Functional TRPV6 channels are crucial for transepithelial Ca\(^{2+}\) absorption

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1Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 2Experimentelle und Klinische Pharmakologie und Toxikologie, Universität des Saarlandes, Homburg, Germany; and 3Pharmakologisches Institut, Universität Heidelberg, Heidelberg, Germany

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Woudenberg-Vrenken TE, Lameris AL, Weiβgerber P, Olausson J, Flockerzi V, Bindels RJ, Freichel M, Hoenderop JG. Functional TRPV6 channels are crucial for transepithelial Ca\(^{2+}\) absorption. Am J Physiol Gastrointest Liver Physiol 303: G879–G885, 2012. First published August 9, 2012; doi:10.1152/ajpgi.00089.2012.—TRPV6 is considered the primary protein responsible for transepithelial Ca\(^{2+}\) absorption. In vitro studies demonstrate that a negatively charged amino acid (D) within the putative pore region of mouse TRPV6 (position 541) is critical for Ca\(^{2+}\) permeation of the channel. To elucidate the role of TRPV6 in transepithelial Ca\(^{2+}\) transport in vivo, we functionally analyzed a TRPV6D541A/D541A knockin mouse model. After weaning, mice were fed a diet (1% wt/wt) or Ca\(^{2+}\)-deficient (0.02% wt/wt) diet and housed in metabolic cages. Blood was sampled for Ca\(^{2+}\) measurements, and the expression of Ca\(^{2+}\) transport proteins was analyzed in kidney and duodenum. Intestinal Ca\(^{2+}\) uptake was measured in vivo by an absorption assay. Challenging the mice with the Ca\(^{2+}\)-deficient diet resulted in hypocalcemia in wild-type and TRPV6D541A/D541A mice. On a low-Ca\(^{2+}\) diet both mouse strains displayed increased expression of intestinal TRPV6, calbindin-D\(_{9K}\), and renal TRPV5. TRPV6D541A/D541A mice showed significantly impaired intestinal Ca\(^{2+}\) uptake compared with wild-type mice, and duodenal TRPV5 expression was increased in TRPV6D541A/D541A mice. On a normal diet, serum Ca\(^{2+}\) concentrations normalized in both mouse strains. Under these conditions, intestinal Ca\(^{2+}\) uptake was similar, and the expression levels of renal and intestinal Ca\(^{2+}\) transport proteins were not affected. We demonstrate that TRPV6D541A/D541A mice exhibit impaired transepithelial Ca\(^{2+}\) absorption. Duodenal TRPV5 expression was increased in TRPV6D541A/D541A mice, albeit insufficient to correct for the diminished Ca\(^{2+}\) uptake. Under normal conditions, when passive Ca\(^{2+}\) transport is predominant, no differences between wild-type and TRPV6D541A/D541A mice were observed. Our results demonstrate a specific role for TRPV6 in transepithelial Ca\(^{2+}\) absorption.

TRPV6 pore mutant mice; intestinal calcium absorption; hypocalcemia; calcium transport proteins

![Image](http://www.ajpgi.org)

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Two pathways of intestinal Ca\(^{2+}\) absorption exist, and the preferred route depends on dietary availability of Ca\(^{2+}\). When dietary Ca\(^{2+}\) levels are high, the paracellular, passive pathway is the predominant absorption mechanism, whereas active, transepithelial transport occurs if Ca\(^{2+}\) supply is restricted (5, 12). Transepithelial Ca\(^{2+}\) absorption requires entry at the luminal side of the intestinal epithelium. The epithelial Ca\(^{2+}\) channel transient receptor potential vanilloid 6 (TRPV6) is hypothesized to be responsible for this luminal Ca\(^{2+}\) uptake. Active Ca\(^{2+}\) absorption occurs at the proximal part of the small intestine, whereas the concentration-dependent paracellular pathway arises gradually throughout the intestine (5, 15).

Within the gastrointestinal tract of human, rat, and mouse, high levels of TRPV6 are observed in the duodenum and colon (14, 20, 24), which is in line with its proposed function in intestinal Ca\(^{2+}\) entry. Inside the cell, Ca\(^{2+}\) binds to a Ca\(^{2+}\)-binding protein, calbindin-D\(_{9K}\), enabling diffusion toward the basolateral side of the cell, where Ca\(^{2+}\) is extruded into the blood via the plasma membrane Ca\(^{2+}\)-ATPase (PMCA1b) (10). Despite the absence of known activity in the kidney, TRPV6 expression has been reported in human and mouse kidney epithelia (20, 23, 24, 26, 35) as well.

