion. Active Mg^{2+} transport is confined to the DCT1 and DCT2, whereas active Ca^{2+} reabsorption mainly occurs in the DCT2 and CNT segments. Thus, DCT2 functions as a transition area between Mg^{2+} and Ca^{2+} reabsorption.

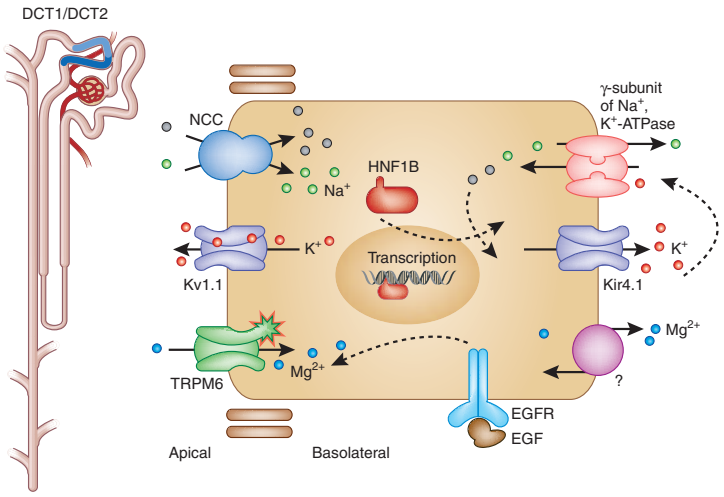

Figure 2. Mechanism of active Mg^{2+} reabsorption in DCT1 and DCT2 segments. Apical membrane TRPM6 channels are located in the apical membrane, which facilitates transport of Mg^{2+} from the tubular fluid into the cell. Mg^{2+} reabsorption is primarily driven by the luminal membrane potential established by the voltage-gated K^+ channel, Kv1.1. The Na^+-K^+-ATPase, situated in the basolateral membrane, provides a sodium (Na^+) gradient that is used by the thiazide-sensitive NCC to facilitate transport of Na^+ from the tubular fluid into the cytoplasm and a K^+ gradient to generate local membrane potential. K^+ is supplied to the Na^+-K^+-ATPase through recycling through Kir4.1. The γ-subunit of the Na^+-K^+-ATPase regulates the function of Na^+ pump. Transcription factor HNF1B (hepatocyte nuclear factor 1 homeobox B) regulates the expression of the γ-subunit of the Na^+-K^+-ATPase. EGF is the first magnesiotropic hormone to regulate active Mg^{2+} reabsorption through the TRPM6 channel.

step and thus the site of regulation. Subsequently, Mg^{2+} diffuses through the cytosol to be extruded actively against an electrochemical gradient across the basolateral membrane. For the Mg^{2+} extrusion, unidentified candidates could be a Na^+-dependent exchange mechanism or an ATP-dependent Mg^{2+} pump. Some of the salient features are described below.

Transient Receptor Potential Melastatin, Subtype 6

The apical epithelial Mg^{2+} channel is known as the transient receptor potential melastatin, subtype 6 (TRPM6). TRPM6 is a cation channel composed of six transmembrane-spanning domains and a conserved pore-forming region that assembles in a tetrameric configuration. Studies of families with autosomal recessive hypomagnesemia with secondary hypocalcemia identified mutations in TRPM6.[10,11] TRPM6 is one of eight members of the identified TRPM cation channel subfamily and is composed of 2022 amino acids encoded by a large gene containing 39 exons.[10–12] TRPM6 displays a restricted expression pattern and is predominantly present in reabsorbing epithelia.[10,11,13] In the kidney, TRPM6 localizes along the apical membrane of the DCT.[13] This channel is a unique bifunctional protein consisting of an Mg^{2+} permeable cation channel with protein kinase activity and is occasionally referred to as chanzymes.[14] Electrophysiologic characterization of TRPM6 shows that TRPM6-transfected human embryonic kidney 293 cells exhibit outwardly rectifying currents. Mg^{2+} itself has a profound effect on the activity of TRPM6. For instance, intracellular Mg^{2+} levels tightly regulate TRPM6 activity with an apparent K_i of 0.5 mM that is comparable to physiologic intracellular Mg^{2+} concentrations.[13] Furthermore, extracellular Mg^{2+} also affects TRPM6, because Mg^{2+} restriction significantly up-regulates levels of mRNA encoding renal TRPM6.[15]

