Localization and Regulation of the Epithelial Ca\(^{2+}\) Channel TRPV6 in the Kidney

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Abstract. The family of epithelial Ca\(^{2+}\) channels consists of two highly homologues members, TRPV5 and TRPV6, which constitute the apical Ca\(^{2+}\) entry mechanism in active Ca\(^{2+}\) (re)absorption in kidney and small intestine. In kidney, TRPV5 expression has been extensively studied, whereas TRPV6 localization and regulation has been largely confined to the small intestine. The present study investigated the renal distribution of TRPV6 and regulation by 1,25-dihydroxyvitamin D\(_3\) (1,25(OH)\(_2\)D\(_3\)). In mouse kidney, TRPV6 was detected by immunohistochemistry at the apical domain of the distal convoluted tubules (DCT2), connecting tubules (CNT), and cortical and medullary collecting ducts (CD). Furthermore, several putative vitamin D–responsive elements were detected upstream of the mouse TRPV6 start codon, and 1,25(OH)\(_2\)D\(_3\) treatment significantly increased renal TRPV6 mRNA and protein expression. In DCT2 and CNT, TRPV6 co-localizes with the other known Ca\(^{2+}\) transport proteins, including TRPV5 and calbindin-D\(_{28K}\). Together, these data suggest a role for TRPV6 in 1,25(OH)\(_2\)D\(_3\)-stimulated Ca\(^{2+}\) reabsorption in these segments. Interestingly, distribution of TRPV6 extended to the CD, where it localized to the apical domain of principal and intercalated cells, which are not generally implicated in active Ca\(^{2+}\) reabsorption. In addition, TRPV6 mRNA levels were quantified in a large set of tissues, and in the order of decreasing expression level were detected: prostate > stomach, brain > lung > duodenum, kidney, bone, cecum, heart > colon > skeletal muscle > pancreas. Therefore, additional physiologic functions for TRPV6 are feasible. In conclusion, TRPV6 is expressed along the apical domain of DCT2, CNT, and CD, where TRPV6 expression is positively regulated by 1,25(OH)\(_2\)D\(_3\).

Active Ca\(^{2+}\) absorption plays a key role in Ca\(^{2+}\) homeostasis and takes place in Ca\(^{2+}\)-transporting tissues, including kidney and intestine. In the mammalian genome, a distinct family of epithelial Ca\(^{2+}\) channels was recently identified, which provides the molecular identity of the apical entry mechanism, facilitating the active Ca\(^{2+}\) transport process (1). This family of highly Ca\(^{2+}\)-selective channels is restricted to two members, TRPV5 (previously ECaC1), which was originally cloned from rabbit kidney, and TRPV6 (previously CaT1/ECaC2), which was identified from rat duodenum (2,3). Genomic analysis revealed that two genes, juxtaposed on human chromosome 7q35 and on mouse chromosome 6, encode these highly homologous but distinct epithelial Ca\(^{2+}\) channels (4–6). In addition to the conserved pore region, these channels share several functional properties, including the permeation profile for monovalent and divalent cations, the high Ca\(^{2+}\) selectivity, and Ca\(^{2+}\)-dependent inactivation (7,8). However, significant differences exist in the N- and C-termini of TRPV5 and TRPV6, which may account for distinct functional and regulatory features.

In the kidney, active transcellular reabsorption of Ca\(^{2+}\) takes place in the distal convoluted tubule (DCT) and the connecting tubule (CNT) and constitutes the fine-tuning mechanism determining net urinary Ca\(^{2+}\) excretion (1,9,10). The renal expression and regulation of TRPV5 has been extensively studied. Briefly, TRPV5 is localized along the apical membrane of DCT2 and CNT and is regulated by vitamin D (1,25(OH)\(_2\)D\(_3\)), dietary Ca\(^{2+}\), and estrogens, substantiating the role of TRPV5 in renal active Ca\(^{2+}\) transport (11–13). Data on the localization and regulation of TRPV6 have been largely confined to the small intestine, where the expression and regulation by 1,25(OH)\(_2\)D\(_3\) strongly supports the involvement of this channel in the absorption of dietary Ca\(^{2+}\) (14,15). Therefore, TRPV5 is generally suggested to be the epithelial Ca\(^{2+}\) channel primarily responsible for renal transcellular Ca\(^{2+}\) transport, whereas TRPV6 would serve as the apical Ca\(^{2+}\) entry mechanism in the small intestine.

