Critical Role of the Epithelial Ca^{2+} Channel TRPV5 in Active Ca^{2+} Reabsorption as Revealed by TRPV5/Calbindin-D_{28K} Knockout Mice

Dimitra Gkika,* Yu-Juei Hsu,*[†] Annemiete W. van der Kemp,* Sylvia Christakos,[‡] René J. Bindels,* and Joost G. Hoenderop*

*Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; †Division of Nephrology, Department of Internal Medicine, Tri-Service General Hospital, Taipei, Taiwan; and ‡Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry, New Jersey Medical School, Newark, New Jersey

The epithelial Ca^{2+} channel TRPV5 facilitates apical Ca^{2+} entry during active Ca^{2+} reabsorption in the distal convoluted tubule. In this process, cytosolic Ca^{2+} remains at low nontoxic concentrations because the Ca^{2+} influx is buffered rapidly by calbindin-D_{28K}. Subsequently, Ca^{2+} that is bound to calbindin-D_{28K} is shuttled toward the basolateral Ca^{2+} extrusion systems. For addressing the *in vivo* role of TRPV5 and calbindin-D_{28K} in the maintenance of the Ca^{2+} balance, single- and double-knockout mice of TRPV5 and calbindin-D_{28K} (TRPV5$^{-/-}$, calbindin-$D_{28K}^{-/-}$, and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$) were characterized. These mice strains were fed two Ca^{2+} diets (0.02 and 2% wt/wt) to investigate the influence of dietary Ca^{2+} content on the Ca^{2+} balance. Urine analysis indicated that TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice exhibit on both diets hypercalciuria compared with wild-type mice. Ca^{2+} excretion in TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice was not significantly different from TRPV5$^{-/-}$ mice, whereas calbindin-$D_{28K}^{-/-}$ mice did not show hypercalciuria. The similarity between TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ and TRPV5$^{-/-}$ mice was supported further by an equivalent increase in renal calbindin-D_{9K} expression and in intestinal Ca^{2+} hyperabsorption as a result of upregulation of calbindin-D_{9K} and TRPV6 expression in the duodenum. Elevated serum parathyroid hormone and 1,25-dihydroxyvitamin D_3 levels accompanied the enhanced expression of the Ca^{2+} transporters. Intestinal Ca^{2+} absorption and expression of calbindin-D_{9K} and TRPV6, as well as serum parameters of the calbindin-$D_{28K}^{-/-}$ mice, did not differ from those of wild-type mice. These results underline the gatekeeper function of TRPV5 being the rate-limiting step in active Ca^{2+} reabsorption, unlike calbindin-D_{28K}, which possibly is compensated by calbindin-D_{9K}.

Ca^{2+} homeostasis is of crucial importance for many physiologic functions, including neuronal excitability, muscle contraction, blood clotting, and bone mineralization. Therefore, the Ca^{2+} balance is tightly controlled through constant regulation of three physiologic processes: Intestinal absorption, renal reabsorption, and exchange of Ca^{2+} from the bone mass (1). Both in intestine and in kidney, Ca^{2+} enters the interstitium by passive paracellular as well as active (re)absorption (2,3). Active Ca^{2+} (re)absorption is critical in this process, because it constitutes the primary target for regulation by calciotropic hormones, including 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] and parathyroid hormone (PTH), enabling the

organism to regulate the extracellular Ca^{2+} concentration on the body's demand (4).

Active absorption of dietary Ca^{2+} occurs primarily in the proximal small intestine, whereas renal active Ca^{2+} reabsorption is restricted to the distal convoluted tubule (DCT) and the connecting tubule (CNT) (5,6). Ca^{2+} absorption occurs also in bone, where it is crucial for bone formation to achieve adequate bone quality and strength, as well as for osteoclastic bone resorption (7). At the cellular level, active Ca^{2+} (re)absorption implies entry of Ca^{2+} across the luminal membrane through the epithelial Ca^{2+} channels, followed by intracellular buffering, facilitated diffusion by Ca^{2+}-binding proteins, and finally extrusion across the basolateral membrane by a Na^+/Ca^{2+} exchanger and/or a plasma membrane Ca^{2+} pump. Ca^{2+} influx occurs through two highly Ca^{2+}-selective members of the transient receptor potential (TRP) cation channel family, TRPV5 and TRPV6, which constitute the gatekeepers of active Ca^{2+} (re)absorption in kidney and intestine, respectively (8,9). Indeed, ablation of TRPV5 (TRPV5$^{-/-}$) in mice impairs renal Ca^{2+} reabsorption, resulting in robust hypercalciuria (10). As a consequence, TRPV5$^{-/-}$ mice develop compensatory dietary

Ca^{2+} hyperabsorption in the intestine. Furthermore, the structure of the bones in these mice is significantly disturbed, showing reduced trabecular and cortical bone thickness (11).

After influx through TRPV5 and TRPV6, Ca^{2+} binds to cytosolic proteins to diffuse toward the basolateral surface of the epithelial cell. Two Ca^{2+}-binding proteins, calbindin-D$_{28K}$ and calbindin-D$_{9K}$, are regarded as key components of Ca^{2+} (re)absorption (4). In mammals, calbindin-D$_{28K}$ is expressed primarily in kidney, whereas calbindin-D$_{9K}$ is abundantly present in small intestine. Only in mouse kidney are both calbindin-D$_{28K}$ and calbindin-D$_{9K}$ expressed in the distal part of the nephron (12). The physiologic importance of calbindin-D$_{28K}$ in renal Ca^{2+}-transporting epithelia is underlined by the consistent co-expression with TRPV5 and their co-regulation by calciotropic hormones, including PTH, 1,25(OH)$_2$D$_3$, and also dietary Ca^{2+} (13,14).