Previous in vivo experiments revealed a role for TRPV6 in placental Ca\(^{2+}\) transport (27). Consistent with this hypothesis is the finding that calbindin-D\(_{9K}\) gene expression is significantly reduced in placenta of TRPV6−/− mice, whereas TRPV5 levels are unaffected, suggesting an association of TRPV6 and calbindin-D\(_{9K}\) in placental Ca\(^{2+}\) transport. Since a considerable amount of Ca\(^{2+}\) transport still occurs in TRPV6−/− mice, it is implied that other channels contribute significantly to this process (27). Recently, gain-of-function polymorphisms of the TRPV6 gene have been found in Ca\(^{2+}\)-stone-forming patients, which might contribute to absorptive hypercalciuria (28). These findings suggest that TRPV6 is one of the key players in intestinal Ca\(^{2+}\) absorption in humans, although further studies are required to elucidate its precise role.

Structural analysis of TRPV6 channels suggested the presence of a pore region between transmembrane domains 5 and 6, in which three negatively charged amino acids are situated (3, 4). An aspartate at position 542 in human TRPV6 (541 in mouse) was hypothesized to determine Ca\(^{2+}\) conductance. In mouse TRPV6, replacement of D541 with an alanine residue abrogated Ca\(^{2+}\) permeation and Mg\(^{2+}\) block in in vitro experiments, and similar results were obtained in vitro with rabbit TRPV5 (21, 34, 36). Expression of human TRPV6 D542A resulted in nonfunctional channels, despite equivalent cell surface expression as for wild-type TRPV6 (4, 34). This suggests that D542 or D541 is critical for Ca\(^{2+}\) permeation and/or channel activity. To address the specific role of TRPV6 in transepithelial Ca\(^{2+}\) permeation in vivo, we analyzed a mouse model with a specific mutation in the pore region: the TRPV6D541A/D541A knockin mouse (36). This mouse model is unique, compared with TRPV6−/− mice, since the mutation creates a nonfunctional TRPV6 channel with no effect on the channel’s structure, rather than a complete knockdown of the channel, which might affect other proteins as well. The TRPV6D541A/D541A knockin mice show a marked reduction of Ca\(^{2+}\) uptake by epididymal epithelial cells and consequently, the mobility and fertility of sperm are significantly reduced (36). Bone microarchitecture and bone matrix mineralization were not affected in these mice (29), suggesting a minor role of TRPV6 in bone metabolism. In the present
study, we determined serum Ca$^{2+}$ concentrations and urinary Ca$^{2+}$ excretion and measured intestinal Ca$^{2+}$ absorption in TRPV6$^{D541A/D541A}$ mice compared with wild-type littermates under both normal dietary circumstances and severe nutritional Ca$^{2+}$ restriction to specifically study the role of TRPV6 in transepithelial transport.

METHODS

Animal studies. Generation of TRPV6$^{D541A/D541A}$ knockin mice is described in Ref. 36. TRPV6 littermates were housed in a temperature- and light-controlled room with standard pellet chow (1% wt/wt SSNIFF Spezialdiäten, Soest, Germany) or a Ca$^{2+}$-deficient diet (0.02% wt/wt Ca$^{2+}$, SSNIFF) and filter sterilized drinking water available ad libitum. They were fed their respective diets from weaning (age 3 wk) until the experiment date. TRPV6 mice on the normal and Ca$^{2+}$-deficient diet, $n = 14$ per group, were housed in metabolic cages, and 24-h urine was collected. Fresh urine was used for the pH determination. The animals were euthanized at an age of 12–14 wk. Blood samples were taken and kidney, duodenum, and colon tissues were sampled and snap frozen in liquid nitrogen. All experiments were performed in compliance with the animal ethics board of the Radboud University Nijmegen (Ethical License number RU-DEC 2008-067, RU-DEC 2008-192).

Genotyping. Genotypes were determined by PCR as described in Ref. 36 to identify animals with wild-type or TRPV6$^{D541A/D541A}$ genotypes.

Analytical procedures. Serum and urine total Ca$^{2+}$ concentrations were measured by using a colorimetric assay kit as described previously (11). Serum albumin concentrations were measured using an ELISA kit according to the manufacturer’s protocol (GenWay Biotech, San Diego, CA). Urine Mg$^{2+}$ concentrations were determined by using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, The Netherlands). Urine phosphate and Na$^{+}$ concentrations were analyzed on a Hitachi autoanalyzer (Hitachi, Laval, Quebec, Canada).

