Hypercalciuria constitutes the main risk factor in urolithiasis. Patients with renal stone disease experience severe pain, a high rate of recurrence, slowly progressing tissue damage, and eventually renal insufficiency, and recurrent clinical treatment is needed.\textsuperscript{1,2} Different types of hypercalciuria are known. In this respect, absorptive, renal, and resorptive hypercalciuria are distinguished: disturbed intestinal Ca\textsuperscript{2+} hyperabsorption, impaired renal Ca\textsuperscript{2+} reabsorption, and increased bone resorption, respectively, are primarily involved.\textsuperscript{3–6} Current treatment strategies of renal stones consist of (pain) medication, dietary adjustments, hydration, extracorporeal shock wave treatment, or surgery.

The transient receptor potential vanilloid member 5 (TRPV5), the epithelial Ca\textsuperscript{2+} channel, which is expressed in the renal distal convoluted tubule (DCT) and the connecting tubule (CNT), constitutes the major apical entry step of Ca\textsuperscript{2+} from the pro-urine into the renal cells.\textsuperscript{7} TRPV5 knockout (TRPV5\textsuperscript{−/−}) mice demonstrate a robust hypercalciuria with hyperphosphaturia, predisposing these mice to an increased risk for renal Ca\textsuperscript{2+}-phosphate stone formation.\textsuperscript{8} Concomitantly, TRPV5\textsuperscript{−/−} mice display profound polyuria and increased urinary
acidification.⁸ Urinary acidification occurs along different segments of the nephron and is of crucial importance in acid/base homeostasis.⁹,¹⁰

In combination with urinary buffers, the activity of acid/base transporters in intercalated cells (ICs) of CNT and collecting duct system (CD) accomplish the fine-tuning of urinary pH. In type A ICs, the multisubunit vacuolar ATP-driven proton pump (H⁺-ATPase) is expressed at the apical membrane, which is mainly responsible for proton excretion.¹¹ Inactivating mutations in the genes encoding individual subunits of the H⁺-ATPase proton pump are associated with distal renal tubular acidosis, characterized by a decrease in net proton secretion.¹²–¹⁴ The formation of less acidic urine predisposes to urolithiasis as the crystallization of Ca²⁺ phosphate occurs via the conversion of phosphate to its divalent form (HPO₄²⁻) in an alkaline rather than in an acidic environment.¹⁵,¹⁶ Previously, insufficient urinary acidification was demonstrated in recurrent stone formers.¹⁷,¹⁸ Currently, no clinical trials have documented the description of the prevention of stone formation by stimulating urinary acidification.

Aquaporin 2 (AQP2) is the water channel localized at the apical membrane of the CNT and CD that is responsible for vasopressin-regulated urinary water reabsorption.¹⁹,²⁰ Previous studies linked Ca²⁺ and water homeostasis, suggesting a functional role for the G-protein-coupled Ca²⁺/polyvalent cation-sensing receptor (CaSR).²¹–²³ Immunolocalization studies of renal CaSR in rats revealed expression on the apical membrane of both proximal tubule and CD, the basolateral membrane of thick ascending limb of Henle (TAL), and a dif-
fuse pattern throughout the DCT. Renal CaSR expression is highest in TAL cells and is absent from the glomerulus. The differential expression and localization of CaSR along the nephron is indicative of the variety of physiologic functions throughout the kidney. The localization of the CaSR along the CD facilitates the activation of the receptor during a hypercalcemic state. In the CD, a CaSR-mediated decrease of the urine concentrating ability could eventually lead to polyuria, assisting the excretion of large amounts of Ca²⁺.

The aim of this study was to elucidate molecular mechanisms involved in the adaptations that prevent renal stone formation during hypercalciuria. The TRPV5⁻/⁻ mouse model was used because polyuria and increased urinary acidification naturally occur in these hypercalciuric mice. The crucial roles of the functional H⁺-ATPase proton pump, the AQP2 water channel, and the CaSR are demonstrated in these hypercalciuria-related adaptations. This study provides new insights regarding the formation and prevention of kidney stones.