Interestingly, TRPV6 mRNA has been detected in the kidney, but little is known about the localization and regulation of TRPV6 in this organ (16). The elucidation of the precise nephron distribution of TRPV6 is crucial in understanding its physiologic role in the kidney in general and particularly in...
evaluating the potential contribution of TRPV6 to renal transcellular Ca\(^{2+}\) reabsorption. Importantly, co-localization of TRPV5 and TRPV6 in the kidney may have significant functional relevance, because it was recently shown that TRPV5 and TRPV6 can form heterotetrameric Ca\(^{2+}\) channels with distinct functionality (17).

The aim of the present study was, therefore, to determine the localization and regulation of TRPV6 in the kidney. Immunohistochemistry was performed using a new TRPV6 antibody to elucidate the tubular and subcellular localization of this epithelial Ca\(^{2+}\) channel. In animal experiments, the effect of 1,25(OH)\(_2\)D\(_3\) on renal TRPV6 mRNA and protein expression was studied using real-time quantitative polymerase chain reaction (PCR) analysis and semiquantitative immunohistochemistry.

Materials and Methods

Generation and Characterization of Affinity-Purified Rabbit TRPV6 Antibody

Antiserum against TRPV6 was obtained by immunization of rabbits with 400 \(\mu\)g of a keyhole limpet hemocyanin-coupled synthetic peptide representing the last 15 amino acids of the C-terminal tail of mouse TRPV6 (NH\(_2\)-INRGLDEDGGEYQI-COOH). The antiserum was affinity-purified according to standard procedures. The specificity of the affinity-purified rabbit TRPV6 antibody was assessed by immunoblot analysis of Xenopus laevis oocytes heterologously expressing TRPV6 or TRPV5. In addition, the efficacy of the antibody to detect endogenous TRPV6 was assessed by immunoblot analyses of mouse kidney and duodenum lysates.

Animal Studies

Experiment 1. To study the nephron localization of TRPV6 by immunohistochemistry, kidneys were isolated from C57BL6 mice (kindly provided by Dr. J. Loffing, University of Zürich, Switzerland). The tissue fixation procedure consisted of anesthetizing the animals, after which the abdominal aorta was clamped downstream of the renal arteries. Tubing was inserted at the level of the iliac bifurcation into the aorta, pushed up to the aortic clamp, and fixed by a ligature. The vena cava was opened, the aortic clamp was removed, and a fixative solution (50 ml) was allowed to flush the mouse vasculature under high pressure. The fixative consisted of 3% paraformaldehyde (vol/vol) and 0.05% picric acid (vol/vol) dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4) and 10% hydroxyethyl starch (vol/vol) in saline (10). After 5-min fixation, the kidneys were rinsed at hydrostatic pressure by perfusion for 5 min with the cacodylate buffer. Kidneys were subsequently collected and stored at \(-80^\circ\)C until further processing.

Experiment 2. To study the effect of 1,25(OH)\(_2\)D\(_3\) on renal TRPV6 mRNA and protein expression, C57BL6 mice were intraperitoneally injected for 2 days with either 1,25(OH)\(_2\)D\(_3\) at a dose of 100 ng daily (n = 6) or vehicle only (n = 6). Thereafter, animals were sacrificed and kidneys were sampled and either immediately frozen in liquid nitrogen or fixed for immunohistochemistry by immersion in 1% (wt/vol) periodate-lysine-paraformaldehyde (PLP) for 2 h and 15% (wt/vol) sucrose in phosphate-buffered saline overnight. Samples were subsequently stored at \(-80^\circ\)C until TRPV6 and TRPV5 mRNA and protein expression levels were determined by real-time quantitative PCR and semiquantitative immunohistochemistry, respectively.