In vivo $^{45}$Ca$^{2+}$ absorption assay. Ca$^{2+}$ absorption was determined by measuring serum $^{45}$Ca$^{2+}$ levels at early time points after oral gavage as described in Ref. 33. Serum $^{45}$Ca$^{2+}$ concentrations were measured using an ELISA kit according to the manufacturer’s protocol (GenWay BioTech, San Diego, CA). Urine Mg$^{2+}$ concentrations were determined by using a colorimetric assay kit according to the manufacturer’s protocol (Roche Diagnostics, Woerden, The Netherlands). Urine phosphate and Na$^{+}$ concentrations were analyzed on a Hitachi autoanalyzer (Hitachi, Laval, Quebec, Canada).

RESULTS

Ca$^{2+}$ deprivation reduces serum Ca$^{2+}$ concentrations in wild-type and TRPV6$^{D541A/D541A}$ mice. Feeding the mice a Ca$^{2+}$-deficient diet, from weaning until the experiment, resulted in a serum Ca$^{2+}$ concentration of 1.7 $\pm$ 0.1 mM for TRPV6$^{D541A/D541A}$ mice and 1.6 $\pm$ 0.1 mM for wild-type mice (Fig. 1A). Ca$^{2+}$ levels in both mouse strains were significantly lower compared with the same genotypes fed the normal-Ca$^{2+}$ diet (1.9 $\pm$ 0.1 mM and 2.0 $\pm$ 0.1 mM respectively, Fig. 1A). Changes in serum Ca$^{2+}$ concentration were not due to alterations in the protein levels caused by a different dietary content, since serum albumin concentrations were similar for the four groups analyzed (Fig. 1B). Ca$^{2+}$ deficiency significantly reduced the body weight of TRPV6$^{D541A/D541A}$ mice to 27.9 $\pm$ 1.0 g compared with the weight of the same genotype on a regular diet of 31.3 $\pm$ 1.0 g (Fig. 1C). Although the trend for wild-type mice was the same, the body weights were not significantly decreased in mice fed the Ca$^{2+}$ deficient diet (28.2 $\pm$ 1.0 vs. 30.9 $\pm$ 0.9 g, Fig. 1C). The volume of urine voided by TRPV6$^{D541A/D541A}$ and wild-type mice on both diets was not significantly different (Fig. 1D), and both mice showed comparable urine pH values (Fig. 1E). Renal Ca$^{2+}$ excretion varied from 3.9 $\pm$ 0.4 to 5.0 $\pm$ 0.7 $\mu$mol/24 h (Fig. 1F) and was similar for the four groups studied. Urinary Mg$^{2+}$ excretion (Fig. 1G) was slightly increased in TRPV6 wild-type mice on the Ca$^{2+}$-deficient diet, but no significant changes could be observed caused by either the genotype or the diet. Ca$^{2+}$ deprivation significantly enhanced urinary P$^{3+}$ excretion 9.5 times in TRPV6 wild-type mice and 11.2-fold change in TRPV6$^{D541A/D541A}$ mice (Fig. 1H). Renal Na$^{+}$ excretion was comparable for the four groups analyzed (Fig. 1I).

Ca$^{2+}$-deficient TRPV6$^{D541A/D541A}$ mice show impaired intestinal Ca$^{2+}$ absorption. To study the functional consequences of Ca$^{2+}$-impermeable pore mutant on intestinal absorption, an in vivo $^{45}$Ca$^{2+}$ absorption assay was performed. A low-Ca$^{2+}$ diet resulted in a significantly increased Ca$^{2+}$ absorption in TRPV6$^{D541A/D541A}$ and wild-type mice compared with mice on a regular diet (Fig. 2A). At all time points studied, TRPV6$^{D541A/D541A}$ mice displayed significantly reduced Ca$^{2+}$ absorption compared with their wild-type littermates (Fig. 2A, open symbols). On the regular diet, uptake of Ca$^{2+}$ from the intestinal lumen was similar for TRPV6$^{D541A/D541A}$ and wild-type mice (Fig. 2A).

