Abstract  Ca\textsuperscript{2+} and Mg\textsuperscript{2+} are essential ions in a wide variety of cellular processes and form a major constituent of bone. It is, therefore, essential that the balance of these ions is strictly maintained. In the last decade, major breakthrough discoveries have vastly expanded our knowledge of the mechanisms underlying epithelial Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport. The genetic defects underlying various disorders with altered Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+} handling have been determined. Recently, this yielded the molecular identification of TRPM6 as the gatekeeper of epithelial Mg\textsuperscript{2+} transport. Furthermore, expression cloning strategies have elucidated two novel members of the transient receptor potential family, TRPV5 and TRPV6, as pivotal ion channels determining transcellular Ca\textsuperscript{2+} transport. These two channels are regulated by a variety of factors, some historically strongly linked to Ca\textsuperscript{2+} homeostasis, others identified in a more serendipitous manner. Herein we review the processes of epithelial Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport, the molecular mechanisms involved, and the various forms of regulation.

Introduction

Serum Ca\textsuperscript{2+} and Mg\textsuperscript{2+} levels are maintained within narrow limits by the human body, despite considerable variations in daily intake and excretion. The ion balance of these divalents is mediated by the coordinated action of the intestine, kidney, and bone. When the body is deprived of Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, the (re)absorption activity in these organs increases accordingly, whereas the reverse action occurs when the plasma levels of these ions threaten to surpass acceptable upper limits. The bone acts as a dynamic storage compartment in this process and contributes to maintain the balance by releasing these ions upon Ca\textsuperscript{2+} or Mg\textsuperscript{2+} deprivation. Herein we will describe in detail the regulation of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport in epithelia and discuss potential avenues for future breakthroughs.
Epithelial Ca\textsuperscript{2+} transport

The maintenance of the body Ca\textsuperscript{2+} balance is of vital importance for several crucial physiological functions including neuronal excitability, muscle contraction, and skeletal integrity. Skeleton growth demands a positive Ca\textsuperscript{2+} balance, whereas a Ca\textsuperscript{2+} deficit is observed in aging and postmenopausal women, ultimately resulting in a loss of Ca\textsuperscript{2+} from bone. The latter is associated with a higher risk for bone fractures. The average daily Ca\textsuperscript{2+} intake of an adult is approximately 1 g, of which roughly 0.35 g is absorbed in the gastrointestinal tract (Greer and Krebs 2006). Approximately 8 g Ca\textsuperscript{2+} is filtered in the kidney on a daily basis, whereas only a fraction is excreted into the urine to balance Ca\textsuperscript{2+} that is absorbed in the intestine. Paracellular and transcellular transport mediates Ca\textsuperscript{2+} (re)absorption. The paracellular component of epithelial Ca\textsuperscript{2+} transport is passive and directly connects the luminal compartment with the blood compartment, whereas the transcellular component is active and involves the passage of at least two membrane barriers. Importantly, the transcellular pathway is the main target site for specific regulation of Ca\textsuperscript{2+} (re)absorption by various calcitropic hormones.

Localization of transcellular Ca\textsuperscript{2+} transport

Ca\textsuperscript{2+} (re)absorption occurs in several organs including kidney, intestine, and bone. Fish have an additional organ for Ca\textsuperscript{2+} uptake, the gills. Furthermore, in pregnancy and lactation, the placenta and mammary glands, respectively, are important tissues in the balance between Ca\textsuperscript{2+} uptake and output.

Kidney

A large amount of Ca\textsuperscript{2+} is filtered at the glomerulus. The proximal tubules (PT), including the proximal convoluted (PCT) and proximal straight (PST) tubules, are responsible for the absorption of the bulk of the Ca\textsuperscript{2+} from the filtrate. Approximately 65% of the filtered Ca\textsuperscript{2+} is reabsorbed here, as was demonstrated using micropuncture experiments (Edwards et al. 1973; Friedman 1999; Sutton and Dirks 1975; Ullrich et al. 1963). This transport is passive and follows the local Na\textsuperscript{+} reabsorption. This site therefore does not provide an independent regulation of Ca\textsuperscript{2+} reabsorption (Suki 1979). In the subsequent segment of the nephron, i.e., the thin descending and ascending loop of Henle (ATL), virtually no Ca\textsuperscript{2+} is reabsorbed (Rocha et al. 1977). However, the thick ascending loop of Henle (TAL) is again permeable for Ca\textsuperscript{2+}, and this segment accounts for approximately 20% of the total Ca\textsuperscript{2+} reabsorption (Bailly et al. 1990; Bourdeau and Burg 1980; Bourdeau et al. 1987; Di Stefano et al. 1989, 1990; Friedman 1988; Friedman and Gesek 1995a; Imai 1978; Ng et al. 1982; Rocha et al. 1977; Suki 1979; Suki et al. 1980; Suki and Rouse 1981). Several studies suggest that Ca\textsuperscript{2+} mainly follows the paracellular pathway in this segment (Bourdeau and Burg 1979; Shareghi and Agus 1982; Wittner et al. 1991, 1993). This was further corroborated when mutations in paracellin-1 (also called claudin-16), localized in the tight junctions of TAL, were associated with renal Ca\textsuperscript{2+} and Mg\textsuperscript{2+} wasting due to impaired paracellular divalent cation reabsorption (Simon et al. 1999). The destination of the filtrated Ca\textsuperscript{2+} remaining at the end of the TAL is determined by the distal part of the nephron, consisting of the distal convoluted tubule (DCT) and connecting tube (CNT) (Costanzo and Windhager 1978; Costanzo et al. 2000). The CNT is situated distal from the DCT, arising abruptly in rabbits and more gradually in other species (Bulger et al. 1967; Crayen and Thoenes 1978; Kaisišing 1982; Loffing et al. 2001). In the CNT and DCT, Ca\textsuperscript{2+} reabsorption takes place against its electrochemical...
gradient, indicating that the transport is active (Costanzo et al. 2000). Furthermore, the tight junctions in DCT and CNT are relatively impermeable to Ca2+, in line with a predominant role for an active transcellular Ca2+ transport pathway. The relative contribution of the initial (DCT1) and later (DCT2) part of DCT and CNT to the active Ca2+ reabsorption is not clear. Microperfusion studies by Costanzo and Windhager showed active Ca2+ transport in both DCT and CNT (Costanzo and Windhager 1978), whereas studies from Greger and coworkers indicated a predominant role for CNT (Greger et al. 1978). Furthermore, the relative contribution of each segment could be regulated; Imai et al. demonstrated that DCT and CNT do not respond to the same extent to parathyroid hormone (PTH) administration (Imai 1981). The cortical collecting duct (CCD) accounts for maximally 3% of the filtered Ca2+ (Ullrich et al. 1963). As net transport occurs against the electrochemical gradient for Ca2+, Ca2+ reabsorption must be active here as well.

Intestine

Intestinal Ca2+ absorption is crucial in the maintenance of the Ca2+ balance, as it primarily mediates the uptake of Ca2+ from the diet. Only about 30% of dietary Ca2+ is absorbed and the remaining 70% is excreted in the feces. Transcellular Ca2+ absorption occurs mainly in duodenum and in the initial part of jejunum and, to a much lesser extent, in ileum and colon, whereas paracellular Ca2+ absorption takes place throughout the entire small intestine (Bronner et al. 1986). The relative contribution of paracellular versus transcellular Ca2+ absorption depends on the dietary Ca2+ content (Bronner 2003; Bronner and Pansu 1999). Chyme moves down the intestinal lumen in approximately 3 h, spending only a short time in the duodenum, but over 2 h in the distal part of the small intestine. When dietary Ca2+ intake is low, transcellular Ca2+ transport accounts for a substantial fraction of the absorbed Ca2+, and vice versa when Ca2+ intake is high. In addition, the contribution of transcellular Ca2+ transport is strongly upregulated by calcitropic hormones including vitamin D, as in “Regulation of epithelial Ca2+ transport”.

Bone

Bone serves as an important storage point for Ca2+, as it contains 99% of the total body Ca2+. There are two types of bone, cortical and trabecular, the former constituting approximately 80% of the total bone mass (Nussey and Whitehead 2001). In the long bones of the skeleton, cortical or compact bone predominates. In the axial skeleton (skull, ribs, vertebrae) there is only a relatively thin layer of circumferential cortical bone with a much greater mass of trabecular or spongy bone. Since the surfaces within bone exposed to the extracellular fluid are higher in trabecular than compact bone it plays a more important role in Ca2+ homeostasis. Bone formation and resorption take place at these surfaces. Formation is carried out by active osteoblasts that extrude collagen into the extracellular space and deposit Ca2+ (Nussey and Whitehead 2001; Rodan 1992). As the osteoblasts become surrounded by mineralized bone these cells lose their activity and become interior osteocytes. However, they remain in contact with the bone surfaces and are supposed to play a role in regulated Ca2+ release in a process termed osteocytic osteolysis (Nussey and Whitehead 2001). Bone resorption also occurs on bone surfaces and is carried out by osteoclasts, which literally tunnel their way into bone, forming resorption pits (Nussey and Whitehead 2001; Teitelbaum 2000). Bone resorption and formation are linked and bone is continuously remodeled (Nussey and Whitehead 2001; Rodan 1992; Rodan and Martin 2000). As the maximum bone density is reached at the age of approximately 30 years, the rate of bone formation exceeds bone resorption.
until this age (Greer and Krebs 2006; Nussey and Whitehead 2001). Later in life, the reversal of this balance can lead to impaired bone thickness (osteoporosis), where the bone is prone to fracture (Greer and Krebs 2006; Harada and Rodan 2003; Nussey and Whitehead 2001; Rodan and Martin 2000). Bone formation and its remodeling are controlled by several calcitropic hormones that will be discussed later. However, it is still unclear in which way bone resorption and formation contributes to blood Ca\(^{2+}\) homeostasis since these processes are relatively slow for the buffering of rapid responses in serum Ca\(^{2+}\). Furthermore, knowledge about the molecular mechanism of bone formation and resorption is limited (Van der Eerden et al. 2005). It is therefore essential to identify and further characterize the Ca\(^{2+}\) transport processes in bone.

**Placenta**

During pregnancy Ca\(^{2+}\) absorption in the placenta is solely responsible for the Ca\(^{2+}\) supply to the developing fetus. Ca\(^{2+}\) is transported across the placenta from the maternal to the fetal circulation via an active transcellular pathway to meet the requirements of the rapidly mineralizing skeleton and to maintain an extracellular level of Ca\(^{2+}\) that is physiologically appropriate for the development of fetal tissues (Belkacemi et al. 2002, 2003, 2004; Brunette 1988; Fukuoka and Satoh 1982; Lafond et al. 1991; Moreau et al. 2002a, b, 2003a, b; Pitkin 1985). Ca\(^{2+}\) is transported by the syncytiotrophoblasts, cells that form the epithelial layer separating the maternal and fetal circulation (Faulk and McIntyre 1983). It has been postulated that the molecular mechanisms of placental Ca\(^{2+}\) transport has considerable similarity with active Ca\(^{2+}\) transfer across the intestinal and renal epithelial cells, as the same Ca\(^{2+}\) transport proteins are expressed (Belkacemi et al. 2002, 2003, 2004, 2005).

**Paracellular Ca\(^{2+}\) transport**

Movement of Ca\(^{2+}\) ions through the tight junctions is a passive process that largely depends on the concentration and electrical gradient across the epithelium. The paracellular transport route must be regulated for the epithelium to remain selectively permeable. Depending on the functional requirements of an epithelium, there may be small or large amounts of solutes flowing passively through this path. Tight epithelia of high resistance can generate and maintain high transepithelial electrical potentials and ionic gradients to form luminal fluids with compositions that deviate significantly from that of interstitial fluid (Schneeberger and Lynch 2004). Furthermore, the paracellular pathway varies in its selectivity for ions and noncharged solutes (Van Itallie et al. 2003).

Tight junctions consist of linear arrays of integral membrane proteins, which include occludin, claudins, and several immunoglobulin superfamily members, such as the junctional adhesion molecule (Ebnet et al. 2003; Goodenough 1999; Martin-Padura et al. 1998). The claudin family consists of at least 20 related integral membrane proteins with four transmembrane domains (TM) and functions as major structural components of the tight junctional complex, while occludin is an accessory protein involved in tight junction formation of which two isoforms have been described (Furuse et al. 2001; Gonzalez-Mariscal et al. 2003; Hirase et al. 1997; McCarthy et al. 1996; Morita et al. 1999; Saitou et al. 1997, 1998; Schneeberger and Lynch 2004; Tang and Goodenough 2003; Tsukita and Furuse 2000).

The molecular nature of the wide variety in electrical resistance and solute permeability of tight junctions is not completely understood. The existence of only two isoforms of occludin and the limited number of charged amino acid residues in the two extracellular
domains of these proteins suggest that occludin is unlikely to contribute directly to the formation of ion-selective pores in the tight junction (Schneeberger and Lynch 2004). Furthermore, disruption of both occludin alleles by homologous recombination resulted in embryonic stem cells that not only differentiated into polarized epithelial cells but also formed an effective barrier to the diffusion of low-molecular-weight molecules (Saitou et al. 1998). Therefore, it is more likely that claudins form the critical component that determines the ion selectivity of tight junctions, particularly considering the large variation of charge in amino acid residues of their extracellular loops.

Although the mechanisms that establish the Ca\textsuperscript{2+} flux via the paracellular pathway are largely unknown, the role of claudins in epithelial Ca\textsuperscript{2+} transport is further supported by the disease mutations documented in these proteins (Cole and Quamme 2000; Wilcox et al. 2001; Wong and Goodenough 1999). Mutations in claudin-16, which are associated with a renal Ca\textsuperscript{2+} and Mg\textsuperscript{2+} wasting syndrome, implicate this particular claudin protein in paracellular reabsorption of Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, but not monovalent ions (Simon et al. 1999). This topic will be more elaborately discussed below (see “Hypomagnesemia with hypercalciiuria and nephrocalcinosis”). Mutations in claudin-14 cause nonsyndromic recessive deafness, and this tight junction protein is essential to maintain the electrochemical gradient between the endolymph and its surrounding tissues (Wilcox et al. 2001). However, it is currently not clear whether this phenotype involves changes in paracellular Ca\textsuperscript{2+} permeability. The role of other claudin family members in determining the rate of Ca\textsuperscript{2+} absorption in epithelia also remains elusive. The rate of paracellular Ca\textsuperscript{2+} transport is mainly determined by the electrochemical gradient across the epithelium (Fig. 1). Therefore, hormones and factors affecting this gradient will indirectly influence the passive Ca\textsuperscript{2+} fluxes through the tight junctions. In addition, the tight junction permeability itself is dynamically regulated (Goodenough 1999; Pappenheimer 1987) and subject to modulation by growth factors, cytokines, bacterial toxins, hormones, and other factors (Benais-Pont et al. 2003; Garcia et al. 1998; Gopalakrishnan et al. 2002; Wang et al. 2004b). In addition, paracellular transport can be mediated by protein kinases, as nicely illustrated by the threonine-serine kinase WNK4, which is present in tight junctions (Kahle et al. 2004; Yamauchi et al. 2004). It was recently demonstrated that WNK4 can bind and phosphate claudins-1 through -4 and that a human disease-causing mutant of WNK4 hyperphosphorylates claudins and increases paracellular Cl\textsuperscript{−} permeability (Kahle et al. 2004; Wilson et al. 2001; Yamauchi et al. 2004). Similarly, it was postulated that phosphorylation of specific claudins (i.e., claudin-16) might provide means to regulate the paracellular Ca\textsuperscript{2+} flux. However, because of the low Ca\textsuperscript{2+} specificity of paracellular transport compared to transepithelial Ca\textsuperscript{2+} movement, specific regulation of the Ca\textsuperscript{2+} flux through tight junctions is likely to play a minor role in the fine-tuning of the Ca\textsuperscript{2+} balance.

Transcellular Ca\textsuperscript{2+} transport

Transcellular Ca\textsuperscript{2+} transport is the pivotal target for specific regulation of Ca\textsuperscript{2+} (re)absorption by various calcitropic hormones (Bawden 1989; Bouillion et al. 2003; Bronner 2003; Bronner and Pansu 1999; Brunette 1988; Friedman and Gesek 1995a; Hoenderop et al. 2005; Wasserman and Fullmer 1995). Transcellular Ca\textsuperscript{2+} (re)absorption can be divided into three consecutive steps. First, Ca\textsuperscript{2+} enters the cells from the luminal compartment. Second, Ca\textsuperscript{2+} bound to specialized Ca\textsuperscript{2+}-binding proteins diffuses to the basolateral side of the cell. Third, Ca\textsuperscript{2+} is extruded into the interstitial fluid by two extrusion mechanisms (Fig. 1). It is essential that the Ca\textsuperscript{2+} influx and efflux mechanism(s) maintains a polar distribution to ensure net Ca\textsuperscript{2+} transport from the apical or luminal side to the
basolateral or serosal compartment. In this respect, it is important to study the Ca\textsuperscript{2+} influx pathway in polarized cell models. Until now, two polarized confluent epithelial cell systems representing duodenal and renal active Ca\textsuperscript{2+} (re)absorption have been studied. First, Caco-2 cells spontaneously differentiate under standard culture conditions into a tissue that exhibits functional duodenal transport properties (Giuliano and Wood 1991). These cells form a polarized epithelial layer and express several markers that are unique to differentiated
Mechanisms of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} reabsorption in the kidney. In TAL, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} are reabsorbed via passive paracellular transport across the tight junctions. This transport is driven by the electrochemical gradient across the epithelium and requires functional claudin-16. Further downstream, Mg\textsuperscript{2+} and Ca\textsuperscript{2+} reabsorption are localized to distinct nephron segments. TRPM6 colocalizes with NCC in DCT1, where the former mediates apical Mg\textsuperscript{2+} influx. Mechanisms underlying the subsequent steps in transcellular Mg\textsuperscript{2+} reabsorption remain elusive. Mg\textsuperscript{2+} buffering is postulated and energy-consuming basolateral Mg\textsuperscript{2+} extrusion is mediated by a putative Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger or ATP-dependent Mg\textsuperscript{2+}-ATPase. The Na\textsuperscript{+} gradient driving the putative Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger is established by the activity of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase that is modulated by the γ-subunit in DCT. In DCT2, a three-step process facilitating active and transcellular Ca\textsuperscript{2+} transport takes place. The first step is the entry of Ca\textsuperscript{2+} at the luminal side of the cell through the (hetero)tetrameric epithelial Ca\textsuperscript{2+} channels TRPV5 and TRPV6. Subsequently, calbindin buffers Ca\textsuperscript{2+} and the Ca\textsuperscript{2+} diffuses to the basolateral membrane. At the basolateral membrane, Ca\textsuperscript{2+} is extruded via an ATP-dependent Ca\textsuperscript{2+}-ATPase (PMCA1b) and a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX1).

Small intestinal epithelium (e.g., high sucrase-isomaltase mRNA and protein levels) (Chantret et al. 1988). Furthermore, Caco-2 cells exhibit net apical-to-basolateral Ca\textsuperscript{2+} transport kinetics, and the rate of transport can be enhanced by pretreatment with vitamin D (Fleet et al. 2002; Fleet and Wood 1999; Giuliano et al. 1991). Second, the use of primary cultures and immortalized cell lines originating from the distal part of the nephron greatly facilitated our understanding of regulated renal Ca\textsuperscript{2+} influx in and transport through these cells. Two groups, Bindels et al. (Bindels et al. 1991, 1993; Hoenderop et al. 1998, 1999a, b; Raber et al. 1997; Van Baal et al. 1996a, b; 1999), and Gesek and Friedman (Bacskaï and Friedman 1990; Friedman 1988; Friedman et al. 1996; Friedman and Gesek 1993; Friedman and Gesek 1994, 1995b; Gesek and Friedman 1992a, b; Magyar et al. 2002; White et al. 1998), have used immunodissected cell lines from rabbit and mouse kidney, respectively, to investigate hormone-stimulated Ca\textsuperscript{2+} transport. In addition, Bindels et al. demonstrated that primary cultures of rabbit CNT and CCD cells exhibit many characteristics of the original epithelium, including calciotropic hormone-stimulated Ca\textsuperscript{2+} transport from the apical to the basolateral compartments (Bindels et al. 1991).

**Luminal Ca\textsuperscript{2+} influx**

In order to identify the apical Ca\textsuperscript{2+} influx channel involved in transcellular Ca\textsuperscript{2+} (re)absorption, Hoenderop and coworkers performed functional expression cloning using a complementary DNA (cDNA) library from rabbit primary CNT and CCD. Injection of the total mRNA from this isolation in *Xenopus laevis* oocytes induced a 45Ca\textsuperscript{2+} uptake 2–3 times above background. Subsequently, the entire cDNA library was screened for 45Ca\textsuperscript{2+} uptake and a single transcript was isolated encoding for a novel epithelial Ca\textsuperscript{2+} channel, named ECaC1 and later renamed as the transient receptor potential channel TRPV5 (Hoenderop et al. 2001b; Montell et al. 2002). Similarly, Hediger and coworkers applied the same approach to screen a cDNA library obtained from rat small intestine. They identified Ca\textsuperscript{2+} transporter 1 (CaT1) that was later renamed into TRPV6 (Montell et al. 2002; Peng et al. 1999).

In the literature TRPV5 is also known as ECaC, ECaC1, and CaT2, whereas TRPV6 has been named previously CaT1, ECaC2, and CaT-Like. TRPV5 and TRPV6 display the defining properties of the long-sought epithelial Ca\textsuperscript{2+} channels, including hormonal regulation, localization, and functional properties as will be discussed below (see “Localization of TRPV5 and TRPV6” through to “Regulation of epithelial Ca\textsuperscript{2+} transport”). TRPV5 and TRPV6 are encoded by two distinct genes, rather than being splice variants (Hoenderop et al. 2001b; Weber et al. 2001a). These genes are juxtaposed on chromosome 7q35, suggesting a gene duplication event during evolution (Muller et al. 2000; Peng et al. 2000b). An iden-
Fig. 2 Overview of TRPV5 and TRPV6 regulatory proteins. a The epithelial Ca\(^{2+}\) channels contain a core domain consisting of six transmembrane segments, with an additional hydrophobic stretch between TM5 and TM6 forming the pore forming region. This core TM segment-containing region is flanked by large amino and carboxyl-termini that face the intracellular compartment. The amino-terminus contains ankyrin repeats, which play a role in channel oligomerization. Calmodulin associates with both the amino and carboxyl-termini. Klotho affects TRPV5 and TRPV6 from the extracellular medium, where it modifies the glycosylated part of the channel. NHERF2 binds to the last three amino acids of the carboxyl-terminus, whereas the remaining channel-associated proteins identified to date, including Rab11a, 80K-H, BSPRY, NHERF4, and the S100A10/annexin 2 complex, bind to more upstream regions. Calbindin-D\(_{28K}\) has been shown to translocate to the apical plasma membrane to associate with TRPV5 at a low intracellular Ca\(^{2+}\) concentration. This enables the local buffering of Ca\(^{2+}\) near the channel, allowing significant Ca\(^{2+}\) influx by preventing rapid channel inactivation.

b The gate and selectivity filter of TRPV5 and TRPV6 are formed by four channel subunits facing the center of the channel with the pore-forming region. This is a hydrophobic region that is flanked by two transmembrane domains (TM5 and TM6) and contains negatively charged amino acids (D542 in rabbit TRPV5) that determine pore size and Ca\(^{2+}\) selectivity. CaBP28K, calbindin-D28K, CaM, calmodulin, NHERF2 Na\(^+\)/H\(^+\) exchanger regulatory factor 2, NHERF4 Na\(^+\)/H\(^+\) exchanger regulatory factor 4, BSPRY B-box and SPRY domain containing protein, ANK ankyrin repeat.

tical situation was observed in mouse, where the two genes are close together on chromosome 6 (Hoenderop et al. 2003a; Weber et al. 2001a). The distinct genes comprise 15 exons encoding proteins of approximately 730 amino acids. TRPV5 and TRPV6 share a predicted topology of six TMs with an additional hydrophobic region between TM5 and TM6, which forms the channel pore (Fig. 2). TRPV5 and TRPV6 have been cloned from a variety of species, including rabbit, rat, mouse, human, and fish (Hoenderop et al. 1999c; Muller et al. 2000; Pan et al. 2005; Peng et al. 1999, 2000a, b). In the latter species, the distinction between TRPV5 and TRPV6 is difficult (Pan et al. 2005; Shahsavarani et al. 2006). The overall similarity of these sequences is approximately 75%. Remarkably, several domains in TRPV5 and TRPV6 are strongly conserved, including the pore region and binding sites involving associated regulatory proteins (Fig. 2). TRPV5 and TRPV6 belong to the superfamily of TRP channels consisting of several groups, including the TRPC, TRPV, and TRPM branches of the family (Clapham 2003). TRPV5 and TRPV6 share the highest homology with members of the TRP vanilloid family (TRPV). This group of cation channels further includes TRPV1–4, which respond to heat, osmolarity, odorants, and mechanical stimuli (for additional information see http://clapham.tch.harvard.edu/trps/TRPVs_2005_web.pdf). The homology of TRPV5 and TRPV6 with these members of the TRP channels is about 30% at the amino acid level.

**Structural properties of TRPV5 and TRPV6** Hoenderop and coworkers addressed the oligomerization of TRPV5 and TRPV6 channels. Cross-linking studies, co-immunoprecipitations, and molecular mass determination of TRPV5 and TRPV6 complexes using sucrose gradient sedimentation showed that the epithelial Ca\(^{2+}\) channels form homo- and heterotetrameric channel complexes (Hoenderop et al. 2003b). The four subunits surround a single pore. Hellwig et al. recently addressed the homo- and heteromultimerization of TRPV channel by analysis of subcellular colocalization, fluorescence resonance energy transfer, and co-immunoprecipitation. TRPV channel subunits preferentially assemble into homomeric complexes, with the exception of TRPV5 and TRPV6, which also readily form heterotetramers (Hellwig et al. 2005).

The molecular determinants of TRPV6 oligomerization were addressed by Erler and coworkers. They identified the ankyrin repeat at position 116–191 of the amino-terminus as a stringent requirement for physical assembly of TRPV6 subunits (Erler et al. 2004). It was proposed that this repeat initiates a molecular zippering process that proceeds past the last
ankyrin repeat and creates an intracellular anchor that is necessary for functional subunit assembly. Structure prediction programs indicated the presence of 3 to 6 ankyrin repeats in the amino-terminus (Erler et al. 2004; Hoenderop et al. 1999c).

Chang and coworkers confirmed the important role of the amino-terminus in channel assembly in general, and in particular demonstrated that a region near the first ankyrin repeat (position 64–77) is critical in the assembly process of TRPV5 (Chang et al. 2004). However, Chang et al. observed slightly increased binding efficiencies using truncated proteins including the first 162 amino acids, indicating that the ankyrin repeat identified for TRPV6 could
also be involved in TRPV5 multimerization. Similarly, the pivotal role of ankyrin repeats in the oligomerization was recently also demonstrated for another TRP channel i.e., TRPV4. They showed that a splice variant of this cation channel that lack ankyrin repeats is impaired in its ability to multimerize (Armiges et al. 2006).

Interestingly, a structural model of the outer pore of TRPV5 and TRPV6 was provided by Dodier et al. (2004) and Voets et al. (2004a), respectively. Mutation of a single aspartate residue at position number 542 (D542), a residue crucial for the high-affinity Ca\(^{2+}\)-binding, altered the apparent pore diameter (Voets et al. 2004a), indicating that this residue lines the narrowest part of the pore. Importantly, mutation of this aspartate residue in the tetrameric channels results in loss of Ca\(^{2+}\) selectivity and voltage-dependence (Hoenderop et al. 2003b). Furthermore, using cysteine-scanning mutagenesis the main structural features of TRPV5 and TRPV6 were determined. Cysteines introduced in a region preceding D542 for TRPV5 and D541 for TRPV6 displayed a cyclic pattern of reactivity to cysteine reacting agents indicative of a pore helix (Dodier et al. 2004; Voets et al. 2004a). The pattern of covalent modification of cysteines supports a 3-D model similar to KcsA K\(^{+}\) channels (Doyle et al. 1998). The external vestibule in TRPV5 and TRPV6 may build up the three structural domains consisting of a coiled structure that is connected to a 15-amino-acid pore helix followed by the selectivity filter (with D542 and D541 forming the narrowest part) and another coiled structure before the beginning of TM6. These are the first structural models of a TRP channel pore.

