Tissue Kallikrein–Deficient Mice Display a Defect in Renal Tubular Calcium Absorption

Nicolas Picard,* Monique Van Abel,† Christelle Campone*‡§ Michelle Seiler,‖ May Bloch-Faure,* Joost G.J. Hoenderop,† Johannes Loffing,‖ Pierre Meneton,* René J.M. Bindels,† Michel Paillard,*‡§ François Alhenc-Gelas,* and Pascal Houillier*‡§

**INSERM, Unité 652, and Institut Fédératif de Recherche 58, Paris, France; †Department of Physiology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands; ‡Université Paris-Descartes, Faculté de Médecine, Paris, France; §AP-HP, Hôpital Européen Georges Pompidou, Département de Physiologie, Paris, France; and ‖Department of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland*

Renal tubular calcium (RTCa) transport is one of the main factors that determine serum Ca concentration and urinary Ca excretion. The distal convoluted and connecting tubules reabsorb a significant fraction (10%) of filtered Ca. These tubule segments also synthesize in large abundance tissue kallikrein (TK), a major kinin-forming enzyme. Tested was the hypothesis that TK and kinins are involved in controlling RTCa transport by studying TK (TK−/−) or kinin B2 receptor (B2−/−)-deficient mice on different Ca diets. On a 0.9% wt/wt Ca diet, 129Sv or C57Bl/6 TK−/− mice excreted significantly more Ca in urine than their wild-type (WT) littermates. There was no difference between TK−/− and WT mice for plasma concentrations of Ca, Mg, creatinine, parathyroid hormone, or 1,25-dihydroxyvitamin D. On a low Ca (LCa) diet (0.01% wt/wt), urinary Ca excretion decreased in both TK−/− and WT mice but still remained higher in TK−/− mice compared with WT. The plasma Ca concentration was unchanged in C57Bl/6 TK−/− mice but decreased significantly in 129Sv TK−/− mice. Taken together, these data demonstrate that TK deficiency led to impaired RTCa absorption. On the LCa diet, renal TK gene expression doubled in WT mice. No change in urinary Ca excretion was observed in B2−/− mice, even after treatment with a kinin B1-receptor antagonist, and these mice adapted normally to the LCa diet. TK deficiency had no effect on the renal abundance of distal Ca transporter mRNA. These data suggest that TK may be a physiologic regulator of RTCa transport, acting through a non–kinin-mediated mechanism.

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Renal tubular Ca (RTCa) reabsorption is a direct determinant of urinary Ca excretion and the concentration of Ca in the extracellular fluid (ECF). Indeed, by matching the amount of Ca that is excreted in urine to the amount of Ca that enters the ECF compartment, the renal tubule keeps the ECF Ca concentration virtually constant (1). Accordingly, any impairment of the ability of the renal tubule to reabsorb the appropriate amount of Ca can affect both urinary Ca excretion and the ECF Ca concentration. As a consequence, in patients with familial benign hypercalcemia and primary hyperparathyroidism, plasma Ca concentration is related to tubular Ca reabsorption (2,3).

Under normal conditions, renal tubular Ca reabsorption is tightly regulated. Nonhormonal factors, such as ECF volume, acid/base status, and plasma Mg and Ca concentrations, influence the handling of Ca in the renal tubule (4–6). Extrarenal hormonal factors, such as parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D, also regulate tubular Ca reabsorption (7). In contrast, relatively little is known about the possible contribution of intrarenal factors in regulating renal tubular Ca transport.

Recently, the molecular basis of the active transcellular transport of Ca in the distal nephron has been unraveled. This process involves the apical influx of Ca through the epithelial Ca channel (TRPV5), which is the rate-limiting step in transcellular Ca transport (8). Accordingly, the lack of TRPV5 leads to a decrease in distal tubule Ca reclamation and hypercalciuria of renal origin (9).

It is noteworthy that one of the major proteins that are synthesized in the distal tubule and secreted into the tubular fluid is tissue kallikrein (TK) (10–12). TK is the main kinin-forming enzyme in mammals (13), but its function in the renal tubule remains largely unknown. Here we show that TK-deficient mice display hypercalciuria of renal origin. The distribution of TK largely overlaps with that of TRPV5 in the distal nephron, and kallikrein gene expression is increased by a low-Ca diet. We therefore suggest that kallikrein may be a physiologic regulator of Ca metabolism.