Experiment 3. To evaluate the mRNA expression of TRPV6 relative to TRPV5 expression in kidney and to determine the quantitative expression in various other tissues, a cDNA panel was constructed. To this end, C57BL6 mice were sacrificed and kidney, bone, prostate, stomach, duodenum, ileum, cecum, colon, pancreas, liver, spleen, brain, lung, heart, and skeletal muscle was sampled. Tissues were subsequently stored at \(-80^\circ\)C. The animal ethics board of the University Medical Center Nijmegen approved all experimental procedures.

Immunohistochemistry

The renal localization of TRPV6 was assessed by immunohistochemistry, including co-localization studies using proteins with established distribution patterns as markers for distinct nephron segments. Immunohistochemical staining was performed as described previously (18). In short, either co-immunohistochemical staining or staining of serial sections for TRPV6 with TRPV5, calbindin-D\(_{28K}\), calbindin-D\(_{32K}\), the thiazide-sensitive Na\(^{+}\)-Cl\(^{-}\) cotransporter (NCC), aquaporin-2 (AQP2), and peanut lectin-binding intercalated cells was performed on 7-\(\mu\)m sections of fixed frozen kidney samples. TRPV5 staining involved immersion of the kidney sections in boiled citrate target retrieval buffer (0.01 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 30 min. Sections were incubated for 16 h at 4\(^\circ\)C with the primary antibodies: affinity-purified rabbit TRPV6 antibody (1:25), affinity-purified guinea pig TRPV5 antibody (1:1000) (18), guinea pig anti-calbindin-D\(_{28K}\) (1:500) (12), mouse anti-calbindin-D\(_{28K}\) (Swant, Bellinzona, Switzerland) (1:750), rabbit anti-NCC (1:200) (19), guinea pig anti-AQP2 (1:1000) (20), and FITC-coupled peanut lectin (1:100), respectively. In addition, immunohistochemistry of kidney sections using pre-immune serum and TRPV6 antisera pre-absorbed for 1 h with the corresponding peptide was performed as negative controls. For detection of TRPV6, calbindin-D\(_{32K}\), calbindin-D\(_{28K}\), NCC, and AQP2 sections were incubated with Alexa-conjugated secondary antibodies. After incubation with biotin-coated goat anti-guinea pig secondary antibody, TRPV5 was visualized using a tyramide signal amplification kit (NEN Life Science Products, Zaventem, Belgium). Images were made using a Nikon Diaphot confocal laser scanning microscope (Tokyo, Japan; MRC-1000; Bio-Rad, Richmond, CA) and a Zeiss fluorescence microscope equipped with a digital photo camera (Nikon DMAX1200). For semiquantitative determination of protein levels, images were analyzed with the Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD), resulting in quantification of the protein levels as the mean of integrated optical density (IOD).

Real-Time Quantitative PCR Analysis

Total RNA was extracted from kidney and other tissues using TriZol Total RNA Isolation Reagent (Life Technologies BRL, Breda, The Netherlands). The obtained RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 2 \(\mu\)g of RNA was reverse transcribed by Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT; Life Technologies BRL) as described previously (18). The obtained cDNA was used to determine TRPV6 and TRPV5 mRNA expression levels as well as mRNA levels of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) as an endogenous control. PCR primers and fluorescence probes were designed using the computer program Primer Express (Applied Biosystems, Foster City, CA) and purchased from Biologen (Malden, The Netherlands). The primer sequences for TRPV6 were 5'-ATCCGCCGGCTATGCACA-3', 5'-AGTTTTTCTCCTGA ATCTTTTCCA-3', and 5'-TTCAGCACAAGAGTGCCCTACTCTGTA-3' for the probe. TRPV5 primer and probe sequences were 5'-CGTTGGTTCTTACGG...
GTTGAAC-3', 5'-GTTTGGAGAACCACAGAGCCTCTA-3', and 5'-TGTTTCTCAGATA GCTGCTCTTGTACTTCCTTTGT-3'. For HPRT, these primers were, respectively, 5'-TTATCAGACTGAGCTACTGTAATGATC-3', 5'-TTACCAGTGGTCAATTATTA CTTCACAAATCTTCGACACTTTTATTGTCCC-3' for the probe. Expression levels were quantified by real-time quantitative PCR on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland).