### Localization of TRPV5 and TRPV6

Expression profiling of TRPV5 and TRPV6 using Northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry showed expression in a variety of tissues including kidney, small intestine, placenta, and bone (Table 1). TRPV5 likely contributes most significantly to transcellular Ca\(^{2+}\) transport in kidney, whereas TRPV6 is more ubiquitously expressed (Hoenderop et al. 2000; Nijenhuis et al. 2003b; Zhuang et al. 2002). In kidney TRPV5 colocalizes with other Ca\(^{2+}\) transport proteins involved in intracellular binding (i.e., calbindins) or basolateral extrusion (i.e., NCX1 and PMCA1b) of Ca\(^{2+}\) (Hoenderop et al. 2000, 1999c). TRPV5 was localized to the apical domain of the late part of the DCT (DCT2) and CNT in the nephron, matching the sites of active Ca\(^{2+}\) transport (Hoenderop et al. 2000). Loffing et al. showed that TRPV5 localization was predominantly apical in DCT2, whereas a more cytosolic localization was observed at the end of the CNT (Loffing et al. 2001). This suggests that channel shuttling to and from the apical plasma membrane plays a role in the regulation of epithelial Ca\(^{2+}\) transport. In mouse kidney, TRPV6 was detected by immunohistochemistry at the apical domain of DCT2, CNT, and cortical and medullary collecting ducts (Nijenhuis et al. 2003b). The significance of the contribution of TRPV6 to Ca\(^{2+}\) reabsorption in the kidney is currently unknown, although the renal phenotype of TRPV5 knockout mice indicates that TRPV6 cannot compensate for loss of TRPV5 activity in the kidney (Hoenderop et al. 2003a).

Furthermore, several groups demonstrated the expression of TRPV6 in the proximal part of duodenum in line with a prominent role for TRPV6 in intestinal Ca\(^{2+}\) absorption (Huybers et al. 2006; Van de Graaf et al. 2003; Walters et al. 2006; Zhuang et al. 2002). However, Wissenbach et al. did not detect the expression of TRPV6 in duodenum and kidney (Wissenbach et al. 2001). TRPV5 and TRPV6 mRNA were both detected in syncytiotrophoblasts (Moreau et al. 2002a). This suggests that TRPV5 and/or TRPV6 mediate basal Ca\(^{2+}\) influx in placenta as these cells mediate Ca\(^{2+}\) transfer to the fetus. In bone, TRPV5 was detected in osteoclasts, in line with a role in Ca\(^{2+}\) resorption in these cells (Van der Eerden et al.
Table 1  Localization of TRPV5 and TRPV6 in Ca\(^{2+}\)-transporting tissues. TRPV5 forms the predominant Ca\(^{2+}\) influx pathway in kidney, whereas TRPV6 is essential for intestinal Ca\(^{2+}\) absorption. The relative contribution of TRPV5 and TRPV6 to epithelial Ca\(^{2+}\) transport in placenta, bone, and mammary gland remains to be established (Hoenderop et al. 2000).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TRPV5</th>
<th>TRPV6</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>DCT2-CNT</td>
<td>DCT2-CD</td>
<td>Hoenderop et al. 2000; Loffing et al. 2001; Nijenhuis et al. 2003b</td>
</tr>
<tr>
<td>Intestine</td>
<td>Duodenum</td>
<td>Duodenum colon</td>
<td>Muller et al. 2000; Weber et al. 2001a; Zhuang et al. 2002</td>
</tr>
<tr>
<td>Placenta</td>
<td>Syncytiotrophoblast</td>
<td>Syncytiotrophoblast</td>
<td>Moreau et al. 2002b; Peng et al. 2001a</td>
</tr>
<tr>
<td>Bone</td>
<td>Osteoclasts</td>
<td>Osteoclasts and osteoblasts</td>
<td>Van der Eerden et al. 2005; Nijenhuis et al. 2003b; Weber et al. 2001a</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>ND</td>
<td>Epithelial cells</td>
<td>Zhuang et al. 2002</td>
</tr>
</tbody>
</table>

DCT2, late distal convoluted tubule; CNT, connecting tubule; CD, collecting duct; ND, not determined

2005). Also TRPV6 was detected in bone (Nijenhuis et al. 2003b; Weber et al. 2001a), although little is known about the exact localization and role of this TRP channel here. Finally, the expression of TRPV6 is not restricted to Ca\(^{2+}\)-transporting cells and is detected in several exocrine tissues including pancreas, salivary gland, stomach, and prostate (Peng et al. 2000b; Wissenbach et al. 2001; Zhuang et al. 2002). The expression of TRPV6 in prostate is elevated in prostate cancer and correlates with the tumor grade (Fixemer et al. 2003; Peng et al. 2001b; Wissenbach et al. 2001). Although the functional consequence of TRPV6 expression in these tissues is unknown, it was postulated that TRPV6 mediates Ca\(^{2+}\) influx under certain conditions during exocytosis or cell proliferation.

Biophysical properties of TRPV5 and TRPV6  TRPV5 and TRPV6 display unique biophysical properties that distinguish them from other TRP channel members (Clapham 2003; Vennekens et al. 2000; Yue et al. 2001). First, TRPV5 and TRPV6 show considerable constitutive activity at low intracellular Ca\(^{2+}\) concentrations and physiological membrane potentials. Most other members of the TRP superfamily display smaller constitutive activity, but are activated upon ligand binding, receptor-mediated phospholipase C (PLC) activation, or temperature shifts (Clapham 2003). Furthermore, TRPV5 and TRPV6 are 100 times more selective for Ca\(^{2+}\) than for Na\(^+\), making them the most Ca\(^{2+}\)-selective TRP channels (Hoenderop et al. 2005; Nilius et al. 2000; Vassilev et al. 2001). At physiological Ca\(^{2+}\) concentrations, the currents passing through TRPV5 and TRPV6 are mainly carried by Ca\(^{2+}\) (Hoenderop et al. 2005). Single channel conductances of TRPV5 and TRPV6 are 40–70 pS, using Na\(^+\) as a charge carrier (Nilius et al. 2000; Vassilev et al. 2001). In addition, both in the inside-out and in the whole-cell configuration, TRPV5 and TRPV6 show a characteristic inward rectification (Hoenderop et al. 2005). So far, reliable single channel measurements have not been performed in the presence of extracellular Ca\(^{2+}\). Although many of the properties are similar between TRPV5 and TRPV6, there are a number of differences between these channels. First, Ba\(^{2+}\) permeates TRPV5 better than TRPV6, e.g., the current ratio for Ba\(^{2+}\) over Ca\(^{2+}\) \((I_{Ba}/I_{Ca})\) is approximately 0.9 for TRPV5 and only approximately
0.4 for TRPV6 (Nilius et al. 2002). Furthermore, the rate of channel inactivation is different between TRPV5 and TRPV6, as is detailed below (Nilius et al. 2002). Remarkably, the structural determinants for these two differences are situated in the intracellular linker region between TM2 and TM3 (Nilius et al. 2002). Another distinction between TRPV5 and TRPV6 is the rate of recovery from Ca\(^{2+}\)-dependent inactivation (see below), which is roughly three times slower in TRPV5 compared to TRPV6 (Hoenderop et al. 2005). Finally, there are pharmacological differences between TRPV5 and TRPV6, as the latter requires higher concentrations of ruthenium red or Cd\(^{2+}\) to be blocked (~100- and ~4-fold increase in IC\(_{50}\), respectively) (Hoenderop et al. 2003b; Nilius et al. 2001b).

TRPV5 and TRPV6 are coexpressed in some tissues, which allows heterooligomerization of these channels in vivo (Hoenderop et al. 2003b). As TRPV5 and TRPV6 exhibit different channel kinetics with respect to Ca\(^{2+}\)-dependent inactivation, Ba\(^{2+}\) selectivity, and sensitivity to inhibition by ruthenium red and Cd\(^{2+}\), heterotetramer composition might influence the functional properties of the formed Ca\(^{2+}\) channel (Hoenderop et al. 2003b). This was investigated using concatemeric constructs consisting of four TRPV5 and/or TRPV6 subunits configured in a head-to-tail fashion. A different ratio of TRPV5 and TRPV6 subunits in these concatemers showed that the phenotype resembles the mixed properties of TRPV5 and TRPV6 (Hoenderop et al. 2003b). A high 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. Consequently, regulation of the relative expression levels of TRPV5 and TRPV6 may be a mechanism to fine-tune the Ca\(^{2+}\) transport kinetics. For instance, immunohistochemical data in kidney clearly demonstrated coexpression of TRPV5 and TRPV6 in DCT and CNT (Hoenderop et al. 2003b). However, coexpression of TRPV5 and TRPV6 in these tissues has been to date quantified only at the mRNA level, indicating that TRPV6 is 100–10,000 times more expressed than TRPV5. Quantification at the protein level of both channels is certainly important to address the stoichiometry in vivo.

Factors affecting channel gating

**Intracellular Ca\(^{2+}\)** Unlike other members of the TRP superfamily, there are no indications that TRPV5 and TRPV6 require a stimulus or ligand to be activated. However, the constitutive activity of these epithelial channels is regulated by various means. First, TRPV5 and TRPV6 are subject to Ca\(^{2+}\)-dependent feedback inhibition (Bodding et al. 2002; Nilius et al. 2001a, 2002; Vennekens et al. 2000, 2001a). Both channels rapidly inactivate during hyperpolarizing voltage steps. This inhibition is dependent on the extracellular Ca\(^{2+}\) concentration and occurs also in cells buffered intracellularly with 10 mM BAPTA. Although TRPV5 and TRPV6 are both rapidly inactivated upon an increase in the intracellular Ca\(^{2+}\) concentration, the initial inactivation is faster in TRPV6 than in TRPV5 (Nilius et al. 2002). As described above, the intracellular region between TM2 and TM3 was identified as a crucial domain for the fast inactivation of TRPV6 (Nilius et al. 2002). Furthermore, two regions in the carboxyl-terminus of TRPV5 contributed to the Ca\(^{2+}\)-dependent inactivation (Nilius et al. 2003). Deletion of the last 30 amino acids of the carboxyl-terminus of TRPV5 (G701X) significantly decreased the Ca\(^{2+}\) sensitivity. Detailed mutation analysis revealed that a domain upstream in the carboxyl-terminus (between E649 and C653) forms a second critical stretch for Ca\(^{2+}\)-dependent inactivation of TRPV5 (Nilius et al. 2001a). Ca\(^{2+}\) influx is a prerequisite for Ca\(^{2+}\)-dependent channel inhibition because the Ca\(^{2+}\)-impermeable D542A mutant lacks a monovalent current decay in response to repetitive stimulation (Nil-
ius et al. 2001c). These data suggest that TRPV5 and TRPV6 channels are downregulated by Ca^{2+} influx through the channel, which increases the Ca^{2+} concentration in a microdomain near the pore region. Considering the high affinity of Ca^{2+}-dependent TRPV5 and TRPV6 inhibition, the presence of intracellular Ca^{2+} buffer proteins such as calbindins plays an important role to maintain channel activity (Lambers et al. 2006b). Measurements of the endogenous TRPV5 or TRPV6 activity in native primary cells that express all proteins involved in transepithelial Ca^{2+} transport would therefore provide another stimulus to our knowledge on the Ca^{2+}-dependent regulation of these channels. Due to the low endogenous expression of the channels, such data remain unavailable to date.

Nilius et al. showed that recovery from inhibition occurred both upon washout of extracellular Ca^{2+} (whole-cell configuration) or by removal of Ca^{2+} from the inner side of the channel (inside-out patches) (Nilius et al. 2001a). However, this process does not simply correlate with the removal of intracellular Ca^{2+}, since full recovery occurs much later than restoration of the basal Ca^{2+} level in non Ca^{2+}-buffered cells, or after removing Ca^{2+} from the inner side of excised membrane patches (Nilius et al. 2001a). Therefore, the recovery from the Ca^{2+}-dependent inactivation seems to be mediated by a mechanism distinct from Ca^{2+}-dependent inactivation. It is currently unknown whether the recovery reflects reopening of channels present in the plasma membrane or insertion of new channels into the plasma membrane.

**Intracellular Mg^{2+} and PIP_2**  
An important feature of TRPV5 and TRPV6 is the voltage-dependent blockage by intracellular Mg^{2+} (Hoenderop et al. 2005; Lee et al. 2005; Voets et al. 2003). Both channels show nearly complete inward rectification, as no outward currents are observed in the presence of intracellular Mg^{2+}. However, the rectification is less pronounced in the absence of intracellular Mg^{2+}, although an intrinsic inward rectification remains (Hoenderop et al. 2005; Voets et al. 2003). Furthermore, in the absence of intracellular Mg^{2+}, hyperpolarizing voltage steps activate inward currents without delay. However, when 1 mM Mg^{2+} is present intracellularly, a slowly rising phase (“gating”) is observed (Hoenderop et al. 2005; Lee et al. 2005). This is due to “unblock” of the Mg^{2+}-dependent channel inhibition. The amount of Mg^{2+}-dependent blockade relies on the voltage that was present just preceding the hyperpolarization. At less negative potentials, partially blocked channels open in a time-dependent manner due to unblock. However, the “unblock” was less pronounced at highly negative potentials and after strong depolarization. These phenomena can be explained as follows: at mild depolarizing potentials, Mg^{2+} moves toward the pore thereby plugging the permeation pathway for monovalent ions. Unblock occurs at hyperpolarizing voltages. At very large depolarization Mg^{2+} is pushed through the pore, which results in a partial unblock of the channels. These three features (i.e., rectification, gating, and voltage-dependence) only appear in the presence of intracellular Mg^{2+}. In the absence, gating and voltage-dependence disappear, whereas rectification is still present, but diminished (Hoenderop et al. 2005; Lee et al. 2005; Voets et al. 2003). This fast voltage-dependent block of TRPV5 was confirmed by Lee et al. However, they also demonstrated a slower Mg^{2+}-dependent channel blockade (tens of seconds compared to milliseconds; Lee et al. 2005). Interestingly, the aspartate at position 542 in the channel pore is responsible for both the fast and slow component of Mg^{2+}-mediated channel blockade (Lee et al. 2005). Phosphatidylinositol bisphosphate (PIP_2) reduces the sensitivity of TRPV5 for this slow Mg^{2+}-induced inhibition (Lee et al. 2005). A role for PIP_2 in the regulation of TRPV5 was also described by Rohacs et al. Application of PIP_2 to inside out patches of TRPV5 expressing *Xenopus* oocytes resulted in a significant current increase (Rohacs et al. 2005).
They illustrated a role for arginine at position 599 of (rat) TRPV5 that seems critical for the direct interaction with PIP2. Mutation of other positively charged residues in this area including K587Q and R600Q (most likely referring to K600Q and R587Q) resulted in only modest amplitude decreases. Lee et al. indeed demonstrated that the rabbit TRPV5 mutant corresponding to R599Q (of rat TRPV5) was more sensitive to inhibition by intracellular Mg2+, in line with a reduced PIP2 binding (Lee et al. 2005). The physiological role of the regulation of TRPV5 by intracellular Mg2+ in a PIP2-modulated fashion needs to be further addressed, but could be related to receptor-activated PLC signaling.

Intra- and extracellular protons Early studies in excitable tissues indicated that acidic pH inhibits voltage-gated Na+ channel activity (Woodhull 1973). Hess and collaborators observed similar findings for L-type Ca2+ channels (Prod’hom et al. 1989). Changes in pH have been found to regulate a number of TRP channels. Extracellular acidic pH increases TRPV1 currents (Caterina and Julius 2001) and decreases the activity of TRPP2 (Gonzalez-Perrett et al. 2002), a distant TRP family member of TRPV5 and TRPV6. Acidification of the apical medium inhibits transcellular Ca2+ absorption across primary cultures of rabbit CNT and CCD cells, providing a pH-dependent activity of the apical Ca2+ influx pathway (Bindels et al. 1994). Importantly, it was previously demonstrated that 45Ca2+ uptake in TRPV5-expressing *Xenopus laevis* oocytes is inhibited by acidification of the incubation medium (Hoenderop et al. 1999c; Peng et al. 2000a). Indeed, extracellular acidification reduced currents through TRPV5 carried by either monovalent or divalent cations (Peng et al. 2000a; Veenekens et al. 2001b). Recently, the mechanism of proton-dependent modulation of TRPV5 channel properties has been addressed by Huang and coworkers. First, Yeh et al. demonstrated that mutation of the glutamate at position 522, preceding the pore region, to glutamine (E522Q) decreases the inhibition of the channel by extracellular protons. Therefore, this residue may act as the “pH sensor” of TRPV5 (Yeh et al. 2003). In a follow-up paper, the same group demonstrated that also intracellular protons inhibit TRPV5 activity (Yeh et al. 2005). Seventeen amino acids surrounding the putative intracellular entrance of the pore were mutated into a nontitratable amino acid and only mutation of lysine-607 to asparagine (K607N) decreased the sensitivity of the channel to inhibition by intracellular acidification. Measurements of the relative permeability of inorganic monovalent cations to Na+ indicated that both intra- and extracellular acidification reduces the estimated TRPV5 pore diameter. This is possibly due to a rotation of the pore helix, as shown by measurements of the accessibility of pore residues to a cysteine-reactive agent, which blocks the channel upon covalent binding (Yeh et al. 2005). Extrapolating the pH-dependence of TRPV5 to the in vivo situation suggests that inhibition of TRPV5 by protons may at least in part provide the molecular basis of acidosis-induced calciuresis. At least two additional mechanisms contribute to the calciuresis. The first is pH-dependent modulation of gene expression of Ca2+ transport proteins, as will be discussed in “Acidosis and alkalosis” (Nijenhuis et al. 2006). Second, Lambers et al. demonstrated a pH-dependent translocation of TRPV5 to the plasma membrane. This mechanism stimulated TRPV5 activity at higher pH due to an increase in the number of TRPV5 channels at the cell surface (Lambers et al. 2006c; Nilius and Mahieu 2006).

**TRPV5 knockout mice** Hoenderop and coworkers generated TRPV5 knockout (TRPV5<sup>−/−</sup>) mice that displayed a number of alterations that could be linked to modification of the Ca2+ balance (Hoenderop et al. 2003a). First, metabolic studies demonstrated that TRPV5<sup>−/−</sup> mice exhibit a robust calciuresis, since significantly more Ca2+ was excreted in
the urine compared to wild-type littermates. Second, serum analysis showed that TRPV5\(^{-/-}\) mice have normal serum Ca\(^{2+}\) concentrations, but significantly elevated levels of active vitamin D (1,25(OH)\(_2\)D\(_3\)) compared to wild-type littermates. Third, Ca\(^{2+}\) absorption in small intestine was significant increased in TRPV5\(^{-/-}\) mice indicating a compensatory role of the small intestine. Fourth, TRPV5\(^{-/-}\) mice show a severe bone phenotype.

One of the most appealing aspects of the phenotype of TRPV5\(^{-/-}\) mice is their massive renal Ca\(^{2+}\) excretion (Hoenderop et al. 2003a). The urinary Ca\(^{2+}\) concentration of TRPV5\(^{-/-}\) mice reached values 4–10 times higher compared to wild-type mice. This hypercalciuria persists during life as it was observed in mice ranging from 10 to 52 weeks (Van Abel et al. 2006). In vivo micropuncture studies were performed in these transgenic mice to pinpoint the defective site of the Ca\(^{2+}\) reabsorption along the nephron. Ca\(^{2+}\) reabsorption in TRPV5\(^{-/-}\) mice was unaffected up to the last surface loop of the late proximal tubule (LPT). However, mean Ca\(^{2+}\) delivery to puncturing sites within DCT and CNT was significantly enhanced in TRPV5\(^{-/-}\) mice (Hoenderop et al. 2003a). This defect in Ca\(^{2+}\) reabsorption along the DCT and CNT is consistent with the localization of TRPV5 in mice (Loffing et al. 2001). Interestingly, polyuria and polydipsia was consistently observed in TRPV5\(^{-/-}\) mice compared to wild-type littermates (Hoenderop et al. 2003a). Polyuria reduces the potential risk of Ca\(^{2+}\) precipitations and thereby facilitates the excretion of large quantities of Ca\(^{2+}\). The hypercalciuria-induced polyuria has been observed in humans (Miller and Stapleton 1989) and animal models (Frick and Bushinsky 2003; Puliyanda et al. 2003). Exactly how the increased luminal Ca\(^{2+}\) concentration induces polyuria is unknown. It is postulated that the high Ca\(^{2+}\) concentration activates the Ca\(^{2+}\)-sensing receptor (CaSR) in the apical membrane of the inner medullary collecting duct (IMCD), which could stimulate the retrieval of aquaporin 2 (AQP2) to reduce water reabsorption (Sands et al. 1997). Furthermore, TRPV5\(^{-/-}\) mice produced urine that was significantly more acidic compared to wild-type mice. Acidification of the urine could also contribute to the prevention of renal stone formation during hypercalciuria, since Ca\(^{2+}\) precipitates will not form at pH 5–6 (Baumann 1998).

A significant increase in the rate of Ca\(^{2+}\) absorption in the small intestine was observed in TRPV5\(^{-/-}\) mice compared to wild-type littermates, indicating an intestinal compensation for renal Ca\(^{2+}\) wasting (Hoenderop et al. 2003a; Renkema et al. 2005). Duodenal Ca\(^{2+}\) absorption gradually decreased upon aging to 52 weeks in wild-type and TRPV5\(^{-/-}\) mice, but remained elevated in the latter compared to age-matched wild-type mice (Van Abel et al. 2006). Intestinal TRPV6 and calbindin-D\(_{9K}\) expression levels were significantly upregulated in TRPV5\(^{-/-}\) mice consistent with this increased Ca\(^{2+}\) absorption (Hoenderop et al. 2003a). To address the role of 1,25(OH)\(_2\)D\(_3\) in Ca\(^{2+}\) hyperabsorption double-knockout mice (TRPV5\(^{-/-}\)/1\(\alpha\)-OHase\(^{-/-}\) mice) were generated that lack the TRPV5 and 25-hydroxyvitamin-D\(_3\)-1\(\alpha\)-hydroxylase genes of which the latter is responsible for the production of 1,25(OH)\(_2\)D\(_3\). Renkema et al. demonstrated that increased serum levels of 1,25(OH)\(_2\)D\(_3\) in TRPV5\(^{-/-}\) mice were essential for this compensatory Ca\(^{2+}\) hyperabsorption. TRPV5\(^{-/-}\)/1\(\alpha\)-OHase\(^{-/-}\) mice have undetectable serum levels of 1,25(OH)\(_2\)D\(_3\) and display a significant hypocalcemia (Renkema et al. 2005). Intestinal TRPV6 and calbindin-D\(_{9K}\) expression levels were decreased compared with wild-type mice. The renal Ca\(^{2+}\) leak, as demonstrated in TRPV5\(^{-/-}\) mice, persisted in TRPV5\(^{-/-}\)/1\(\alpha\)-OHase\(^{-/-}\) mice, but a compensatory upregulation of intestinal Ca\(^{2+}\) transporters was abolished (Renkema et al. 2005). From these results, it was concluded that hypervitaminosis D is of crucial importance in TRPV5\(^{-/-}\) mice to maintain normocalcemia despite impaired renal Ca\(^{2+}\) reabsorption.

The molecular mechanisms involved in bone resorption and formation are largely elusive, although both processes likely require transcellular transport of Ca\(^{2+}\). Detailed analyses
of femurs demonstrated that trabecular thickness in the femoral head of TRPV5−/− mice was drastically reduced compared with TRPV5+/+ mice (Hoenderop et al. 2003a). Furthermore, the cortical bone volume, cortical volume fraction, and cortical bone thickness were decreased in TRPV5−/− versus wild-type mice. Van der Eerden et al. further investigated the functional role of TRPV5 in bone. TRPV5 mRNA was expressed in human and murine bone samples and in osteoclasts along with other genes involved in transcellular Ca2+ transport, including calbindin-D9K and calbindin-D28K, Na+/Ca2+ exchanger 1, and plasma membrane Ca2+-ATPase 1b (Van der Eerden et al. 2005). TRPV5 shows predominant localization to the ruffled border membrane of murine osteoclasts. However, TRPV5 was absent in osteoblasts (Van der Eerden et al. 2005). Analyses of femoral bone sections and an in vitro bone marrow culture system revealed increased osteoclast numbers and osteoclast area in TRPV5−/− mice, whereas a urinary bone resorption marker was reduced compared to TRPV5+/+ mice. Using a functional resorption pit assay, it was, however, found that bone resorption was nearly absent in osteoclast cultures from TRPV5−/− mice, supporting the impaired resorption observed in vivo (Van der Eerden et al. 2005). Although this study clearly showed that TRPV5 is essential for osteoclastic bone resorption and demonstrates the significance of transcellular Ca2+ transport in osteoclasts, it does not explain the bone phenotype of TRPV5−/− mice. As osteoclastic function is impaired, one would expect increased bone thickness. However, TRPV5−/− mice show reduced bone thickness suggesting functional Ca2+ resorption from bone. Furthermore, in TRPV5−/−/1α-OHase−/− mice, rickets was even more pronounced than observed in single TRPV5 or 1α-OHase−/− mice, suggesting that the high vitamin D levels do not cause the reduced bone thickness in TRPV5−/− mice (Renkema et al. 2005). Perhaps the answer lies in the role of bone formation by osteoblasts. This process remains largely elusive, and the role of transcellular Ca2+ transport and TRPV5 and/or TRPV6 therein should be one of the first topics of future investigation.

In summary, targeted ablation of the TRPV5 gene seriously disturbs renal Ca2+ handling, causing increased 1,25(OH)2D3 serum levels, Ca2+ hyperabsorption, and reduced bone formation. These data from TRPV5−/− mice convincingly demonstrate that TRPV5 is the gatekeeper in active Ca2+ reabsorption. Similarly, TRPV6 provides a good candidate as the apical Ca2+ influx channel involved in intestinal Ca2+ absorption. However, the creation of TRPV6 knockout mice to obtain the most direct and ultimate proof for this hypothesis has not been achieved to date.

**Intracellular Ca2+ transport**

The process of transcellular Ca2+ transport places a substantial and continuous challenge on epithelial cells, as substantial amounts of Ca2+ traffic through the cytosol, while the cytosolic free Ca2+ concentration ([Ca2+]i) needs to be maintained at low levels. Results from mathematical modeling (Bronner and Stein 1988) have indicated that diffusion of free Ca2+ at physiological cytosolic concentrations can only account for about 1/70th of the observed rate of transcellular transport (Costanzo and Windhager 1978). To date, two models have been proposed to explain this discrepancy.

**Facilitated diffusion** In the first model, termed the “facilitated diffusion model,” intracellular Ca2+-binding proteins keep the free Ca2+ concentration low, while the diffusible cellular Ca2+ concentration is high enough to support massive transcellular Ca2+ rates (Bronner et al. 1986; Bronner and Stein 1988; Feher 1983; Feher and Wasserman 1979; Feher et al. 1992; Larsson and Nemere 2002). The abundance of these Ca2+-buffering proteins is regulated by calcitropic hormones, i.e., vitamin D and PTH, to ensure sufficient Ca2+...
(re)absorption capacity in conditions when this is required. Indeed, there are two major subclasses of vitamin D-dependent Ca\(^{2+}\)-binding proteins, calbindin-D\(_{9K}\) and calbindin-D\(_{28K}\). Calbindin-D\(_{28K}\) is highly conserved during evolution and present in kidney, small intestine (only birds), pancreas, placenta, bone, and brain. Calbindin-D\(_{9K}\) is present in highest concentrations in small intestine as well as in kidney (only mouse). The expression level of these calbindins in kidney and intestine is closely correlated with the efficiency of Ca\(^{2+}\) (re)absorption; calbindins, therefore, play a central role in the facilitated diffusion model. Even though these buffering proteins diffuse more slowly than the Ca\(^{2+}\) ion (inversely related to the square root of the molecular weight), the intracellular concentration of calbindins (submillimolar range) is sufficient to raise the total diffusible Ca\(^{2+}\) concentration to the level needed to attain the experimental diffusion rates (Bronner and Stein 1988). Importantly, due to the relatively slow binding kinetics of these Ca\(^{2+}\)-binding proteins, Ca\(^{2+}\) signaling can occur independently of transcellular Ca\(^{2+}\) movement mediated by calbindin-D\(_{9K}\) and calbindin-D\(_{28K}\) (Koster et al. 1995). In addition, the Ca\(^{2+}\)-buffering activity of the calbindins plays an important role to maintain TRPV5 and TRPV6 channels in an open conformation. These channels are rapidly inactivated upon a rise in the [Ca\(^{2+}\)]\(_i\), and therefore it is pivotal to keep [Ca\(^{2+}\)]\(_i\) low. Recent data show that calbindin-D\(_{28K}\) can fulfill this role for TRPV5 by direct interaction with the channel. Therefore, calbindin-D\(_{28K}\) provides epithelial cells with both local and general Ca\(^{2+}\) buffering to support high rates of transcellular Ca\(^{2+}\) transport (Lambers et al. 2006b).

**Vesicular transport** Previous studies have also proposed a vesicular model in which the Ca\(^{2+}\)-transporting cells use lysosomes to sequester Ca\(^{2+}\) and facilitate its movement to the basolateral membrane (Larsson and Nemere 2002; Nemere et al. 1986). Formation of Ca\(^{2+}\)-enriched vesicles is initiated by influx of Ca\(^{2+}\) through Ca\(^{2+}\) channels in the apical or luminal membrane. The rapid increase in [Ca\(^{2+}\)] in close vicinity to the apical membrane disrupts the actin filaments near the Ca\(^{2+}\) channels and initiates the formation of endocytic vesicles. The formed Ca\(^{2+}\)-containing vesicles are transported by microtubules and fuse with lysosomes (Larsson and Nemere 2002; Nemere and Norman 1990). While calbindins have been found to associate with lysosomes, the role of these Ca\(^{2+}\)-binding proteins in this latter model is at present unclear.