The aim of our study was to investigate whether calbindin-D$_{28K}$ deficiency is critical for active reabsorption in the presence or absence of TRPV5. To this end, single- and double-knockout mice of calbindin-D$_{28K}$ and TRPV5 (calbindin-D$_{28K}{}^{-/-}$, TRPV5$^{-/-}$, and TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{-/-}$) were generated. These mice were functionally characterized, including measurements of expression of the Ca^{2+} transporter proteins at mRNA and protein levels.

Materials and Methods
Animal Experiments

TRPV5$^{-/-}$ mice were generated as described previously (10). Calbindin-D$_{28K}{}^{-/-}$ mice were provided by Dr. Michael Meyer (Physiologisches Institut, Ludwig Maximilians Universität München, Munich, Germany) (15). Cross-breeding of TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{+/+}$ with TRPV5$^{+/+}$/calbindin-D$_{28K}{}^{-/-}$ mice resulted in offspring that were heterozygous for both TRPV5 and calbindin-D$_{28K}$ (TRPV5$^{+/-}$/calbindin-D$_{28K}{}^{+/-}$). This heterozygous offspring displayed the wild-type phenotype and subsequently was intercrossed to obtain TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{-/-}$ mice. Genotypes were determined by PCR analysis using specific primers for *Trpv5* (gene for TRPV5) as described previously (10,16) and for *Calb1* (gene for calbindin-D$_{28K}$): Two sense primers 5'-tgcagcggctagtttgagagtg-3' to detect the wild-type allele and 5'-tgactaggggaggagtagaag-3' to detect the null allele in combination with a common antisense primer 5'-gcaagtaactaatggcatcg-3'. At the age of 4 wk, mice were fed *ad libitum* two diets that contained either 0.02 or 2% (wt/wt) Ca^{2+} for 5 wk and subsequently placed in metabolic cages (Techniplast, Buggiate, Italy), which enabled 24-h collection of urine. At the end of the experiment, blood samples were taken and the mice were killed. Subsequently, kidney and duodenum tissue was sampled. Urine and serum Ca^{2+} concentrations were analyzed using a colorimetric assay kit (Roche, Mannheim, Germany). Serum PTH was measured using an immunoradiometric assay (Immutopics Inc., San Clemente, CA). Serum vitamin D levels were determined by an [I^{125}]1,25(OH)$_2$D$_3$ RIA assay (IDS Inc., Fountain Hills, AZ). The animal ethics board of Radboud University Nijmegen approved all animal experimental procedures.

Real-Time Quantitative PCR Analysis

Renal and duodenal mRNA expression levels of calbindin-D$_{28K}$, calbindin-D$_{9K}$, TRPV5, and TRPV6 were quantified by real-time quantitative PCR as described previously (17), using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). The expression level of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase was used as an internal control to normalize differences in RNA extractions and reverse transcription efficiencies.

Immunoblotting

Total kidney and duodenum lysates of all mouse groups were prepared as described previously (17). Briefly, protein concentrations of the homogenates were determined by the Bio-Rad protein assay (Bio-Rad, München, Germany), and 10 μg of each sample was loaded on 12 or 16.5% (wt/vol) SDS-PAGE gels and blotted to polyvinylidene difluoride nitrocellulose membranes (Immobilon-P, Millipore Corp., Bedford, MA). Blots were incubated with a rabbit anti–calbindin-D$_{28K}$ polyclonal antibody (1:10,000; Sigma, St. Louis, MO), a rabbit anti–calbindin-D$_{9K}$ polyclonal antibody (1:5000; Swant, Bellinzona, Switzerland), or a rabbit β-actin polyclonal antibody (1:20,000; Sigma) at 4°C for 16 h. Subsequently, blots were incubated with a goat anti-rabbit peroxidase-labeled secondary antibody (1:10,000; Sigma). Immunoreactive protein was detected by the chemiluminescence method (Pierce, Rockford, IL). The immunopositive protein bands were scanned and the pixel density was determined by using the Molecular Analyst Software of Bio-Rad Laboratories (Hercules, CA).

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

Ca^{2+} absorption was assessed by measuring serum $^{45}Ca^{2+}$ at early time points after oral gavage as described previously (10). Briefly, mice were fasted 16 h (overnight) before the test and a $^{45}Ca^{2+}$ solution was administrated by oral gavage. Blood samples were obtained at indicated time intervals, and serum (10 μl) was analyzed by liquid scintillation counting. Differences in serum Ca^{2+} concentration were calculated from the $^{45}Ca^{2+}$ content in the samples and the specific activity of the administrated $^{45}Ca^{2+}$.

Statistical Analyses

Values are expressed as means ± SEM. Statistical significance ($P < 0.05$) between groups was determined by one-way ANOVA. In case of significance, the Tukey-Kramer multiple comparisons test was applied. All analyses were performed using the Statview Statistical Package Software (Power PC, version 4.51; Berkeley, CA).