Kv1.1

The voltage-gated K^+ channel, Kv1.1, is a new protein thought to regulate Mg^{2+} influx through TRPM6. Recently, there has been evidence that a mutation in *KCNA1* encoding Kv1.1 causes autosomal dominant hypomagnesemia.[16] The phenotype detectable from infancy consists of recurrent muscle cramps, tetany, tremor, muscle weakness, cerebellar at-

rophy, and myokymia. The K^+ channel co-localizes with TRPM6 along the luminal membrane of the DCT. The identified mutation results in a nonfunctional channel with a dominant-negative effect on wild-type channel function.[17] Thus, Kv1.1 is a new luminal K^+ channel in the DCT that establishes favorable luminal membrane potential controlling TRPM6-mediated Mg^{2+} reabsorption.

γ-Subunit of the Na^+-K^+-ATPase

We also identified *FXYD2* as being involved in hypomagnesemia.[18] *FXYD2* encodes the γ-subunit of the basolateral Na^+-K^+-ATPase and is mutated in patients with autosomal dominant renal hypomagnesemia associated with hypocalciuria. Currently, the exact molecular mechanism by which the γ-subunit controls Mg^{2+} handling in the DCT remains elusive. It is postulated this transmembrane protein facilitates the basolateral extrusion of Mg^{2+} in renal epithelial cells.[19] Others suggest the γ-subunit regulates additional transport mechanisms that localize to the basolateral membrane such as the Na^+-K^+-ATPase, Kir4.1/5.1, or the unidentified basolateral Mg^{2+} extrusion mechanism (Figure 2).[1,5]

Hepatocyte Nuclear Factor 1B

Further support for an active role of the γ-subunit in Mg^{2+} reabsorption is suggested by the observation that a transcription factor, hepatocyte nuclear factor 1B (HNF1B), modulates the *FXYD2* gene.[20] Hypomagnesemia, hypermagnesuria, and hypocalciuria are observed in one half of the *HNF1B* mutation carriers. Analyses of the *FXYD2* promoter region identify two highly conserved HNF1B recognition sites. Future studies should confirm the role of HNF1B in the regulation of *FXYD2* and possibly other components of the molecular machinery involved in renal Mg^{2+} handling.

Kir4.1

Two independent studies recently described a mutation within the *KCNJ10* gene as the underlying cause of a hypomagnesemia syndrome.[21,22] The first study described two nonrelated consanguineous families with a disorder characterized by epilepsy, ataxia, sensorineurol deafness, and tubulopathy (also referred to as SeSAME), whereas the other study described four kindreds with similar clinical findings. The *KCNJ10* gene encodes a K^+ channel called Kir4.1, expressed in brain, ear, and kidney, in keeping with the phenotype observed in these patients. The renal phenotype of EAST syndrome (a syndrome characterized by epilepsy, ataxia, sensorineural deafness, and tubulopathy) is similar to the Gitelman's syndrome phenotype and consists of polyuria, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria.[22] In kidney, Kir4.1 is expressed along the basolateral membrane of DCT cells with the Na^+-K^+-ATPase. Kir4.1 is thought to recycle K^+ into the interstitium to allow a sufficient supply of K^+ for optimal Na^+-K^-ATPase activity.