**Calbindin-D\(_{28K}\) knockout mice** Homozygous calbindin-D\(_{28K}\) knockout (calbindin-D\(_{28K}\)^\(-/-\)) mice were previously generated, which developed normally (Airaksinen et al. 1997a, b; Barski et al. 2003; Sooy et al. 1999, 2000). Calbindin-D\(_{28K}\)^\(-/-\) mice fed a regular Ca\(^{2+}\) diet displayed an approximately twofold increase in the urinary Ca\(^{2+}\) excretion compared to wild-type littermates (Sooy et al. 2000; Zheng et al. 2004). However, these mice displayed no significant differences in serum Ca\(^{2+}\) or PTH levels. This could indicate that intestinal hyperabsorption compensates for the hypercalciuria induced by calbindin-D\(_{28K}\) deficiency. Furthermore, only in mice is calbindin-D\(_{9K}\) expressed in renal epithelia. This suggests that calbindin-D\(_{9K}\) could mediate a significant amount of Ca\(^{2+}\) buffering, not only in intestine (as in all other species), but also in the mouse kidney where it colocalizes with TRPV5 (Hoenderop et al. 2002). In this respect, it is important to note that ablation of the TRPV5 gene results in a greater than sixfold increase in calciuria (Hoenderop et al. 2003a), whereas inactivation of the calbindin-D\(_{28K}\) results only in roughly twofold increase (Zheng et al. 2004), on a high Ca\(^{2+}\) diet, indicating that the renal defect in Ca\(^{2+}\) handling is submaximal in calbindin-D\(_{28K}\)^\(-/-\) mice. This suggests that TRPV5 plays a more pivotal role in Ca\(^{2+}\) reabsorption compared to calbindin-D\(_{28K}\) that could also be explained by a possible
redundancy for this calbindin. The relative contribution of TRPV5 and calbindin-D
was investigated using mice lacking both the TRPV5 and calbindin-D28K gene (Gkika et
al. 2006). This study suggests that TRPV5 is the most crucial factor of the two proteins,
as removal of the calbindin-D28K gene did not further deteriorate the Ca2+ hyperexcretion
in TRPV5-lacking mice. It is, however, also possible that calbindin-D9K now functions as
an alternative Ca2+ buffer since this protein is also expressed in mice DCT. Mutant mice
that lack the calbindin-D9K gene have recently been generated (Kutuzova et al. 2006)
and they show normal serum Ca2+ levels. However, no information about the intestinal
Ca2+ absorption or urine Ca2+ excretion in these animals was provided. Further studies
using mice lacking both calbindins would provide further insight into the role of these
Ca-buffering proteins in epithelial Ca2+ transport. Li et al. had previously demonstrated that
 genetic inactivation of the vitamin D receptor (VDR) gene leads to a 90% reduction in renal
calbindin-D9K expression, but little change in calbindin-D28K (Li et al. 2001). To address
whether calbindin-D9K compensates for the role of calbindin-D28K in Ca2+ homeostasis,
Zheng et al. generated VDR/calbindin-D28K double knockout mice, which expressed no
calbindin-D28K and only 10% of calbindin-D9K in kidney. VDR knockout mice suffer from
hypocalcemia, secondary hyperparathyroidism, rickets, and osteomalacia (Zheng et al.
2004). However, ablation of the calbindin-D28K gene further deteriorates the phenotype, as
the double knockout mice were even more growth-retarded, significantly smaller in body
weight than VDR−/− mice, and died prematurely at a few months of age. Compared with
VDR−/− mice, the VDR−/−/calbindin-D28K−/− mice had higher urinary Ca2+ excretion and
developed more severe secondary hyperparathyroidism and rachitic skeletal phenotype,
which were manifested by larger parathyroid glands, higher serum PTH levels, and much
lower bone mineral density (Zheng et al. 2004). Using histomorphometry and microcom-
puter tomography, a recent study from Margolis and coworkers showed that the femora
of calbindin-D28K−/− mice have significantly increased cortical bone volume compared to
wild-type mice (Margolis et al. 2006). These results directly suggest that calbindin-D28K
plays a distinct role in maintaining Ca2+ homeostasis and skeletal mineralization.

Ca2+ extrusion mechanisms

The energy-consuming step of transcellular Ca2+ transport lies in the Ca2+ efflux pathways.
This step transports intracellular Ca2+ to the serosal side across the basolateral membrane
against a considerable electrochemical gradient. Two Ca2+ transporters have been implicated
in this extrusion process, a Na+/Ca2+ exchange mechanism (NCX1) and a Ca2+-ATPase
(PMCA1b).

Na+/Ca2+ exchanger Counter transport of Na+ for Ca2+ across the plasma membrane
accounts for the maintenance of low [Ca2+]i in a wide variety of cells (Linck et al. 1998).
To date, three genes for NCX, designated NCX1, NCX2, and NCX3, have been identified
in mammals. Similarities between NCX1–3 include a homology of around 70% sequence
identity at the protein level, the presence of an amino-terminal signal sequence, two sets of
multiple transmembrane α-helices near the ends of the protein, and a large intracellular loop
(Blaustein and Lederer 1999; Schulze et al. 2002). A functional comparison of the three iso-
forms of NCX stably expressed in baby hamster kidney (BHK) cells failed to detect striking
differences (Linck et al. 1998).

The genes encoding NCX1, NCX2, and NCX3 have been mapped to mouse chromo-
somes 17, 7, and 12, respectively (Nicoll et al. 1996). At the posttranscriptional level, at
least 12 NCX1 and 3 NCX3 proteins are generated through alternative splicing (Kofuji et
al. 1994). These variants arise from a region of the large intracellular f loop, are encoded by six small exons defined A to F, and are used in different combinations in a tissue-specific manner (Lee et al. 1994; Nakasaki et al. 1993). All splice variants include either exon A or B (Quednau et al. 1997). Excitable tissues, such as those of the brain and heart, are usually characterized by the presence of exon A, whereas kidney, stomach, and skeletal muscle tissues comprise NCX with exon B (Quednau et al. 1997). Reilly and Shugrue identified the sequence of the rabbit kidney NCX1 (Reilly and Shugrue 1992) and in kidney the expression of this transporter is restricted to the distal part of the nephron where it is predominantly localized along the basolateral membrane (Biner et al. 2002; Hoenderop et al. 2000; Loffing et al. 2001). NCX1 is widely distributed in many different mammalian tissues, whereas NCX2 and NCX3 are only expressed in brain and skeletal muscle (Li et al. 1994; Nicoll et al. 1996). Bindels and coworkers suggested that in DCT and CNT NCX1 is the primary extrusion mechanism, whereas only a minor amount of Ca2+ is extruded by the plasma membrane Ca2+ pump (Bindels et al. 1992; Van Baal et al. 1996b). NCX1 is also expressed in the basolateral membrane of enterocytes (Hildmann et al. 1982; Kikuchi et al. 1988; Van Abel et al. 2003). In fish enterocytes, NCX appears to be the main mechanism by which Ca2+ is extruded from the cells at the basolateral surface, whereas in mammals PMCA is the predominant extrusion mechanism (Flik et al. 1990; Hildmann et al. 1982; Van Abel et al. 2003). Together, these studies suggest that in kidney basolateral Ca2+ efflux is mainly mediated by NCX1, whereas Na+/Ca2+ exchange seems less important in the small intestine. Recently, it was demonstrated that NCX1 knockout mice do not have a spontaneously beating heart and die in utero (Koushik et al. 2001; Reuter et al. 2002). Unfortunately, this animal model is, therefore, not suitable to verify the importance of NCX1 in renal epithelial Ca2+ transport. In addition, K+-dependent Na+-Ca2+ exchangers (NCKX) could play a role in cellular Ca2+ efflux (Philipson and Nicoll 2000). Northern blot analysis demonstrated that some isoforms [i.e., NCKX4 (Li et al. 2002) and NCKX6 (Cai and Lytton 2004)] of this family are expressed in epithelia including small intestine and kidney. Therefore, it remains to be established whether the NCKX transporters play a role in epithelial Ca2+ transport or NCX1 is indeed the pivotal extrusion mechanism in renal Ca2+ reabsorption.

The stoichiometry of NCX is generally accepted to be three Na+ ions per one Ca2+ ion. However, it has recently been demonstrated that ion flux ratio can vary from 1:1 to a maximum of 4:1, depending on the intracellular concentration of Na+ and Ca2+ ions (Fujioka et al. 2000; Kang and Hilgemann 2004). This variation might be most relevant in excitable cells. In resting excitable cells, when [Ca2+]i rises and the cells require the return of [Ca2+]i to resting levels (Carafoli 1985), NCX-mediated transport couples the extrusion of Ca2+ to the influx of Na+ ions into the cells down the electrochemical Na+ gradient. This mode of operation, defined as forward mode (Blaustein and Santiago 1977), maintains the steep Ca2+ gradient across the cell membrane. However, when the transmembrane Na+ electrochemical gradient is reduced, i.e., upon membrane depolarization, NCX can operate in reverse mode and mediates the extrusion of [Na+]i and the influx of Ca2+ ions (Baker et al. 1969; DiPolo 1979). Although this latter feature is an important topic in the field of neuroscience and cardiac function, the role of NCX in epithelial Ca2+ transport is probably limited to Ca2+ efflux.

**Plasma membrane Ca2+-ATPase** PMCs are high-affinity Ca2+ efflux pumps present in virtually all eukaryotic cells, wherein they are responsible for the maintenance and resetting of the resting [Ca2+]i levels (Blaustein et al. 2002). Four PMCA isoforms (PMCA1–4;
human gene symbols ATP2B1–4) have been identified in mammalian tissues (Strehler and Zacharias 2001). In addition, alternative splicing of the transcripts of these genes yields a large variety of splice variants differing mainly in their carboxyl-terminal amino acid sequence (Stauffer et al. 1993; Strehler and Zacharias 2001). PMCA1 and PMCA4 are expressed in all tissues, suggesting that they are housekeeping genes, involved in the maintenance of cellular Ca\(^{2+}\) homeostasis (Greeb and Shull 1989; Stauffer et al. 1993). In contrast, the limited tissue distribution of PMCA2 and PMCA3 suggests that they have tissue-specific functions. The relevance for PMCA as a system for the extrusion of Ca\(^{2+}\) in Ca\(^{2+}\)-transporting epithelial cells is variable between tissues. In the kidney, PMCA 1b, 2b, and 4b isoforms are present in all nephron segments (Caride et al. 1998; Magosci et al. 1992). Compared to other nephron segments, DCT shows the strongest immunocytochemical reactivity for PMCA protein expression and the highest Ca\(^{2+}\)-ATPase activity (Borke et al. 1989; Borke et al. 1987; Doucet and Katz 1982; Magosci et al. 1992). PMCA1b transcripts were demonstrated in rabbit CNT and collecting duct (CD), whereas expression of the PMCA2 isoform was not detected (Hoenderop et al. 2000; Kip and Strehler 2003). In addition, Strehler and coworkers demonstrated in Madin-Darby canine kidney (MDCK) cells that PMCA4b plays a significant role in basolateral Ca\(^{2+}\) extrusion (Kip and Strehler 2003, 2004). However, in renal epithelia, NCX mediates the majority (~70%) of the Ca\(^{2+}\) efflux in Ca\(^{2+}\)-transporting cells in DCT and CNT, whereas PMCA mediates the extrusion of the remaining 30% (Bindels et al. 1992). In addition, PMCA probably serves a general role in the maintenance of cellular Ca\(^{2+}\) homeostasis of all other nephron segments. Interestingly, in the small intestine PMCA1b is abundantly expressed, whereas NCX1 is expressed only at low levels. This suggests that PMCA1b is the principal Ca\(^{2+}\) extrusion mechanism in intestinal Ca\(^{2+}\) absorption (Van Abel et al. 2002; Wasserman and Fullmer 1995).

Recent studies of mice carrying PMCA1, PMCA2, or PMCA4 null mutations reveal the in vivo functions of these isoforms. Mice that are deficient in PMCA3 have not been generated yet. The PMCA1 gene was disrupted by removal of sequences encoding the catalytic phosphorylation site (Okunade et al. 2004). When heterozygous mutant mice were bred, only wild-type and heterozygous mutants were observed at birth, whereas null mutants showed early embryolethality. Heterozygous PMCA1 mutants exhibit no apparent disease phenotype. These observations suggest that PMCA1 serves a critical housekeeping function. Because PMCA1b is ubiquitous (Keeton et al. 1993; Stauffer et al. 1993), it is likely to be the major housekeeping form of the enzyme. Furthermore, the role of PMCA2 was demonstrated using two mouse strains [deafwaddler (Street et al. 1998) and wriggle mouse Sagami (Takahashi and Kitamura 1999)], carrying PMCA2 mutations, and in PMCA2 knockout mice (Kozel et al. 1998), all displaying phenotypes with deafness and having problems keeping their balance related to Ca\(^{2+}\) handling in the inner ear. PMCA4 null mutants survive and appear healthy (Okunade et al. 2004; Schuh et al. 2004). However, an in vitro apoptosis phenotype was identified in smooth muscle of isolated portal veins from PMCA4 knockout mice on a mixed 129SvJ and black Swiss background (Okunade et al. 2004), indicating that loss of PMCA4 could lead to Ca\(^{2+}\) overload and apoptotic cell death under some conditions, in particular when the loss of PMCA4 was combined with the loss of a single copy of the PMCA1 gene. This suggests that PMCA1 is the major housekeeping isoform required for maintenance of [Ca\(^{2+}\)]\(_i\) but that PMCA4 can contribute to this function in certain tissues. Moreover, PMCA4 plays an important role in testis, as male PMCA4 null mutant mice are infertile, because the sperm cells are unable to achieve hyperactivated motility (Okunade et al. 2004; Schuh et al. 2004).
In conclusion, ATP driven Ca\(^{2+}\) extrusion and Na\(^{+}\)-Ca\(^{2+}\) exchange both play a role in epithelial Ca\(^{2+}\) extrusion with PMCA1 and NCX1, respectively forming the molecular identity of this process.

Regulation of epithelial Ca\(^{2+}\) transport

1,25-dihydroxyvitamin D\(_{3}\)

The vitamin D\(_{3}\) endocrine system is critical for the proper development and maintenance of the Ca\(^{2+}\) balance (Jones et al. 1998). There are two sources of vitamin D\(_{3}\) in the body. It is either ingested with the diet or synthesized in the skin from its precursor 7-dehydrocholesterol in the presence of sunlight (Neer 1975). Vitamin D\(_{3}\) itself is physiologically inactive. It will undergo a modification process, starting with 25-hydroxylation in the liver. Subsequently, the active form of vitamin D, 1,25-dihydroxyvitamin D\(_{3}\) (1,25(OH)\(_{2}\)D\(_{3}\)), is synthesized in the PT by the renal cytochrome P450 enzyme 25-hydroxyvitamin D\(_{3}\)-1\(\alpha\)-hydroxylase (1\(\alpha\)-OHase) (Fraser and Kodicek 1970; Jones et al. 1998).

The biological effects of 1,25(OH)\(_{2}\)D\(_{3}\) on target organs are mediated by both genomic and rapid posttranscriptional mechanisms (Jones et al. 1998). 1,25(OH)\(_{2}\)D\(_{3}\) transcriptionally controls the expression of a particular set of target genes (Table 2). The genomic mechanism of action is similar to that of other steroid hormones and is mediated by stereospecific interaction of 1,25(OH)\(_{2}\)D\(_{3}\) with a nuclear VDR. Upon binding of 1,25(OH)\(_{2}\)D\(_{3}\), the VDR undergoes a conformational change and forms a complex with a retinoid X receptor (RXR). This VDR–RXR complex binds to DNA elements in the promoter regions of target genes described as vitamin D response elements (VDREs). Binding to these VDREs controls the rate of gene transcription. Importantly, VDR is expressed in epithelia that play a role in Ca\(^{2+}\) (re)absorption. The intestine and kidney are the main target organs for the calciotropic action of this hormone, although vitamin D affects many processes i.e., notably in the skin and immune system, but also directly in bone and parathyroid gland (DeLuca 2004). The rapid response of vitamin D presumably utilizes a VDR-independent signal transduction pathway that is probably linked to putative plasma membrane receptors for 1,25(OH)\(_{2}\)D\(_{3}\). The physiological relevance of rapid actions of vitamin D is not well understood.

A number of human diseases are associated with the role of active vitamin D. These disorders are often characterized by defective bone mineralization and clinical features of rickets, poor growth as an infant, and hypocalcemia and can have at least two distinct genetic causes (Jones et al. 1998; Van de Graaf et al. 2004). Vitamin D-dependent rickets type I (VDDR I) is due to an enzymatic defect in synthesis of the active form of vitamin D (Kitanaka et al. 1998; Panda et al. 2001). It has also been referred to as “pseudovitamin D-deficiency rickets” (Prader et al. 1961). VDDR I is an autosomal recessive disorder caused by mutations in the gene encoding 25-hydroxyvitamin D\(_{3}\)-1\(\alpha\)-hydroxylase on chromosome 12q14 (Kitanaka et al. 1998). Patients with this disease show markedly decreased serum 1,25(OH)\(_{2}\)D\(_{3}\), hyperparathyroidism, and normal serum 25-hydroxyvitamin D\(_{3}\). A similar disease is caused by end-organ unresponsiveness of active vitamin D due to recessive mutations in the gene encoding the vitamin D receptor (Hughes et al. 1988; Pike et al. 1984). Vitamin D-dependent rickets type II (VDDR II) is caused by a defect in the vitamin D receptor gene. This defect leads to an increase in the circulating ligand, 1,25(OH)\(_{2}\)D\(_{3}\). Furthermore, alopecia (hair loss) is often observed in these patients. Often mutations had been found in the highly conserved amino-terminal DNA-binding domain of the VDR, a location that does not affect the ligand-binding properties of the receptor. This explains that the
Table 2 Regulation of the epithelial Ca\(^{2+}\) channels

<table>
<thead>
<tr>
<th>Regulatory factor</th>
<th>TRPV5</th>
<th>TRPV6</th>
<th>Affected process</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D</td>
<td>+</td>
<td>+</td>
<td>Transcription</td>
<td>Hoenderop et al. 2001a; Hoenderop et al. 2005; Van Cromphaut et al. 2001</td>
</tr>
<tr>
<td>PTH</td>
<td>+</td>
<td>=</td>
<td>Transcription</td>
<td>Van Abel et al. 2005</td>
</tr>
<tr>
<td>Dietary Ca(^{2+})</td>
<td>+(^a)</td>
<td>+(^a)</td>
<td>Transcription</td>
<td>Hoenderop et al. 2002; Van Abel et al. 2003</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>–</td>
<td>ND</td>
<td>Transcription</td>
<td>Nijenhuis et al. 2004</td>
</tr>
<tr>
<td>Acidosis</td>
<td>–</td>
<td>ND</td>
<td>Transcription/channel activity/ trafficking</td>
<td>Nijenhuis et al. 2006; Vennekens et al. 2001b; Yeh et al. 2005</td>
</tr>
<tr>
<td>Klotho</td>
<td>+</td>
<td>+</td>
<td>Trafficking</td>
<td>Chang et al. 2005</td>
</tr>
<tr>
<td>S100A10/annexin 2</td>
<td>+</td>
<td>+</td>
<td>Trafficking</td>
<td>Van de Graaf et al. 2003</td>
</tr>
<tr>
<td>Rab11a</td>
<td>+</td>
<td>+</td>
<td>Trafficking</td>
<td>Van de Graaf et al. 2006a</td>
</tr>
<tr>
<td>FKBP52</td>
<td>–</td>
<td>ND</td>
<td>Not known</td>
<td>Gkika et al. 2006</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>=</td>
<td>+</td>
<td>Channel activity</td>
<td>Lambers et al. 2004; Niemeyer et al. 2001</td>
</tr>
<tr>
<td>80K-H</td>
<td>+</td>
<td>ND</td>
<td>Channel activity</td>
<td>Gkika et al. 2004</td>
</tr>
<tr>
<td>[Ca(^{2+})](^i)</td>
<td>–</td>
<td>–</td>
<td>Channel activity</td>
<td>Hoenderop et al. 2001b</td>
</tr>
<tr>
<td>NHERF2/SGK1</td>
<td>+</td>
<td>ND</td>
<td>Not known</td>
<td>Embark et al. 2004</td>
</tr>
<tr>
<td>NHERF4</td>
<td>=</td>
<td>=</td>
<td>Not known</td>
<td>Van de Graaf et al. 2006c</td>
</tr>
<tr>
<td>BSPRY</td>
<td>+</td>
<td>ND</td>
<td>Not known</td>
<td>Van de Graaf et al. 2006d</td>
</tr>
</tbody>
</table>

\(^a\) In vitamin D-depleted mice

“+” and “−” indicate stimulation or inhibition, respectively, of the indicated process including transcription, trafficking and channel activity of TRPV5/6. ND means not determined and “=’” indicates no effect.

TRPV5, transient receptor potential cation channel subfamily V member 5; TRPV6, transient receptor potential cation channel subfamily V member 6; PTH, parathyroid hormone; FKBP52, FK binding protein 52; [Ca\(^{2+}\)]\(^i\), intracellular Ca\(^{2+}\) concentration; NHERF2, Na\(^+/\)H\(^+\) exchanger regulatory factor 2; SGK1, serum and glucocorticoid-regulated kinase 1; NHERF4 Na\(^+/\)H\(^+\), exchanger regulatory factor 4; BSPRY, B-box and SPRY domain containing protein. See text for explanation.

receptor can sometimes still bind vitamin D, while the calcitropic genomic consequences are absent (Malloy et al. 1997). Furthermore, vitamin D-dependent rickets type II can be associated with a normal vitamin D receptor cDNA sequence. The VDR suppressive effect in these patients was due to overexpression of a heterogeneous nuclear ribonucleoproteins (hnRNPs) that specifically interacted with a DNA response element known to bind retinoid X receptor-VDR heterodimers, interfering with the vitamin D receptor–DNA interaction (Chen et al. 2003).

Targeted deletion of genes encoding 1α-OHase (Dardenne et al. 2001; Panda et al. 2001) and of the nuclear VDR (Li et al. 2001; Takeyama et al. 1997; Van Cromphaut et al. 2001; Yoshizawa et al. 1997) have provided useful mice models of inherited human diseases of VDDR I and VDDR II. St. Arnaud and coworkers generated 1α-OHase knockout (1α-OHase\(^{-/-}\)) mice that represent a unique animal model for VDDR I since these mice display undetectable 1,25(OH)\(_2\)D\(_3\) concentrations, hypocalcemia, and secondary hyperparathyroidism. On a normal diet, 1α-OHase\(^{-/-}\) mice have an average lifespan of approximately 12 weeks (Dardenne et al. 2001; Hoenderop et al. 2002). In addition, the 1α-OHase\(^{-/-}\) mice developed distinct histological evidence of rickets and osteomalacia (Dardenne et al. 2001; Panda et al. 2001). Previous studies indicated that daily injections of 1,25(OH)\(_2\)D\(_3\) com-
pletely rescued these 1α-OHase−/− mice (Dardenne et al. 2003b). Bone histology and histomorphometry confirmed that the rickets and osteomalacia were cured by this 1,25(OH)2D3 supplementation. Blood analysis further revealed that the rescue treatment corrected the hypocalcaemia and secondary hyperparathyroidism.

Interestingly, in this VDDR I mouse model, there was a positive correlative relationship between the expression level of TRPV5, calbindin-D28K, and NCX1 proteins in kidney, TRPV6, calbindin-D9K, and PMCA1b in duodenum, and the serum Ca2+ concentration (Hoenderop et al. 2002; Van Abel et al. 2002, 2003). Normalization of the serum Ca2+ concentration by 1,25(OH)2D3 supplementation was associated with a restoration of the expression level of the Ca2+ transporters, confirming the essential role of these proteins in active 1,25(OH)2D3-mediated Ca2+ (re)absorption. Analogous observations were made from experiments performed with VDR knockout mice (Van Cromphaut et al. 2001; Weber et al. 2001a). In these hypocalcemic mice, urinary Ca2+ excretion is inappropriately high, suggesting renal Ca2+ wasting due to disturbed Ca2+ reabsorption. Furthermore, it has been demonstrated in this mouse model that duodenal TRPV5 and TRPV6 levels are dramatically downregulated (Van Cromphaut et al. 2001; Weber et al. 2001a). Calbindin-D9K expression was also downregulated, although to a lesser extent.

Finally, more recent evidence for a role of vitamin D in the positive regulation of the epithelial Ca2+ channel comes from two distinct double knockout models. First, it was shown that the increased vitamin D levels observed in TRPV5−/− mice are pivotal for the compensatory intestinal hyperabsorption seen in these mice. This was demonstrated using mice TRPV5−/−/1α-OHase−/− double knockout mice (Renkema et al. 2005). Second, 1α-OHase−/− and PTH double knockout mice were created to eliminate a possible role of PTH during vitamin D administration. Administration of 1,25(OH)2D3 upregulated mRNA and protein levels of the renal TRPV5, calbindin-D28K, calbindin-D9K and NCX1, increased serum Ca2+ concentration and stimulated bone formation (Xue et al. 2006).

The correlation between vitamin D and the expression level of the Ca2+ transport proteins has also been addressed in several cell models. Wood and coworkers observed the correlation between the 1,25(OH)2D3-induced expression of TRPV6, calbindin-D9K, and PMCA1b and transcellular Ca2+ transport in Caco2 cells, a model duodenal cell line (Fleet et al. 2002; Wood et al. 2001). Furthermore, in controlled tissue culture conditions using primary cultures from the distal part of the nephron including DCT and CNT, a direct relationship between 1,25(OH)2D3-induced expression of Ca2+ transport proteins and transcellular Ca2+ transport was also shown (Bindels et al. 1991; Van Baal et al. 1996b). In contrast, Barley et al. could not confirm the generally observed vitamin D-dependent sensitivity of TRPV6 in duodenal biopsies from 20 normal volunteers. However, samples were taken from individuals that formed a very variable population of men and women of age 25–71 years (Barley et al. 2001). It is hypothesized that both vitamin D levels and the expression of Ca2+ transport proteins is age-dependent and possibly gender-sensitive, which could explain the tenfold variation between the lowest and the highest level of TRPV6 expression. This variation made it hard to disclose a relationship between TRPV6 expression and vitamin D metabolites in this study. TRPV5 and TRPV6 promoter analysis indicated that there are functional VDREs located upstream of the start codon (Hoenderop et al. 2001a; Meyer et al. 2006; Peng et al. 2000b; Weber et al. 2001a). Mutagenesis of the VDREs within the −2.1-kb and −4.3-kb region and the VDRE at −1.2 kb abrogated all response to 1,25(OH)2D3 when examined within the TRPV6 promoter (Meyer et al. 2006). Taken together, vitamin D-deficient animal models and epithelial cell lines demonstrated a consistent 1,25(OH)2D3-sensitivity of TRPV5, TRPV6, and the calbindins and to a lesser extent the basolateral extrusion systems NCX1 and PMCA1b.
Parathyroid hormone

The parathyroid glands play a key role in maintaining the extracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_{e}$) through their secretion of PTH (Potts 2005). PTH controls the extracellular Ca$^{2+}$ balance by activation of the PTH receptor, regulating concerted Ca$^{2+}$ transport in bone, intestine, and kidney. Parathyroid cells sense decreases in [Ca$^{2+}$]$_{e}$ by means of the CaSR, to increase PTH secretion. Brown et al. identified CaSR by expression cloning in Xenopus laevis oocytes using parathyroid cell cDNA (Brown et al. 1993). CaSR comprises a large extracellular region, forming the Ca$^{2+}$ binding site, followed by a seven-membrane-spanning domain typical for members of the G protein-coupled receptor superfamily. In addition to parathyroid tissue, the receptor was also expressed in regions of the kidney involved in regulated Ca$^{2+}$ and Mg$^{2+}$ reabsorption (Lee et al. 1996; Yang et al. 1997). PTH itself acts primarily on kidney and bone, where it activates the PTH receptor (Juppner et al. 1991; Mannstadt et al. 1999). This receptor can also bind the parathyroid-related protein (PTHrP). PTHrP was isolated from a human lung cancer cell line (Moseley et al. 1987). Although a separate gene encodes PTHrP, eight of the first 13 amino acids in the mature peptide are identical to those of PTH. Distinct pathophysiological manifestations are associated with changes in PTH levels in the blood. Garfield and Karaplis reviewed the various causes and clinical forms of hypoparathyroidism (Garfield and Karaplis 2001). Hypoparathyroidism is characterized by hypocalcemia and hyperphosphatemia, the first causing the most severe symptoms, including tetany (Potts 2005). In contrast, hyperparathyroidism is characterized by hypercalcemia and often severe bone loss (Potts 2005). Ca$^{2+}$ handling by the kidney is also abnormal in individuals with hyperparathyroidism, who fail to show a normal hypercalciuric response to hypercalcemia. There are multiple factors that can lead to hyper- or hypoparathyroidism. Hypoparathyroidism manifests when insufficient PTH is secreted from the parathyroid glands to maintain normal [Ca$^{2+}$]$_{e}$ or less commonly when PTH is unable to function optimally in target tissues, despite adequate circulating levels. The latter is due to mutations in the PTH-receptor (Pearce et al. 1995, 1996; Pollak et al. 1993). Low plasma PTH levels are caused by trauma to the parathyroids during neck surgery or mutations of the PTH or CaSR (CASR) genes (Lovlie et al. 1996; Parkinson and Thakker 1992). Furthermore, the gene causing the X-linked recessive form of hypoparathyroidism remains to be identified, although Bowl et al. recently suggested a role for the transcription factor SOX3 in the development of this disease (Bowl et al. 2005). Hyperparathyroidism is observed in patients with chronic renal insufficiency. Furthermore, excessive PTH secretion can be due to parathyroid tumor. In addition, PTHrP is responsible for most cases of hypercalcemia of malignancy of other cells (Strewler 2000). Individuals with mutations in CaSR have an altered relation between PTH secretion and serum Ca$^{2+}$ concentration. Mutations in CaSR can result in reduced PTH secretion or in hypersecretion of PTH, depending whether the mutations activate or inactivate the receptor, respectively.