TRPV6 Promoter Analysis

The general mechanism by which 1,25(OH)2D3 induces gene transcription involves direct interaction of the vitamin D receptor with regulatory domains in the promoter region of the gene known as vitamin D responsive elements (VDRE). To identify putative VDRE, the nucleotide sequence upstream of the ATG codon in the mouse TRPV6 (mTRPV6) gene was analyzed (Genbank accession no. NT039341).

Statistical Analyses

Data are expressed as means ± SEM. Statistical comparisons were tested by one-way ANOVA and Fisher multiple comparison. P values less than 5% were considered statistically significant. All analyses were performed using the Statview Statistical Package software (Power PC version 4.51; Statview, Berkeley, CA) on a Macintosh computer.

Results

Generation and Characterization of Affinity-Purified Rabbit TRPV6 Antibody

Figure 1 shows immunoblots of lysates of Xenopus laevis oocytes heterologously expressing TRPV6 or TRPV5. The affinity-purified TRPV6 antibody detected two specific bands of approximately 75 kD and 85 to 100 kD in size corresponding to the core and complex glycosylated proteins, respectively, as shown previously (17), whereas the affinity-purified TRPV5 antibody failed to detect these specific bands. Conversely, in lysates of TRPV5 expressing Xenopus laevis oocytes, specific signals were not detected using the affinity-purified TRPV6 antibody. However, the affinity-purified TRPV5 antibody recognized two specific bands of 75 kD and 85 to 100 kD, corresponding to the core and complex glycosylated protein, respectively. Therefore, the applied antibodies did not cross-react, indicating that both antibodies were channel-specific. In addition, immunoblot analysis of mouse kidney and duodenum lysates did not result in a specific immunopositive signal using either affinity-purified antibody, probably due to the relatively low abundance of these channels (data not shown).

Immunohistochemical Localization of TRPV6 in Kidney

To elucidate the renal segmental localization of TRPV6, immunohistochemical staining of mouse kidney sections with the TRPV6 antibody was performed. Distinct immunopositive staining for TRPV6 was detected in kidney cortex (Figure 2, A and B) and, in addition, in renal medulla (Figure 2, C and D). Importantly, in both cortex and medulla, TRPV6 immunolabeling was predominantly confined to the apical domain of the tubular cells, suggesting apical membrane localization. All negative controls, including immunohistochemistry on kidney sections with either antiserum pre-absorbed for 1 h with 10 μg/ml peptide (Figure 2E), pre-immune serum, or omission of the primary antibody (data not shown), were devoid of staining.

Staining of serial kidney sections for TRPV5 and TRPV6 demonstrated that these epithelial Ca2+ channels show considerable co-expression in renal cortex. All TRPV5-immunopositive tubules expressed TRPV6, but a substantial number of TRPV6-immunopositive tubules lacked TRPV5 immunoreactivity (Figure 3A). In mice, TRPV5 expression is known to start at the transition from DCT1 to DCT2, continuing into CNT (10,18). Calbindins are also expressed in DCT2 and the principal cells of CNT, extending farther into CCD, and are involved in intracellular buffering and facilitated diffusion of Ca2+ (10). Figure 3B shows that TRPV6 co-localizes with calbindin-D28K, although a portion of TRPV6-positive tubules did not express calbindin-D28K (data not shown). Co-staining for TRPV6 and calbindin-D28K also showed a considerable co-expression of both proteins (Figure 4D). However, a number of TRPV6-immunopositive cells lacked calbindin-D28K. In line with previous studies, renal medulla was devoid of any
TRPV5, calbindin-D_9K, or calbindin-D_28K immunopositive signal (data not shown).