Materials and Methods

Animals and Diets

TK-deficient mice (TK−/−) were produced in our laboratory as described previously (13). The consequence of kallikrein deficiency in

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Address correspondence to: Dr. Pascal Houillier, Département de Physiologie, Hôpital Européen Georges Pompidou, 20 rue Leblanc, 75015 Paris, France. Phone: +33-1-5609-3974; Fax: +33-1-5609-3995; E-mail: pascal.houillier@egp.ap-hop-paris.fr

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mice was studied in two different genetic backgrounds. To do this, mice that bear the kallikrein gene–inactivating mutation were backcrossed for >10 generations with pure strains of 129Sv or C57Bl/6 mice (IFFA Credo, L'Arbresle, France) (13,14). Kinin B2 receptor–deficient mice (B2−/−) were also studied (15). These mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and backcrossed for >10 generations with C57Bl/6 mice (14).

For each experiment, the mutated (TK−/− or B2−/−) mice and their wild-type (WT) littermates were produced by heterozygous crossing. The studies were performed in 3- to 6-mo-old female or male mice. They were fed two different semisynthetic diets (UAR, Epinay sur Orge, France). Both diets contained 0.3% wt/wt sodium (Na), 0.9% wt/wt potassium (K), but differing Ca contents. The normal Ca (NCa) diet contained 0.9% wt/wt Ca, and the low Ca (LCa) diet contained 0.01% wt/wt Ca. The mice had free access to food and distilled water. All of the experimental procedures were performed in accordance with the European Guidelines for the care and use of laboratory animals.

Experimental Protocols

Study of Urinary Ca Excretion under Basal (NCa diet) Conditions. In three separate experiments, the female 129Sv TK−/− mice (n = 19) and their female WT littermates (n = 20), the female C57Bl/6 TK−/− (n = 23) mice and their female WT littermates (n = 26), or the male C57Bl/6 TK−/− mice (n = 15) and their male WT littermates (n = 14) were equilibrated for 14 d on the NCa diet. The mice then were housed individually in metabolic cages (Marty Technology, Marcilly sur Eure, France) for a 24-h period of urine collection; the mice were allowed to adapt to these cages for 1 d before collection began. The animals then were anesthetized by an intraperitoneal injection of ketamine (0.1 mg/g body wt) and xylazine (0.01 mg/g body wt), and blood was collected by orbital puncture in heparinized capillaries tubes. The urine and plasma samples were frozen at −20°C until analyzed.

Effect of a LCa Diet. In other sets of experiments, designed to study the ability of mice to adapt to a LCa diet, either female 129Sv TK−/− (n = 11) and WT (n = 11) mice or female C57Bl/6 TK−/− (n = 9) mice and WT (n = 10) mice were equilibrated for 14 d on the NCa diet and then switched to the LCa diet for an additional 14 d. At the end of the LCa period, 24-h urine and blood were collected as described above. At the end of the collection, the kidneys were removed and immediately frozen in liquid nitrogen for subsequent molecular studies.

Effect of Kinin B2-Receptor Inactivation (B2−/−) and of Kinin B1-Receptor Blockade in B2−/− Mice. Female B2−/− mice were equilibrated for 7 d on the NCa diet and then treated for an additional 7 d, while maintained on the same diet, with either the specific, nonpeptidic, kinin B1-receptor antagonist SSR240612, 1 mg/kg intraperitoneally (n = 12), daily (gift from Dr. J. Gougat, Sanofi-Synthelabo, Montpellier, France) (16), or its vehicle, saline + 2% vol/vol DMSO (n = 10). WT littermates (n = 10) were equilibrated in the same way as the B2−/− mice on the NCa diet and then treated with the vehicle for 7 d. Urine and blood were collected as above. The SSR240612 was provided by Dr. J. Gougat. In another experiment, B2−/− mice (n = 6) and their WT littermates (n = 8) were studied while on the LCa diet, as described above.

Measurement of Biologic Parameters in Plasma and Urine

Plasma Ca and Mg concentrations were measured by atomic absorption spectrophotometry (Perkin Elmer Apparatus, Model 3110, Cortabeuf, France). Plasma creatinine was measured by an automatic enzymatic method (Kodak Biolyzer, Eastman Kodak, Rochester, NY). The plasma concentration of 1,25-dihydroxyvitamin D was measured by a receptor competition assay (Dia Sorin, Stillwater, MN), and that of PTH was measured by an ELISA (Mouse Intact PTH ELISA kit; Immutopics, San Clemente, CA).