Recent investigations on PTH focus also on its role as a therapy in osteoporosis (Hodsmman et al. 2003; Potts 2005). Paradoxically, although hyperparathyroidism is associated with severe bone loss, administration of PTH restores bone mass and strength and reduces fracture incidence in the treatment of postmenopausal osteoporosis (Potts 2005). This discrepancy might lie in delicate timing differences. Although PTH ultimately activates osteoclasts, these cells express no PTH receptors. Instead, PTH is sensed by osteoblasts, which in turn increase osteoclast activity. It is hypothesized that transient elevations of PTH (due to injection) favor the osteoblasts anabolic action on bone, whereas chronically high PTH levels favor the catabolic osteoclast activity (Dobnig and Turner 1997).
In addition to the effects on bone, PTH stimulates the activity of 1α-OHase in proximal tubules (Fraser and Kodicek 1973). Thereby, PTH increases the 1,25(OH)₂D₃-dependent (re)absorption of Ca²⁺. In addition, activation of the PTH/PTHrP receptor directly enhances the Ca²⁺ (re)absorption in kidney and intestine. Immunohistochemical analysis of rat duodenal sections showed localization of the PTH/PTHrP receptor in epithelial cells along the villus (Gentili et al. 2003). Interestingly, the receptor is absent in goblets cells. The first indication of a direct effect of PTH on the intestine was accomplished by a perfusion experiment of isolated duodenal loops, showing increased Ca²⁺ transport with addition of PTH (Nemere and Norman 1986; Nemere and Szego 1981). These findings were confirmed by Picotto and coworkers who demonstrated that PTH directly stimulates enterocyte Ca²⁺ influx (Picotto et al. 1997). Several groups localized PTH/PTHrP receptor mRNA in rat kidney to glomerular podocytes, PCT, PST, cortical segment of the TAL (cTAL), and DCT, but the receptor was not detected in the thin limb of Henle’s loop or in CD (Lee et al. 1996; Yang et al. 1997). PTH directly stimulates active Ca²⁺ reabsorption in the distal part of the nephron (Greger et al. 1978). In TAL, it was shown that PTH increases the transepithelial driving force for Ca²⁺ reabsorption, enhancing paracellular Ca²⁺ transport (Wittner et al. 1993). Various mechanisms of PTH action have been proposed for the effect in DCT, including membrane insertion of apical Ca²⁺ channels (Bacskai and Friedman 1990), opening of basolateral chloride channels resulting in cellular hyperpolarization (Friedman and Gesek 1994), and modulation of PMCA activity (Tsukamoto et al. 1992). Van Abel et al. recently reported that PTH stimulates renal Ca²⁺ reabsorption through the coordinated expression of renal transcellular Ca²⁺ transport proteins. They showed that parathyroidectomy in rats resulted in decreased serum PTH levels and hypocalcemia, which was accompanied by reduced levels of TRPV5, calbindin-D₂₈K, and NCX1 (Van Abel et al. 2005). Supplementation with PTH restored serum Ca²⁺ concentrations and abundance of the Ca²⁺ transport proteins (Table 2). Similarly, infusion of a calcimimetic compound (chemical that activates CaSR at low serum Ca²⁺ concentrations) decreased PTH levels, resulted in reduced expression of TRPV5, calbindin, and NCX1, which is consistent with diminished Ca²⁺ reabsorption, and in line with the observed hypocalcemia in these mice (Van Abel et al. 2005). Importantly, serum 1,25(OH)₂D₃ levels and renal VDR or CaSR mRNA abundance did not significantly change during these treatments (Van Abel et al. 2005). Furthermore, PTH injection in mice increased both TRPV5 and TRPV6 mRNA expression in kidney (Okano et al. 2004). This demonstrates the important role for PTH in epithelial Ca²⁺ transport.

**Estrogen**

Previous studies indicated that estrogen affects Ca²⁺ handling by kidney, intestine, and bone. Estrogen deficiency results in a negative Ca²⁺ balance and has been strongly associated with bone loss in postmenopausal women (Nordin et al. 1979, 1991; Prince et al. 1995; Young et al. 1968; Young and Nordin 1967). In addition, estrogen plays an essential role in bone handling in men (Carani et al. 1997; Lorentzon et al. 2006; Smith et al. 1994). Aromatase is the key enzyme in the conversion of testosterone to estradiol. A malfunctioning aromatase enzyme has been shown to impair the normal development of the (male) skeleton (Carani et al. 1997). Furthermore, mutations in the estrogen receptor gave rise to a similar phenotype, including low bone mineral density and long stature. The latter phenotype points to a role of estrogen in the determination of bone size (Lorentzon et al. 2006).

It has been generally described that the rise in serum and urine Ca²⁺ upon estrogen deficiency are secondary to an increase in bone resorption. However, there is increasing evidence that, besides bone, the intestine and kidney are also sites for estrogen action on Ca²⁺
handling and regulation. Menopausal estrogen deficiency in humans is associated with reduced duodenal Ca\textsuperscript{2+} absorption (Heaney et al. 1989), whereas estrogen replacement therapy helps prevent bone loss in postmenopausal women and corrects a decline in Ca\textsuperscript{2+} absorption efficiency at the onset of menopause (Gennari et al. 1990). However, the mechanism by which 17β-estradiol (17βE\textsubscript{2}) stimulates Ca\textsuperscript{2+} absorption could be direct via estrogen receptors (ER\textsubscript{α} and ER\textsubscript{β}) or indirect via increasing 1,25(OH)\textsubscript{2}D\textsubscript{3} or the VDR. Several studies were carried out to examine the mechanism of action of estrogen on intestinal Ca\textsuperscript{2+} absorption. Ten Bolscher et al. treated ovariectomized rats with estradiol or 1,25(OH)\textsubscript{2}D\textsubscript{3} and measured intestinal Ca\textsuperscript{2+} absorption in vivo using single pass perfusion of the duodenum. A pharmacological dose of estradiol caused a significant increase in intestinal absorption of Ca\textsuperscript{2+} (Ten Bolscher et al. 1999). This estrogen-induced rise in intestinal Ca\textsuperscript{2+} absorption was completely blocked by an ER antagonist, whereas this antagonist did not block vitamin D-enhanced intestinal Ca\textsuperscript{2+} absorption (Ten Bolscher et al. 1999). This suggests a direct effect of estrogen on duodenal Ca\textsuperscript{2+} absorption. In contrast, Cotter et al. did not observe an increase in Ca\textsuperscript{2+} uptake in Caco2 cells upon estrogen treatment (Cotter and Cashman 2006). Further evidence for a direct role of estrogen in kidney and intestine was recently provided by Van Abel et al. and Van Cromphaut et al. Van Abel et al. demonstrated that estrogen regulates the expression of TRPV5 in kidney in a 1,25(OH)\textsubscript{2}D\textsubscript{3}-independent manner (Table 2). Estrogen replacement in ovariectomized rats resulted in significant increased renal mRNA levels of TRPV5, calbindin-D\textsubscript{28K}, NCX1, and PMCA1b and increased the protein abundance of TRPV5 (Van Abel et al. 2002). Furthermore, 17βE\textsubscript{2} upregulated TRPV5 mRNA and protein expression in 1α-OHase\textsuperscript{-/-} mice, demonstrating a vitamin D-independent regulation by estrogen (Van Abel et al. 2002). Moreover, 17βE\textsubscript{2} treatment partially restored serum Ca\textsuperscript{2+} levels in these hypocalcemic mice, suggesting that 17βE\textsubscript{2} is directly involved in renal Ca\textsuperscript{2+} reabsorption via the upregulation of TRPV5 and possibly other Ca\textsuperscript{2+} transport proteins. Van Cromphaut et al. corroborated the direct role of estrogen on transepithelial Ca\textsuperscript{2+} transport. They showed that duodenal TRPV6 expression was reduced in ER\textalpha knockauthouse mice and induced by estrogen treatment, pregnancy, or lactation (Van Cromphaut et al. 2003). These latter effects occurred both in VDR knockout and wild-type mice. Therefore, estrogens or hormonal changes during pregnancy or lactation have distinct, vitamin D-independent effects at the genomic level on active duodenal Ca\textsuperscript{2+} absorption mechanisms, mainly through a major upregulation of the Ca\textsuperscript{2+} influx channel TRPV6. The expression of TRPV6 was not altered in ER\textbeta knockout mice, suggesting that the estrogen effects on duodenum are mediated by ER\textalpha (Van Cromphaut et al. 2003).

Dietary Ca\textsuperscript{2+} intake

Diets containing high amounts of Ca\textsuperscript{2+} have been implicated in the reduction of risk in osteoporosis (Greer and Krebs 2006). Obesity (Dixon et al. 2005) and hypertension (Karppanen et al. 2005) are some less well-known areas in which increasing dietary Ca\textsuperscript{2+} has a positive outcome. Even in cases of kidney stone formation, restricted Ca\textsuperscript{2+} intake is generally not advised (Borghi et al. 2002; Martini and Wood 2000; Moe 2006; Straub and Hautmann 2005), illustrating the importance of adequate Ca\textsuperscript{2+} intake. Recommended daily intake of Ca\textsuperscript{2+} is 1,000 mg/day for adults and 1,300 mg/day for adolescents, although both groups often do not reach these values, which could have negative consequences on bone density (Greer and Krebs 2006). The power of Ca\textsuperscript{2+} supplementation is best illustrated by the use of VDR and 1α-OHase knockout models. The bone phenotype of VDR-ablated mice can be completely rescued by feeding the animals a high Ca\textsuperscript{2+}, high phosphorus, high lactose diet (Van Cromphaut et al. 2001). Similarly, healing of rickets was demonstrated in a patient with
vitamin D resistance by long-term nocturnal Ca\textsuperscript{2+} infusions (Balsan et al. 1986). In addition, the VDDR I phenotype of mice deficient for the 1\alpha-OHase gene has been rescued by feeding them with a high Ca\textsuperscript{2+} diet. Dietary Ca\textsuperscript{2+} normalized the hypocalcemia, secondary hyperparathyroidism, and biomechanical properties of the bone tissue (Dardenne et al. 2003a; Hoenderop et al. 2002, 2004). Other studies indicated, however, that exogenous Ca\textsuperscript{2+} may not entirely compensate for 1,25(OH)\textsubscript{2}D\textsubscript{3} deficiency in mice and piglets (Goltzman et al. 2004; Schlumbohm and Harmeyer 2004).

To investigate the mechanism(s) underlying the effects of dietary Ca\textsuperscript{2+}, the expression level of several Ca\textsuperscript{2+} transport proteins was studied in various mice models. Importantly, the reduced expression level of renal TRPV5, calbindin-D\textsubscript{28K}, and NCX1 in 1\alpha-OHase\textsuperscript{−/−} mice was restored by high dietary Ca\textsuperscript{2+} intake and accompanied by normalization of the serum Ca\textsuperscript{2+} concentration (Hoenderop et al. 2002). Likewise, the expression of the intestinal Ca\textsuperscript{2+} transport proteins, TRPV6, calbindin-D\textsubscript{9K}, and PMCA1b, was normalized by this rescuing Ca\textsuperscript{2+} diet (Van Abel et al. 2003). Comparable observations were made in VDR knockout mice, where dietary Ca\textsuperscript{2+} upregulated duodenal TRPV5 and TRPV6 mRNA levels (Van Cromphaut et al. 2001). These findings suggest that dietary Ca\textsuperscript{2+} can affect active Ca\textsuperscript{2+} (re)absorption via vitamin D-independent modulation of the expression of Ca\textsuperscript{2+} transport proteins. However, the molecular mechanism of this vitamin D-independent Ca\textsuperscript{2+}-sensitive pathway remains to be further elucidated.

Diuretics

Thiazide diuretics are commonly used in the treatment of patients with hypertension. These diuretics enhance renal Na\textsuperscript{+} excretion through inhibition of the Na\textsuperscript{+}/Cl\textsuperscript{−} cotransporter (NCC) present in the apical membrane of DCT cells (Gamba et al. 1993). Importantly, these diuretics have, in contrast to loop diuretics, the unique characteristic of decreasing Na\textsuperscript{+} reabsorption, while increasing Ca\textsuperscript{2+} reabsorption resulting in hypocalciuria (Costanzo et al. 2000; Lamberg and Kuhlback 1959; Seitz and Jaworski 1964). This hypocalciuric effect provides therapeutic opportunities, for instance, in idiopathic hypercalciuria and nephrolithiasis. Furthermore, long-term effects of thiazides include increased bone mineral density and decreased fracture risk (Ray et al. 1989). Mutations in the gene encoding NCC were shown to cause Gitelman’s syndrome, a recessive disorder with a phenotype resembling chronic thiazide administration including hypocalciuria (Ellison 2000; Gitelman et al. 1966; Ray et al. 1989; Reilly and Ellison 2000; Simon et al. 1996). Gitelman’s syndrome is characterized by hypermatriuria and hypocalciuria, paralleled by an increase in bone mineral density. These symptoms are also present in NCC knockout mice, which therefore form a suitable mouse model for Gitelman’s syndrome (Loffing et al. 2004; Schultheis et al. 1998).

However, the exact molecular mechanism responsible for thiazide-induced hypocalciuria has been debated strongly. The hypocalciuric effect was suggested to result from direct stimulation TRPV5-mediated Ca\textsuperscript{2+} entry in transcellular Ca\textsuperscript{2+} transport in the DCT (Friedman 1998; Reilly and Ellison 2000). Alternatively, hypocalciuria was proposed to result from enhancement of passive paracellular Ca\textsuperscript{2+} reabsorption in the PT secondary to extracellular volume (ECV) contraction (Biner et al. 2002; Friedman 1998; Friedman and Bushinsky 1999; Loffing et al. 2001), distinct from any effect on transcellular Ca\textsuperscript{2+} transport (Wilson and Freis 1959). Recent evidence strongly supports the latter model. First, it was reported that thiazide-induced hypocalciuria occurs in spite of reduced renal expression of Ca\textsuperscript{2+} transport proteins in rat (Nijenhuis et al. 2003a). In addition, Nijenhuis et al. showed that the thiazide-induced hypocalciuria was accompanied by a significant decrease in body weight compared to controls, illustrating that extra cellular volume contraction occurred.
Furthermore, it was shown that ECV contraction mimics the hypocalciuria, and volume repletion completely reverses thiazide-induced hypocalciuria in these rats (Nijenhuis et al. 2005). Recent micropuncture experiments demonstrated that reabsorption of Na\(^+\) and, importantly, Ca\(^{2+}\) in the PT is increased during chronic hydrochlorothiazide (HCTZ) treatment, whereas Ca\(^{2+}\) reabsorption in DCT and CNT appeared unaffected (Nijenhuis et al. 2005). Importantly, chronic HCTZ administration still induces hypocalciuria in TRPV5\(^{-/-}\) mice, in which active Ca\(^{2+}\) reabsorption is abolished. HCTZ did not affect renal expression of the proteins involved in active Ca\(^{2+}\) transport, including TRPV5 mRNA and protein expression in wild-type mice (Nijenhuis et al. 2005). Lee et al. confirmed that thiazide treatment in mice does not affect renal TRPV5 expression, except when thiazide treatment is combined with salt repletion (Lee et al. 2004). However, salt repletion alone induced TRPV5 mRNA expression to a similar extent, suggesting that this effect is not thiazide-specific. Loffing and coworkers recently demonstrated that renal TRPV5 expression is unaffected in NCC knockout mice (Loffing et al. 2004). In accordance, micropuncture experiments in these mice showed that active Ca\(^{2+}\) reabsorption is unaltered in DCT and CNT, and indicated increased fractional absorption of both Na\(^+\) and Ca\(^{2+}\) upstream of DCT (Loffing et al. 2004). In conclusion, these studies demonstrated that chronic thiazide treatment induces hypovolemia that subsequently stimulates proximal Na\(^+\) and Ca\(^{2+}\) reabsorption. The latter explains the Ca\(^{2+}\)-sparring during thiazide treatment and Gitelman’s syndrome.

**Acidosis and alkalosis**

Acid-base homeostasis is known to affect renal Ca\(^{2+}\) handling (Canzanello et al. 1990; Sutton et al. 1979). For instance chronic metabolic acidosis is associated with increased renal Ca\(^{2+}\) excretion. Long-standing metabolic acidosis can lead to Ca\(^{2+}\) loss from bone and ultimately results in metabolic bone disorders, including osteomalacia and osteoporosis (Lehmann et al. 2003). In contrast, chronic metabolic alkalosis is known to decrease urine Ca\(^{2+}\) excretion (Bushinsky et al. 1989; Sutton et al. 1979). However, the molecular mechanisms that explain the altered renal divalent excretion during these disturbances of acid–base balance remain unknown.

It has been shown by several groups that extracellular protons inhibit TRPV5 channel activity (Peng et al. 2000a; Vennekens et al. 2001b; Yeh et al. 2005; Yeh et al. 2003). Furthermore, earlier studies, including micropuncture experiments, suggested that systemic acid–base disturbances specifically affect Ca\(^{2+}\) reabsorption in DCT and CNT (Sutton et al. 1979; Wong et al. 1986). It has, therefore, been suggested that acidification of the DCT and CNT luminal fluid during chronic metabolic acidosis and subsequent inhibition of TRPV5 explains the decreased Ca\(^{2+}\) reabsorption in vivo (Vennekens et al. 2001b; Yeh et al. 2003). Nijenhuis et al. recently addressed the mechanism(s) underlying acid–base balance on renal Ca\(^{2+}\) handling in more detail. Metabolic alkalosis was induced by oral NaHCO\(_3\) loading and metabolic acidosis by NH\(_4\)Cl loading, as well as by acetazolamide administration in wild-type and TRPV5\(^{-/-}\) mice (Nijenhuis et al. 2006). Acetazolamide specifically inhibits proximal tubular bicarbonate reabsorption, resulting in a self-limiting metabolic acidosis with, in contrast to NH\(_4\)Cl loading, an alkaline urine pH (Dirks et al. 1966; Soleimani 2002; Soleimani and Aronson 1989). This further enabled evaluation of the role of luminal pH during acidosis.

Chronic metabolic acidosis that was induced by NH\(_4\)Cl loading enhanced Ca\(^{2+}\) excretion and decreased the expression of the epithelial Ca\(^{2+}\) channel TRPV5 and calbindin-D\(_{28K}\) in wild-type mice. Importantly, although 0.14 M NH\(_4\)Cl administration induced a similar metabolic acidosis in TRPV5\(^{-/-}\) mice compared with wild-type mice, it did not further in-
crease Ca\(^{2+}\) excretion in these knockout mice. These results point to a primary renal Ca\(^{2+}\) leak, in contrast to increased Ca\(^{2+}\) mobilization from bone as a previously suggested explanation for the Ca\(^{2+}\) wasting (Krieger et al. 2004; Lemann et al. 2003). Furthermore, this indicates that, besides a direct effect on TRPV5 activity, downregulation of Ca\(^{2+}\) transport proteins that are present in DCT and CNT could be an explanation for the observed acidosis-induced hypercalciuria. Importantly, acetazolamide-induced acidosis also downregulated the expression of the Ca\(^{2+}\) transport proteins.

Acetazolamide treatment induces metabolic acidosis by diminishing the proximal tubular bicarbonate-reabsorptive capacity (Dirks et al. 1966; Soleimani and Aronson 1989). This results in an increased luminal pH at more distal nephron segments, including DCT and CNT, which is reflected by urinary alkalinization in contrast to NH\(_4\)Cl loading-induced urine acidification. Despite the alkaline pH at the site of TRPV5 expression, this treatment resulted in a significant hypercalciuria. Therefore, luminal pH in DCT and CNT does not seem to be a dominant factor in the long-term in vivo hypercalciuric effect of chronic metabolic acidosis. Instead, acidosis-induced hypercalciuria is in accordance with downregulation of Ca\(^{2+}\) transport proteins, including TRPV5. The mechanism underlying the Ca\(^{2+}\)-sparing action of chronic metabolic alkalosis is not simply the reverse of this effect. Chronic metabolic alkalosis increased renal expression of the Ca\(^{2+}\) transport proteins in wild-type mice. However, chronic NaHCO\(_3\) administration induced metabolic alkalosis in wild-type as well as TRPV5\(^{-/-}\) mice (Nijenhuis et al. 2006). These findings suggest that upregulation of Ca\(^{2+}\) transport proteins in DCT and CNT is not the sole explanation for the alkalosis-induced hypocalciuria. Li\(^+\) clearance studies suggested that NaHCO\(_3\)-treated mice show increased proximal tubular Na\(^{+}\) reabsorption and therefore possibly display enhanced passive Ca\(^{2+}\) reabsorption compared with NaCl-treated controls. Therefore, the Ca\(^{2+}\) sparing effect during alkalosis could be explained by increased passive Ca\(^{2+}\) reabsorption independent of DCT and CNT.

Klotho: novel insight in hormonal regulation of Ca\(^{2+}\) reabsorption

In 1997 Kuro-o et al. described a transgenic mouse with several age-related disorders caused by the single insertion of a transgene. The affected gene was named klotho, for one of the Fates, the Greek goddess who spins the thread of life (Kuro-o et al. 1997). Mice homozygous for the affected klotho gene (effectively klotho knockout mice) show a phenotype resembling those in patients with premature-aging syndromes: arteriosclerosis, osteoporosis, age-related skin changes, and ectopic calcifications, together with short lifespan and infertility (Kuro-o et al. 1997). Re-introduction of the klotho gene normalized the phenotype. Furthermore, overexpression of klotho in mice resulted in a significant extension of lifespan and a suppression of phenotypes associated with aging (Kurosu et al. 2005). In humans, allelic variations are related to life expectancy and coronary artery disease (Arking et al. 2005; Arking et al. 2002, 2003). The klotho gene encodes a single-pass transmembrane protein of 1,014 amino acids with a putative signal sequence at the amino-terminus and a single transmembrane helix near the carboxyl-terminus. Klotho is secreted and activated by cleavage of the amino-terminal extracellular domain, and this secreted form of klotho exhibits β-glucuronidase activity (Imura et al. 2004; Tohyama et al. 2004).

Several observations connect klotho to a role in Ca\(^{2+}\) metabolism. First, klotho-deficient mice have a slight hypercalcemia that was associated with high levels of 1,25(OH)\(_2\)D\(_3\), caused by increased expression of renal 1α-hydroxylase (Yoshida et al. 2002). Furthermore, administration of 1,25(OH)\(_2\)D\(_3\) induces klotho expression in the kidney (Tsujikawa et al. 2003). Second, klotho\(^{-/-}\) mice show bone abnormalities including an approximately
20% lower bone mineral density than control mice (Kuro-o et al. 1997). In humans, allelic variants of klotho are associated with osteoporosis, confirming this phenotype (Kawano et al. 2002; Ogata et al. 2002). Third, klotho is strongly expressed in DCT of the kidney and the parathyroid gland, further supporting a role in epithelial Ca\(^{2+}\) handling (Chang et al. 2005; Kuro-o et al. 1997). Fourth, Chang et al. recently demonstrated a novel mechanism employed by klotho to directly stimulate active Ca\(^{2+}\) reabsorption. They demonstrated that incubation of TRPV5-expressing HEK293 cells with preconditioned culture medium from klotho-expressing cells resulted in strongly increased TRPV5-mediated Ca\(^{2+}\) uptake (Chang et al. 2005). This effect was mimicked by β-glucuronidase indicating that the enzymatic activity of klotho is responsible for the increased TRPV5 activity. Mutation of the conserved N-glycosylation site of TRPV5 (N358Q) abolished both klotho- and β-glucuronidase-mediated activation of TRPV5, indicating that klotho may work by affecting the extracellular glycosylation status of the channel (Chang et al. 2005). Membrane protein biotinylation indicated a significant increase in plasma membrane localization of TRPV5 after klotho or β-glucuronidase stimulation. Together, these indicate that klotho traps the channels in the plasma membrane, thereby increasing TRPV5-mediated Ca\(^{2+}\) influx activity. Interestingly, disruption of the klotho gene in mice is also associated with hyperphosphatemia (Kuro-o et al. 1997). It has been reported that klotho increases the affinity of fibroblast growth factor 23 (FGF-23) binding to its receptor in the proximal tubule (Razzaque et al. 2006). Knockout of the FGF-23 gene or overexpression of FGF-23 in mice resulted in significant alteration of phosphate (P\(_i\)) transport (Razzaque et al. 2006). In humans, fibroblast growth factor 23 mutations are responsible for hypophosphatemic rickets (Jonsson et al. 2003). This suggests that klotho is also a novel phosphaturic factor, possibly forming a hormonal link between Ca\(^{2+}\) and P\(_i\) homeostasis.

**Concerted regulation of epithelial Ca\(^{2+}\) transport proteins**

An increasing number of studies combine the investigation of multiple Ca\(^{2+}\) transport proteins under various (patho)physiological and pharmacological circumstances. Remarkably, virtually all of the results point to a concerted regulation of these Ca\(^{2+}\) transport proteins including TRPV5, TRPV6, NCX1, PMCA, and the calbindins. The concomitant regulation of the renal Ca\(^{2+}\) transport proteins was observed in studies exploring the regulatory role of 1,25(OH)\(_2\)D\(_3\), estrogens, PTH, and dietary Ca\(^{2+}\) (Hoenderop et al. 2002; Van Abel et al. 2002, 2003, 2005). Furthermore, calbindin-D\(_{28K}\) and NCX1 showed downregulation in kidneys of TRPV5 –/– mice despite elevated levels of 1,25(OH)\(_2\)D\(_3\) (Hoenderop et al. 2003a). This suggests that TRPV5 is primarily involved in the regulation of the Ca\(^{2+}\) transport proteins expression in kidney independent of 1,25(OH)\(_2\)D\(_3\) (Van Abel et al. 2005). Furthermore, chronic metabolic alkalosis enhances calbindin-D\(_{28K}\) abundance in wild-type mice, but not in TRPV5 –/– mice, in line with TRPV5-dependent regulation of this Ca\(^{2+}\)-transporting protein (Nijenhuis et al. 2006). An interesting question is how the expression of TRPV5 specifically coordinates the Ca\(^{2+}\) transport machinery. Because TRPV5 is the gatekeeper controlling apical Ca\(^{2+}\) influx in the kidney, it was postulated that the magnitude of Ca\(^{2+}\) influx through TRPV5 determines the expression of the other Ca\(^{2+}\) transport proteins (Lambers et al. 2006a; Van Abel et al. 2005). This hypothesis was recently investigated using primary cultures of rabbit CNT and CCD. Long-term exposure to PTH stimulated transepithelial Ca\(^{2+}\) transport in these epithelial cells and concomitantly elevated the expression of TRPV5, calbindin-D\(_{28K}\), and NCX1 (Van Abel et al. 2005). Inhibition of TRPV5 channel activity by ruthenium red eliminated this PTH-stimulated transepithelial Ca\(^{2+}\) transport, which was accompanied by a reduction in NCX1 and calbindin-D\(_{28K}\) expression (Van Abel...
et al. 2005). These findings support the hypothesis that the magnitude of the Ca\(^{2+}\) influx through TRPV5 modulates the expression of the other proteins that are required for transepithelial Ca\(^{2+}\) transport. An important question that remains to be answered is how the flux of Ca\(^{2+}\) through TRPV5 adjusts the expression of the other Ca\(^{2+}\) transport proteins. It is possible that Ca\(^{2+}\)-sensitive transcription factors/promoters play a role in this process (Ashby and Tepikin 2002). A 40-bp Ca\(^{2+}\)-responsive element has been identified in the promoter sequence of calbindin-D\(_{28K}\) that partly underlies the Purkinje cell-specific expression of calbindin-D\(_{28K}\) (Arnold and Heintz 1997). This element is also present in the calmodulin II promoter. However, future studies are needed to test whether this element is active in the kidney and/or additional intracellular signaling molecules are involved.

TRPV5/6 regulatory proteins

Recently, several proteins have been identified that associate with TRPV5, TRPV6, or both. These include calmodulin (CaM), S100A10-annexin 2, Rab11a, 80K-H, NHERF2, NHERF4, FKBP52, and BSPRY (Van de Graaf et al. 2006b). The identification of these TRPV5/TRPV6-binding proteins has significantly improved our knowledge of the molecular pathways modulating epithelial Ca\(^{2+}\) transport, as will be discussed below.