RESULTS

Metabolic Studies

At 8-wk-old TRPV5⁻/⁻ and littermate control mice were housed in metabolic cages for 24 h and killed. Genetic ablation of TRPV5 resulted in hypercalciuria, and these mice remained normocalcemic (Table 1). In addition, TRPV5⁻/⁻ mice displayed hyperphosphaturia with normal serum phosphate levels and a significant polyuria with decreased urinary osmolality. Urinary citrate and oxalate excretion levels were not different between the two mouse strains. Urinary pH was significantly lower in TRPV5⁻/⁻ mice compared with control mice. The 24-h ammonium and HCO₃⁻ excretion levels were unchanged (Table 2). Furthermore, urinary titratable acidity (TA) was significantly increased in TRPV5⁻/⁻ mice. Consequently, renal net acid excretion (NAE), which is the sum of renal ammonium excretion and TA minus HCO₃⁻ excretion, was elevated in TRPV5⁻/⁻ mice. Ca²⁺ excretion levels correlated linearly with urinary volume as well as pH (Figure 1, A and B, respectively). To address the contribution of H⁺-ATPase activity to the acidified urine in TRPV5⁻/⁻ mice, we generated TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice that were genetically ablated for both TRPV5 and the IC-specific B1 subunit of H⁺-ATPase (Atp6v1b1). Hypercalciuria remained present in 5-wk-old TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice, although less than in TRPV5⁻/⁻ mice (Figure 1C). Urinary pH was normalized (Figure 1D) whereas urinary volume was further increased in TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice in comparison with TRPV5⁻/⁻ mice (Figure 1E).

Figure 2. Analysis of TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice. (A) Growth curves comparing body weights of mouse genotypes, representing a litter containing one TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mouse that died at 14 d of age (dotted line) and four littermates (solid lines). (B) Growth retardation in TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice, demonstrating 5-wk-old control (left) and TRPV5⁻/⁻/Atp6v1b1⁻/⁻ (right) mice. (C) Mortality rate of TRPV5⁻/⁻/Atp6v1b1⁻/⁻ (solid line; n = 10) and littermate (dotted line; n = 55) mice. (D) Kidneys from 5-wk-old control (left) and TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice (right). (E) Renal histology of a 5-wk-old TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mouse. (F) Von Kossa staining in renal sections of a 1-wk-old TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mouse. (G) Transmission electron microscopy image of a tubular precipitate in the medullary collecting duct of a 1-wk-old TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mouse. (H) STEM image of (1) a precipitate, x-ray maps separately depicting (2) Ca²⁺, and (3) phosphorus content. (I) Energy dispersive x-ray microanalysis of the renal precipitate (arrow in H) demonstrating Ca²⁺ and phosphorus content. This spectrum represents all spot measurements performed (n = 35 spots). Magnifications: ×100 in E (left), F (left); ×250 in E (right), F (right); ×2500 in G.

Phenotypic Analysis of the TRPV5⁻/⁻/Atp6v1b1⁻/⁻ Mice

TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice were retarded in their growth directly after birth, compared with littermate mice (Figure 2A).
At 5 wk of age, TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice were easily identified because of severe growth retardation (Figure 2B). Moreover, 80% of the TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice died within 6 wk after birth (Figure 2C). These double-knockout mice showed bilateral hydronephrosis (Figure 2D) and abnormal dilation of the CD (Figure 2E). Von Kossa staining in kidneys of 1-wk-old TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice showed massive tubular Ca$^{2+}$ precipitations in the medullary CD (Figure 2F and G). Subsequently, kidney samples that included the observed precipitates were analyzed by energy-dispersive x-ray microanalysis using a Jeol 1200/STEM (Jeol Ltd., Tokyo, Japan) in combination with a Thermo Noran microanalysis SIX system (Thermo Fisher Scientific Inc., Waltham, MA) (Figure 2H). The x-ray maps and spectrum unequivocally showed the presence of Ca$^{2+}$ and phosphorus (Figure 2, H, 2 and 3, and I) in the precipitates present in the lumen of the CD suggesting Ca$^{2+}$-phosphate crystals.