Results
Serum Parameters

Wild-type, 4-wk-old TRPV5$^{-/-}$, calbindin-D$_{28K}{}^{-/-}$, and TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{-/-}$ mice were fed a diet that contained 0.02 or 2% (wt/wt) Ca^{2+} for 5 wk. All mice strains were fertile and had similar average litter sizes (Table 1). Furthermore, serum analysis showed that TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{-/-}$ mice that were on the 0.02% (wt/wt) Ca^{2+} diet exhibit increased PTH and 1,25(OH)$_2$D$_3$ levels compared with wild-type mice. In contrast, serum PTH and 1,25(OH)$_2$D$_3$ levels in calbindin-D$_{28K}{}^{-/-}$ mice were not significantly different from those of wild-type mice. The increased PTH and 1,25(OH)$_2$D$_3$ levels were normalized in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-D$_{28K}{}^{-/-}$ mice that were fed the high-Ca^{2+} diet. Serum Ca^{2+} levels were not significantly altered between the mice genotypes, regardless of the dietary treatment (Table 1).

Table 1. Characteristics of TRPV5 and calbindin-D_{28K} single- and double-knockout mice

| | Ca^{2+} Diet (wt/wt) | | | | | | | |
| | Wild-Type | | TRPV5$^{-/-}$ | | Calbindin-$D_{28K}^{-/-}$ | | TRPV5$^{-/-}$/Calbindin-$D_{28K}^{-/-}$ | |
	0.02%	2%	0.02%	2%	0.02%	2%	0.02%	2%
Body weight (g)	25.6 ± 1.4	26.1 ± 1.4	25.5 ± 1.7	25.5 ± 1.4	23.2 ± 1.7	22.3 ± 1.9	23.6 ± 0.9	24.7 ± 1.4
Serum Ca^{2+} (mM)	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.1 ± 0.1	2.0 ± 0	1.9 ± 0	2.0 ± 0.1	2.2 ± 0.1
PTH[a] (pg/ml)	25.3 ± 4.5	6.4 ± 1.9	182.1 ± 51.0[b]	2.6 ± 0.9[c]	47.9 ± 22.5	6.5 ± 2.8	114.6 ± 30.4[b]	2.4 ± 1.2[c]
1,25(OH)$_2$D$_3$ (pmol/L)	561 ± 94	139 ± 26	1302 ± 207[b]	283 ± 74[c]	595 ± 86	155 ± 33	2190 ± 192[b,d]	659 ± 160[c]

[a]PTH, parathyroid hormone.
[b]$P < 0.05$ *versus* wild-type mice on the same diet.
[c]$P < 0.05$ *versus* the same mice group on 0.02% (wt/wt) Ca^{2+} diet.
[d]$P < 0.05$ *versus* TRPV5$^{-/-}$ mice on 0.02% (wt/wt) Ca^{2+} diet.

mRNA Expression of Epithelial Ca^{2+} Transporters

To evaluate the regulation of mRNA expression levels of the Ca^{2+} transporters in kidney and duodenum, we applied quantitative real-time PCR assays. In kidney, calbindin-D_{9K} mRNA expression levels were increased in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice that were fed the 0.02 (wt/wt) Ca^{2+} diet compared with wild-type mice. Furthermore, renal calbindin-D_{9K} expression was similar in calbindin-$D_{28K}^{-/-}$ compared with wild-type mice. Exposure of the mice to the high-Ca^{2+} diet resulted in downregulation of renal calbindin-D_{9K} mRNA in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice (Figure 1A). Conversely, dietary Ca^{2+} content did not affect renal expression of calbindin-D_{28K} mRNA, which was significantly reduced in TRPV5$^{-/-}$ compared with wild-type mice (Figure 1B). In duodenum, TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice that were fed the 0.02% (wt/wt) Ca^{2+} diet demonstrated an upregulation of calbindin-D_{9K} and TRPV6 mRNA expression in comparison with wild-type mice. On the same Ca^{2+} diet, duodenal calbindin-D_{9K} and TRPV6 expression remained unchanged in calbindin-$D_{28K}^{-/-}$ compared with wild-type mice. The high-Ca^{2+} diet reduced intestinal calbindin-D_{9K} and TRPV6 mRNA expression in all mouse strains (Figure 2).

Protein Expression of Epithelial Ca^{2+} Transporters

For validation of whether the changes in renal and duodenal mRNA levels of the Ca^{2+} transporters resulted in altered protein expression, the abundance of the Ca^{2+} transporters was semiquantified by immunoblot analysis. In kidney, calbindin-D_{9K} protein expression was increased in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice that were fed the 0.02% (wt/wt) Ca^{2+} diet compared with wild-type mice. On the same diet, calbindin-$D_{28K}^{-/-}$ mice expressed wild-type levels of calbindin-D_{9K} protein in kidney. However, an increase in dietary Ca^{2+} content from 0.02 to 2% (wt/wt) resulted in downregulation of renal calbindin-D_{9K} in wild-type, TRPV5$^{-/-}$, and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice (Figure 3, A and B). Furthermore, renal calbindin-D_{28K} protein abundance was significantly decreased in TRPV5$^{-/-}$ mice in accordance with the downregulated mRNA levels. Variations in dietary Ca^{2+} did not affect renal calbindin-D_{28K} protein expression in both wild-type and TRPV5$^{-/-}$ mice (Figure 3, C and D). In duodenum,

calbindin-D_{9K} protein expression was increased in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice compared with wild-type and calbindin-$D_{28K}^{-/-}$ mice, in line with measured mRNA expression levels. Finally, dietary Ca^{2+} restriction resulted in a significant increase of duodenal calbindin-D_{9K} protein in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice, which was consistent with the calbindin-D_{9K} mRNA expression data (Figure 4).