Serial kidney sections were stained for TRPV6 and the thiazide-sensitive Na^+\text{-Cl}^- cotransporter (NCC) that is exclusively expressed in DCT and functionally characterizes this segment (Figures 3, C and D) (9,10). In addition to tubules in which both proteins co-localized, NCC expression was not detected in a significant number of tubules that exhibited strong apical TRPV6 immunoreactivity. Furthermore, some NCC-positive tubules lacked TRPV6 immunostaining, substantiating that TRPV6 is not expressed in DCT1. Taken together, these data demonstrate that TRPV6 expression starts together with TRPV5 at the transition from DCT1 to DCT2. In contrast to TRPV5 expression, TRPV6 distribution extended into consecutive nephron segments.

To establish the identity of the additional TRPV6 positive tubules, co-staining of mouse kidney sections for TRPV6 and AQP2, characterizing the CNT and collecting ducts, was performed (9,21). A bright immunopositive TRPV6 signal co-localized with AQP2 in the cortical collecting ducts (CCD) (Figure 4A), outer medullary collecting ducts (OMCD), and inner medullary collecting ducts (IMCD) (Figure 4B). Higher
magnification of these AQP2/TRPV6 co-expressing cells showed that, although AQP2 is expressed strictly at the apical membrane, TRPV6 shows additional intracellular staining at the apical side in some of these cells. Taken together, these data indicate that TRPV6 distribution extends from DCT2 into IMCD. In addition, Figures 4C and 4D show that TRPV6 was
detected along the apical domain in the intercalated cells of the CD and CNT, respectively (arrows). Co-immunohistochemical staining of TRPV6 with peanut lectin, identifying over 90% of the intercalated cell population, confirmed the strictly apical TRPV6 expression in this cell type (Figure 4F) (22). Therefore, in addition to the principal cells, TRPV6 was clearly expressed in the intercalated cells, which characteristically lack expression of Ca2+ transport proteins.

Identification of Putative VDRE in the TRPV6 Promoter Region

To identify putative VDRE, the mTRPV6 5’UTR was analyzed for the presence of putative hexameric VDRE consensus sequence repeats, known to be involved in the transcriptional regulation of genes by 1,25(OH)2D3. VDRE have been reported to consist of two imperfect repeats separated by a limited number of nucleotide pairs known to contain the hexameric RRKNSA (R = A or G, K = G or T, S = C or G) consensus sequence (23,24). Figure 5 depicts four putative VDRE that were detected within 1500 bp upstream of the mTRPV6 ATG codon (black boxes).

Regulation of Renal TRPV6 Expression by 1,25(OH)2D3

The regulation of renal TRPV6 expression by 1,25(OH)2D3 was investigated in mice. TRPV6 mRNA copy numbers, as determined by real-time quantitative PCR analysis, were significantly increased in 1,25(OH)2D3-treated animals relative to controls (Figure 6A). This increased mRNA expression was accompanied by a significantly enhanced TRPV6 immunofluorescence in kidney cortex (Figure 6B) and medulla (Figure 6C), indicating that cortical and medullary TRPV6 expression is positively regulated by 1,25(OH)2D3. Figure 6D shows representative immunohistochemical staining for TRPV6, illustrating the upregulation of TRPV6 protein expression by 1,25(OH)2D3 in kidney cortex. Furthermore, in concordance with previously reported data, TRPV5 mRNA and proteins levels were significantly increased by 1,25(OH)2D3 (Figure 6, E and F).