EGF

We identified EGF as the first magnesiotropic hormone directly stimulating TRPM6 activity.[23] Genetic analyses showed that a point mutation in the *pro-EGF* gene causes a rare inherited autosomal recessive form of renal hypomagnesemia. EGF acts as an autocrine/paracrine magnesiotropic hormone, specifically increasing TRPM6 activity by engagement of its receptor along the basolateral membrane of DCT cells. This activation relies on both the Src family of tyrosine kinases and the downstream effector, Rac1. Activation of Rac1 increases the mobility of TRPM6, assessed by fluorescence recovery after photobleaching, and a constitutively active mutant of Rac1 mimics the stimulatory effect of EGF on TRPM6 mobility and activity. Ultimately, TRPM6 activation results from increased cell surface abundance.[24] These findings provide the first insight into the molecular regulation of TRPM6 by extracellular EGF. Moreover, it shows the molecular basis for the hypomagnesemia after treatment with cetuximab, an EGF receptor blocking antibody used in the treatment of colorectal cancer, and indicates TRPM6 is a potential pharmacologic target during cetuximab therapy.[23,25]

COMPREHENSIVE MODEL OF TRANSCELLULAR Mg^{2+} REABSORPTION ALONG THE DCT

The recent knowledge concerning the molecular nature of Mg^{2+} transporting proteins offers for the first time a comprehensive cellular model for transepithelial Mg^{2+} reabsorption (Figure 2). The epithelial Mg^{2+} channel TRPM6 facilitates Mg^{2+} entry from tubular fluid through an energized local electrochemical gradient. Importantly, the DCT cell lacks a substantial chemical gradient for Mg^{2+}. The luminal membrane potential in the DCT favoring luminal Mg^{2+} influx is approximately −70 mV and likely established by the luminal Kv1.1. channel. The basolateral extrusion mechanism for Mg^{2+} remains elusive and is a subject for further study. The Na^+-K^+-ATPase in the basolateral membrane generates opposing K^+ and Na^+ gradients. Importantly, Kir4.1 enables the basolateral recirculation of K^+, thereby supplying sufficient K^+ during high transport rates of the Na^+-K^+-ATPase; the basolateral γ-subunit in all likelihood supports the Na^+ pump. Apparently, these special features are necessary to enable the substantial transport of NaCl by NCC and Mg^{2+} by TRPM6 in the DCT cell. Finally, EGF stimulates transcellular transport of Mg^{2+}. Activation of the basolateral EGF receptor promotes the insertion of TRPM6 channels into the luminal membrane to stimulate this Mg^{2+} reabsorption. Thus, in the last two decades, many Mg^{2+} transport proteins have been identified and characterized by several research groups; the next step will be to develop specific therapeutics to treat the corresponding forms of hypomagnesemia.

Ca^{2+} REABSORPTION IN DCT2 AND CNT SEGMENTS

In DCT2 and CNT segments, Ca^{2+} reabsorption takes place against its chemical gradient, indicating that the transport is active.[26] In addition to the ubiquitously expressed Na^+-K^+-ATPase, the Na^+/Ca^{2+} exchanger (NCX1) and the plasma

membrane ATPase type 1b (PMCA1b) are also found along the basolateral site of the DCT2 and CNT segments.[2] DCT2 shares similarities with the CNT segment, because both segments express the transient receptor potential vanilloid subtype 5 (TRPV5) channel and the Ca^{2+}-binding protein, calbindin-D_{28K}. Transepithelial transport of Ca^{2+} is a three-step procedure and is outlined in more detail below (Figure 3).

Ca^{2+} influx across the apical membrane is mediated by TRPV5. Subsequently, the specialized intracellular carrier protein, calbindin-D28K, sequesters Ca^{2+} entering the cell, and this complex diffuses toward the basolateral membrane. Finally, transporter proteins, such as NCX1 and PMCA1b, extrude Ca^{2+} from the epithelial cell back into the circulation.[2]