Functional Analysis of Ca^{2+} (re)Absorption

The various mouse strains were functionally characterized by measurement of their urinary Ca^{2+} excretion and intestinal Ca^{2+} absorption. On both Ca^{2+} diets, urinary Ca^{2+} excretion was increased in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice compared with wild-type mice. Conversely, Ca^{2+} excretion was not significantly different in calbindin-$D_{28K}^{-/-}$ compared with wild-type mice. Dietary Ca^{2+} restriction did not affect the amount of Ca^{2+} excreted in the urine (Figure 5A). Subsequently, intestinal Ca^{2+} absorption was investigated by measurement of serum ^{45}Ca^{2+} at early time points after oral gavage. On the 0.02% (wt/wt) Ca^{2+} diet, the time curves of ^{45}Ca^{2+} absorption did not differ among the four mouse strains (Figure 5B). On the 2% (wt/wt) Ca^{2+} diet, intestinal ^{45}Ca^{2+} absorption was significantly reduced in all groups compared with mice that were fed the low-Ca^{2+} diet. However, the amount of ^{45}Ca^{2+} absorption remained significantly higher in TRPV5$^{-/-}$ and TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ mice compared with wild-type and calbindin-$D_{28K}^{-/-}$ mice (Figure 5C).

Discussion

Our study demonstrates that TRPV5 may constitute a more critical component of active Ca^{2+} reabsorption in kidney than calbindin-D_{28K}. This conclusion is based on the following experimental data. First, TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ and TRPV5$^{-/-}$ mice showed a comparable hypercalciuria and compensatory Ca^{2+} hyperabsorption in comparison with wild-type mice. Second, the expression of calbindin-D_{9K} in kidney as well as calbindin-D_{9K} and TRPV6 in duodenum increased equally in TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ and TRPV5$^{-/-}$ mice compared with wild-type mice. Third, upregulation of TRPV6 and calbindin-D_{9K} in TRPV5$^{-/-}$/calbindin-$D_{28K}^{-/-}$ and TRPV5$^{-/-}$
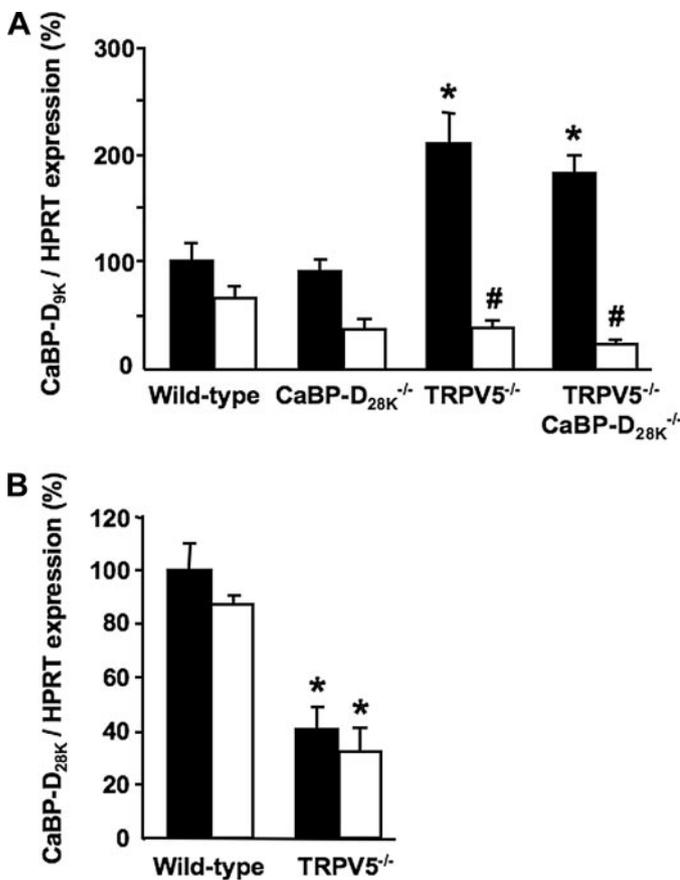
Figure 1. Renal mRNA expression of Ca^{2+} transporters. Expression of calbindin (CaBP)-D$_{9K}$ (A) and CaBP-D$_{28K}$ (B) mRNA in kidney of wild-type, TRPV5$^{-/-}$, CaBP-D$_{28K}$$^{-/-}$, and TRPV5$^{-/-}$/CaBP-D$_{28K}$$^{-/-}$ mice (n = 10) was analyzed by quantitative real-time PCR analysis. Mice were fed a 0.02% (wt/wt; ■) or 2% (wt/wt; □) Ca^{2+} diet. Values are calculated as a ratio of hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression in relative percentages compared with the wild-type mice on 0.02% (wt/wt) Ca^{2+} diet. Data are means ± SEM. *$P <$ 0.05 *versus* wild-type on the same diet; #$P <$ 0.05 *versus* the same group on 0.02% (wt/wt) Ca^{2+} diet.