Next, the gene expression of the Ca$^{2+}$ transporters in the duodenum of these mice was examined by quantitative PCR analysis. Ca$^{2+}$ deprivation resulted in a 20- to 25-fold induction of TRPV6 expression (Fig. 2B) in wild-type and TRPV6$^{D541A/D541A}$ mice. Interestingly, duodenal TRPV5 expression was significantly increased 6-times in TRPV6$^{D541A/D541A}$ mice on the Ca$^{2+}$-deficient diet (Fig. 2C), whereas a threefold change was induced by Ca$^{2+}$ deficiency in wild-type mice (Fig. 2C). Calbindin-D$^{9K}$ mRNA levels were 2.3 and 3.5 times upregulated in wild-type and TRPV6$^{D541A/D541A}$ mice, respectively (Fig. 2D). PMCA1b gene expression was not changed by the diet in either genotype (Fig. 2E). Intestinal mRNA levels of TRPV6, TRPV5, calbindin-D$^{9K}$, and PMCA1b were similar for TRPV6$^{D541A/D541A}$ and wild-type mice on the regular diet (Fig. 2, B–E). Next, we analyzed TRPV6 mRNA levels in the colon, to study differences in expression in the large intestine. Ca$^{2+}$ deficiency significantly increased
TRPV6 expression in TRPV6<sup>D541A/D541A</sup> and wild-type mice (23,000- and 18,000-fold, respectively, Fig. 2F).

Regulation of the renal Ca<sup>2+</sup> transporters by Ca<sup>2+</sup> deprivation. Expression of renal Ca<sup>2+</sup> transport proteins was then investigated. Ca<sup>2+</sup> deficiency resulted in a twofold up-regulation of TRPV5 mRNA levels (Fig. 3A) in both TRPV6<sup>D541A/D541A</sup> and wild-type mice. Renal TRPV6 expression, which is quite low, was not increased compared with the mice on the regular diet (Fig. 3B). Calbindin-D<sub>28K</sub> mRNA levels were significantly reduced in wild-type mice (Fig. 3C); the trend was the same for TRPV6<sup>D541A/D541A</sup> knockin mice, but no significant effect was detected in the kidneys of TRPV6<sup>D541A/D541A</sup> mice. NCX1 expression was decreased by almost 50% in both TRPV6<sup>D541A/D541A</sup> and wild-type mice (Fig. 3D), but the levels of PMCA1b were not reduced compared with the mice on the regular diet (Fig. 3E). Besides the different regulation of calbindin-D<sub>28K</sub> mRNA levels (Fig. 3C), gene expression of renal Ca<sup>2+</sup> transporters was identical in TRPV6<sup>D541A/D541A</sup> and wild-type mice (Fig. 3, A–E). On a regular diet, renal mRNA levels of TRPV5, TRPV6, calbindin-D<sub>28K</sub>, NCX1, and PMCA1b were equal in TRPV6<sup>D541A/D541A</sup> and wild-type mice (Fig. 3, A–E). Expression of renal 1αOHase was studied to indicate changes in vitamin D homeostasis. In both TRPV6<sup>D541A/D541A</sup> and wild-type mice, 1αOHase expression was significantly increased by Ca<sup>2+</sup> deprivation (Fig. 3F); however, no changes in expression between TRPV6<sup>D541A/D541A</sup> and wild-type mice were observed (Fig. 3F).

**DISCUSSION**

This is the first study characterizing Ca<sup>2+</sup> homeostasis in a mouse model in which TRPV6 channels are functionally inactivated by mutating the channel pore. Our results show that TRPV6 is specifically involved in transepithelial small intestinal Ca<sup>2+</sup> transport. TRPV6<sup>D541A/D541A</sup> mice exhibit de-
increased duodenal Ca\textsuperscript{2+} uptake and increased intestinal TRPV5 gene expression compared with wild-type mice. Functional consequences on serum Ca\textsuperscript{2+} and the net urinary Ca\textsuperscript{2+} excretion were not observed, and the expression of Ca\textsuperscript{2+} transporters in the kidney was not affected.