Figure 5. Putative vitamin D–responsive elements (VDRE) in the mouse TRPV6 promoter region. Nucleotide sequence upstream of the start codon of the mouse TRPV6 gene (Genbank accession no. NT039341), which was probed for VDRE. Putative VDRE are indicated by black boxes.

Figure 6. Regulation of TRPV6 expression by 1,25(OH)2D3 in mouse kidney. TRPV6 mRNA expression was quantified by real-time quantitative polymerase chain reaction (PCR) analysis (A). TRPV6 protein expression in kidney cortex (B) and medulla (C) as determined by immunohistochemistry. Representative immunohistochemical staining of kidney cortex showing the effect of 1,25(OH)2D3 on TRPV6 expression (D). TRPV5 mRNA (E) and protein (F) expression in kidney cortex. Protein expression is presented as integrated optical density (IOD). Data are presented as means ± SEM. * P < 0.05 versus controls.
Quantification of TRPV6 mRNA Expression in Kidney and Other Tissues

To study the expression of TRPV6 and TRPV5 in various mouse tissues, including kidney, a mouse cDNA panel was constructed. Subsequently, TRPV6 and TRPV5 mRNA levels were quantified in this panel by real-time PCR analysis and normalized for cDNA input. Figure 7A demonstrates that TRPV6 mRNA was identified in various tissues and, in the order of decreasing expression level, were detected: prostate > stomach, brain > lung > duodenum, cecum, heart, kidney, bone > colon > skeletal muscle > pancreas. Likewise, TRPV5 was expressed in kidney > bone > stomach > duodenum, cecum > prostate, brain, liver > heart, pancreas, spleen (Figure 7B).

Discussion

The present study demonstrated that the epithelial Ca\(^{2+}\) channel TRPV6 is expressed along the apical domain of DCT2 through IMCD in mouse kidney. Together with the extensive co-localization with the proteins known to be involved in renal active Ca\(^{2+}\) transport, the positive regulation of TRPV6 by 1,25(OH)\(_2\)D\(_3\) suggests a role for TRPV6 in Ca\(^{2+}\) reabsorption. However, TRPV6 was also clearly detected in nephron segments and extrarenal tissues not known to accommodate active Ca\(^{2+}\) absorption. Therefore, additional physiologic functions for TRPV6 are feasible.

By detailed immunohistochemical analysis, the renal segmental and subcellular localization of TRPV6 was elucidated; the results are summarized in Figure 8. In both renal cortex and medulla, TRPV6 expression was predominantly confined to the apical membrane. TRPV6 co-localized with TRPV5 and the calbindins in DCT2 and CNT. It was previously shown that the Na\(^+/\)Ca\(^{2+}\) exchanger (NCX1) and the plasma membrane Ca\(^{2+}\)-ATPase (PMCA), constituting the basolateral Ca\(^{2+}\) extrusion machinery, are also confined to these nephron segments (9,10). In addition, TRPV6 co-localized with AQP2 in the principal cells of CCD, OMCD, and IMCD. Interestingly, the intercalated cells in CNT and collecting duct also displayed apical TRPV6 expression. Thus, it was concluded that TRPV6 is consistently present along the luminal membranes of DCT2 through IMCD.

1,25(OH)\(_2\)D\(_3\) is one of the most important regulators of Ca\(^{2+}\) homeostasis, and vitamin D receptors are primarily expressed in kidney, small intestine, and bone (25–28). The regulation of TRPV6 expression by 1,25(OH)\(_2\)D\(_3\) was therefore investigated in kidney. In mice exposed for 48 h to

Figure 7. Quantification of tissue distribution of TRPV6 and TRPV5. TRPV6 (A) and TRPV5 (B) mRNA expression levels in a large set of mouse tissues were determined by real-time quantitative PCR analysis. Copy numbers were normalized for the cDNA concentration of the input. Data are presented as means ± SEM.