Calmodulin

CaM is a ubiquitous protein encoded by three separate genes, all resulting in the same protein (Means and Dedman 1980). CaM consists of four Ca\(^{2+}\)-binding structures, localized in the amino- and carboxyl-terminus. Ca\(^{2+}\) binding to CaM is highly cooperative with Ca\(^{2+}\) binding first to the carboxyl-terminal EF-hands, which have the highest affinity for Ca\(^{2+}\), followed by Ca\(^{2+}\) binding to lower affinity sites located in the amino-terminus (Wang 1985).

CaM is involved in a plethora of processes, many related to Ca\(^{2+}\)-related signaling. The protein is well-known to be involved in Ca\(^{2+}\)-dependent feedback regulation of several ion channels (Levitan 1999), including multiple TRP channels (Harteneck 2003). The first connection between CaM and the canonical TRPs was established upon the expression cloning of the second TRPC (TRP-Like) from Drosophila photoreceptors, for which CaM was used as the probe (Phillips et al. 1992). Upon Ca\(^{2+}\) influx, CaM inactivates the TRPL-mediated currents (Scott et al. 1997). Subsequently, it has been shown that many members of the TRPC family bind to and/or are regulated by CaM (Abeele et al. 2003; Boulay 2002; Ordaz et al. 2005; Scott et al. 1997; Shi et al. 2004; Singh et al. 2002; Tang et al. 2001; Trost et al. 2001; Yildirim et al. 2003; Zhang et al. 2001).

Importantly, CaM also binds to several TRPV channels, including TRPV6. Niemeyer and coworkers initially demonstrated that TRPV6 interacts in a Ca\(^{2+}\)-dependent manner with CaM (Niemeyer et al. 2001). They showed that CaM binding to a 21-amino-acid sequence in the carboxyl-terminus of TRPV6 is competitively regulated by protein kinase C (PKC) phosphorylation of a threonine residue. This phosphorylated residue is not conserved and absent from mouse TRPV6. However, Hirnet et al. demonstrated that mouse TRPV6 protein is also capable of Ca\(^{2+}\)-dependent CaM binding, using a synthetic peptide encompassing the stretch of amino acid residues of the mouse protein corresponding to the CaM binding site within the human TRPV6 protein (Hirnet et al. 2003). The apparent dissociation constant of CaM with the carboxyl-terminal peptide of mouse TRPV6 (43 nM) corresponds to the value obtained for human TRPV6 (65 nM) (Hirnet et al. 2003). Furthermore, Lambers et al. showed that CaM associates in a Ca\(^{2+}\)-dependent manner with specific regions in TRPV5 and TRPV6.
Two separate approaches demonstrate a functional role for CaM in the regulation of TRPV6 (Lambers et al. 2004; Niemeyer et al. 2001). First, removal of the CaM binding-site in the carboxyl-terminus of TRPV6 resulted in a significant reduction of the slow component of channel inactivation, revealing a role of CaM in TRPV6 regulation (Niemeyer et al. 2001). Second, HEK293 cells heterologously coexpressing Ca\(^{2+}\)-insensitive CaM mutants along with TRPV5 or TRPV6 showed a significantly reduced Ca\(^{2+}\) current through TRPV6. Remarkably, no functional effect was demonstrated on TRPV5 channel activity (Lambers et al. 2004), even though TRPV5 contains CaM-binding sites at similar locations. This functional effect on TRPV6 was localized to the high Ca\(^{2+}\)-affinity EF-hand structures of CaM.

These data demonstrated a regulatory role of CaM in TRPV6-mediated Ca\(^{2+}\) influx. It remains to be established whether CaM functions as a general Ca\(^{2+}\) sensor in TRPV5 and TRPV6 channels or, alternatively, can explain the differences in Ca\(^{2+}\)-dependent inactivation between the epithelial Ca\(^{2+}\) channels.

80K-H  
Gkika et al. identified 80K-H in a microarray screen designed to identify proteins that respond similarly to vitamin D and/or altered dietary Ca\(^{2+}\) intake as TRPV5 (Gkika et al. 2004). 80K-H was originally cloned as a PKC substrate of 80 kDa (Sakai et al. 1989) and was further shown to form a component of a cytosolic signal transduction complex (Goh et al. 1996; Kanai et al. 1997), a receptor for advanced glycation end products (Li et al. 1996) and the \(\beta\)-subunit of endoplasmic reticulum glucosidase II (Trombetta et al. 1996, 2001). In addition, 80K-H has been implicated in the insulin-stimulated translocation of the glucose transporter 4 (GLUT4)-containing vesicles to the plasma membrane cells. This effect is mediated by the interaction of 80K-H with a complex of PKC\(\zeta\) and Munc18c in an insulin-dependent manner (Hodgkinson et al. 2005). Importantly, mutations in 80K-H were suggested as the probable cause of polycystic liver disease (Drenth et al. 2003, 2004; Peces et al. 2005), a dominantly inherited condition characterized by the presence of multiple liver cysts of biliary epithelial origin. This is the reason that 80K-H is also referred to as hepatocystin (Drenth et al. 2004).

80K-H contains two putative EF-hand structures, a highly negatively charged glutamate stretch, and a putative ER-targeting signal (His–Asp–Glu–Leu). Using glutathione S-transferase (GST) pull-down assays and coimmunoprecipitations, a physical interaction between 80K-H and TRPV5 was demonstrated (Gkika et al. 2004). Furthermore, both proteins colocalized in a subset of tubular segments in the kidney indicating that regulation of TRPV5 by 80K-H could occur in vivo. Furthermore, similar transcriptional regulation of both proteins by 1,25(OH)\(_2\)D\(_3\) and dietary Ca\(^{2+}\) was shown (Gkika et al. 2004). Electrophysiological studies using 80K-H mutants demonstrated that three domains of 80K-H (the two EF-hand structures, the glutamate stretch, and the His–Asp–Glu–Leu sequence) are critical determinants of TRPV5 activity (Gkika et al. 2004). The Ca\(^{2+}\) binding properties of 80K-H are abolished upon inactivation of its two EF-hand structures. Importantly, this modification of the EF-hand pair in 80K-H also reduces the TRPV5-mediated Ca\(^{2+}\) current and increased the TRPV5 sensitivity to intracellular Ca\(^{2+}\), accelerating the feedback inhibition of the channel (Gkika et al. 2004). Therefore, it is hypothesized that 80K-H acts a Ca\(^{2+}\) sensor to regulate TRPV5 activity at the plasma membrane.

B-box and SPRY-domain containing protein  
Recently, we identified BSPRY (B-box and SPRY-domain containing protein) as a novel factor involved in the control of TRPV5 activity (Van de Graaf et al. 2006d). BSPRY contains a B-box and SPRY domain, whose tentative functions are protein–protein interaction modules (Borden 1998; Ponting et al. 1997).
RT-PCR and Northern blot analysis showed expression of this novel protein in several tissues including kidney, small intestine, prostate, lung, and uterus in mice. BSPRY was less abundantly expressed in heart, whereas skeletal muscle and liver were negative (Van de Graaf et al. 2006d). Rat BSPRY (also called zetin 1) has a shorter amino-terminus compared to mouse and human BSPRY and is ubiquitously expressed in a variety of tissues, with highest expression being found in testis. In adult brain, high levels of BSPRY mRNA were observed in the hippocampus, cerebral cortex, and piriform cortex (Birkenfeld et al. 2003). Using an antibody directed against a conserved peptide in the carboxyl-terminus of BSPRY, the presence of BSPRY was demonstrated along the apical domain of all TRPV5-immunopositive tubules in mouse kidney (Van de Graaf et al. 2006d). These tubules were previously identified as the second part of DCT2 and CNT (Hoenderop et al. 2000). Furthermore, expression of BSPRY in Madin-Darby canine kidney cells stably expressing TRPV5 resulted in a significant reduction of the Ca$^{2+}$ influx without affecting channel cell surface abundance (Van de Graaf et al. 2006d).

As described in “1,25-dihydroxyvitamin D$_3$” above, TRPV5 expression is strongly regulated by 1,25(OH)$_2$D$_3$. Therefore, the role of vitamin D on the abundance of BSPRY was assessed in wild-type and 1α-OHase$^{-/-}$ mice, which are unable to synthesize 1,25(OH)$_2$D$_3$. Quantitative real-time PCR and computerized analysis of the immunohistochemical BSPRY staining in kidney showed significantly enhanced BSPRY mRNA expression in the 1α-OHase$^{-/-}$ mice compared to wild-type mice, demonstrating the inverse regulation of BSPRY expression by circulating vitamin D (Van de Graaf et al. 2006d). Together with the inhibitory function of BSPRY on TRPV5 activity and the striking colocalization of both proteins, this suggests that BSPRY operates as a negative modulator for TRPV5, and that this mechanism will be downregulated when vitamin D levels increase to stimulate active Ca$^{2+}$ transport (Van de Graaf et al. 2006d). These data provide the first evidence of a functional role of BSPRY. However, the mechanism of these functional effects remains unclear. So far, only two other studies provided information about BSPRY. BSPRY was initially identified in a yeast two-hybrid screen using zyxin as bait (Schenker and Trueb 2000). In epithelial cells zyxin is involved in the formation of cell–cell contacts, which require actin cytoskeleton rearrangements (Vasioukhin et al. 2000). This might hint at a role of the cytoskeleton in the BSPRY-mediated regulation of TRPV5. Second, it was shown that BSPRY interacts with 14-3-3 proteins (Birkenfeld et al. 2003). It has been demonstrated that 14-3-3 proteins bind to specific motifs containing a phosphorylated serine residue and have been implicated in the binding to and activation of signaling proteins (Muslin et al. 1996; Yaffe et al. 1997). Furthermore, a role of 14-3-3 proteins in K$^+$ channel trafficking was postulated (O’Kelly et al. 2002). However, cell surface biotinylation did not provide evidence for TRPV5 trafficking as an explanation for the observed inhibitory function of BSPRY (Van de Graaf et al. 2006d). Therefore, it is currently hypothesized that BSPRY is involved in inhibitory signaling cascades controlling the activity of the epithelial Ca$^{2+}$ channels at the cell surface.

**S100A10** Van de Graaf et al. identified S100A10 (also known as p11 or annexin 2 light chain) as an auxiliary protein for TRPV5 and TRPV6 using a yeast two-hybrid system (Van de Graaf et al. 2003). S100A10 is a member of the S100 superfamily that is present in a large number of organisms including vertebrates, insects, nematodes, and plants. The two EF-hands of S100A10 carry deletions and substitutions that render it Ca$^{2+}$ insensitive. S100A10 is predominantly present as a heterotetrameric complex with annexin 2, which has been implicated in several biological processes including endocytosis, exocytosis, and membrane-cytoskeleton interactions (Gerke et al. 2005).
It was shown that S100A10, annexin 2, and TRPV5 or TRPV6 are coexpressed in Ca\(^{2+}\)-transporting cells of the kidney and small intestine (Van de Graaf et al. 2003). The association of S100A10 with TRPV5 and TRPV6 was restricted to a short peptide sequence, VATTV, located in the carboxyl-termini of these channels (Van de Graaf et al. 2003). This stretch is conserved among all identified species of TRPV5 and TRPV6. Interestingly, the TTV sequence in the S100A10-binding site resembles an internal type I PDZ (postsynaptic density 95/disk-large/zonula occludens-1) consensus binding sequence, which is S/TXV (Songyang et al. 1997). However, S100A10 does not contain PDZ domains, indicating that the TRPV5-S100A10 interaction is structurally distinct. The first threonine of the S100A10 interaction motif is a crucial residue. Both the S100A10 binding capacity and the activity of TRPV5 and TRPV6 are largely abolished when this particular threonine is mutated, demonstrating that this motif is essential for channel function (Van de Graaf et al. 2003). Malfunctioning of these mutant channels is accompanied by a major disturbance in their subcellular localization, indicating that the S100A10-annexin 2 heterotetramer facilitates the translocation of TRPV5 and TRPV6 channels to the plasma membrane.

The importance of annexin 2 in this process was demonstrated by small interference RNA. Downregulation of annexin 2 significantly inhibited the currents through TRPV5 and TRPV6 (Van de Graaf et al. 2003). The expression of S100A10 was also downregulated by this approach, indicating that annexin 2, in conjunction with S100A10, is crucial for TRPV5 activity. The association of annexin 2 with TRPV5 was only detectable in the presence of S100A10, demonstrating that annexin 2 binds indirectly to the channel, with S100A10 most likely operating as a molecular bridge between TRPV5 and annexin 2 (Van de Graaf et al. 2003). These findings provide the first functional evidence for a regulatory role of S100A10/annexin 2 controlling Ca\(^{2+}\) channel trafficking and therefore the Ca\(^{2+}\) balance. Interestingly, previous and later studies indicated that several ion channels and receptors associate with S100A10. It was reported that the background K\(^+\) channel (TASK1) is associated with S100A10 via its carboxyl-terminal sequence SSV (Girard et al. 2002). The S100A10 interaction blocks an ER-retention signal that promotes the translocation of TASK1 to the plasma membrane producing functional K\(^+\) channels (Girard et al. 2002). This sequence resembles the binding motif in TRPV5 and TRPV6 identified in the present study, suggesting a shared structural S100A10 binding pocket. However, another study suggests that S100A10 binding is located at a different binding site in TASK1 formed by a 40-amino-acid region in the proximal carboxyl-terminus, and the authors proposed that S100A10 binding inhibits TASK-1 targeting to the plasma membrane (Renigunta et al. 2006).

In addition to TRPV5 and TASK1, a number of other ion channels have recently been shown to be regulated by S100A10 binding. All these proteins show a requirement for S100A10 binding for their trafficking toward the plasma membrane (Donier et al. 2005; Okuse et al. 2002; Svenningsson et al. 2006). Initially, Okuse et al. identified the tetrodotoxin-insensitive voltage-gated Na\(^+\) channel (Nav1.8), as the first ion channel to associate with S100A10 (Okuse et al. 2002). Nav1.8 was shown to bind S100A10 via its amino-terminus (Poon et al. 2004). The binding of S100A10 is essential for plasma membrane trafficking of this Na\(^+\) channel (Okuse et al. 2002). Furthermore, Donier et al. have recently demonstrated the association of S100A10 with an acid-sensing ion channel (ASIC1) and confirmed this association in rat dorsal root ganglion neurons by coimmunoprecipitation (Donier et al. 2005). Finally, Svenningsson recently reported the interaction of S100A10 with the serotonin 1B (5-HT1B) receptor. S100A10 increases cell surface abundance of 5-HT1B receptors (Svenningsson et al. 2006). The interaction between S100A10 and the 5-HT1B receptor is associated with the pathophysiology of depression, as was shown using S100A10 knockout mice or S100A10 overexpressing...
mouse models (Svenningsson et al. 2006). On the whole, the S100A10-annexin 2 complex seems a significant component for the regulation of cell surface abundance of several ion channels and receptors, including TRPV5 and TRPV6.

Rab11a  Rab11a was recently identified as a novel TRPV5- and TRPV6-associated protein (Van de Graaf et al. 2006a). The Rab family of small guanosine triphosphatases (GTPases) has a well-recognized role in membrane trafficking (Zerial and McBride 2001). They are localized to specific organelles within the cell and have been implicated in distinct transport steps including vesicle budding, targeting, and tethering. Although the role of Rab GTPases in protein trafficking has long been recognized, the underlying mechanism is far from understood. Rab11a is a small GTPase involved in cargo trafficking via recycling endosomes (Brown et al. 2000; Casanova et al. 1999; Wang et al. 2000). Van de Graaf et al. demonstrated that Rab11a colocalizes with TRPV5 and TRPV6 in Ca\(^{2+}\)-transporting epithelial cells of the kidney. Here, both TRPV5 and Rab11a are present in vesicular structures below the apical plasma membrane (Van de Graaf et al. 2006a). Using a combination of GST pull-down and coimmunoprecipitation assays, the direct and specific interaction between Rab11a and the epithelial Ca\(^{2+}\) channels was shown (Van de Graaf et al. 2006a). Association of cargo with Rab GTPases has recently received much attention with the identification of an association between Rab3 and the polymeric IgA receptor (Smythe 2002; van IJzendoorn et al. 2002) and between Rab11a and the thromboxane A2 receptor (TP\(\beta\)) (Hamelin et al. 2005). The binding of TRPV5 and TRPV6 to Rab11a provides the first evidence for an ion channel directly associating with a Rab GTPase. Furthermore, it was demonstrated that TRPV5 and TRPV6 preferentially interact with Rab11a in its GDP-bound conformation (Van de Graaf et al. 2006a). Interestingly, although the identified Rab11a-binding regions in TRPV5 and TP\(\beta\) are not homologous, both proteins interact with the GDP-bound form of Rab11, suggesting a common binding mechanism. Expression of a mutant Rab11a protein, locked in the GDP-bound state, results in a marked decrease of TRPV5 and TRPV6 channels at the cell surface, indicating a direct role of Rab11a in the trafficking of TRPV5 and TRPV6 toward the plasma membrane (Van de Graaf et al. 2006a). Similarly, it was demonstrated that direct binding of Rab11a is a determinant factor in controlling the recycling to the cell surface of TP\(\beta\) (Hamelin et al. 2005). The association with Rab11a is essential in directing the intracellular trafficking of the receptor from the Rab5-positive intracellular compartment to the perinuclear recycling endosome. It is possible that also TRPV5/6 channels, present on the (apical) plasma membrane, are continuously exchanged with TRPV5 and TRPV6 channels from the intracellular (recycling) endosomes in a Rab11-dependent manner. The molecular mechanisms determining the distribution of TRPV5 between the plasma membrane and the intracellular pool are currently unknown.

NHERF family members  The Na\(^{+}\)-H\(^{+}\) exchanger regulatory factors 1 and 2 (NHERF1 and NHERF2) form a family of adaptor proteins characterized by the presence of two tandem PDZ protein interaction domains and a carboxyl-terminal domain that binds the cytoskeleton proteins ezrin, radixin, moesin, and merlin (Weinman et al. 2006a). These proteins were initially characterized as facilitating the formation of a multiprotein complex that mediates protein kinase A (PKA) phosphorylation of the renal Na\(^{+}\)-H\(^{+}\) exchanger 3 (NHE3) and downregulation of its activity (Lamprecht et al. 1998; Weinman et al. 1995; Yun et al. 1997; Zizak et al. 1999). In general, these proteins are known to operate as adapter proteins responsible for organizing a multiprotein complex involved in the regulation of receptors, including the PTH receptor (Mahon et al. 2002) and ion channels, for instance the cystic
fibrosis transmembrane conductance regulator CFTR (Li et al. 2005; Liedtke et al. 2002; Sun et al. 2000) and several TRP channels (Obukhov and Nowycky 2004; Odell et al. 2005; Tang et al. 2000).

Interestingly, the first hint for a physiological role of NHERF in the regulation of TRPV5 and/or TRPV6 came from studies using NHERF1 knockout (NHERF1−/−) mice. NHERF1−/− mice display a threefold increase in urinary phosphate excretion compared with wild-type animals. In addition, NHERF1−/− mice display increased urine Ca2+ excretion compared with wild-type controls, which persists during life (Shenolikar et al. 2002; Weinman et al. 2006b). The molecular mechanism underlying the effect on Ca2+ handling is currently unknown, although a secondary effect resulting from hyperphosphaturia seems the most straightforward explanation (Beck et al. 1998; Shenolikar et al. 2002). Subsequently, Embark and coworkers demonstrated that TRPV5 activity increases upon coexpression with NHERF2 and SGK1 or SGK3 (serum and glucocorticoid inducible kinase 1 or 3) in Xenopus laevis oocytes. Coexpression of TRPV5 with NHERF2 or SGK1/3 alone did not stimulate TRPV5-mediated currents, indicating that both NHERF2 and SGK1/3 are required (Embark et al. 2004).

Deletion of the second, but not the first, PDZ domain in NHERF2 abrogates the stimulating effect of SGK1/3/NHERF2 on TRPV5 activity (Palmada et al. 2005). Furthermore, TRPV5 activity was not stimulated with a kinase-dead point mutant of SGK1 (K127N), suggesting a phosphorylation-mediated effect (Embark et al. 2004). Using GST pull-down and overlay assays, the specific interaction of NHERF2 with the last three amino acids (YHF) of the carboxyl-terminus of TRPV5 was demonstrated (Van de Graaf et al. 2006c). Furthermore, TRPV6 did not bind NHERF2 (Palmada et al. 2005). These findings suggest that regulation of the epithelial Ca2+ channels by NHERF2-SGK is limited to TRPV5 and operates via direct interaction with the channel.

The coexpression of SGK1 with NHERF2 also stimulated the activity of the renal outer medullary K+ channel (ROMK1), a K+ channel involved in renal K+ handling. This results from a stabilization of ROMK1 in the plasma membrane (Yun et al. 2002). Therefore, it is postulated that NHERF2/SGK stimulates the activity of TRPV5 via a comparable mechanism. Furthermore, it was shown that TRPV5 and TRPV6 associate with another PDZ domain-containing protein, NHERF4 (Van de Graaf et al. 2006c). Other names for this protein are intestine and kidney enriched PDZ protein (IKEPP), PDZK1, and Napi-Cap2 (Gisler et al. 2001; Scott et al. 2002).

In contrast to NHERF1 and 2, this PDZ protein possesses four PDZ domains. Yeast two-hybrid, GST pull-down and coimmunoprecipitation assays identified NHERF4 as a novel auxiliary protein for both TRPV5 and TRPV6 (Van de Graaf et al. 2006c). NHERF4 utilizes PDZ domain 1 and 4 to bind the carboxyl-terminus of TRPV5 at a site distinct from NHERF2. Furthermore, NHERF4 is coexpressed with TRPV6 in Caco-2 cells, an intestinal epithelial cell line, whereas limited colocalization with TRPV5 was observed in the kidney (Van de Graaf et al. 2006a). This suggests that NHERF2 predominantly regulates TRPV5, whereas NHERF4 operates on TRPV6.

Calbindin-D28K As discussed above, in Ca2+-transporting, epithelial calbindins act as cytosolic Ca2+ buffers, facilitating the intracellular diffusion of Ca2+, while keeping the free Ca2+ concentration at physiological levels. Calbindin-D28K is highly expressed in Ca2+-transporting epithelia where it colocalizes with TRPV5 (Hoenderop et al. 2000; Hoenderop et al. 1999c). Interestingly, several animal studies exploring the effect of various treatments (including vitamin D, dietary Ca2+ or PTH depletion or administration, chronic acidosis)
showed that the expression of TRPV5 and calbindin-D28K is concomitantly regulated (Hoen-derop et al. 2002; Nijenhuis et al. 2006; Van Abel et al. 2005). In a recent study, Lambers et al. demonstrated that calbindin-D28K is a dynamic Ca^{2+} buffer that is functionally and physically tangled together with TRPV5 (Lambers et al. 2006b). The dynamic nature of the buffer was illustrated by evanescent wave microscopy, used to excite fluorophore-coupled calbindin only in the vicinity of the plasma membrane. They showed that calbindin-D28K translocates toward the plasma membrane upon decreases in [Ca^{2+}]_i. This effect was only observed when TRPV5 was present in these cells, and confirmed using endogenous expression of TRPV5 and calbindin-D28K. Importantly, calbindin-D28K directly associated with TRPV5 under conditions of low [Ca^{2+}]_i (Lambers et al. 2006b). The functional relevance of this dynamic association between TRPV5 and calbindin-D28K was elucidated using three approaches (Lambers et al. 2006b). First, coexpression of calbindin-D28K with TRPV5 increased the TRPV5-mediated 45Ca^{2+} influx in HEK293 cells. This effect was due to the Ca^{2+} buffering capacity of calbindin-D28K, as a mutant protein with affected EF-hands was not able to increase TRPV5 activity. Second, using a photolysable Ca^{2+} chelator to experimentally control [Ca^{2+}]_i, it was demonstrated that coexpression of calbindin-D28K with TRPV5 does not alter the Ca^{2+}-dependent channel inactivation characteristics, but likely operates as a local Ca^{2+} buffer to keep the [Ca^{2+}]_i at the vicinity of the channel pore low. Third, overexpression of Ca^{2+}-binding-deficient mutant calbindin resulted in a reduced Ca^{2+} transport in primary cultures of rabbit CNT and CCD, a model for transepithelial Ca^{2+} transport. This Ca^{2+}-binding-deficient calbindin mutant associated with TRPV5, even at high [Ca^{2+}]_i. Together these findings constitute the first direct evidence that calbindin-D28K operates as a dynamic channel-associated Ca^{2+} buffer, essential for transepithelial Ca^{2+} transport (Lambers et al. 2006b). At a low [Ca^{2+}]_i, calbindin-D28K translocates toward the plasma membrane and associates with TRPV5. At the apical plasma membrane, it buffers Ca^{2+} that enters the cell via TRPV5, thereby avoiding local accumulation of free Ca^{2+} near the pore and subsequent inactivation of the channel. Upon Ca^{2+}-binding, calbindin-D28K releases from TRPV5 and subsequently facilitates diffusion of Ca^{2+} to the basolateral membrane. This illustrates a mechanism of localized dynamic Ca^{2+} buffering mediated by protein–protein interaction, which could operate in various tissues where Ca^{2+} transport or signaling is abundant (Lambers et al. 2006b).

The binding site(s) of TRPV5/6 auxiliary proteins A remarkable feature of the identified auxiliary proteins is the promiscuous binding site in TRPV5/6. The binding of Rab11 was localized to a conserved stretch in the carboxyl-termius of TRPV5/6 in close proximity of the last TM. Five amino acid residues (MLERK) within this area were identified as a critical region for binding of Rab11 (Van de Graaf et al. 2006a). However, the interaction with 80K-H is localized to the same region, suggesting a possible common binding site in TRPV5/6 (Gkika et al. 2004). Remarkably, Chang and coworkers have also identified this 5-amino-acid motif as one of the components that plays a role in assembly of the tetrameric channel (Chang et al. 2004). Furthermore, this region was shown to be involved in the binding of PIP2 to TRPV5, as was demonstrated using point-mutants (Rohacs et al. 2005). Finally, the binding of NHERF4 was also impaired upon deletion of this region (Van de Graaf et al. 2006c), whereas S100A10, NHERF2, and CaM associated with distinct regions of the carboxyl-terminus (Lambers et al. 2004; Niemeyer et al. 2001; Van de Graaf et al. 2003, 2006c). This suggests that the MLERK region is either a critical element for the general folding of the carboxyl-terminus of TRPV5/6 or that several of these associated proteins indeed recognize motifs within this region. The latter hypothesis could imply...
that these proteins compete for binding at this region. The occupancy of this site would depend on the relative concentrations and affinities of the proteins. Future examination of the channel structure or studies that investigate binding competition among multiple channel associated proteins are required to clarify this issue.

Epithelial Mg$^{2+}$ transport

Magnesium (Mg$^{2+}$) is the second most abundant intracellular cation, the fourth most abundant extracellular cation, and a cofactor in more than 300 enzymatic reactions varying from energy metabolism to protein and nucleic acid synthesis (Elin 1994; Flatman 1984). Approximately 50% of the total body Mg$^{2+}$ is present intracellularly in soft tissue, and the other half is present in bone. Less than 1% of the total body Mg$^{2+}$ is circulating in blood (Elin 1994). In healthy individuals, plasma Mg$^{2+}$ levels are maintained in a narrow range (0.7–1.1 mmol/l; Konrad et al. 2004) by the balance between intestinal absorption, renal excretion, and dynamic exchange with the stored Mg$^{2+}$ in bone. Several regulatory processes control these Mg$^{2+}$ transport processes in kidney and intestine to account for variations in dietary Mg$^{2+}$ content (Quamme and de Rouffignac 2000). In analogy with renal and intestinal Ca$^{2+}$ transport, Mg$^{2+}$ is transported via a paracellular and a transcellular pathway. First, the various tissues will be discussed where Mg$^{2+}$ is transported and the pathway(s) involved in this process.

Localization of epithelial Mg$^{2+}$ transport

Gastrointestinal tract

Gastrointestinal Mg$^{2+}$ absorption in healthy adults is balanced by the renal excretion of Mg$^{2+}$ into the urine. The fate of intestinal Mg$^{2+}$ in human volunteers was quantified by Fine and coworkers (1991). They measured net Mg$^{2+}$ absorption after ingestion of a standard meal supplemented with various amounts of Mg-acetate. The relationship between absorption and intake was curvilinear, which could be fitted perfectly to the sum of an unsaturable linear component and a saturable component. Saturation kinetics of the transcellular transport system are explained by the limited transport capacity of active transport. This suggests that intestinal Mg$^{2+}$ absorption is mainly transcellular (forming the saturable component of the sum) in conditions of low Mg$^{2+}$ intake and that the relative role of paracellular absorption (forming the unsaturable ~7% of the intake) increases with increasing Mg$^{2+}$ intake.