The mice were challenged by a Ca\textsuperscript{2+}-deficient diet to address the specific function of TRPV6 in transcellular Ca\textsuperscript{2+} transport. This resulted in a failure to thrive, emphasizing the importance of in vivo absorption assays to study the functional consequences. Dietary Ca\textsuperscript{2+} restriction reduced intestinal ab-
sorption in TRPV6 \textsuperscript{541A/D541A} knockin mice, whereas the homeostasis of other electrolytes, measured by urinary excretion, was not significantly affected in TRPV6 \textsuperscript{541A/D541A} knockin mice. This implies a critical role for TRPV6 in transcellular Ca\textsuperscript{2+}/H\textsuperscript{+} transport specifically. This conclusion is supported by a gain-of-function haplotype in TRPV6, which is suggested to contribute to absorptive hypercalciuria in patients (28). Parallel to our study, Benn et al. (1) described a downregulation of intracellular Ca\textsuperscript{2+}/H\textsuperscript{+} transport proteins in TRPV6\textsuperscript{-/-} mice on a 0.02% wt/wt Ca\textsuperscript{2+}-deficient diet but no differences in the same mice on a regular diet. However, impaired intestinal Ca\textsuperscript{2+} uptake for TRPV6\textsuperscript{-/-} mice on a normal diet has been reported as well (2). This model is different from ours in that Bianco et al. (2) studied knockout mice, whereas we analyzed knockin mice with an inactivated Ca\textsuperscript{2+} permeation, achieved by a single amino acid replacement in the pore region of TRPV6. Their study described TRPV6\textsuperscript{-/-} mice by a reduction in body weight, polyuria, and hypercalciuria (2), whereas we showed that the phenotype of the TRPV6\textsuperscript{D541A/D541A} mice on a regular diet is indistinguishable from wild-type mice. This is supported by the study of Benn et al. (1), who described the TRPV6\textsuperscript{-/-} mouse on a regular diet as normocalcemic with Ca\textsuperscript{2+} absorption comparable to wild-type mice. Both studies confirm the specific role of TRPV6 in transepithelial Ca\textsuperscript{2+} transport. Ca\textsuperscript{2+} uptake was not completely absent in TRPV6\textsuperscript{D541A/D541A} mice, implying that other mechanisms are critically involved. Vitamin D was found to regulate paracellular transport in vitro via direct effects on the expression of the tight junction complexes claudin-2 and claudin-12 and on the cell adhesion molecules claudin-3 and cadherin 17 (6, 7, 16). In the duodenum of TRPV6\textsuperscript{-/-} mice, vitamin D administration slightly increased expression of claudin-12 and reduced levels of cadherin 17, whereas no changes in claudin-2 expression were detected. This suggests some stimulation of paracellular absorption, but in vivo proof is still not definite (1, 17). Therefore, other yet-unidentified transport mechanisms might be compensating for the inactivation of TRPV6 in these TRPV6\textsuperscript{D541A/D541A} mice, such as caveolin1.3 (Cav1.3). Cav1.3 is highly expressed in the luminal membrane in jejunum and ileum of rat (19) and is supposed to function in glucose-mediated transcellular Ca\textsuperscript{2+} absorption under normal dietary conditions (18). It is interesting to study the effects of Ca\textsuperscript{2+} deprivation on Cav1.3-mediated transport.

Changes in the expression of renal and intestinal Ca\textsuperscript{2+} transporters mediated by Ca\textsuperscript{2+} deficiency in the TRPV6\textsuperscript{D541A/D541A} and wild-type mice has been described before (1, 8).

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**Fig. 3.** mRNA expression of renal Ca\textsuperscript{2+} transport proteins in TRPV6\textsuperscript{D541A/D541A} pore mutant mice is comparable to wild-type mice. Renal mRNA expression of TRPV5 (A), TRPV6 (B), calbindin-D\textsubscript{28k} (C), NCX1 (D), PMCA1b (E), and 1\textalpha\textsubscript{OHase (F) of wild-type and TRPV6\textsuperscript{D541A/D541A} mice on a regular 1% wt/wt Ca\textsuperscript{2+} diet (solid bars) or on a Ca\textsuperscript{2+}-deficient diet (0.02% wt/wt Ca\textsuperscript{2+}, open bars) were measured and are presented as ratio to HPRT1. Data are shown as means ± SE. N = 6–8 per group. *P < 0.05 vs. wild-type mice on the regular diet. †P < 0.05 vs. TRPV6\textsuperscript{D541A/D541A} mice on the regular diet.
intestinal TRPV5 expression, which is generally low compared with TRPV6 expression in the duodenum of rodents (9, 13, 22, 32), was significantly enhanced in TRPV6D541A/D541A mice on the Ca\(^2\+)

expression and urinary Ca\(^2\+)

excretion levels; e.g., within the normal range, despite being hypocalcemic. This phenomenon has been observed in knockout models associated with secondary hyperparathyroidism D (25). In addition, similar findings were previously described in wild-type mice, as well as in CaBP-D28K knockout mice, TRPV5 knockout mice, and CaBP-D28K-TRPV5 double knockout mice, none of which were found to have altered renal Ca\(^2\+)

excretion on a 0.02% compared with a 2% Ca\(^2\+)

deficient diet, and a minor, but not significant, in-


Hoenderop JG, Hartog A, Stuiver M, Doucet A, Willems PH, Bindels RJ. Localization of the epithelial Ca\(^2\+)


Hoenderop JG, van der Kemp AW, Hartog A, van de Graaf SF, van Os CH, Willems PH, Bindels RJ. Molecular identification of the apical Ca\(^2\+)