Figure 8. Renal distribution of TRPV6 in mouse kidney. Summary of the renal distribution of TRPV6 as determined by immunohistochemistry. DCT, distal convoluted tubule; CNT, connecting tubule; CCD, cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; NCC, Na\(^+\)-Cl\(^-\) cotransporter; AQP2, aquaporin-2.
1,25(OH)₂D₃, a modest but significant upregulation of renal TRPV6 mRNA expression was observed, accompanied by a marked increase in tubular TRPV6 protein abundance in both kidney cortex and medulla. These data demonstrated that 1,25(OH)₂D₃ stimulates renal TRPV6 transcription and suggested that additional translational regulation by 1,25(OH)₂D₃ occurs. In general, the genomic mechanism of 1,25(OH)₂D₃ action has been shown to involve direct interaction of the activated vitamin D receptor complex with regulatory domains in the promoter region of genes, known as VDRE (23,24). Promoter sequence analysis described in the present study identified several putative VDRE upstream of the mTRPV6 start codon. The promoter sequences of the genes encoding the vitamin D–responsive Ca²⁺ transport proteins TRPV5, calbindin-D₉K and calbindin-D₂₈K, were previously shown to contain several VDRE, and stimulation of the expression of these transporters in the kidney by 1,25(OH)₂D₃ has been repeatedly confirmed (11,12,29,30).

The predominantly apical localization of TRPV6, which has previously been shown to constitute a highly Ca²⁺-selective ion channel, is in line with a role as apical Ca²⁺ entry mechanism in kidney (8). The co-localization of TRPV6 with the other key players in active Ca²⁺ transport in DCT2 and CNT, the main sites of active Ca²⁺ reabsorption, in conjunction with the positive regulation by 1,25(OH)₂D₃ in kidney clearly supports our hypothesis that TRPV6 could have functional importance in 1,25(OH)₂D₃-regulated transcellular Ca²⁺ reabsorption (10). TRPV6 was originally cloned from duodenum and was recently localized to the brush border membrane of the small intestine, in which calbindin-D₉K and PMCA are also known to be expressed (3,14,18). The regulation of TRPV6 expression by dietary Ca²⁺ and 1,25(OH)₂D₃ in duodenum and intestinal cell lines has further emphasized the involvement of this channel in dietary Ca²⁺ absorption (15,30–33). Previously, an essential role was not attributed to TRPV6 in kidney, while TRPV5 was generally considered as the rate-limiting Ca²⁺ entry step in renal transcellular Ca²⁺ reabsorption. It is, however, tempting to speculate on the basis of the present renal localization data that TRPV6 could facilitate active Ca²⁺ (re)absorption in both kidney and intestine.

We recently demonstrated that the epithelial Ca²⁺ channels are present in the plasma membrane as functional tetrameric complexes (17). Moreover, TRPV5/TRPV6 heterotetramers were shown to form functional Ca²⁺ channels in mammalian and Xenopus laevis oocyte expression systems. Depending on their exact subunit composition, these heterotetramers exhibited distinct electrophysiologic characteristics. The co-localization of TRPV6 and TRPV5 along the apical membrane of DCT2 and CNT, which was demonstrated in this study, suggests that these heterotetramers can potentially be formed in the kidney. Hypothetically, co-expression of both epithelial Ca²⁺ channels may provide these Ca²⁺ transporting tubules with a pleiotropic set of heterotetrameric channels to cover a broad range of Ca²⁺ transport kinetics.