However, the data provided limited information on the localization of the uptake process within the gastrointestinal tract. In contrast to the kidney, functional data on the distribution of Mg$^{2+}$ absorption in the stomach and gut is relatively scarce. In ruminating animals, most of the required Mg$^{2+}$ is absorbed from the forestomachs by active, transcellular mechanisms (Schweigel and Martens 2000; Schweigel et al. 2006). However, in other mammals, the localization of the Mg$^{2+}$ absorption in the gastrointestinal tract is less clear. The main reason for this lack of data is the absence of a suitable isotope of Mg$^{2+}$, as $^{28}$Mg$^{2+}$ has low specific activity and a short half-life (Avioli and Berman 1966; Dai et al. 2001). It was, however, shown that colectomy in rats and humans results in decreased urinary Mg$^{2+}$ excretion, normal plasma Mg$^{2+}$ levels, and decreased bone Mg$^{2+}$ content, suggesting an important role for this intestinal segment in Mg$^{2+}$ absorption (Croner et al. 2000; Fagan and Phelan 2001). Kayne and Lee have reviewed the available literature and suggested a prominent role for the
distal segments of the small intestine, in particular the ileum and colon in Mg\textsuperscript{2+} absorption (Kayne and Lee 1993). However, they indicated that this information is mainly derived from isolated segments and may not adequately reflect absorption. Schweigel and Martens studied the sites of intestinal Mg\textsuperscript{2+} absorption in various animals and concluded that in dogs, cats, and pigs, Mg\textsuperscript{2+} is predominantly absorbed from the ileum and colon, whereas in horses and probably rabbits, Mg\textsuperscript{2+} is mainly absorbed from the small intestine (Schweigel and Martens 2000).

Intestinal Ca\textsuperscript{2+} absorption was not affected by increased Mg\textsuperscript{2+} intake in humans (Fine et al. 1991), suggesting that these cations utilize distinct, noncompeting uptake mechanisms. Recently, Groenestege and coworkers confirmed this in animal studies. Ca\textsuperscript{2+} absorption, as measured by \textsuperscript{45}Ca\textsuperscript{2+} uptake in the blood, was unaffected by the Mg\textsuperscript{2+} content of the food (Groenestege et al. 2006). However, both in humans and mice, the renal handling of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} pointed to competitive reabsorption of the two divalent cations, suggesting a common transport pathway (Fine et al. 1991; Groenestege et al. 2006). In addition, the distribution of the Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport proteins further pinpoints the localization of transepithelial absorption of divalents (Groenestege et al. 2006). Transepithelial absorption of Ca\textsuperscript{2+} takes place predominantly in duodenum and colon as illustrated by the robust expression of the epithelial Ca\textsuperscript{2+} channel TRPV6 in these particular intestinal segments. However, the luminal Mg\textsuperscript{2+} channel TRPM6 was mainly expressed in colon while being virtually absent in duodenum (Groenestege et al. 2006). Therefore, active Mg\textsuperscript{2+} and Ca\textsuperscript{2+} absorption occurs in the distal part of the intestine, whereas in duodenum only active Ca\textsuperscript{2+} absorption takes place.

### Kidney

Fine-tuning of the Mg\textsuperscript{2+} balance mainly resides within the kidney (Dai et al. 2001; Quamme 1997). Approximately 80% of the total plasma Mg\textsuperscript{2+} is filtered by the glomerulus. Along the nephron 95% of this filtrate is being reabsorbed. Of the ultrafilterable Mg\textsuperscript{2+}, 5–15% is reabsorbed by passive transport in the PT. Remarkably, Lelievre-Pegorier et al. reported that the permeability for Mg\textsuperscript{2+} of the PT dramatically changes during development. The immature kidney of the newborn can absorb up to 70% of the filtered Mg\textsuperscript{2+} in this segment (de Rouffignac and Quamme 1994; Lelievre-Pegorier et al. 1983). Later in development (early childhood), PT reabsorbs only 5–15% of the filtered Mg\textsuperscript{2+}, whereas the fractional reabsorption of Na\textsuperscript{+} and Ca\textsuperscript{2+} remains high (70%). The cTAL plays a major role in the determination of Mg\textsuperscript{2+} excretion, as it accounts for approximately 70% of Mg\textsuperscript{2+} reabsorption (Mandon et al. 1993; Shareghi and Agus 1982), whereas the medullary segment (mTAL) is Mg\textsuperscript{2+} impermeable. Transport in the cTAL is passive and paracellular in nature, driven by the lumen-positive transepithelial potential difference (Mandon et al. 1993). Processes that affect the transepithelial voltage (i.e., rate of Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport) or alter the permeability of the paracellular pathway will therefore alter Mg\textsuperscript{2+} reabsorption in this segment. Finally, 10–15% of the Mg\textsuperscript{2+} that is filtered at the glomerulus will be delivered distally from TAL. The remaining reabsorption takes place in DCT with no evidence for significant Mg\textsuperscript{2+} transport inCNT or CD. Mg\textsuperscript{2+} reabsorption in DCT is mediated by the active transcellular pathway and plays a pivotal role in determining the final urinary Mg\textsuperscript{2+} excretion (Dai et al. 2001). Interestingly, Wilson et al. identified a mutation in mitochondrial DNA resulting in a cluster of metabolic effects including hypomagnesemia due to renal Mg\textsuperscript{2+} wasting. They suggested that impaired mitochondrial function and consequent lower ATP production in DCT has significant effects on the Mg\textsuperscript{2+} transport capacities of this highly energy-consuming nephron.
segment (Bastin et al. 1987; Wilson et al. 2004). Given an average Mg\(^{2+}\) intake, less than 5% of the filtered Mg\(^{2+}\) appears in the urine.

**Bone**

Several studies demonstrated a positive correlation between dietary Mg\(^{2+}\) intake and bone density as indicated by increased bone loss in the situation of deficient Mg\(^{2+}\) intake (New et al. 2000; Tucker et al. 1999). In a rat model, this effect was already observed even in situations of moderate Mg\(^{2+}\) deficiency as demonstrated by Rude et al. Here, only a 50% reduction of the advised dietary Mg\(^{2+}\) intake resulted in bone Mg\(^{2+}\) deficiency, although no significant change in plasma Mg\(^{2+}\) was observed (Rude et al. 2006). This suggests that bone operates as a Mg\(^{2+}\) storage in situations of low Mg\(^{2+}\) supply. The increase in trabecular bone mineral content and bone volume normally observed in rats between 3 and 6 months of age was reduced in the group with dietary Mg\(^{2+}\) restriction. Furthermore, osteoclast number was also significantly increased with Mg\(^{2+}\) depletion. Dietary Mg\(^{2+}\) deficiency was accompanied by low 1,25(OH)\(_2\)D\(_3\) and high PTH levels in plasma and elevated tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) and interleukin 1\(\beta\) (IL-1\(\beta\)) levels in osteoclasts and megakaryocytes, suggesting that these may contribute to bone loss. (Rude et al. 2006; Vidal et al. 2006). However, PTH levels in humans with hypomagnesemia and hypocalcemia have been either low or even undetectable (Agus 1999; Chase and Slatopolsky 1974; Rude and Gruber 2004; Rude et al. 1978).

Similar results were obtained in dogs that were fed a Mg\(^{2+}\)-free diet for 4–6 months (Freitag et al. 1979). This effect might be due to impaired PTH production or secretion. The short-term restoration of plasma PTH levels on Mg\(^{2+}\) supplementation suggests that the latter is mainly affected (Agus 1999). In addition, Mg\(^{2+}\) depletion diminished the cyclic AMP (cAMP) production during PTH administration in isolated bone in line with an organ resistance to PTH (Chase and Slatopolsky 1974; Freitag et al. 1979; Rude et al. 1976). As described, several studies illustrated the important role of Mg\(^{2+}\) in bone formation and integrity. In contrast, in which manner bone affects the Mg\(^{2+}\) balance is mechanistically not clear. Mg\(^{2+}\) exists abundantly in bone (0.5–1%, Rude et al. 2006), suggesting significant Mg\(^{2+}\) transport in this tissue. However, the Mg\(^{2+}\) entry pathway in bone and the molecular mechanism of release is poorly understood. With the current insight of the molecular identity of the players involved in epithelial Mg\(^{2+}\) transport, more information on these mechanisms should become available.

**(Patho)physiology of Mg\(^{2+}\) influx in kidney and intestine**

The genetic basis of a number of inherited renal Mg\(^{2+}\) wasting disorders has been elucidated in recent years. This yielded the identification of novel proteins involved in epithelial Mg\(^{2+}\) transport (Table 3). Furthermore, the various inherited diseases described to date often affect distinct nephron segments and lead to variable phenotypic presentations. As a whole, this novel information has significantly boosted our understanding of epithelial Mg\(^{2+}\) handling.

**Hypomagnesemia with hypercalciuria and nephrocalcinosis**

Hypomagnesemia with hypercalciuria and nephrocalcinosis (HHN, OMIM #248250) is mainly characterized by hypomagnesemia with inappropriately high urinary Mg\(^{2+}\) excretion. It is an autosomal-recessive disorder that is further characterized by hypercalciuria.
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Inheritance</th>
<th>OMIM</th>
<th>Affected protein</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypomagnesemia with hypercalciuria and nephrocalcinosis (HHN)</td>
<td>AR</td>
<td>248250</td>
<td>Claudin-16, claudin-19</td>
<td>Konrad et al. 2006; Simon et al. 1999</td>
</tr>
<tr>
<td>Dominant isolated hypomagnesemia with hypocalciuria (IDH)</td>
<td>AD</td>
<td>154020</td>
<td>γ-Subunit Na⁺, K⁺-ATPase</td>
<td>Meij et al. 2000</td>
</tr>
<tr>
<td>Dominant isolated hypomagnesemia with hypocalciuria</td>
<td>AD</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Hypomagnesemia with normocalciuria</td>
<td>AR</td>
<td>–</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Autosomal dominant hypoparathyroidism (ADH)</td>
<td>AD, AR</td>
<td>146200</td>
<td>CaSR, Activating mutations</td>
<td>Lovlie et al. 1996</td>
</tr>
<tr>
<td>Familial hypomagnesemia/ neonatal severe hyperparathyroidism</td>
<td>AR</td>
<td>239200</td>
<td>CaSR, Inactivating mutations</td>
<td>Pollak et al. 1993</td>
</tr>
<tr>
<td>Familial hypocalciuric hypercalcemia (FHH/ or HHC1) and neonatal severe hyperparathyroidism (NSHPT)</td>
<td>AR</td>
<td>145980</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gitelman’s syndrome (GS)</td>
<td>AR</td>
<td>263800</td>
<td>NCC</td>
<td>Simon et al. 1996</td>
</tr>
<tr>
<td>Hypomagnesemia, hypertension and hypercholesterolemia</td>
<td>M</td>
<td>500005</td>
<td>MTTI</td>
<td>Wilson et al. 2004</td>
</tr>
<tr>
<td>Familial hypomagnesemia with secondary hypocalcemia (HSH)</td>
<td>AR</td>
<td>602014</td>
<td>TRPM6</td>
<td>Schlingmann et al. 2002; Walder et al. 2002</td>
</tr>
</tbody>
</table>

AR, autosomal-recessive; AD, autosomal-dominant; NCC, Na⁺/Cl⁻ cotransporter; MTTI, mitochondrial transfer RNA; TRPM6, transient receptor potential cation channel subfamily M member 6; CaSR, Ca²⁺ sensing receptor; ND, not determined. See text for explanation.
often leading to nephrocalcinosis (Manz et al. 1978; Milazzo et al. 1981; Nicholson et al. 1995; Praga et al. 1995; Richard and Freycon 1992; Torralbo et al. 1995). Furthermore, several patients with this disorder displayed ocular disorders (Torralbo et al. 1995). Renal transplantation corrected the abnormal Mg\(^{2+}\) and Ca\(^{2+}\) handling and normalizes plasma Mg\(^{2+}\) and Ca\(^{2+}\) concentrations (Praga et al. 1995), further substantiating the role of the kidney in the etiology of the disease. Using whole genome analysis in 12 kindreds with recessive renal hypomagnesemia, Simon et al. demonstrated linkage to a segment at chromosome 3q27 and subsequently identified the responsible gene, which was called PCLN-1 or CLDN-16 (Simon et al. 1999). Subsequently, several other groups demonstrated patients with mutations in this gene (Muller et al. 2003; Weber et al. 2000, 2001b). The PCLN-1 gene encodes a protein of 305 amino acids with 4 TMs and intracellular amino- and carboxy-termini named paracellin-1 (Simon et al. 1999).

Paracellin-1 shows sequence and structural similarity to members of the claudin family and was therefore renamed claudin-16. Most claudins have an amino-terminus of only 6 to 7 amino acids (Morita et al. 1999). However, claudin-16 contains a cytoplasmic amino-terminus of 73 amino acids. Northern blot analysis demonstrated that claudin-16 is exclusively expressed in kidney (Simon et al. 1999). Here, its expression was localized to TAL and DCT (Figs. 1 and 3). Significant colocalization between claudin-16 and occludin was demonstrated by confocal microscopy, indicating that this protein is a component of tight junctions (Schneeberger and Lynch 2004; Simon et al. 1999). This was corroborated by Muller et al. who identified a novel homozygous mutation in the CLDN-16 gene (T233R) in

![Image](image-url)

**Fig. 3** Localization of transport proteins involved in active and passive Ca\(^{2+}\) and Mg\(^{2+}\) reabsorption along the nephron. TRPV5 transient receptor potential cation channel subfamily V member 5, TRPV6 transient receptor potential cation channel subfamily V member 6, NCX1 Na\(^{+}\)/Ca\(^{2+}\) exchanger type 1, PMCA1b ATP-dependent Ca\(^{2+}\)-ATPase type 1b, TRPM6 transient receptor potential cation channel subfamily M member 6, \(\gamma\)-subunit \(\gamma\)-subunit of the Na\(^{+}\)/K\(^{+}\) ATPase, NKCC2 Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter type 2 ROMK1 renal outer medullary K\(^{+}\) channel.
two families (Müller et al. 2003). They showed that the mutation results in an activation of a PDZ-domain binding motif in claudin-16, disabling the association of claudin-16 with the tight junction protein ZO1. The mutant claudin-16 was no longer localized to tight junctions in kidney epithelial cells, but instead accumulated in lysosomes. Furthermore, these patients displayed serious childhood hypercalciuria, in contrast to more classic symptoms as hypomagnesemia with hypercalciuria (Müller et al. 2003). Thus, mutations at different sites in the claudin gene may lead to particular clinical phenotypes with a distinct prognosis.

Konrad and coworkers recently described several families with a similar renal phenotype as patients with CLDN16 mutations but the affected individuals also showed severe visual impairment (Konrad et al. 2006). The CLDN16 gene in these patients was not mutated pointing to a distinct affected gene. Genome-wide linkage and subsequent mutation analyses pointed to disease causative mutations in CLDN19, the gene encoding claudin-19. Claudin-19 is a tight junction protein (Lee et al. 2006b) expressed in the eye and in the kidney where it prominently colocalizes with claudin-16 in TAL (Konrad et al. 2006). On the whole, this shows that at least two distinct tight junction proteins of the claudin family are involved in Mg^{2+} homeostasis and explains HHN with ocular defects in several families with unaffected CLDN16.

**Dominant isolated hypomagnesemia with hypocalciuria**

Geven et al. described two unrelated families in which hypomagnesemia due to renal Mg^{2+} wasting was inherited as an autosomal dominant trait (Geven et al. 1987a) (OMIM #154020). Mg^{2+} infusions performed in two patients showed a reduced renal transport maximum for Mg^{2+} and Ca^{2+}. Subsequently, Meij et al. performed a genome-wide linkage study in these families (Meij et al. 1999). Linkage was demonstrated to locus 11q23. Detailed haplotype analyses suggested the existence of a single, hypomagnesemia-causing mutation in both families. In a follow-up study Meij et al. identified a putative dominant-negative mutation in the FXYD2 gene encoding the Na\(^+\),K\(^+\)-ATPase \(\gamma\)-subunit (\(\gamma\)-subunit Na\(^+\),K\(^+\)-ATPase) (Meij et al. 2000). It was shown that the mutant \(\gamma\)-subunit accumulates in the cytoplasm, whereas the wild-type protein localizes predominantly to the plasma membrane (Meij et al. 2000).

The \(\gamma\)-subunit of Na\(^+\),K\(^+\)-ATPase was originally cloned by Kim et al. and encodes a small (58 amino acids), type I membrane protein (Kim et al. 1997). The protein is localized in DCT, the main site of active renal Mg^{2+} reabsorption. Two individuals with an 11q23.3-qter deletion including FXYD2 had normal plasma Mg^{2+} levels, showing that the hypomagnesemia results from the presence of mutant \(\gamma\)-subunit Na\(^+\),K\(^+\)-ATPase, rather than from haploinsufficiency, consistent with a dominant-negative inheritance pattern (Meij et al. 2000). This was corroborated in mice lacking the FXYD2 gene, which show no disturbances in the Mg^{2+} balance (Jones et al. 2005).

The Na\(^+\)-K\(^+\)-ATPase complex consists of three subunits. The catalytic \(\alpha\)-subunit hydrolyzes ATP and transports the cations, whereas the \(\beta\)- and \(\gamma\)-subunits function in the membrane insertion of the \(\alpha\)-subunit and modulate its transport properties (Levenson 1994). There is some controversy whether the presence of the mutant \(\gamma\)-subunit results in the impaired trafficking of the entire Na\(^+\)-K\(^+\)-ATPase complex to the plasma membrane or the affected subunit (with normal membrane insertion of the \(\alpha\)- and \(\beta\)-subunits) (Meij et al. 2000; Pu et al. 2002). Furthermore, the precise cellular mechanism of decreased Mg^{2+} reabsorption and the hypocalciuria remains to be further refined. Kantorovich et al. described a family with a similar phenotype as patients with mutated \(\gamma\)-subunit Na\(^+\),K\(^+\)-ATPase, but without linkage to the 11q23 locus. Genetic analysis suggested an autosomal dominant in-
heritance, although a X-linked dominant inheritance could not be excluded (Kantorovich et al. 2002). This shows that dominant inheritance of hypomagnesemia can be caused by a gene other than \textit{FXYD2} and points to the existence of another protein involved in \textit{Mg}\textsuperscript{2+} homeostasis.

**Autosomal-recessive hypomagnesemia with normocalciuria**

Another variant of hypomagnesemia that is more consistent with isolated renal \textit{Mg}\textsuperscript{2+} loss with autosomal-recessive inheritance was described (Geven et al. 1987b; Meij et al. 2002). Unlike several other \textit{Mg}\textsuperscript{2+} wasting diseases, no abnormalities in plasma or urine \textit{Ca}\textsuperscript{2+} concentrations were observed in these patients. However, despite the hypomagnesemia, \textit{Mg}\textsuperscript{2+} excretion in the urine was in the normal range, indicating a renal defect in these patients. Meij et al. excluded linkage to the \textit{FXYD2} locus and mutations in any other previously reported loci associated with hypomagnesemia, indicating a distinct disease (Meij et al. 2003). Therefore, this suggests that a second so-far-unidentified genetic cause for hypomagnesemia exists. Identification of the etiology of this disease should provide novel insight into renal \textit{Mg}\textsuperscript{2+} handling and maintenance of the \textit{Mg}\textsuperscript{2+} balance.

**Disorders associated with abnormal extracellular \textit{Mg}\textsuperscript{2+}/\textit{Ca}\textsuperscript{2+} sensing**

Malfunctioning of CaSR is frequently associated with disturbed \textit{Mg}\textsuperscript{2+} handling (OMIM 239200 and 146200). The CaSR is sensitive to both \textit{Ca}\textsuperscript{2+} and \textit{Mg}\textsuperscript{2+}, although the relative affinity for \textit{Ca}\textsuperscript{2+} is higher (Brown 1991). In the parathyroid glands, CaSR senses the plasma levels of these divalent ions to regulate the PTH production and secretion. Both activating and inactivating mutations in the CaSR have been identified. Activating mutations are dominant and lead to hypoparathyroidism (Pollak et al. 1994), which is mainly characterized by hypocalcemia and hypercalciuria. However, hypomagnesemia is observed in up to about 50\% of affected individuals (Okazaki et al. 1999; Pearce et al. 1996). This is explained by a shift of the set point of the receptor, resulting in decreased PTH secretion by the parathyroid glands and inhibition of divalent cation reabsorption in the kidney (Brown and MacLeod 2001). On the other hand, patients with inactivating CaSR mutations display hypocalciuric hypercalcemia (Pollak et al. 1993). Furthermore, affected individuals also show a tendency toward hypermagnesemia (Marx et al. 1981). Inactivation of both alleles of the CaSR gene requires parathyroidectomy early in life to prevent fatal consequences of the severe hyperparathyroidism (Pollak et al. 1993).

**Gitelman’s syndrome**

In Gitelman’s syndrome, hypomagnesemia is accompanied by hypocalciuria, hypokalemia, and metabolic alkalosis (OMIM #263800). The low urinary \textit{Ca}\textsuperscript{2+} excretion is the biochemical parameter to distinguish between Gitelman’s and Bartter’s syndrome, genetically distinct tubular transport disorders that share a hypokalemic metabolic alkalosis (Knoers et al. 2003). Dissociation of renal \textit{Ca}\textsuperscript{2+} from \textit{Mg}\textsuperscript{2+} transport together with exaggerated natriuresis after furosemide treatment in these patients indicated the presence of a defect in DCT rather than in TAL. Furthermore, hydrochlorothiazide administration had almost no effect in patients with Gitelman’s syndrome compared to controls (Colussi et al. 1997).

The molecular explanation of Gitelman’s syndrome was provided by the demonstration of linkage of the disease to the \textit{Na}\textsuperscript{+}-\textit{Cl}\textsuperscript{−} cotransporter (NCC) gene on 16q13 (Pollak et al. 1996; Simon et al. 1996). This cotransporter is the target of thiazide diuretics, one
of the major classes of agents used in the treatment of hypertension (Gamba et al. 1993), and expressed in the apical membrane of DCT (Bachmann et al. 1995; Loffing et al. 2001; Plotkin et al. 1996). The correlation between Gitelman’s syndrome and NCC was confirmed in a mouse model (Loffing et al. 2004; Morris et al. 2006; Schultheis et al. 1998) that shows all the characteristics of the disease. More than 100 different, putative loss-of-function mutations in the NCC encoding gene (SLC12A3) have been identified in Gitelman’s patients (Knoers et al. 2003). Functional expression studies and results of immunocytochemistry in Xenopus laevis oocytes showed that most disease-causing NCC mutants are impaired in their trafficking to the plasma membrane (de Jong et al. 2002).

The mechanisms of hypocalciuria and hypomagnesemia in Gitelman’s syndrome remain unclear. It has been suggested that inactivating mutations of NCC cause hypocalciuria by the same mechanisms as postulated for chronic thiazide administration (Nijenhuis et al. 2005). Hypomagnesemia has been suggested to be associated with hypokalemia, a hypothesis disputed by studies in NCC knockout mice that develop severe hypocalciuria and hypomagnesemia despite the absence of hypokalemia or alkalosis (Knoers et al. 2003), although this might be dependent on the amount of potassium in the diet (Morris et al. 2006). An alternative hypothesis to explain hypomagnesemia in Gitelman’s syndrome is based on the observation in rats that blockage of NCC by chronic thiazide treatment results in an increased rate of apoptosis in DCT cells (Loffing et al. 1996). Furthermore, remodeling of DCT has been observed in mouse models of Gitelman’s syndrome, with a significant reduction in parvalbumin positive (DCT1) tubules (Loffing et al. 2004). It is, therefore, possible that reduced number of DCT cells in Gitelman’s syndrome compromises Mg\(^{2+}\) reabsorption in this nephron segment resulting in hypomagnesemia. This is in line with recent data from Nijenhuis et al. who showed a significant reduction of the expression of the epithelial Mg\(^{2+}\) channel TRPM6 during chronic thiazide treatment (Nijenhuis et al. 2005). However, this latter study does not discriminate whether the DCT cells are reduced in number or that the cells display lower expression of proteins involved in transepithelial Mg\(^{2+}\) transport. This latter would be another explanation for the observed hypomagnesemia during thiazide treatment or in Gitelman’s patients.

**Hypomagnesemia with secondary hypocalcemia**

Familial hypomagnesemia with secondary hypocalcemia (HSH) is an autosomal recessive disease due to defective intestinal and renal Mg\(^{2+}\) (re)absorption (OMIM #602014). The disease can be fatal or results in permanent neuronal damage if untreated. As the passive component of intestinal Mg\(^{2+}\) transport is not affected, the disease can be controlled with high oral Mg\(^{2+}\) supplements.

The mechanisms leading to hypocalcemia are not completely understood, but might be related to Mg\(^{2+}\)-dependent effects on PTH secretion or PTH resistance as described in “Disorders associated with abnormal extracellular Mg\(^{2+}\)/Ca\(^{2+}\) sensing”. However, plasma Ca\(^{2+}\) and PTH levels are usually restored by administration of high doses of Mg\(^{2+}\). In addition to the observed decreased intestinal Mg\(^{2+}\) absorption in HSH, there may be a renal leak, deteriorating the efficacy of oral Mg\(^{2+}\) supplements to normalize plasma Mg\(^{2+}\) or the symptoms of the hypomagnesemia (Konrad et al. 2004; Matzkin et al. 1989). It was postulated that the renal defect was due to impaired Mg\(^{2+}\) reabsorption in DCT (Cole and Quamme 2000). HSH was initially postulated to be a X-linked recessive disorder based on its initial predominant occurrence in males (Skyberg et al. 1967; Stromme et al. 1969; Vainsel et al. 1970), and on the basis of a case of X-autosome translocation t(9;X) (Chery et al. 1994; Walder et al. 1997). The proposed X-linked inheritance of this disorder was later questioned, as an
autosomal recessive inheritance was suggested (Garty et al. 1983; Hennekam and Doncker-wolce 1983; Pronicka and Gruszczynska 1991; Walder et al. 1997).

Finally, Walder et al. convincingly demonstrated that the disorder is, in fact, autosomal recessive and is determined by a mutation in a gene located on 9q12-q22.2 (Walder et al. 1997). The disease segregated with a single affected haplotype in three inbred Bedouin kindreds from Israel, suggesting that hypomagnesemia is caused by a common ancestral mutation. Walder et al. suggested that a likely candidate gene for HSH would be a receptor or ion channel involved in the absorption of intestinal Mg$^{2+}$ (Walder et al. 1997). Indeed, using positional cloning, Schlingmann et al. and Walder et al. identified HSH causing mutations in a gene within this region encoding a novel member of the TRP superfamily of cation channels, TRPM6 (Schlingmann et al. 2002; Walder et al. 2002).

Subsequently, many novel mutations in several families with HSH were elucidated, further confirming the role of TRPM6 in this disease (Schlingmann et al. 2005). TRPM6 (also known as CHAK2) is a member of the transient receptor potential melastatin (TRPM) cation channel family. Remarkably, TRPM6 comprises both an ion channel domain and a protein kinase and will be described in detail in section “TRPM6”.

Molecular mechanism of epithelial Mg$^{2+}$ transport

Elucidation of the genetic basis of a number of diseases that involve disturbances of the Mg$^{2+}$ balance has increased our understanding of epithelial Mg$^{2+}$ transport. Some of the molecular players that are mutated in certain pathological states affect mainly paracellular transport of Mg$^{2+}$, whereas other diseases are caused by disturbances in transcellular transport. In this section our current knowledge and some hypothesis are summarized about the molecular mechanisms of these Mg$^{2+}$ transport pathways.

Paracellular Mg$^{2+}$ transport

Paracellular Mg$^{2+}$ reabsorption accounts for a significant fraction of the total Mg$^{2+}$ (re)absorption in intestine and kidney. In TAL paracellular Mg$^{2+}$ absorption is driven by the lumen-positive transepithelial voltage (Mandon et al. 1993). This transepithelial potential is mainly created by the luminal K$^{+}$ conductance of the ROMK channel and drives the positively charged Mg$^{2+}$ and Ca$^{2+}$ ions from the lumen through the paracellular pathway into the interstitium (Fig. 1).

The high paracellular Mg$^{2+}$ and Ca$^{2+}$ conductance contrasts with the low water permeability of this segment (Kokko 1974). As described in “Hypomagnesemia with hypercalcuria and nephrocalcinosis”, the identification of genetic defects in claudin-16 revealed that this protein governs the divalent ion conductance of the tight junction complex (Simon et al. 1999). Wild-type claudin-16 resides on the plasma membrane in HeLa, MDCK, and LLC-PK1 cells. In these latter two cell types it is confined to tight junctions where it colocalizes and associates with ZO-1 (Hou et al. 2005; Muller et al. 2003). Disease-causing mutations in claudin-16 can lead to the intracellular retention of this protein or affect its capacity to facilitate paracellular Mg$^{2+}$ transport (Kausalya et al. 2006; Muller et al. 2006). Kausalya et al. reported that several claudin-16 mutants are retained in the endoplasmic reticulum, where they undergo proteasomal degradation or accumulate in the Golgi complex (Kausalya et al. 2006). In addition, two mutants were delivered to lysosomes, one via clathrin-mediated endocytosis following transport to the cell surface and the other without appearing on the
plasma membrane (Kausalya et al. 2006). This indicates that claudin-16 mutations have distinct cellular consequences. Cell surface localization of some of these mutants was rescued by inhibiting endocytosis (Muller et al. 2006) or using compounds acting as pharmacological chaperones (Kausalya et al. 2006). These compounds include glycerol, dimethylsulfoxide, thapsigargin, curcumin, and 4-phenylbutyrate (4-PBA) and have been postulated to aid in the correct folding of transmembrane proteins (Ulloa-Aguirre et al. 2004), including mutant forms of the cystic fibrosis transmembrane regulator (CFTR) (Egan et al. 2002, 2004; Sato et al. 1996), the V2 vasopressin receptor (Robben et al. 2006; Tan et al. 2003), and aquaporin 2 (Tamarappoo et al. 1999). However, these chaperones did not restore paracellular Mg$^{2+}$ permeability, suggesting that this claudin-16 mutant has additional defects on paracellular Mg$^{2+}$ transport capacities (Kausalya et al. 2006). Remarkably, in LLC-PK1 cells, the G121R claudin-16 mutant was localized in tight junctions even without pharmacological intervention, further supporting a transport defect of this mutant protein (Hou et al. 2005).