Figure 2. Duodenal mRNA expression of Ca^{2+} transporters. Expression of CaBP-D$_{9K}$ (A) and TRPV6 (B) mRNA in duodenum of wild-type, TRPV5$^{-/-}$, CaBP-D$_{28K}$$^{-/-}$, and TRPV5$^{-/-}$/CABP-D$_{28K}$$^{-/-}$ mice (n = 10) was assessed by quantitative real-time PCR analysis. Mice were fed a 0.02% (wt/wt; ■) or 2% (wt/wt; □) Ca^{2+} diet. Values are calculated as a ratio of HPRT expression in relative percentages compared with the wild-type mice on 0.02% (wt/wt) Ca^{2+} diet. Data are means ± SEM. *$P <$ 0.05 *versus* wild-type on the same diet; #$P <$ 0.05 *versus* the same group on 0.02% (wt/wt) Ca^{2+} diet.

mice was accompanied by an analogous increase in serum PTH and 1,25(OH)$_2$D$_3$ levels. Fourth, urinary Ca^{2+} excretion, intestinal Ca^{2+} absorption, expression levels of the epithelial Ca^{2+} transporters, and serum parameters in calbindin-D$_{28K}$$^{-/-}$ mice were not different from those in wild-type mice. Fifth, dietary Ca^{2+} restriction did not influence the Ca^{2+} excretion in the evaluated mice strains, whereas it enhanced intestinal Ca^{2+} absorption in TRPV5$^{-/-}$/calbindin-D$_{28K}$$^{-/-}$ and TRPV5$^{-/-}$ mice. The observed hyperabsorption is in line with the upregulation of duodenal calbindin-D$_{9K}$ and TRPV6 expression.

Calbindin-D$_{28K}$ contains six high-affinity binding sites for Ca^{2+} and is present predominantly in kidney, intestine (birds only), pancreas, placenta, bone, and brain (4,12). In these tissues, calbindin-D$_{28K}$ is widely regarded as a key component in cellular Ca^{2+} handling by acting as a cytosolic Ca^{2+} buffer to protect cells against large fluctuations in the intracellular Ca^{2+} concentration (18), as well as a shuttle that facilitates Ca^{2+}