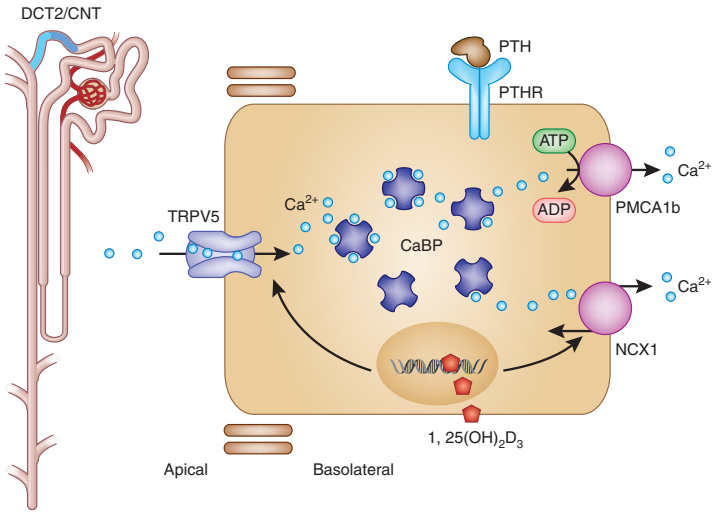

Figure 3. Mechanism of active Ca^{2+} reabsorption in DCT2 and CNT. A three-step process facilitates active and transcellular Ca^{2+} transport. The first step is entry of luminal Ca^{2+} at the apical side of the cell through the TRPV5 channel. Subsequently, calbindin (CaBP) buffers Ca^{2+}, and the Ca^{2+} diffuses to the basolateral membrane. At the basolateral membrane, Ca^{2+} is extruded by PMCA1b and NCX1. This process is controlled by calciotropic hormones including parathyroid hormone and 1,25$(OH)_2D_3$.

Apical Entry of Ca^{2+} by TRPV5

To identify the apical Ca^{2+} influx channel involved in transcellular Ca^{2+} reabsorption, we performed functional expression cloning using a cDNA library from rabbit primary CNT and the cortical collecting duct.[27] Injection of total mRNA from this isolation into *Xenopus laevis* oocytes induces a $^{45}Ca^{2+}$ uptake two to three times above background. Subsequently, the entire cDNA library was screened for $^{45}Ca^{2+}$ uptake, and a single transcript was isolated that encodes for a novel epithelial Ca^{2+} channel called eCaC1 and later renamed TRPV5, as a member of the TRP channel superfamily.[27,28] This channel comprises intracellular amino and carboxyl-terminal tails flanking six transmembrane domains and an additional hydrophobic stretch between domains 5 and 6, predicted to be the pore-forming region. Furthermore, the first extracellular loop between transmembrane domains 1 and 2 contains an evolutionary conserved asparagine (N_{358}) crucial for its complex-glycosylation, and in turn, for regulating channel activity.[29–31] The carboxyl and amino-terminal tails contain several regulatory sites including protein kinase C and A sites, which suggests an important role for phosphorylation in the regulation of channel activity. Moreover, in cultured mammalian cells, as well as in oocytes, TRPV5 assembles into homotetramers to acquire an active conformational state.[29,32]

The TRPV5-null (*TRPV*$^{-/-}$) mouse provides compelling evidence for the physiologic function of this channel. Active Ca^{2+} reabsorption in DCT2 and CNT segments is severely impaired in these null animals, because *TRPV5*$^{-/-}$ mice excrete ~10-fold more Ca^{2+} than their wild-type littermates, in line with a postulated gatekeeper function for TRPV5 in active Ca^{2+} reabsorption.[33] Electrophysiologic studies showed constitutive activity of TRPV5 at low intracellular Ca^{2+} concentrations and physiologic membrane potentials.[34] The current–voltage relationship of TRPV5 shows strong inward rectification.[2,34] Another important functional feature is that TRPV5 is the most Ca^{2+}-selective member of the TRP superfamily.[34] The single channel conductance, P_o, and the number of channels at the plasma membrane determines cellular TRPV5 activity. This activity is under the control of various factors like hormones, intracellular Ca^{2+}, and other intracellular messengers.