Renal and Intestinal Expression of Water, Acid, and Phosphate Transporters

To address renal AQP2 expression levels in control and TRPV5$^{-/-}$ mice, we performed immunoblot analyses. AQP2 was significantly downregulated in TRPV5$^{-/-}$ mice compared with control mice at 8 wk of age (Figure 3A). Moreover, a further AQP2 downregulation was detected in TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice compared with TRPV5$^{-/-}$ mice, analyzed at 5 wk of age (Figure 3B). Membrane fractions were isolated from renal medulla tissue of 8-wk-old TRPV5$^{-/-}$ and control mice. Analysis of AQP2 protein expression in the membrane fractions revealed a significant downregulation in kidneys of TRPV5$^{-/-}$ compared with control littermate mice (Figure 3C). Immunoblotting for the B1 subunit of H$^+$/ATPase revealed no differences in expression levels between TRPV5$^{-/-}$ and control mice (Figure 4A). Furthermore, a significant downregulation of the renal type IIa NaPi co-transporter (NaPi-IIa) was revealed in TRPV5$^{-/-}$ mice (Figure 4B). In contrast, expression of the intestinal type IIb NaPi co-transporter (NaPi-IIb) was increased in brush border membrane vesicles isolated from ileum of TRPV5$^{-/-}$ mice compared with control mice (Figure 4C).

Effect of Ca$^{2+}$ on H$^+$/ATPase Activity of Outer Medullary CDs

We enzymatically isolated outer medullary CDs (OMCDs) from mouse renal tissue to perform pH$_i$ measurements. The activity of H$^+$/ATPase was determined from pH$_i$ recovery rate (ΔpH$_i$/min) after intracellular acidification by NH$_4$Cl.$^{25}$ We investigated the effect on H$^+$/ATPase activity of low (0.1 mM), normal (1.0 mM), and high (5.0 mM, mimicking the hypercalcicuric state of TRPV5$^{-/-}$ mice) extracellular [Ca$^{2+}$].

Representative recordings are depicted in Figure 5, A and C. Exposure of OMCDs to 5.0 mM [Ca$^{2+}$] significantly increased H$^+$/ATPase–mediated pH$_i$ recovery rates in control (n = 178 cells; 15 OMCDs; five mice; Figure 5B) and TRPV5$^{-/-}$ mice (n = 124 cells; nine OMCDs; four mice; Figure 5D) compared with OMCDs exposed to 0.1 mM [Ca$^{2+}$] from control mice (n = 159 cells; 12 OMCDs; four mice) and TRPV5$^{-/-}$ mice (n = 149 cells; 13 OMCDs; five mice). Comparison of pH$_i$ recovery rates in control and TRPV5$^{-/-}$ mouse OMCDs exposed to 0.1 mM [Ca$^{2+}$] revealed no significant differences in H$^+$/ATPase activity (Figure 5, B and D). To confirm the specific involvement of the H$^+$/ATPase proton pump, we performed the following experiments. First, OMCDs were incubated with 100 nM concanamycin, a specific H$^+$/ATPase inhibitor, which prevented the stimulatory action of 5.0 mM [Ca$^{2+}$] on the pH$_i$ recovery rate in OMCDs of control as well as
that are crucial adaptations in the prevention of renal stone formation during hypercalciuria. This conclusion is based on the following observations. First, TRPV5\(^{-/-}\) mice display hypercalciuria as a result of impaired active Ca\(^{2+}\) reabsorption, concomitant hyperphosphaturia, polyuria, and increased urinary acidification, whereas renal Ca\(^{2+}\) precipitations are not detected. Second, additional gene ablation of Atp6v1b1 in TRPV5\(^{-/-}\) mice prevents the increased urinary acidification and evokes massive Ca\(^{2+}\)-phosphate precipitation. Third, activation of the renal CaSR by elevated luminal Ca\(^{2+}\) levels stimulates H\(^+\)-ATPase–mediated H\(^+\) excretion and renal AQP2 protein downregulation, responsible for the consistent increased urinary acidification and polyuria, respectively, in hypercalciuric TRPV5\(^{-/-}\) mice.