diffusion from the luminal to the basolateral surface (4). In mouse kidney, calbindin-D$_{28K}$ strikingly co-localizes with TRPV5, which constitutes the apical Ca^{2+} entry mechanism in DCT and CNT (4,19). Taking into account that calbindin-D$_{28K}$ expression is regulated by calciotropic hormones in a similar way as TRPV5 (4,20), both proteins could be functionally linked in the process of active Ca^{2+} reabsorption. Indeed, TRPV5$^{-/-}$ mice displayed a profound renal Ca^{2+} wasting combined with significant reduction of renal calbindin-D$_{28K}$ expression levels. This suggested that the impaired TRPV5-mediated Ca^{2+} influx suppresses the expression of calbindin-D$_{28K}$. Our previous experiments in primary cultures of rabbit CNT and CCD cells demonstrated that blockage of TRPV5-mediated Ca^{2+} influx by the channel inhibitor ruthenium red downregulates calbindin-D$_{28K}$ expression, indicating that regulation of the latter protein is highly dependent on the magnitude of the Ca^{2+} influx through TRPV5 (14). Arnold and Heintz (21) showed that Ca^{2+} is important for gene transcription. A Ca^{2+}-responsive element
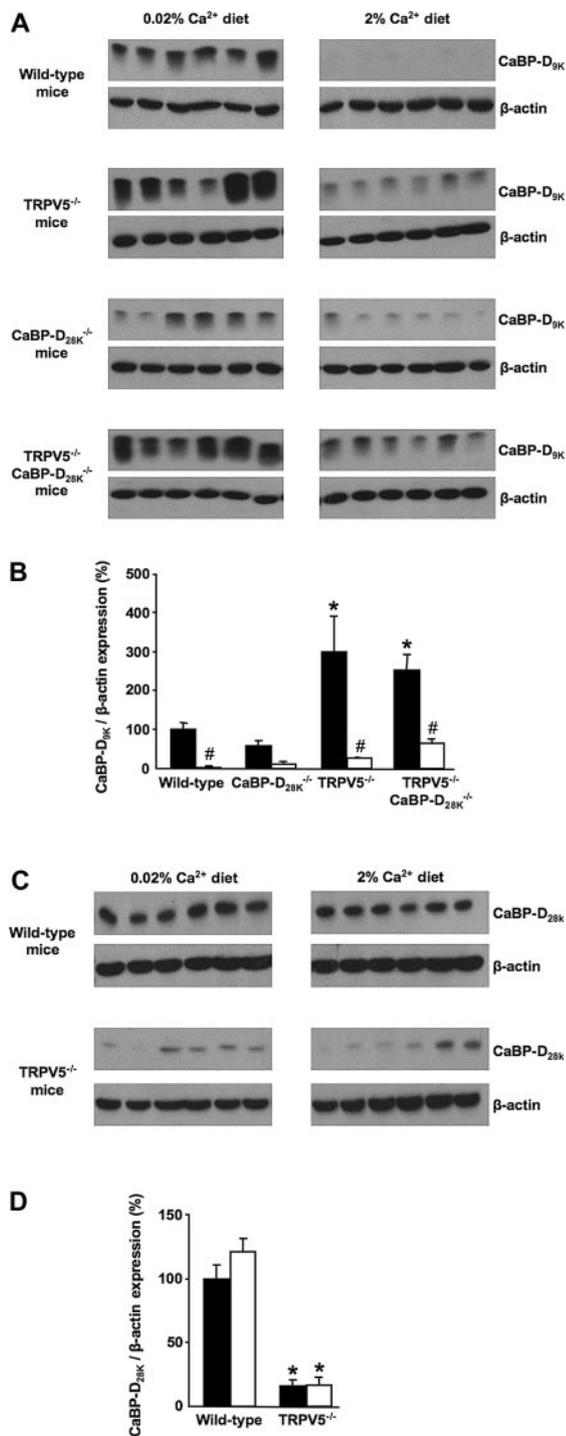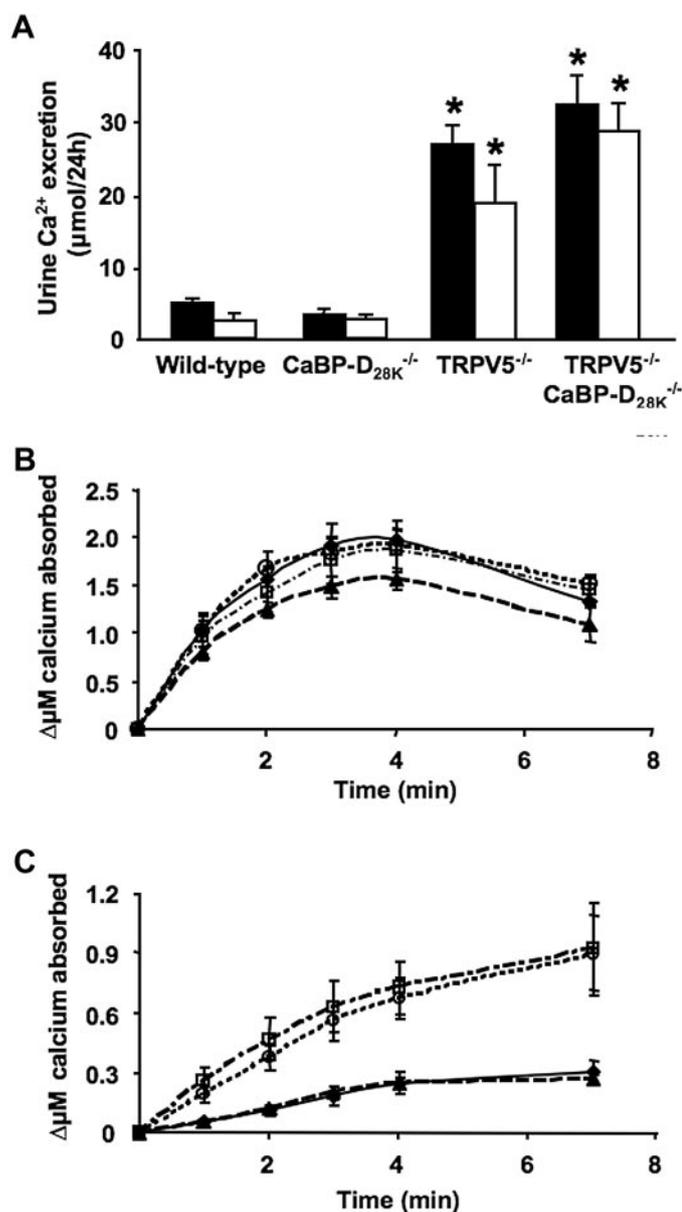
Figure 3. Renal protein expression of CaBP-D$_{28K}$ and CaBP-D$_{9K}$. Immunoblot of total kidney homogenates from wild-type, TRPV5$^{-/-}$, CaBP-D$_{28K}$$^{-/-}$, and TRPV5$^{-/-}$/CaBP-D$_{28K}$$^{-/-}$ mice ($n = 6$) that were on a 0.02% (wt/wt) and a 2% (wt/wt) Ca^{2+} diet and probed with anti–CaBP-D$_{9K}$ antibody (A) or anti–CaBP-D$_{28K}$ antibody (C). The intensities of the CaBP-D$_{9K}$ (B) and the CaBP-D$_{28K}$ (D) immunopositive bands were quantified by densitometry and presented as a ratio to β-actin expression in relative percentages compared with wild-type mice that were fed a 0.02% (wt/wt) diet. Mice were fed a 0.02% (wt/wt; ■) or 2% (wt/wt; □) Ca^{2+} diet. Data are means ± SEM. *$P < 0.05$ *versus* wild-type on the same diet; #$P < 0.05$ *versus* the same group on 0.02% (wt/wt) Ca^{2+} diet.
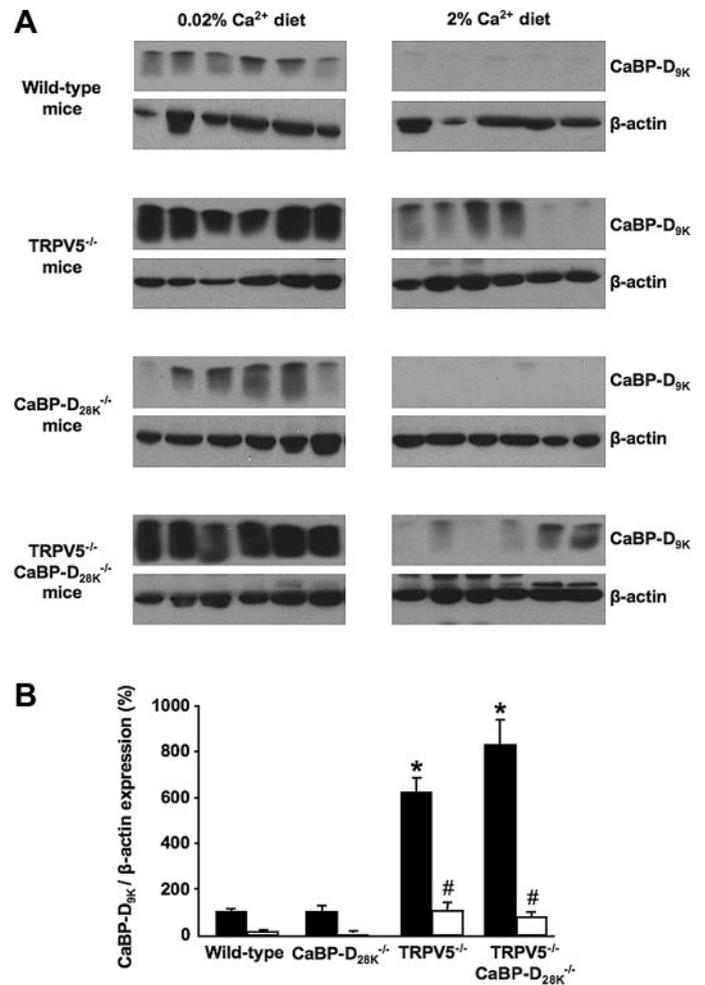
Figure 4. Duodenal protein expression of CaBP-D$_{9K}$. Immunoblot analysis of duodenum homogenates from wild-type, TRPV5$^{-/-}$, CaBP-D$_{28K}$$^{-/-}$, and TRPV5$^{-/-}$/CaBP-D$_{28K}$$^{-/-}$ mice ($n = 6$) that were on a 0.02% (wt/wt) and a 2% (wt/wt) Ca^{2+} diet and probed with anti–CaBP-D$_{9K}$ antibody (A). The intensity of the CaBP-D$_{9K}$ immunopositive bands was quantified by densitometry and presented as a ratio to β-actin expression in relative percentages compared with wild-type mice that were fed a 0.02% (wt/wt) diet (B). Mice were fed a 0.02% (wt/wt; ■) or 2% (wt/wt; □) Ca^{2+} diet. Data are means ± SEM. *$P < 0.05$ *versus* wild-type on the same diet; #$P < 0.05$ *versus* the same group on 0.02% (wt/wt) Ca^{2+} diet.