Vitamin D

Ample evidence of a direct role for vitamin D in the positive regulation of TRPV5 comes from several animal studies, particularly those involving 25-hydroxyvitamin D_3-1α-hydroxylase– and vitamin D receptor–null mice.[35,36] A direct relationship between 1,25$(OH)_2D_3$-induced expression of Ca^{2+} transport proteins and transcellular Ca^{2+} transport is known from studies of cultured cells from DCT and CNT cells.[37,38] Together these studies suggest a consistent 1,25$(OH)_2D_3$ sensitivity of TRPV5 and the calbindins, and, to a lesser extent, the basolateral extrusion systems involving NCX1, a Na^+/Ca^{2+}exchanger, and PMCA1b, a plasma membrane Ca^{2+}-ATPase.

Thiazide Diuretics

Thiazide diuretics, in contrast to loop diuretics, have the unique characteristic of decreasing Na^+ reabsorption while increasing Ca^{2+} reabsorption. In addition, mutations in the *NCC* gene encoding the NaCl co-transporter cause Gitelman's syndrome. Patients with Gitelman's syndrome exhibit hypovolemia, hypokalemic alkalosis, hypomagnesemia, and hypocalciuria.[39] Intriguingly, the molecular mechanisms responsible for the hypocalciuria and hypomagnesemia with thiazide administration or in Gitelman's syndrome remain elusive. Two hypotheses exist with respect to the Ca^{2+}-sparing effect of thia-

zides.[40,41] First, renal salt and water loss caused by thiazide treatment results in contraction of the extracellular volume (ECV), which triggers a compensatory increase of proximal Na^+ reabsorption. This in turn enhances the electrochemical gradient driving passive Ca^{2+} transport in proximal tubular segments.[6,39,42] Second, thiazide treatment stimulates Ca^{2+} reabsorption in DCT, possibly through the TRPV5 channel, that could explain the Ca^{2+}-sparing effect.[40] We showed in rats that hydrochlorothiazide-induced hypocalciuria is accompanied by a significant decrease in body weight compared with controls, confirming ECV contraction.[43,44] Because sodium depletion results in a similar hypocalciuria, it is likely that the ECV contraction by itself is responsible for the thiazide-induced hypocalciuria. Further evidence supporting this notion is the finding that sodium repletion during thiazide treatment, thereby preventing the ECV contraction, normalizes the calciuresis. A direct role for TRPV5 in the thiazide-induced hypocalciuria seems unlikely, because thiazides also have a hypocalciuric effect in *TRPV5*$^{-/-}$ mice, and the overlap in the expression of NCC and TRPV5 in the distal part of the nephron is restricted to DCT2.[44] Taken together, enhanced proximal tubular Na^+ transport as a consequence of ECV contraction stimulates paracellular Ca^{2+} transport and best explains the tubular mechanism for thiazide-induced hypocalciuria.

Activation of the Ca^{2+}-Sensing Receptor Prevents Nephrolithiasis

TRPV5$^{-/-}$ mice display hypercalciuria from impaired active Ca^{2+} reabsorption but also hyperphosphaturia, polyuria, and increased urinary acidification.[33] The latter two adaptations seem highly beneficial because there are no renal calcium precipitates. Polyuria also diminishes the risk of renal stone formation by reducing urinary Ca^{2+} concentration. In mice, calciuresis linearly correlates with urinary volume because an increase in Ca^{2+} excretion leads to an enhanced urinary volume. The consistent polyuria in hypercalciuric *TRPV5*$^{-/-}$ mice, noted by a substantial decrease in urinary osmolality, is caused by downregulation of renal AQP2 water channels, possibly a result of activating the Ca^{2+}-sensing receptor along the luminal membrane of the collecting duct.[45] Furthermore, gene ablation of the collecting duct-specific B1 subunit of H^+-ATPase in *TRPV5*$^{-/-}$ mice abolishes enhanced urinary acidification, which resulted in severe tubular precipitations of Ca^{2+}-phosphate in the renal medulla.[45] Thus, in *TRPV5*$^{-/-}$ mice, activation of the renal Ca^{2+}-sensing receptor promotes H^+-ATPase–mediated H^+ excretion and downregulation of AQP2, leading to urinary acidification and polyuria, respectively (Figure 4).