Fine-tuning of renal acid excretion is generally accomplished in the CD, where the H\(^+\)-ATPase proton pump is localized at the apical side of type A ICs. Exposure to a 5.0 mM Ca\(^{2+}\) concentration significantly enhanced H\(^+\)-ATPase activity in OMCDs, a specific stimulatory effect that was absent in OMCDs from Atp6v1b1\(^{-/-}\) mice. Also, the CaSR agonist neomycin increased H\(^+\)-ATPase activity, emphasizing that CaSR activation modulates urinary acid excretion. In TRPV5\(^{-/-}\) mice, an increased luminal Ca\(^{2+}\) concentration is consistently present in the renal DCT, CNT, and CD.\(^{8}\) This could activate the apical CaSR, increasing H\(^+\)-ATPase activity and thereby initiating the observed acidic urinary pH. Importantly, genetic ablation of TRPV5 increased NAE, which is attributed to a significant rise in urinary TA as a result of hyperphosphaturia. In this perspective, the hyperphosphaturia observed in TRPV5\(^{-/-}\) mice could potentially present an acid load to the animals, initially caused by an increase in NaPi-IIb–mediated phosphate absorption; however, previous investigations showed that phosphate administration in healthy volunteers evokes metabolic alkalosis with a concomitant increase of urinary pH and remarkably only transient increases of TA and NAE.\(^{26}\) Although a higher dietary acid load cannot be completely ruled out, it did not cause a significant change in the acid-base homeostasis of the TRPV5\(^{-/-}\) mice. A mild metabolic acidosis would also have increased urinary ammonium excretion, which was not observed; therefore, the decline of the urinary pH in TRPV5\(^{-/-}\) mice represents a significant renal acid loss in the presence of hyperphosphaturia. Previously, Nijenhuis \textit{et al.}\(^{27}\) showed that TRPV5\(^{-/-}\) mice display normal blood pH and HCO\(_3^-\) levels under basal conditions. These mice become even more alkalotic than wild-type mice when given an NaHCO\(_3\) diet \textit{via} drinking water, which indicates that TRPV5\(^{-/-}\) mice do not have a metabolic acidosis. Together with these observations, our data demonstrate that TRPV5\(^{-/-}\) mice have increased urinary acidification without an altered systemic acid-base status.

Additional gene ablation of Atp6v1b1 in TRPV5\(^{-/-}\) mice normalized urinary pH and caused tubular precipitation of Ca\(^{2+}\)-phosphate in the medullary CD. These observations in-

DISCUSSION

As illustrated in this study, activation of the CaSR by increased urinary Ca\(^{2+}\) levels triggers urinary acidification and polyuria
activate enhanced H⁺-K⁺-ATPase activity in isolated parietal cells of gastric glands, allowing maximal ionization of dietary Ca²⁺ and establishing enhanced Ca²⁺ absorption from the intestine. Furthermore, Farajov et al. showed that the activation of the CaSR at the basolateral membrane of medullary TAL cells causes increased acid secretion in a hypercalcemic state. Together with our data, this strongly suggests a key role of the CaSR in linking Ca²⁺ metabolism and acid secretion.