was identified in the promoter sequence of calbindin-D$_{28K}$ that partly underlies the Purkinje cell–specific expression of calbindin-D$_{28K}$. However, it is not known whether this element is active in kidney or whether additional intracellular signaling molecules are involved. Together, these findings underline the TRPV5-coordinated expression of calbindin-D$_{28K}$ and suggest that TRPV5 constitutes the rate-limiting step of active Ca^{2+} reabsorption in kidney.

In contrast to TRPV5$^{-/-}$ mice that displayed a significant hypercalciuria, calbindin-D$_{28K}$$^{-/-}$ mice exhibit normal Ca^{2+} excretion values. In line with our data are two previous studies that showed that genetic ablation of calbindin-D$_{28K}$ does not modulate Ca^{2+} excretion in mice that are fed a regular rodent

Figure 5. Functional characterization of single- and double-knockout mice for TRPV5 and CaBP-D$_{28K}$. Twenty-four-hour urinary Ca^{2+} excretion in wild-type, TRPV5$^{-/-}$, CaBP-D$_{28K}$$^{-/-}$, and TRPV5$^{-/-}$/CaBP-D$_{28K}$$^{-/-}$ mice ($n = 10$) that were fed a 0.02% (wt/wt; ■) or 2% (wt/wt' □) Ca^{2+} diet (A). Changes in serum Ca^{2+} ($\Delta\mu$M) within 10 min after ^{45}Ca^{2+} administration by oral gavage in wild-type (♦), TRPV5$^{-/-}$ (□), CaBP-D$_{28K}$$^{-/-}$ (▲), and TRPV5$^{-/-}$/CaBP-D$_{28K}$$^{-/-}$ (○) mice ($n = 6$) that were fed a 0.02% (wt/wt; B) or 2% (wt/wt; C) Ca^{2+} diet. Data are means ± SEM. *$P < 0.05$ *versus* wild-type on the same diet.

diet that contains 1% (22) or 0.02% (wt/wt) Ca^{2+} (23,24). In contrast, Lee *et al.* (23) and Sooy *et al.* (24) fed calbindin-D$_{28K}$$^{-/-}$ mice a defined diet that contained 1% (wt/wt) Ca^{2+} and showed a two- to three-fold increase in urinary Ca^{2+} excretion compared with wild-type controls. In addition, compared with vitamin D receptor (VDR) knockout mice, mice that lack both VDR and calbindin-D$_{28K}$ and are fed a regular diet

have significantly higher urinary Ca^{2+} excretion (1.7-fold), more severe hyperparathyroidism, and rachitic skeletal phenotype (22). Ca^{2+} excretion in TRPV5$^{-/-}$ mice, however, was 10-fold higher than in wild-type mice and, therefore, more severe compared with calbindin-D$_{28K}$$^{-/-}$ mice or mice that lack both the VDR and calbindin-D$_{28K}$. Furthermore, we showed that the renal Ca^{2+} leak in TRPV5$^{-/-}$ mice is not increased in the TRPV5$^{-/-}$/calbindin-D$_{28K}$$^{-/-}$ mice. These findings suggest that TRPV5 acts as the gatekeeper in the process of Ca^{2+} reabsorption in the DCT and CNT.