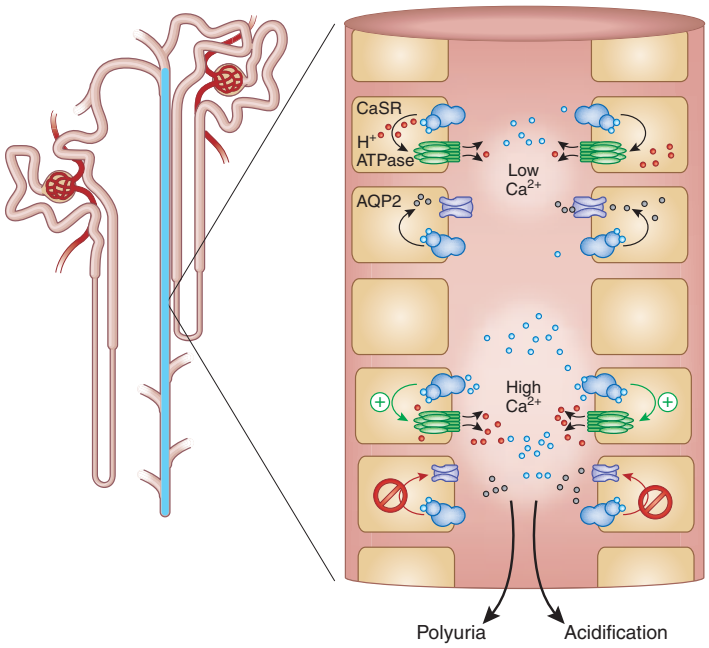

Figure 4. Molecular mechanism of Ca^{2+}-induced polyuria and urinary acidification. The Ca^{2+}-sensing receptor (CaSR) is localized at the apical site of principal and intercalated cells of the collecting duct. AQP2 proteins are responsible for water reabsorption, whereas H^+-ATPases pump H^+ into the tubular fluid. During hypercalciuria, increased urinary Ca^{2+} levels activate the CaSR. CaSR activation leads to AQP2 downregulation and polyuria. Furthermore, the CaSR triggers urinary acidification by increasing the H^+-ATPase activity. Both polyuria and increased urinary acidification prevent the precipitation of renal Ca^{2+}-phosphate.

FUTURE PERSPECTIVES ON RENAL Ca^{2+} HANDLING

Ca^{2+} reabsorption in the kidney, and particularly in the distal DCT2 and CNT segments, is crucial for the maintenance of the Ca^{2+} balance. The identification and characterization of the proteins mediating this active Ca^{2+} transport provides novel insight and means to study molecular relationships. In these segments, TRPV5 facilitates the gatekeeper function of Ca^{2+} entry, and therefore, a tight control of its activity enables the organism to adjust Ca^{2+} reabsorption according to the demands of Ca^{2+} load. The molecular mechanism of Ca^{2+} shuttling between calbindin-D_{28K} on one site and NCX1 and PMCA1b on the other site is not clear. Another interesting and unaddressed question is the regulation of NCX1 and PMCA1b in DCT2 and CNT cells. Whether there is a crosstalk between apical Ca^{2+} entry and basolateral Ca^{2+} extrusion regulatory systems is not known. The next step is to investigate how these Ca^{2+} transport proteins communicate with each other to facilitate optimal and regulated Ca^{2+} reabsorption under conditions of disturbed Ca^{2+} homeostasis. Finally, the role of TRPV5 in Ca^{2+}-related disorders needs further study.

ACKNOWLEDGMENTS

There are many people I would like to acknowledge and thank for their invaluable contribution to my work. It has been an honor and privilege to be working with each one of them over the last 25 years. In particular, I would like to thank my colleague, Joost Hoenderop, for critical reading of this manuscript before submission and for his continuous support during our long-term collaboration. Furthermore, I would like to acknowledge the contribution of many colleagues who spent some time in my laboratory including technicians, PhD students, postdocs, and staff members. Finally, I would like to thank all my collaborators for their seminal help and making science so much fun. It was and is a great pleasure to work with you all.

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