There are currently two models explaining the molecular mechanism by which claudin-16 mediates paracellular Mg$^{2+}$ transport. Upon identification of mutations, it was initially postulated that claudin-16 directly promotes paracellular Mg$^{2+}$ and Ca$^{2+}$ movement by creating a selective paracellular conductance for these divalent cations, allowing paracellular fluxes of Mg$^{2+}$ and Ca$^{2+}$ down their electrochemical gradients (Simon et al. 1999; Wong and Goodenough 1999). This hypothesis was supported by studies on two other claudins (claudin-4 and 14), showing that these proteins also influence ion selectivity by forming a charge-selective passage through the tight-junction barrier (Colegio et al. 2002; Van Itallie et al. 2001).

A recent report by Hou et al., however, suggested that in LLC-PK1 epithelial cells claudin-16 modulates the ion selectivity of the tight junction by significantly and selectively increasing the permeability of Na$^+$ (with no effects on Cl$^-$) and generating a high permeability ratio of Na$^+$ to Cl$^-$ (Hou et al. 2005). Remarkably, Mg$^{2+}$ flux across cell monolayers showed a far less-pronounced change (compared to monovalent cations) following claudin-16 expression, suggesting that this protein does not form Mg$^{2+}$-selective paracellular channels (Hou et al. 2005). Therefore, a second hypothesis was postulated to explain the phenotype of patients with defects in the claudin-16 gene by causing a reduction in driving force for transepithelial Mg$^{2+}$/Ca$^{2+}$ movement without directly affecting a divalent-selective shunt (Hou et al. 2005).

Early microperfusion studies (Greger and Schlatter 1983) have indicated that the transepithelial voltage is lumen positive (+3–10 mV) when TAL is perfused with isotonic solutions. This is mainly due to apical ROMK1-mediated membrane K$^+$ secretion. However, in conditions where the lumen content in TAL is hypotonic compared to the interstitial fluid (as is the physiological condition in the TAL) the transepithelial potential in TAL increases to as much as +30 mV (lumen positive) (Greger 1981; Rocha and Kokko 1973). This results from the back-flow of Na$^+$ from the interstitium to the lumen down its concentration gradient via the paracellular pathway. Claudin-16 dysfunction in TAL, with a concomitant loss of cation selectivity, could therefore contribute to a reduction of the lumen-positive potential that constitutes the driving force for the reabsorption of Mg$^{2+}$ and Ca$^{2+}$ (Hou et al. 2005). Future studies that address the transmembrane potential in the TAL in the presence and absence of functional claudin-16 are needed to distinguish between these two models.

**Physiological regulation of paracellular Mg$^{2+}$ transport**

Paracellular Mg$^{2+}$ movement is affected by two distinct mechanisms. First, all factors that affect the transepithelial potential also control the amount of Mg$^{2+}$ transported via the para-
cellular pathway, for reasons described above. Second, the expression of claudins is subject to regulation (reviewed in Balkovetz 2006). So far, little information is available on the molecular regulation of claudin-16, which seems the most relevant claudin for epithelial Mg²⁺ transport. However, the role of other claudins in paracellular Mg²⁺ movement, i.e., in the intestine, cannot be excluded. In vitro analysis of the human claudin-16 (using luciferase reporter vectors) indicated promoter activity in renal cell lines only (Efrati et al. 2005). Interestingly, a high ambient Mg²⁺ concentration ([Mg²⁺]) increased, whereas low [Mg²⁺] reduced the promoter activity (Efrati et al. 2005). Furthermore, 1,25(OH)₂D₃ decreased claudin-16 promoter activity, and this action appeared to be mediated through the single peroxisome-proliferator-responsive element (PPRE) within the promoter region (Efrati et al. 2005). It is currently unclear to what extent these findings correlate with a physiological regulation of claudin-16 expression and/or paracellular Mg²⁺ transport by the circulating Mg²⁺ and 1,25(OH)₂D₃ concentration.

Transcellular Mg²⁺ transport

In analogy with active Ca²⁺ (re)absorption (as described in “Transcellular Ca²⁺ transport”), the process of transcellular Mg²⁺ transport is envisaged by a series of sequential steps. This transport starts with the entry into the epithelial cell through the luminal epithelial Mg²⁺ channel TRPM6. This step is driven by a favorable transmembrane potential. Subsequently, Mg²⁺ diffuses through the cytosol to reach the basolateral plasma membrane. Here, Mg²⁺ is actively extruded into the interstitium that is in contact with the blood compartment. It was postulated that luminal Mg²⁺ entry forms the rate-limiting step (Dai et al. 2001; Groenestege et al. 2006; Voets et al. 2004b) and therefore the major site of regulation (Fig. 1).

Mg²⁺ influx

In contrast to the steep chemical gradient for Ca²⁺, mammalian cells lack a significant chemical gradient driving Mg²⁺ influx, since [Mg²⁺]ᵢ is typically in the submillimolar range (Grubbs 2002; Romani and Maguire 2002; Wolf et al. 2003). Consequently, the negative membrane potential primarily drives the movement of Mg²⁺ into the cell. Until recently, the molecular nature of the luminal Mg²⁺ influx pathway remained elusive. Goytain and Quamme attempted to elucidate the characteristics of the epithelial Mg²⁺ transporter and identified a number of proteins with similarity to bacterial Mg²⁺ transporting proteins. Moreover, with the recent elucidation of TRPM6, a novel TRP channel required for renal and intestinal Mg²⁺ absorption, and TRPM7, a ubiquitously expressed cellular Mg²⁺ channel, our understanding of Mg²⁺ influx pathways has significantly advanced.

MagT1, SLC41A1, SLC41A2, and ACDP2

Two approaches have recently been applied to identify proteins with Mg²⁺-transporting capacities. First, eukaryotic proteins were cloned based on homology with the prokaryote CorA Mg²⁺ transport protein family. A Mg²⁺ transport protein family, termed Mrs2, was isolated from yeast, mouse, and human mitochondria (Bui et al. 1999; Zsurka et al. 2001). However, Mrs2 does not seem to operate as a cellular Mg²⁺ influx channel, but is merely involved in Mg²⁺ transport in mitochondria (Kolisek et al. 2003). In addition, homology search with prokaryotic Mg²⁺ transport proteins yielded the elucidation of members of the solute carrier 41 (SLC41) family as novel Mg²⁺ transporters (Goytain and Quamme 2005b; Goytain and Quamme 2005c; Wabakken et al. 2003). The second approach to find novel Mg²⁺ transport proteins was performed by Goytain et al.
who screened for genes that are upregulated by low extracellular Mg\(^{2+}\) in a DCT-like cell line and in mouse kidney. They identified SLC41A1 (Goytain and Quamme 2005b), a Mg\(^{2+}\) transporter called MagT (Goytain and Quamme 2005d), and the ancient conserved domain protein 2 (ACDP2) (Goytain and Quamme 2005a). SLC41 proteins consist of 10 putative TMs, and SLC41A1 has a predicted molecular mass of 56 kDa (Wabakken et al. 2003). Goytain et al. showed that expression of members of the SLC41 family, including SLC41A1 and SLC41A2, in *Xenopus* oocytes resulted in currents that are dependent on extracellular Mg\(^{2+}\) (Goytain and Quamme 2005b, c). Both SLC41A1 and SLC41A2 also transport a variety of other divalent cations. Expression of SLC41A1 resulted in transport of a range of divalent cations: Mg\(^{2+}\), Sr\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\), Co\(^{2+}\), Ba\(^{2+}\), and Cd\(^{2+}\). The divalent cations Ca\(^{2+}\), Mn\(^{2+}\), and Ni\(^{2+}\) and the trivalent ion Gd\(^{3+}\) did not induce currents nor did they inhibit Mg\(^{2+}\) transport, whereas La\(^{3+}\) abolished Mg\(^{2+}\) uptake (Goytain and Quamme 2005b). In addition to Mg\(^{2+}\), cells overexpressing SLC41A2 transported a range of other divalent cations: Ba\(^{2+}\), Ni\(^{2+}\), Fe\(^{2+}\), or Mn\(^{2+}\), but not Ca\(^{2+}\), Zn\(^{2+}\), or Cu\(^{2+}\). Mg\(^{2+}\) transport was inhibited by high concentrations of Ca\(^{2+}\) (Goytain and Quamme 2005c).

MagT comprises 335 amino acids with a relative molecular mass of approximately 38 kDa. Hydrophathy profile analysis suggested that MagT1 is an integral membrane protein containing five hydrophobic transmembrane-spanning (TM) \(\alpha\)-helical regions, the first of which is likely cleaved to form the final product with four TM domains (Goytain and Quamme 2005d). MagT proteins form a novel family without major similarity to other transport proteins. This family consists of two members, MagT1 and MagT2, of which MagT1 is selective for Mg\(^{2+}\), whereas MagT2 is also permeable for other divalent cations including Fe\(^{2+}\), Cu\(^{2+}\), and Mn\(^{2+}\) (Goytain and Quamme 2005d).

The ancient conserved domain gene, ACDP2, is responsive to Mg\(^{2+}\) and encodes a protein of 874 amino acids with a postulated 4 TM topology (Wang et al. 2004a). ACDP2 is part of a family of four homologous ubiquitous proteins with homology to the microbial CorC protein, which is involved in bacterial Co\(^{2+}\) resistance (Gibson et al. 1991; Wang et al. 2003). The cellular localization of the endogenous ACDP proteins is unclear, although plasma membrane localization was suggested for ACDP1 in neurons (Wang et al. 2004a). However, overexpressed ACDP1 in HeLa cells primarily localized to the nucleus (Wang et al. 2003). When expressed in oocytes, ACDP2 mediated saturable Mg\(^{2+}\) uptake with a Michaelis constant of 0.56 mM (Goytain and Quamme 2005a). ACDP2 has a low substrate selectivity as it transports a range of divalent cations: Mg\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\), Cu\(^{2+}\), and Fe\(^{2+}\). The cations Ca\(^{2+}\), Cd\(^{2+}\), Zn\(^{2+}\), and Ni\(^{2+}\) did not induce currents (Goytain and Quamme 2005a).

Although a role for these novel proteins in maintaining the Mg\(^{2+}\) balance was postulated by Goytain et al., several lines of evidence remain untouched. The ubiquitous tissue distribution does not support a specialized role in epithelial Mg\(^{2+}\) transport. Likewise, the localization of these transporters in kidney and intestine remains unknown, as well as the cellular distribution of these proteins. Finally, the phenotype of patients with mutations in TRPM6 showed that neither of these putative Mg\(^{2+}\) transporters is able to compensate the physiological role of TRPM6.

### TRPM6

The TRPM6 gene contains 39 exons encoding a protein of 2,022 amino acids with a calculated molecular mass of 234 kDa. (Clapham 2003; Schlingmann et al. 2002; Voets et al. 2004b; Walder et al. 2002). TRPM6 shares the highest (approximately 50%) sequence homology with TRPM7, which was identified by Runnels et al. in a screen for phospholipase C-associated proteins (Nadler et al. 2001; Runnels et al. 2001). TRPM6 and
TRPM7 share the predicted topology of 6 TM domains with a putative pore region between TM5 and TM6. However, unlike other members of the TRP family, TRPM6 and TRPM7 contain long carboxyl-terminal domains with similarity to the atypical α-kinases (Runnels et al. 2001; Schlingmann et al. 2002; Walder et al. 2002). The combination of channel and enzyme domains in TRPM6 and TRPM7, also known as chanzymes, is unique among known proteins and raises intriguing questions concerning the physiological role(s) of these chanzymes (Montell 2003). TRPM6 has a restricted expression pattern and is predominantly present in absorbing epithelia, whereas TRPM7 is ubiquitously expressed and implicated in cellular Mg\(^{2+}\) homeostasis (Groenestege et al. 2006; Nadler et al. 2001; Schlingmann et al. 2002; Schmitz et al. 2003; Voets et al. 2004b; Walder et al. 2002). In kidney, immunohistochemical studies using NCC as a marker of DCT showed that the localization of TRPM6 was restricted to this segment (Voets et al. 2004b; Fig. 3).

Importantly, this localization strongly supports a function in transcellular Mg\(^{2+}\) reabsorption, which is restricted to DCT. The localization in the apical domain of DCT cells is further in line with TRPM6 as the gatekeeper of Mg\(^{2+}\) influx. In small intestine, TRPM6-positive signal was detected in absorptive epithelial cells by in situ hybridization and immunohistochemistry (Schlingmann et al. 2002; Voets et al. 2004b). In these cells, TRPM6 was localized along the brush-border membrane (Voets et al. 2004b). Groenestege et al. recently addressed the relative expression of TRPM6 and TRPM7 (Groenestege et al. 2006). They showed that TRPM6 is expressed predominantly in kidney, lung, and intestine, whereas TRPM7 is distributed ubiquitously. Several other tissues including spleen, heart, brain, and liver, were virtually negative for TRPM6. In intestine, highest TRPM6 mRNA levels were measured in cecum and colon, in line with a predominant localization of the active component of intestinal Mg\(^{2+}\) absorption in these latter segments.

Voets and coworkers expressed full-length TRPM6 in HEK293 cells to functionally characterize this novel ion channel. They showed that TRPM6-transfected HEK293 cells perfused with an extracellular solution containing 1 mM Mg\(^{2+}\) or Ca\(^{2+}\) exhibit characteristic outwardly rectifying currents upon establishment of the whole-cell configuration (Voets et al. 2004b). These results were similar to those obtained upon overexpression of TRPM7 (Nadler et al. 2001; Runnels et al. 2001, 2002). In contrast, other groups reported that TRPM6 alone does not yield any additional currents compared to mock-transfected cells (Chubanov et al. 2004; Schmitz et al. 2005). Chubanov and coworkers suggested that association with TRPM7 is a prerequisite for proper plasma membrane localization of TRPM6, and therefore for functional activity (Chubanov et al. 2004). The S141L TRPM6 missense mutation that causes HSH abrogated the oligomeric assembly of TRPM6 and therefore provides a molecular explanation for this disease. This further demonstrates a role for the amino-terminus in the oligomerization of TRPM6. Furthermore, during the procedure to isolate full-length TRPM6, the human TRPM6 gene was found to encode for multiple mRNA isoforms (Chubanov et al. 2004). Therefore, it seems likely that subtle differences are present between the various TRPM6 constructs used in the literature. This notion is further supported by Li and coworkers who characterized homomeric and heteromeric TRPM6 and TRPM7. This latter group used the TRPM6 construct employed in the study by Voets et al. and confirmed that expression of TRPM6 alone yields functional channels in both HEK293 and CHOK1 cells, which exhibit lower endogenous TRPM7 expression than HEK203 cells (Li et al. 2006).

At physiological membrane potentials, significant inward currents were observed in TRPM6-expressing HEK293 and CHOK1 cells with all tested divalent cations as the sole charge carrier, including Ca\(^{2+}\) and Mg\(^{2+}\). The linkage of mutations in TRPM6 with the HSH phenotype indicated that this current is essential and perhaps sufficient for epithelial Mg\(^{2+}\)
It is possible that the TRPM6-mediated Mg\(^{2+}\) inward current is more pronounced in native DCT and intestinal cells as a result of specific cofactors, such as intracellular Mg\(^{2+}\) buffers, that are missing in overexpression systems used to date. Voets et al. evaluated the effect of [Mg\(^{2+}\)]\(_i\) on TRPM6 activity, using flash photolysis of the photolabile Mg\(^{2+}\) chelator DM-nitrophen to rapidly alter the [Mg\(^{2+}\)] in a spatially uniform manner (Voets et al. 2004b). The TRPM6-mediated current was significantly inhibited by increased levels of [Mg\(^{2+}\)]\(_i\) (K\(_D\) \~0.5 mM) indicating that the channel is tightly regulated by [Mg\(^{2+}\)]\(_i\). Similarly, TRPM7 channel activity is strongly reduced by millimolar concentrations of Mg\(^{2+}\)-ATP (Hermosura et al. 2002; Nadler et al. 2001). Kozak and Cahalan demonstrated that TRPM7 inhibition is mediated by intracellular Mg\(^{2+}\) rather than ATP (Kozak and Cahalan 2003).

Importantly, data obtained by Voets et al. indicated that the TRPM6 pore has a higher affinity for Mg\(^{2+}\) than for Ca\(^{2+}\) (Voets et al. 2004b). This is physiologically important as micropuncture studies have shown that the luminal concentration of free Mg\(^{2+}\) in DCT ranges from 0.2 to 0.7 mM (Dai et al. 2001), whereas the luminal Ca\(^{2+}\) concentration is in the millimolar range. Therefore, luminal Mg\(^{2+}\) influx should exhibit a higher affinity for Mg\(^{2+}\) than for Ca\(^{2+}\) to ascertain specific divalent cation transport in DCT. TRPM6 uniquely fulfills this role as all known Ca\(^{2+}\)-permeable channels, including members of the TRP superfamily, generally display a 10 to 1,000 times lower affinity for Mg\(^{2+}\) than for Ca\(^{2+}\) (Voets et al. 2004b).

TRPM7  TRPM7 is the protein with the highest homology to TRPM6 and was cloned independently by two groups (Nadler et al. 2001; Runnels et al. 2001). Similar to TRPM6, TRPM7 forms a cation channel conducting both Mg\(^{2+}\) and Ca\(^{2+}\) ions (Monteilh-Zoller et al. 2003), exhibits constitutive activity, and is both an ion channel and a kinase (Nadler et al. 2001). TRPM7 activity is regulated by the free [Mg\(^{2+}\)]\(_i\) (Matsushita et al. 2005; Schmitz et al. 2003; Takezawa et al. 2004). However, the regulation by Mg\(^{2+}\)-nucleotides including ATP proposed by Nadler et al. has been challenged by Cahalan and colleagues, who suggested that the inhibitory effect of Mg\(^{2+}\)-nucleotides can be explained by free Mg\(^{2+}\) alone (Kozak and Cahalan 2003). A recent report by Demeuse et al. suggested that TRPM6 is regulated by both free Mg\(^{2+}\) and by Mg\(^{2+}\)-nucleotides and this dual mode of regulation could be modulated by its kinase domain (Demeuse et al. 2006). Furthermore, modulation of TRPM7 activity by various factors including PIP\(_2\) (Runnels et al. 2002) and PKA (Takezawa et al. 2004) has been suggested.

Recent studies have been performed to address the similarities and differences in the physiological function and electrophysiological properties of the structurally analogous channels TRPM6 and TRPM7. Particular emphasis has initially been placed on the function of the atypical protein \(\alpha\)-kinase domain located in the carboxy-terminus. \(\alpha\)-Kinases are a recently discovered family of proteins that have low sequence homology to conventional protein kinases (Drennan and Ryazanov 2004). Both the TRPM7 and TRPM6 \(\alpha\)-kinase domain display autophosphorylation activity when expressed in bacteria (Ryazanova et al. 2004). The TRPM7 \(\alpha\)-kinase is specific for ATP and cannot use GTP as a substrate, requires Mg\(^{2+}\) or Mn\(^{2+}\) for optimal activity, and has been shown to phosphorylate several substrates such as myelin basic protein and histone H3 on serine and threonine residues in vitro (Ryazanova et al. 2004). Dorovkov and Ryazanov identified annexin 1 as a novel substrate for the TRPM7 \(\alpha\)-kinase. TRPM7 \(\alpha\)-kinase phosphorylated annexin 1 at a conserved serine residue (Ser5) located within the amino-terminal amphipathic \(\alpha\)-helix of annexin 1 (Dorovkov and Ryazanov 2004). This region plays a crucial role in the interaction of annexin 1 with other proteins, lipids, and phosphatidylinositol, suggesting that TRPM7
modulates the function of annexin 1. In addition, Clark et al. demonstrated that TRPM7 phosphorylates myosin IIA heavy chain. Association of TRPM7 with myosin IIA was regulated by Ca\(^{2+}\) entering the cell via TRPM7, and both activation of TRPM7 and inhibition of myosin II resulted in actomyosin remodeling (Clark et al. 2006).

It is currently unclear to what extent the substrate-specificity of TRPM6 and TRPM7 are conserved. Future studies should address the question whether the α-kinase domain, present in TRPM6, has specific cellular targets that might modulate ion channel activity or transepithelial Mg\(^{2+}\) transport and, therefore, the Mg\(^{2+}\) balance. In a comprehensive study aiming to compare the functional properties of TRPM6 and TRPM7, Li and coworkers convincingly demonstrated that TRPM6 alone forms functional channels with biophysical properties distinct from TRPM7 or TRPM6/TRPM7 hetero-oligomers (Li et al. 2006). Heterologous expression of TRPM6 produced functional channels with a divergent permeability profile, pH sensitivity and unitary conductance that were distinct from those of TRPM7 channels or from cells heterologously coexpressing TRPM6 and TRPM7 (called TRPM6/7 currents). The relative permeability for Ni\(^{2+}\) over Ca\(^{2+}\) was significantly higher for TRPM7 than for TRPM6 or TRPM6/7. The relative permeability for other cations, including Ba\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\), Sr\(^{2+}\), and Cd\(^{2+}\) of TRPM6 was similar to TRPM7. Furthermore, both TRPM6 and TRPM7 currents were increased upon extracellular acidification. However, the relative increase of currents at low pH compared to currents at pH 7.4 was much greater for TRPM7 than for TRPM6.

Li et al. also measured the single channel conductance of TRPM6, TRPM6/7, and TRPM7 in the outside-out configuration and demonstrated that these conditions yielded distinct unitary conductances (Li et al. 2006). TRPM7 showed a unitary conductance of approximately 40 pS, whereas TRPM6 channels reveal higher current amplitudes at all measured voltages, displaying a unitary conductance of approximately 84 pS. Coexpressing TRPM6 with TRPM7 in a 1:1 ratio yielded a single channel conductance of about 57 pS. When TRPM6 and TRPM7 are coexpressed oligomeric channels may be formed at random yielding a mixture of single channel conductances. Indeed, in patches that contained two or more channels, single channel events indicative of distinct amplitudes were observed. These amplitudes matched with the conductances of TRPM6 or TRPM7, but also a single novel conductance with an intermediate value was obtained, suggesting that heteromeric TRPM6/7 channels have a preferred configuration with a distinct single channel conductance. Only one type of channels was observed when TRPM6 or TRPM7 were expressed alone.

Finally, Li et al. also identified 2-aminoethoxydiphenyl borate (2-APB) as a novel pharmacological tool to distinguish between TRPM6 and TRPM7 currents, as micromolar levels of this compound maximally increased TRPM6, but significantly inhibited TRPM7 channel activities. In contrast, millimolar concentrations of 2-APB potentiated TRPM6/7 and TRPM7 channel activities.

Not only the biophysical properties, but also the physiological roles of TRPM6 and TRPM7 are distinct. TRPM6 and TRPM7 have different expression patterns, with TRPM6 present mainly in absorbing epithelia, whereas TRPM7 is ubiquitously expressed (Groenestege et al. 2006). TRPM7 has been implicated in several processes including anoxic neuronal death (Aarts et al. 2003), regulation of actomyosin contractility and cell adhesion (Clark et al. 2006), influx of toxic divalent metals (Monteilh-Zoller et al. 2003), and maintenance of cellular Mg\(^{2+}\) homeostasis (Schmitz et al. 2003). However, the physiological role of TRPM6 seems to be more specialized as the gatekeeper of epithelial Mg\(^{2+}\) influx (Schlingmann et al. 2002; Voets et al. 2004b; Walder et al. 2002). Furthermore, several lines of evidence suggest that both channels are functionally nonredundant. Deletion of the TRPM7 gene in chicken DT40 B-lymphocytes is lethal (Nadler et al. 2001) and TRPM7
downregulation using siRNA impairs viability of human neuroblastoma cells. The viability of TRPM7-deficient DT40 cells cannot be complemented by heterologously expressed TRPM6 (Schmitz et al. 2005). Similarly, mutations in TRPM6 result in HSH despite the ubiquitous presence of TRPM7 (Schlingmann et al. 2002; Walder et al. 2002).

Intracellular Mg\(^{2+}\) transport

The second step of transcellular Mg\(^{2+}\) transport is the diffusion of cytosolic Mg\(^{2+}\) from the luminal entry site toward the basolateral membrane. Importantly, \([\text{Ca}^{2+}]_i\) is typically in the 0.1 µM range, which the cell maintains for signaling purposes, while \([\text{Mg}^{2+}]_i\) with estimated values of 0.8 mM is substantially higher (Grubbs 2002; Romani and Maguire 2002; Wolf et al. 2003). This has considerable consequences for buffering of \([\text{Mg}^{2+}]_i\) with respect to the process of epithelial Mg\(^{2+}\) transport. Transcellular Ca\(^{2+}\) transport requires a massive Ca\(^{2+}\) buffering capacity to maintain low \([\text{Ca}^{2+}]_i\) during large luminal to basolateral transport rates. Given the much higher \([\text{Mg}^{2+}]_i\), this requirement seems less strict for Mg\(^{2+}\). Thus, the question is whether the molecular mechanism of transcellular Mg\(^{2+}\) transport indeed requires intracellular Mg\(^{2+}\) buffers? If so, which proteins could fulfill this buffering function? Certainly, the physiological role of putative Mg\(^{2+}\) buffers needs to be calculated using mathematical models for epithelial Mg\(^{2+}\) transport and experimentally determined, i.e., using animal models lacking certain Mg\(^{2+}\) buffering proteins. However, the strong regulation of TRPM6 by \([\text{Mg}^{2+}]_i\) points to the importance of a significant local Mg\(^{2+}\) buffering capacity (Voets et al. 2004b). Therefore, we postulate that specific Mg\(^{2+}\)-binding proteins play an important role in the process of transcellular Mg\(^{2+}\) transport in general and in particular in the Mg\(^{2+}\) influx step.

It is interesting to mention that parvalbumin and calbindins in addition to Ca\(^{2+}\) also bind Mg\(^{2+}\) (Eberhard and Erne 1994; Yang et al. 2002) with dissociation constants that are in the same order of magnitude as the respective cellular concentrations for these two ions. Importantly, TRPM6 colocalizes with parvalbumin in DCT1 and with calbindin-D\(_{28K}\) in DCT2 (Voets et al. 2004b; Fig. 3). Therefore, these proteins are candidates to fulfill the role of intracellular Mg\(^{2+}\) buffers in Mg\(^{2+}\) reabsorption in DCT; however, other buffers including members of the S100 family (Gribenko and Makhadadze 1998) cannot be excluded. A role of calbindins and parvalbumin in neuronal Ca\(^{2+}\) signaling has been studied using mouse knockout models, but possible effects on the Mg\(^{2+}\) balance in these mice have not yet been addressed (Caillard et al. 2000; Servais et al. 2005).

Mg\(^{2+}\) extrusion mechanisms

To date, little experimental data are available on the extrusion of Mg\(^{2+}\) across the basolateral membrane. The chemical gradient for Mg\(^{2+}\) across this plasma membrane is negligibly small. However, the negative membrane potential dictates the participation of primary (ATP consuming Mg\(^{2+}\)-pump) or secondary (Mg\(^{2+}\) efflux-coupled to Na\(^{+}\) influx) active transport processes governing the exit of Mg\(^{2+}\). At present, experimental data only support the involvement of the latter transport mechanism. Schweigel et al. addressed the Mg\(^{2+}\) efflux step of epithelial cells of the bovine forestomachs. This organ fulfills the Mg\(^{2+}\) absorption in ruminant animals (cow, sheep, goat, etc.) similar to intestinal Mg\(^{2+}\) absorption in monogastric animals and humans (Schweigil and Martens 2000). Mg\(^{2+}\) efflux of isolated rumen epithelial cells required the presence of extracellular Na\(^{+}\) (Schweigel et al. 2006). The activation of the Mg\(^{2+}\) efflux by extracellular Na\(^{+}\) followed a simple Michaelis-Menten relationship with a \(K_m\) of 24 mM, in line with observations in other cell types (Gunther and Vormann 1985).
Previously, it was shown that inhibition of the Na\(^+-\)K\(^+-\)ATPase by ouabain reduces transcellular Mg\(^{2+}\) transport across isolated sheep rumen epithelia by 90\% (Martens and Harmeyer 1978). Therefore, a Na\(^+-\)linked mechanism was suggested utilizing the electrochemical gradient of Na\(^+\) (generated by Na\(^+-\)K\(^+-\)ATPase) to extrude Mg\(^{2+}\) via a Na\(^+\)/Mg\(^{2+}\) exchanger. Na\(^+\)/Mg\(^{2+}\) exchange has been proposed to regulate [Mg\(^{2+}\)]\(_i\) of a variety of other cells types including human red blood cells (Feray and Garay 1986), suggesting that the Na\(^+\)/Mg\(^{2+}\) exchanger has a more ubiquitous expression. Schweigel et al. generated monoclonal antibodies using material from red blood cells as an antigen. This yielded an antibody that blocks Na\(^+\)/Mg\(^{2+}\) exchange in rumen epithelial cells. Importantly, this antibody recognized a product that is upregulated by high extracellular Mg\(^{2+}\) and detected a protein of approximately 70 kDa in lysates of bovine rumen epithelial cells and porcine red blood cells. This size is distinct from the size of the Na/Ca\(^{2+}\) exchanger, suggesting the presence of a specific Mg\(^{2+}\) efflux system. However, the molecular identity of this protein remains unknown.