Polyuria can diminish the risk for renal stone formation by reducing the urinary Ca²⁺ concentration. In mice, calciuresis linearly correlated with urinary volume, because an increase of Ca²⁺ excretion leads to an enhanced urinary volume. The consistent polyuria in hypercalciuric TRPV5⁻/⁻ mice, assessed by a substantial decrease in urinary osmolality, was caused by downregulation of renal AQP2 water channels. Specifically, immunoblot analysis revealed a significant downregulation of AQP2 protein expression levels in isolated renal medullary membrane fractions from TRPV5⁻/⁻ compared with control mice. Importantly, this is in line with previous studies performed on humans and rats that suggested that activation of the apical CaSR reduces the CD water permeability when the luminal Ca²⁺ level rises. This involves the CaSR-mediated retrieval of AQP2 from the apical membrane into endocytic compartments. Recently, Bustamante et al. showed an attenuated AQP2 expression in a mouse CD cell line after exposure to extracellular Ca²⁺, neomycin, or Gd³⁺. Thus, hypercalciuria activates the apical CaSR in the CD and reduces AQP2-mediated water reabsorption, permitting the disposal of excess urinary Ca²⁺ and preventing the formation of stones.

The B1 subunit of the renal H⁺-ATPase proton pump, encoded by the Atp6v1b1 gene, is important for urinary acidification in the renal CD. Atp6v1b1⁻/⁻ mice demonstrated hypocalciuria, which is an important difference in phenotype between mice and humans with a genetic Atp6v1b1 defect, although it confirms previous observations of Finberg et al. The presence of hypocalciuria in Atp6v1b1⁻/⁻ has not been explained yet and needs further investigation. Moreover, Atp6v1b1⁻/⁻ mice displayed a higher urinary pH compared with control mice. Consequently, gene ablation of the Atp6v1b1 gene in TRPV5⁻/⁻ mice normalized urinary pH. Interestingly, TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice evoked a more severe polyuria than displayed by the TRPV5⁻/⁻ mice. To explain the increased urinary volume in TRPV5⁻/⁻/Atp6v1b1⁻/⁻ mice, the CaSR response as a function of the extracellular pH was previously investigated, showing the functional modulation of the CaSR by extracellular pH. The increased urinary
volume in TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice compared with TRPV5$^{-/-}$ mice was explained by a further AQP2 down-regulation, demonstrated by immunoblotting. We postulate that the extended decrease of AQP2 expression in the TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice is evoked by the higher sensitivity for Ca$^{2+}$ of the CaSR at a neutral urinary pH. On the basis of previous studies, we conclude that, in the absence of increased urinary acidification in TRPV5$^{-/-}$ mice, a more severe reduction in water reabsorption is induced in an attempt to prevent renal stone formation. Stehberger et al. demonstrated a decreased urinary osmolality in AE1-deficient (AE1$^{-/-}$) mice as a result of the retrieval of AQP2 water channels to intracellular vesicles in CD cells. This effect could be the result of increased pH sensitivity of the CaSR at an alkaline environment. Despite the severe polyuria, TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice developed renal Ca$^{2+}$-phosphate precipitations that obstructed the CD system at an early age. Thus, these results underscore the crucial importance of increased urinary acidification in the local prevention of renal Ca$^{2+}$-phosphate stones.

TRPV5$^{-/-}$ mice also displayed, besides the hypercalciiuric, a profound hyperphosphaturia, increasing the risk for Ca$^{2+}$-phosphate precipitation. For maintaining normocalcemia, TRPV5$^{-/-}$ mice present with a hypervitaminosis D. Previous investigations showed that increased circulating 1,25-dihydroxyvitamin D$_3$ levels caused compensatory Ca$^{2+}$ hyperabsorption by upregulation of intestinal Ca$^{2+}$ transporters. Moreover, 1,25-dihydroxyvitamin D$_3$ is an important key player in phosphate homeostasis, increasing the abundance of intestinal NaPi-IIb transporters. TRPV5$^{-/-}$ mice displayed a significant upregulation of NaPi-IIb proteins in the ileum, which might evoke phosphate hyperabsorption. Here, we postulate that downregulation of renal NaPi-IIa proteins results in hyperphosphaturia in TRPV5$^{-/-}$ mice, possibly as a compensatory response to the increased phosphate absorption. Whether the NaPi-IIa downregulation could also be an effect of the hypervitaminosis D needs further investigation.

TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice exhibited growth retardation and died before 6 wk of age. The lethal phenotype is likely to be affected by the hydronephrosis, which could be explained by the premature formation of renal stones that physically obstruct the urine flow. This resembles the occurrence of hydronephrosis in congenital nephropathy that is often caused by a functional obstruction in the renal pelvis. Another explanation could be that the hydronephrosis arises in response to the pressure produced by the severe polyuria, dilating the CD and leading to kidney damage. This might also clarify the individual differences in severity of the hydronephrosis, polyuria, and early age of death observed in TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice. A similar observation was previously made in the NKCC2 knockout mice, a mouse model of Bartter syndrome that displayed polyuria and concomitant hydronephrosis as well. Li et al. previously investigated the presence of uremia subsequent to urinary tract obstruction in rats, which might have occurred in the TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice as well. The less robust hypercalciiuria observed in the TRPV5$^{-/-}$/Atp6v1b1$^{-/-}$ mice in comparison with the single TRPV5$^{-/-}$ mice could be due to the severe increase in urinary volume resulting in a slight dehydration in these mice. This could evoke increased proximal tubular Ca$^{2+}$ reabsorption as a compensatory mechanism, decreasing Ca$^{2+}$ wasting via the urine.

In conclusion, the increased urinary acidification and polyuria in response to hypercalciiuria are prerequisites to prevent the formation of renal Ca$^{2+}$-phosphate stones in TRPV5$^{-/-}$ mice. Interestingly, these adaptations involve luminal CaSR activation by increased urinary Ca$^{2+}$ levels, inducing enhanced H$^+$-ATPase activity and AQP2 downregulation (Figure 6). Renal Ca$^{2+}$-phosphate stone formers could benefit from the induction of a decrease in urinary pH as well as polyuria that is accomplished by increased water intake. Knowledge of these adaptive mechanisms is relevant for the therapy and prevention of kidney stones.
CONCISE METHODS

Animal Experiments and Metabolic Data
Please see supplemental online information for detailed methods.

Renal Histology
Von Kossa staining of kidney tissue was performed on fixed 5- to 7-μm cryosections to stain Ca2+-containing deposits, and further analysis of renal tubular Ca2+ precipitations was performed by transmission electron microscopy. Please see supplemental information for detailed methods.

Protein Isolations and Immunoblotting
To quantify the renal AQP2, the H+-ATPase B1 subunit, and the NaPi-IIa protein expression levels in 8-wk-old control and littermate TRPV5 knockout mice, we prepared renal protein lysates as described previously. For renal AQP2 quantification in the control, TRPV5−/−, Atp6v1b1−/−, and littermate TRPV5+/−/Atp6v1b1−/− mice, kidneys were dissected at 5 wk of age. Ileum brush border membrane vesicles were isolated to obtain renal OMCDs from control, TRPV5−/−, and Atp6v1b1−/− mice for H+-ATPase activity measurements. BCECF AM (1 μM; Molecular Probes, Invitrogen AG, Basel, Switzerland) was used to monitor pHi. Cells were acidified using the NH4Cl (20 mM) pre-pulse technique, as described previously. Please see supplemental information for detailed methods.

Tubule Preparations and pH Measurements
Mice were transcardially perfused with a digestion buffer containing 1 mg/ml collagenase (Sigma-Aldrich Chemie, Buchs, Switzerland) to obtain renal OMCDs from control, TRPV5−/−, and Atp6v1b1−/− mice for H+-ATPase activity measurements. BCECF AM (1 μM; Molecular Probes, Invitrogen AG, Basel, Switzerland) was used to monitor pHi. Cells were acidified using the NH4Cl (20 mM) pre-pulse technique, as described previously. Please see supplemental information for detailed methods.

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DISCLOSURES

None.