Although previous studies demonstrated increased Ca^{2+} excretion in calbindin-D$_{28K}$$^{-/-}$ mice, our data indicate no significant differences in serum Ca^{2+}, PTH, and 1,25(OH)$_2$D$_3$ levels in calbindin-D$_{28K}$$^{-/-}$ mice compared with wild-type mice (24). A compensatory intestinal Ca^{2+} hyperabsorption or increased high bone turnover could occur in these knockout mice. In contrast, we found similar intestinal ^{45}Ca^{2+} absorption rates as well as intestinal TRPV6 and calbindin-D$_{9K}$ expression in calbindin-D$_{28K}$$^{-/-}$ and wild-type mice. Previous studies by Sooy *et al.* (24) and Zheng *et al.* (22) are in line with our data on intestinal calbindin-D$_{9K}$ expression. Zheng *et al.* (22) demonstrated a modest decrease in bone mineral density in calbindin-D$_{28K}$$^{-/-}$ mice. In addition, detailed structural analysis of teeth and bones showed that mineralization was unaffected in calbindin-D$_{28K}$$^{-/-}$ mice (24). Consequently, neither a disturbed Ca^{2+} absorption nor an abnormal bone phenotype can account for the excess of urinary Ca^{2+} that was observed in their calbindin-D$_{28K}$$^{-/-}$ mice. Theoretically, ablation of calbindin-D$_{28K}$ should seriously impair the Ca^{2+} buffering capacity of the TRPV5-expressing cells in DCT and CNT, which in turn should inhibit the activity of TRPV5. However, the lack of a general hypercalciuria in calbindin-D$_{28K}$$^{-/-}$ mice suggests that calbindin-D$_{28K}$ deficiency might be compensated for by other renal Ca^{2+}-binding proteins. It is interesting that the specific coexpression of calbindin-D$_{9K}$ and calbindin-D$_{28K}$ in mouse DCT cells hints to a comparable function of calbindin-D$_{9K}$ in Ca^{2+} reabsorption (13). In the VDR$^{-/-}$ mice, there is a 90% decrease in the level of renal calbindin-D$_{9K}$ compared with wild-type mice (22). Therefore, in mice that lack both VDR and calbindin-D$_{28K}$, the increased urinary Ca^{2+} excretion may reflect the loss of compensation by calbindin-D$_{9K}$ (22). However, we cannot exclude the possibility that other molecular mechanisms could compensate for the deficiency of calbindin-D$_{28K}$ or that downstream reabsorptive nephron segments balance an impaired Ca^{2+} transport capacity of DCT that lack calbindin-D$_{28K}$.

In this study, we observed that the expression of renal and duodenal Ca^{2+} transporters is regulated by the dietary Ca^{2+} content. However, it is difficult to investigate the direct effects of dietary Ca^{2+} without affecting serum PTH and 1,25(OH)$_2$D$_3$ levels. Indeed, dietary Ca^{2+} restriction was accompanied by a compensatory increase in serum PTH and 1,25(OH)$_2$D$_3$ levels. Ample studies indicate that Ca^{2+} transporter genes are transcriptionally controlled by circulating 1,25(OH)$_2$D$_3$ (4). For instance, renal and intestinal calbindin-D$_{9K}$ abundance correlated positively with serum 1,25(OH)$_2$D$_3$ levels as consistently shown in various mouse models (17,25,26). Conversely, intestinal cal-

bindin-D_{9K} and plasma membrane Ca^{2+} ATPase expression was suppressed by alterations of dietary Ca^{2+} content in $VDR^{-/-}$ mice [27]. It is interesting that we demonstrated previously that a reduction in the expression of duodenal calbindin-D_{9K} but also TRPV6 can be normalized by a high-Ca^{2+} diet in 1α-OHase$^{-/-}$ mice, which lack circulating $1,25(OH)_2D_3$ [28]. Furthermore, dietary Ca^{2+} controls the renal abundance of TRPV5, calbindin-D_{28K}, and Na^+/Ca^{2+} exchanger in this latter knockout model [28]. Altogether, these findings suggest that the abundance of Ca^{2+} transport proteins can be controlled by vitamin D–dependent and –independent means.

Conclusion

TRPV5 and calbindin-D_{28K} are functionally coupled and play an important role in renal Ca^{2+} handling, where TRPV5 constitutes the rate-limiting step of active Ca^{2+} reabsorption in DCT and CNT. In contrast to TRPV5$^{-/-}$ mice, calbindin-$D_{28K}^{-/-}$ mice display normal serum parameters, intestinal Ca^{2+} absorption, and renal Ca^{2+} excretion. Ablation of calbindin-D_{28K} in TRPV5$^{-/-}$ mice does not aggravate the TRPV5$^{-/-}$ phenotype, indicating that the role of calbindin-D_{28K} possibly can be compensated for by calbindin-D_{9K}.

Acknowledgments

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See the related editorial, "Who Wins the Competition: TRPV5 or Calbindin-D$_{28K}$?," on pages 2954–2956.