Regulation of epithelial Mg\(^{2+}\) transport

Initially, Mg\(^{2+}\) transport was generally described as functionally characterized using electrophysiological methods employing isolated tissues or immortalized nonpolarized cells (Dai et al. 2001). Quamme and coworkers have performed several experiments using nonpolarized immortalized cells with a partial DCT phenotype. Their investigations on the regulation of Mg\(^{2+}\) influx have mainly relied on fluorescence measurements of [Mg\(^{2+}\)]\(_i\) (Dai et al. 2001). To this end, cells were depleted for Mg\(^{2+}\) by incubation in Mg\(^{2+}\)-deficient medium for 16 h and subsequent exposure of the cells to Mg\(^{2+}\)-containing medium to measure the rate of Mg\(^{2+}\) influx. They suggested that Mg\(^{2+}\) entry into cultured DCT-like cells is mediated by a specific and regulated Mg\(^{2+}\) channel (Dai et al. 2001). However, electrophysiological measurements have not been performed, making it difficult to compare the Mg\(^{2+}\) influx properties of these cells with measured TRPM6 currents. In addition, several studies addressed the regulation of epithelial Mg\(^{2+}\) (re)absorption using micropuncture and microperfusion methods. Finally, the recent identification of TRPM6 allowed for studies performed at the molecular level, yielding novel insight into the regulation of epithelial Mg\(^{2+}\) transport. In the following we will present an overview of the regulation of epithelial Mg\(^{2+}\) transport and its possible molecular rationalization obtained using these various methods.

Diet-dependent Mg\(^{2+}\) (re)absorption

Mg\(^{2+}\) (re)absorption in the intestine and kidney is load dependent. In intestine, dietary Mg\(^{2+}\) content has been shown to affect the relative contribution of transcellular and paracellular Mg\(^{2+}\) transport. The fraction of transcellular Mg\(^{2+}\) absorption is saturable whereas paracellular Mg\(^{2+}\) absorption is not (Fine et al. 1991). In addition, Groenestege et al. have recently addressed the effect of the dietary Mg\(^{2+}\) content on the expression of Mg\(^{2+}\) transporters in intestine and kidney (Groenestege et al. 2006). Expression levels of TRPM6 mRNA in colon were upregulated by the Mg\(^{2+}\)-enriched diet, whereas Mg\(^{2+}\) restriction did not significantly affect TRPM6 mRNA expression levels. This suggests that mice can increase their transcellular Mg\(^{2+}\) absorption capacity when fed a Mg\(^{2+}\)-enriched diet (Groenestege et al. 2006). In contrast, in the same study an increased TRPM6 expression level upon dietary Mg\(^{2+}\) restriction was demonstrated in the kidney. Therefore, it was suggested that an excess of Mg\(^{2+}\) absorption as a result of high dietary Mg\(^{2+}\) intake together with TRPM6 upregulation in colon can be corrected by the kidney. Indeed, although the kidney normally excretes only 2–
4% of the filtered Mg\textsuperscript{2+}, it is capable of increasing fractional excretion to nearly 100% in the face of increased plasma Mg\textsuperscript{2+} levels (Sutton and Domrongkitchaiporn 1993). Furthermore, the unaltered expression levels of TRPM6 mRNA in colon during Mg\textsuperscript{2+} restriction indicates that the Mg\textsuperscript{2+} absorptive capacity is sufficient to obtain maximal transepithelial Mg\textsuperscript{2+} transport.

The load dependence of Mg\textsuperscript{2+} reabsorption in the kidney has been investigated by microperfusion (Massry et al. 1969; Quamme and Dirks 1980). Mg\textsuperscript{2+} absorption in PT is not saturable and increases linear with the luminal Mg\textsuperscript{2+} concentration or the delivered load, in line with the paracellular Mg\textsuperscript{2+} transport. In the loop of Henle, similar results were obtained. Interestingly, even in conditions when Mg\textsuperscript{2+} is absent from the luminal fluid, back-flow of Mg\textsuperscript{2+} from the blood side into the lumen was not detectable, indicating that paracellular Mg\textsuperscript{2+} transport in the TAL is strictly unidirectional (Quamme and Dirks 1980). Finally, the load dependence of Mg\textsuperscript{2+} absorption in DCT was measured. Although the absolute amount of Mg\textsuperscript{2+} that was absorbed in DCT increased with the enlarged load, the relative amount strongly decreased. This suggests that the Mg\textsuperscript{2+} absorptive capacity of DCT has a maximum. Interestingly, the fractional Mg\textsuperscript{2+} reabsorption in DCT decreased with hypermagnesemia. This is in line with the results of Groenestege et al. demonstrating an inverse relation between plasma Mg\textsuperscript{2+} levels and TRPM6 expression in DCT (Groenestege et al. 2006). Furthermore, it was postulated that elevated extracellular Mg\textsuperscript{2+} or Ca\textsuperscript{2+} inhibits fractional Mg\textsuperscript{2+} reabsorption in DCT through activation of the CaSR (Bapty et al. 1998a; Bapty et al. 1998b).

**Hormonal control of epithelial Mg\textsuperscript{2+} transport**

Although the significance of a strict Mg\textsuperscript{2+} balance is clearly reflected by the severity of pathology associated with hypomagnesemia and hypermagnesemia, a specific “magnesiotropic” hormone has not been identified (Kelepouris and Agus 1998). Several hormones, including PTH, calcitonin, 1,25(OH)\textsubscript{2}D\textsubscript{3}, insulin, glucagons, antidiuretic hormone, aldosterone, and sex steroids have been reported to influence the Mg\textsuperscript{2+} balance (Bailly et al. 1984; Dai et al. 1999, 2001; Elalouf et al. 1983, 1984; Harris et al. 1979). PTH stimulated renal Mg\textsuperscript{2+} reabsorption in parathyroidectomized animals (Bailly et al. 1985; Harris et al. 1979). This stimulation was localized to TAL and DCT. In addition, Dai and Quamme showed that PTH enhances Mg\textsuperscript{2+} influx in immortalized immortalized DCT-like cells (Dai et al. 1999). This effect was accompanied by increased cAMP values, suggesting that PTH acts via PKA. Other cellular stimulations that are associated with increases in cytosolic cAMP levels, including prostaglandin E\textsubscript{2}, vasopressin, glucagon, and insulin treatment, also enhanced Mg\textsuperscript{2+} influx in these cells (Dai et al. 2001). It was further shown that the signaling pathway underlying the effect of PTH on Mg\textsuperscript{2+} influx in immortalized DCT-like cells also involves PKC, as both inhibitors for PKA and PKC pathways blocked the PTH-mediated increase in Mg\textsuperscript{2+} influx (Dai et al. 2001). In contrast, PTH had no effect on TRPM6 and TRPM7 expression level in kidney, as the expression of these channels was not affected by parathyroidectomy alone or parathyroidectomy with subsequent pharmacological PTH supplementation (Groenestege et al. 2006). Similarly, 1,25(OH)\textsubscript{2}D\textsubscript{3} enhanced the influx of Mg\textsuperscript{2+} in a mouse DCT cell line (Ritchie et al. 2001), but this calcitriotropic hormone did not upregulate renal TRPM6 expression levels (Groenestege et al. 2006). The expression of TRPM6 was identical in wild-type mice, 25-hydroxyvitamin D\textsubscript{3}-1α-hydroxylase knockout mice (showing no detectable 1,25(OH)\textsubscript{2}D\textsubscript{3} synthesis), and in the same knockout mice supplemented with 1,25(OH)\textsubscript{2}D\textsubscript{3}. In addition, Karbach showed that cellular Mg\textsuperscript{2+} transport in rat colon is not responsive to 1,25(OH)\textsubscript{2}D\textsubscript{3} (Karbach
In contrast to 1,25(OH)$_2$D$_3$ and PTH, estrogen displayed significant effects on the expression level of TRPM6 in kidney. In ovariectomized rats, the renal TRPM6 mRNA level was significantly reduced and subsequently normalized by 17β-estradiol (17β-E2) supplementation (Groenestege et al. 2006). These findings indicate a role for estrogens in Mg$^{2+}$ homeostasis via regulation of TRPM6. Postmenopausal estrogen loss is associated with hypermagnesuria, which is corrected after estrogen substitution therapy (McNair et al. 1984). This finding could be explained by estrogen-mediated enhancement of renal TRPM6 expression resulting in increased Mg$^{2+}$ reabsorption. The stimulatory effect of 17β-E2 could be due to enhanced transcriptional activity or mRNA stabilization. Thus far, sequence analysis indicated 17β-E2-responsive elements in the putative promoter sequence of human and mouse TRPM6.

**Tacrolimus**

Tacrolimus (also FK-506 or Fujimycin, tradename Prograf) was discovered as a novel immunosuppressant in 1984 from the fermentation broth of the bacteria *Streptomyces tsukubaensis* (Kino et al. 1987; Wallemacq and Reding 1993). Tacrolimus interacts with the immunophilin FKBP-12 (FK506 binding protein) (Liu et al. 1991). This complex binds and inhibits calcineurin, resulting in decreased T cell receptor signal transduction (Liu et al. 1991). Tacrolimus is mainly used as an immunosuppressive drug to reduce the risk of organ rejection after transplant. Hypomagnesemia is a significant side effect of tacrolimus, even at relatively low doses (Lote et al. 2000; Nijenhuis et al. 2004). The effect of tacrolimus on plasma [Mg$^{2+}$] is mediated via decreased renal tubular Mg$^{2+}$ reabsorption (Lote et al. 2000; Nijenhuis et al. 2004). The underlying principle for the inappropriate hypermagnesuria, however, was unknown. Nijenhuis et al. have recently addressed the effect of tacrolimus on renal TRPM6 expression in male Wistar rats. These rats received tacrolimus by oral gavage for 7 days. Analysis of serum and urine samples showed a robust hypomagnesemia in the tacrolimus treated group (Nijenhuis et al. 2004). In line with a defect in renal Mg$^{2+}$ reabsorption, a significant increase in urinary Mg$^{2+}$ excretion was observed. Interestingly, these effects were accompanied by a significant reduced TRPM6 expression in kidney. The authors further addressed whether this downregulation reflects a general nephrotoxic effect on DCT, or a more specific effect on TRPM6 expression (Nijenhuis et al. 2004). The expression of several proteins with marked expression in DCT, including kallikrein (Zolotnitskaya and Satlin 1999) and NCC, did not significantly differ between controls and the tacrolimus-treated group. This indicates that no overt tacrolimus-mediated nephrotoxicity is present in DCT. Furthermore, no signs of a general deleterious effect of tacrolimus were detected, glomerular filtration rate was unaffected and enzymuria was not increased (Nijenhuis et al. 2004). Together, these factors suggest that tacrolimus has a direct effect on the expression of TRPM6 via an unknown regulatory pathway.

**Thiazides**

Thiazide diuretics are among the most commonly prescribed drugs, particularly in the treatment of hypertension. These compounds inhibit NCC present in the apical membrane of DCT to enhance renal Na$^+$ excretion (Gamba et al. 1993). Besides this diuretic effect, thiazides are known to cause hypocalciuria and hypomagnesemia. Several features of chronic thiazide treatment are mimicked in NCC knockout mice, which form an animal model for Gitelman’s syndrome (Loffing et al. 2004; Schultheis et al. 1998). Intriguingly, the molecular mechanisms responsible for hypomagnesemia during thiazide administration and Gitel-
man's syndrome have remained elusive. Thiazides have opposing effects on the Mg$^{2+}$ and Ca$^{2+}$ balance. Therefore, direct inhibitory actions on active Mg$^{2+}$ absorption in DCT have been proposed, as Ca$^{2+}$ and Mg$^{2+}$ are generally mutually reabsorbed in other segments of the nephron.

Identification of TRPM6 provides a powerful new tool to study the mechanism of active Mg$^{2+}$ transport at the molecular level. A recent study by Voets et al., demonstrating complete colocalization of NCC with TRPM6, provided the first indication of a role of TRPM6 in thiazide-mediated hypomagnesemia (Voets et al. 2004b). Subsequently, Nijenhuis et al. addressed the mechanism of thiazide-induced hypomagnesemia. They showed that, although a single dose of thiazides resulted in a clear natriuresis within 24 h after administration, urinary Mg$^{2+}$ excretion remained unaltered, which contradicts the hypothesis that Mg$^{2+}$ reabsorption is directly inhibited by thiazide-mediated reduction in Na$^{+}$-Cl$^{-}$ influx in DCT (Nijenhuis et al. 2005). Furthermore, the renal expression level of TRPM6 in NCC knockout mice, and in mice chronically receiving thiazides, was analyzed. Renal TRPM6 mRNA expression was significantly reduced in NCC knockout mice compared to control littermates. Furthermore, immunohistochemical analysis revealed that TRPM6 protein abundance along the apical membrane of the DCT is profoundly decreased in these mice. Similarly, renal TRPM6 expression was diminished in thiazide-treated animals. NCC expression was enhanced in these animals, illustrating that TRPM6 downregulation is a specific nondeleterious effect (Nijenhuis et al. 2005). On the whole, this demonstrates that chronic application of thiazide diuretics results in specific downregulation of renal TRPM6, resulting in inappropriate high renal Mg$^{2+}$ excretion and hypomagnesemia.

**Acid–base balance**

Metabolic acidosis induces hypermagnesuria in several animal models (Nijenhuis et al. 2006; Shapiro et al. 1987; Wong et al. 1986) and in humans (Ariceta et al. 2004; Blumberg et al. 1998). Micropuncture experiments indicated that this effect was localized beyond the late PT (Wong et al. 1986). Likewise, metabolic alkalosis has a renal Mg$^{2+}$ sparing effect and results in hypermagnesemia.

The role of TRPM6 in the acid–base effects on Mg$^{2+}$ balance was recently addressed (Nijenhuis et al. 2006). NH$_4$Cl-induced chronic metabolic acidosis decreased renal TRPM6 abundance accompanied by increased Mg$^{2+}$ excretion and hypomagnesemia. Conversely, chronic metabolic alkalosis increased TRPM6 expression as well as renal Mg$^{2+}$ reabsorption, resulting in hypermagnesemia (Nijenhuis et al. 2006). These data indicate that regulation of TRPM6 explains the effects of acid–base status on renal Mg$^{2+}$ handling. However, other studies support the existence of additional mechanisms to explain acid–base-mediated effects on epithelial Mg$^{2+}$ transport.

First, an alkaline extracellular pH was shown to enhance Mg$^{2+}$ uptake in immortalized mouse DCT cells, and conversely a low pH diminished this uptake. This effect was immediate and did not require preincubation of the cells with low or high pH (Dai et al. 1997). Therefore, pH-mediated effects on TRPM6 expression seem unlikely at this timescale. Furthermore, Li et al. recently indicated that TRPM6 currents are increased upon a shift of the extracellular medium pH from 7.0 to lower values with a half-maximal activation at pH 4.3 (Li et al. 2006). At present, it is unclear how these findings reconcile with the reduced epithelial Mg$^{2+}$ transport in acidosis. Furthermore, it is possible that the acidosis-mediated downregulation of TRPM6 provides the determining factor for Mg$^{2+}$ transport, as the proton-dependence of TRPM6 channel activity cannot explain the hypermagnesuria during acidosis. Interestingly, inhibition of carbonic anhydrase by acetazolamide displayed
a Mg\(^{2+}\)-sparing effect in mice (Nijenhuis et al. 2006) and humans (Sutton and Walker 1980) via an unknown mechanism. Treatment with this drug resulted in acidosis. However, the urine pH was alkaline, in contrast to situations with NH\(_4\)Cl-induced acidosis. Importantly, TRPM6 expression was significantly diminished during chronic acetazolamide treatment (Nijenhuis et al. 2006). This suggests that chronic metabolic acidosis results in TRPM6 downregulation, irrespective of the luminal pH.

Downregulation of TRPM6 does not support, however, the stimulated active Mg\(^{2+}\) reabsorption as a likely explanation for the decreased Mg\(^{2+}\) excretion during acetazolamide exposure. This treatment was associated with a larger urine volume and Na\(^{+}\) excretion, which has been shown to result in increased mRNA and protein abundance (Attmane-Elakeb et al. 1998) and activity (Kwon et al. 2003) of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC2) in the TAL. Therefore, it was postulated that these additive effects of acetazolamide enhance passive Mg\(^{2+}\) reabsorption in TAL, and that this dominates the effect of TRPM6 downregulation in DCT (Nijenhuis et al. 2006).

Other factors affecting epithelial Mg\(^{2+}\) transport

Although our understanding of epithelial Mg\(^{2+}\) transport has greatly improved in recent years, information concerning the molecular regulation of this process is still relatively scarce. It is anticipated that the molecular mechanism of multiple factors related to changes in the Mg\(^{2+}\) balance will be elucidated in the near future. For instance, it is currently unclear why hypomagnesemia is observed so frequently in the clinical setting, as this occurs in up to 12% of hospitalized patients, a number which rises to about 60% in the intensive care setting (Aglio et al. 1991; Agus 1999). Furthermore, disturbances in the Mg\(^{2+}\) balance are associated with diabetes mellitus. Urinary Mg\(^{2+}\) excretion in diabetic adolescents is significantly higher than in healthy persons, with higher excretion in boys than in girls (Driziene et al. 2005). In addition, it has been suggested that Mg\(^{2+}\) intake may be inversely related to the risk of hypertension and type 2 diabetes mellitus and that decreased cellular and plasma Mg\(^{2+}\) concentration is related to impaired insulin efficacy (He et al. 2006; Huerta et al. 2005; Kao et al. 1999; Paolisso and Barbagallo 1997).

Although several studies point to an association of diabetes with renal Mg\(^{2+}\) wasting, the etiology of the hypomagnesemia is largely unknown. In a recent study Lee et al. investigated the effect of streptozotocin-induced diabetes on the expression of claudin-16 and TRPM6 (Lee et al. 2006a). They showed that diabetic rats have a significant increase in the fractional excretion of Mg\(^{2+}\) and Ca\(^{2+}\), but not of Na\(^{+}\). Remarkably, a significant increase in mRNA levels of TRPM6 was observed. No change was found in claudin-16 mRNA or protein levels. Furthermore, several transport proteins including TRPV5, TRPV6, calbindin-D\(_{28K}\), and also NCC were upregulated. Insulin administration completely corrected the hyperglycemia-associated hypercalciuria and hypermagnesuria, and normalized the augment of Ca\(^{2+}\) transporter and TRPM6 abundance (Lee et al. 2006a). These findings suggest that the increased TRPM6 expression might reflect an adaptation to the higher Mg\(^{2+}\) load present in the lumen of DCT. Furthermore, this could indicate a compensatory mechanism for the diabetes-induced Mg\(^{2+}\) wasting, which was mainly localized to TAL (Garland 1992; Garland et al. 1991). The etiology of this transport defect in TAL is unknown and could be related to several factors including the level of hyperglycemia (Djurhuus et al. 2000), insulin concentration, or tubular alterations in osmolarity, pH, or membrane potential.
Mutual regulation of epithelial Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transport

A coupling of the Ca\textsuperscript{2+} and Mg\textsuperscript{2+} balance is observed in certain pathological conditions and animal models (Groenestege et al. 2006; Hebert et al. 1997; Simon et al. 1999). For several situations, there is consensus in the field on the molecular mechanism that links transport of these divalent ions, whereas in some conditions the reason for this coupling remains unknown.

To explain the HHN phenotype, it was proposed that claudin-16 either controls the Ca\textsuperscript{2+} and Mg\textsuperscript{2+} permeability of the paracellular pathway in TAL, or the driving force for the reabsorption of both ions. Therefore, mutations in claudin-16 affect both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} reabsorption, although the effect is more prominent for Mg\textsuperscript{2+}. In addition, mutations in the CaSR are associated with disturbed Ca\textsuperscript{2+} and Mg\textsuperscript{2+} handling. Mutations in the CaSR resulted in a lower set point for plasma Ca\textsuperscript{2+} and Mg\textsuperscript{2+} to activate the receptor. Consequently, renal Ca\textsuperscript{2+} and Mg\textsuperscript{2+} reabsorption and PTH secretion are suppressed, resulting in inappropriately low plasma PTH levels, and increased Ca\textsuperscript{2+} and Mg\textsuperscript{2+} excretion.

However, in other conditions Ca\textsuperscript{2+} and Mg\textsuperscript{2+} reabsorption are oppositely affected. Patients with mutations in TRPM6, the \(\gamma\)-subunit of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, or NCC all exhibit hypermagnesuria, while renal Ca\textsuperscript{2+} excretion is reduced. The expression of these particular Na\textsuperscript{+}, Ca\textsuperscript{2+} and Mg\textsuperscript{2+} transporting proteins is restricted to DCT, suggesting a direct regulatory connection in this nephron segment.

The nature of this interaction between transcellular Ca\textsuperscript{2+} and Mg\textsuperscript{2+} pathways in the distal part of the nephron, however, is still unclear. There is limited overlap in expression between the Ca\textsuperscript{2+} transport proteins and TRPM6, \(\gamma\)-subunit of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, or NCC (Fig. 3). Furthermore, it is interesting that hypocalcemia in HSH patients can be corrected only by supplementation of the diet with high amounts of Mg\textsuperscript{2+}, probably linked to restoration of PTH secretion and efficacy (Konrad and Weber 2003). Similarly, alterations of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} excretion in Gitelman’s syndrome have been attributed to distinct mechanisms. Renal Mg\textsuperscript{2+} loss in patients with chronic thiazide treatment, with Gitelman’s syndrome, and in NCC knockout mice is most likely due to reduced TRPM6 expression, whereas the increased Ca\textsuperscript{2+} reabsorption is mediated by adaptive mechanisms in the PT to compensate for the hypovolemia resulting from reduced or abolished NCC function (Nijenhuis et al. 2005). Furthermore, mutations in the \(\gamma\)-subunit of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase are the cause of dominant isolated hypomagnesemia with hypocalciuria. It was proposed that the mutated \(\gamma\)-subunit impairs the activity of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, resulting in reduced [K\textsuperscript{+}], increased [Na\textsuperscript{+}], or depolarization of the plasma membrane (Meij et al. 2000). This might subsequently lead to reduced Mg\textsuperscript{2+} influx through the apical TRPM6 channel, resulting in Mg\textsuperscript{2+} wasting. However, the molecular mechanism of the decreased Mg\textsuperscript{2+} reabsorption and the associated hypocalciuria remains to be further substantiated in this disorder.

On the whole, many diseases show disturbances in both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} balance. In some cases, there is an explanation for the mutual disorder in divalent renal handling, but in the majority of the diseases, the origin of this coupling is still unclear. Particularly, the limited segmental overlap between the Mg\textsuperscript{2+} transport (DCT1-DCT2) and Ca\textsuperscript{2+} transport (DCT2-CNT) machinery suggests that additional mechanisms might be involved in the kidney.
Future perspective

In the last decade, significant advances were made in the field of epithelial Ca\(^{2+}\) and Mg\(^{2+}\) (re)absorption. The identification of the proteins mediating this transport, including proteins involved in paracellular Ca\(^{2+}\)/Mg\(^{2+}\) transport (claudin-16), active Ca\(^{2+}\) transport (TRPV5/6 and novel channel associated proteins), and active Mg\(^{2+}\) transport (TRPM6) has provided novel insight and means to study the molecular aspects of divalent ion transport. Several questions on the molecular mechanisms of divalent ion transport remain unknown. One example is the unknown etiology of hypomagnesemia in several diseases, suggesting that novel molecular players involved in epithelial Mg\(^{2+}\) transport remain to be elucidated. Although the identification of TRPM6 provided a first view on the luminal Mg\(^{2+}\) influx pathway, molecular data explaining the diffusion and basolateral extrusion of Mg\(^{2+}\) are still elusive (Fig. 1). Furthermore, in view of life-threatening consequences of large deviations in the plasma [Mg\(^{2+}\)], it is surprising how little we know about the maintenance of the Mg\(^{2+}\) balance. In conclusion, the timely area of epithelial Ca\(^{2+}\) and Mg\(^{2+}\) transport is very dynamic and will likely remain so for the years to come. The large number of recent novel developments and the ones to be expected in the near future will further increase our understanding of epithelial ion homeostasis and provide new insights in the diagnoses and management of corresponding diseases.

Acknowledgements. S.F.G. was supported by an EMBO long-term fellowship (ALTF 700–2005). The authors were further supported by grants of the Dutch Organization of Scientific Research (Zon-Mw 016.006.001, ZonMW 9120.6110, NWO-ALW 814.02.001), the Stomach Liver Intestine foundation (MWO 03–19), Human Frontiers Science Program (RGP32/2004), and the Dutch Kidney foundation (C02.2030, C03.6017, C05.2134).

References


Dardenne O, Prud’homme J, Hacking SA, Glorieux FH, St-Arnaud R (2003a) Correction of the abnormal mineral ion homeostasis with a high-calcium, high-phosphorus, high-lactose diet rescues the PDDR phenotype of mice deficient for the 25-hydroxvitamin D-1alpha-hydroxylase (CYP27B1). Bone 32:332–340


DiPolo R (1979) Calcium influx in internally dialyzed squid giant axons. J Gen Physiol 73:91–113


Goytain A, Quamme GA (2005b) Functional characterization of human SLC41A1, a Mg2+ transporter with similarity to prokaryotic MgtE Mg2+ transporters. Physiol Genomics 21:337–342


Goytain A, Quamme GA (2005d) Identification and characterization of a novel mammalian Mg2+ transporter with channel-like properties. BMC Genomics 6:48


Gunter T, Vormann J (1985) Mg\(^{2+}\) efflux is accomplished by an amiloride-sensitive Na\(^+\)/Mg\(^{2+}\) antiport. Biochem Biophys Res Commun 130:540–545


Kozak JA, Cahalan MD (2003) MIC channels are inhibited by internal divalent cations but not ATP. Biophys J 84:922–927


Liedtke CM, Yun CH, Kyle N, Wang D (2002) Protein kinase C epsilon-dependent regulation of cystic fibrosis transmembrane regulator binding to a receptor for activated C kinase (RACK1) and RACK1 binding to Na⁺/H⁺ exchange regulatory factor. J Biol Chem 277:22925–22933


of the intracellular loop located between transmembrane segments 2 and 3. J Biol Chem 277:30852–30858
The carboxyl terminus of the epithelial Ca\textsuperscript{2+} channel EC\textsubscript{a}C\textsubscript{1} is involved in Ca\textsuperscript{2+}-dependent inactivation. Pflugers Arch 455:584–588
Pan TC, Liao BK, Huang CJ, Lin LY, Hwang PP (2005) Epithelial Ca\textsuperscript{2+} channel expression and Ca\textsuperscript{2+} uptake in developing zebrafish. Am J Physiol Regul Integr Comp Physiol 289:R1202–R1211
Peng JB, Brown EM, Hediger MA (2001a) Structural conservation of the genes encoding CaT1, CaT2, and related cation channels. Genomics 76:99–109
Rohacs T, Lopes CM, Michailidis I, Logothetis DE (2005) PIP2 regulates the activation and desensitization of TRPM8 channels through the TRP domain. Nat Neurosci 8:626–634


Shapiro RJ, Yong CK, Quamme GA (1987) Influence of chronic dietary acid on renal tubular handling of magnesium. Pflugers Arch 409:492–498


Tan CM, Nickols HH, Limbird LE (2003) Appropriate polarization following pharmacological rescue of V2 vasopressin receptors encoded by X-linked nephrogenic diabetes insipidus alleles involves a conformation of the receptor that also attains mature glycosylation. J Biol Chem 278:35678–35686


Wood RJ, Tchack L, Taparia S (2001) 1,25-Dihydroxyvitamin D3 increases the expression of the CaT1 epithelial calcium channel in the Caco-2 human intestinal cell line. BMC Physiol 1:11

Woodhall AM (1973) Ionic blockage of sodium channels in nerve. J Gen Physiol 61:687–708

Xue Y, Karaplis AC, Hendy GN, Goltzman D, Miao D (2006) Exogenous 1,25-dihydroxyvitamin D3 exerts a skeletal anabolic effect and improves mineral ion homeostasis in mice which are homozygous for both the lalpaha-hydroxylase and parathyroid hormone null alleles. Endocrinology 147:4801–4810


Zhuang L, Peng JB, Tou L, Takanaga H, Adam RM, Hediger MA, Freeman MR (2002) Calcium-selective ion channel, CaT1, is apically localized in gastrointestinal tract epithelia and is aberrantly expressed in human malignancies. Lab Invest 82:1755–1764