Interestingly, TRPV6 expression was also detected in nephron segments not generally implicated in active Ca²⁺ reabsorption. First, principal cells in cortical and medullary collecting ducts, which are responsible for the fine-tuning of renal Na⁺ excretion and are highly permeable to water in the presence of vasopressin, displayed distinct apical TRPV6 expression. Second, intercalated cells, known to be involved in urinary acidification, also expressed TRPV6. The absence of supportive Ca²⁺ transport proteins along the larger part of the CD, as confirmed in this study by the lack of immunopositive staining for calbindins in OMCD and IMCD, precludes involvement of TRPV6 in transcellular Ca²⁺ reabsorption in these tubules (10). The consistent predominant apical localization would, however, imply that TRPV6 has a functional role as apical Ca²⁺ entry channel. Hypothetically, TRPV6-mediated Ca²⁺ influx could affect transport processes in these cells, for instance as part of a hormonal signaling cascade. Vasopres- sin was shown to induce Ca²⁺ influx across the apical membrane of renal collecting duct cells, and alterations in intracellular Ca²⁺ levels are known to affect Na⁺ reabsorption in these tubules (34–36). In addition, it has been suggested that the action of other hormones, including atrial natriuretic peptide (ANP), parathyroid hormone (PTH), bradykinin, and prostaglandins on transport processes involve Ca²⁺ signaling (37–39). The toxicity of the pre-urine osmotically challenges tubular epithelial cells constantly, and the resulting cell volume regulation is facilitated by Ca²⁺ signaling, which has been shown to encompass Ca²⁺ release from intracellular stores followed by Ca²⁺ influx from the extracellular compartment (40,41). The identity of the apical Ca²⁺ entry mechanism in the aforementioned processes has not been elucidated. Therefore, TRPV6 might be a potential candidate.

In this study, TRPV6 and TRPV5 expression levels were quantified in a large set of tissues. Our results showed that TRPV6 mRNA is expressed in nearly all tissues studied but that its expression levels vary greatly. A robust TRPV6 expression was detected in prostate. Although the exact function in this organ remains to be elucidated, previous reports have suggested that TRPV6 expression correlates with prostate carcinoma tumor grade (42,43). In addition, TRPV6 was expressed at lower levels in both kidney and duodenum. The functional relevance of TRPV6 in dietary Ca²⁺ absorption has been previously illustrated. Therefore, the detection of comparable expression levels in kidney further substantiated that renal TRPV6 expression is indeed quantitatively significant. Of note, substantial TRPV6 expression was detected in bone, an important reservoir of rapidly exchangeable Ca²⁺, suggesting a role in bone mineralization. Interestingly, TRPV6 mRNA was also abundantly expressed in stomach, which is not known as a site of Ca²⁺ absorption. This might point to an additional role for TRPV6 in the gastrointestinal tract, distinct from transcellular Ca²⁺ absorption. Furthermore, TRPV6 was detected at significant levels in brain, lung, and heart, but the functional relevance in these tissues is not clearly envisaged. TRPV5 expression levels were remarkably high in kidney, but TRPV5 was detected in various other tissues as well, including bone and intestine, albeit at considerably lower levels. Given the recent finding that both epithelial Ca²⁺ channels can combine into heterotetramers with novel functional properties, the variation in relative expression of TRPV6 and TRPV5 in the
tissues studied might reflect differential regulation to fine-tune Ca\(^{2+}\) channel kinetics (17). Altogether, these data indicate that, in addition to facilitating active Ca\(^{2+}\) transport in duodenum and kidney, the epithelial Ca\(^{2+}\) channels might also have additional functional roles in the body.

In conclusion, the localization of TRPV6 to the apical domain of cells lining DCT2 to IMCD and the positive regulation by 1,25(OH)\(_2\)D\(_3\) substantiate the functional relevance of TRPV6 in kidney. The co-expression of TRPV6 and TRPV5 implies that a pleiotropic set of heterotetrameric Ca\(^{2+}\) channels could facilitate renal active Ca\(^{2+}\) reabsorption. However, the precise role of TRPV6 in kidney remains to be established, particularly because this epithelial Ca\(^{2+}\) channel was also detected in nephron segments not involved in Ca\(^{2+}\) reabsorption. Given the widespread distribution of TRPV6 throughout the body, additional functions for TRPV6 are indeed conceivable, including a role in Ca\(^{2+}\) signaling.

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