The urinary Na concentration (UNa) was measured by flame-spectrophotometry (Flame Photometer 943; Instrumentation Laboratory, Paris, France), the urinary Ca (UCa) and magnesium (UMg) concentrations were measured by atomic absorption spectrophotometry as above, and the urinary creatinine (UCr) concentration was measured by a modified, kinetic Jaffé colorimetric method (RAXT; Bayer, Leverkusen, Germany). Urinary free deoxypyridinoline (DPyr) and cAMP were measured by RIA (Nichols Institute Diagnostics, San Juan Capistrano, CA; and Amersham, Little Chalfont, UK, respectively). UNa, UCa, UMg, DPyr, and cAMP excretions were expressed as a ratio to the UCr excretion to take into account the variations in urine collection. Plasma and urinary phosphate concentrations were measured by a colorimetric method.

Measurement of Renal Kallikrein mRNA by Northern Blotting

Total RNA was extracted from one kidney of each mouse by the phenol/guanidium isothiocyanate method (Trireagent; Molecular Research Center, Cincinatti, Ohio) and checked for quality on agarose gel (17). Kallikrein mRNA was quantified by Northern blotting with a mouse kallikrein cDNA probe, as described previously (13).

Measurement of TRPV5, TRPV6, NCX1, PMCA1b, and Calbindins Gene Expression by Quantitative Real-Time PCR

Kidney RNA, prepared as described above, was DNase treated and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Cergy Pontoise, France). Quantitative, real-time PCR reactions were performed on an ABI-PRISM 7700 sequence detector (Gene-AmpPCR system 9600; PE Biosystems, Foster City, CA). The primers and fluorescence probes for mouse TRPV5, TRPV6, NCX1, PMCA1b, Calbindin-D_{9k}, Calbindin-D_{28k}, and hypoxanthine-guanine phosphoribosyl transferase and the PCR conditions have been described elsewhere (8,18). Standard curves were used to calculate the copy number of the target genes in each sample, expressed as a ratio to the hypoxanthine-guanine phosphoribosyl transferase gene.

Immunohistochemical Studies

Fixation of mouse kidneys and the subsequent immunohistochemistry were performed as described previously (19). Serial cryosections were taken and incubated at 4°C overnight with previously described guinea pig/anti-rabbit TRPV5 diluted 1:400 (20), rabbit/anti-rat TK diluted 1:10,000 (as described [13]). Binding sites of the primary antibodies were detected using Cy3-conjugated goat/anti–guinea pig and goat/anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. To determine the nonspecific antibody binding, the primary antibodies were omitted or replaced by nonimmune guinea pig and rabbit sera, respectively. Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung, Vienna, Austria), and digital images were acquired with a charged coupled device camera.

Statistical Analyses

Values are expressed as mean ± SEM. Comparisons were made by using t test for unpaired values or the Mann-Whitney test, as appropriate, and ANOVA for multiple groups (Statview 5.0 statistical software; SAS, Cary, NC). $P < 0.05$ was considered to be statistically significant.

Results

Immunolocalization of Kallikrein and the Epithelial Ca Channel (TRPV5) in the Distal Nephron

As described previously (19,21), TRPV5 and TK were detectable by immunohistochemistry in tubular profiles of the late distal convoluted tubule (DCT) and the connecting tubule (CNT) in the kidneys of WT mice. No TK was detectable in the tubular profiles of the TK−/− mice. As shown in Figure 1, the distribution of TRPV5 and TK overlapped to a considerable extent, although the two distribution patterns were not identical. TRPV5 immunostaining was bright in late DCT profiles and more faint in CNT profiles. The subcellular localization of TRPV5 shifted progressively from a predominantly apical localization in late DCT to a predominantly intracellular localization in the late portions of the CNT. In contrast, TK immunostaining was faint in late DCT but strong in early CNT. The immunofluorescent signal for TK diminished along the CNT and the late, TRPV5-positive, CNT segments were consistently not stained by the TK antibody (Figure 1).

Renal Ca Handling in TK-Deficient Mice

The data for the female TK−/− mice and their WT littermates are shown in Table 1 and Figure 2 for 129Sv mice and Table 2 and Figure 2 for C57Bl/6 mice. Body weight, food intake, urinary volume, creatinine, and Na excretions did not differ between the WT and TK−/− mice on either diet in both strains. Differences between 129Sv and C57bl/6 mice were observed for food intake and the excretion of urinary ions. The plasma Ca concentration was the same in C57Bl/6 and 129Sv mice, but urinary Ca excretion was lower in C57Bl/6 mice. Taken together, these data suggest important interstrain differences in intestinal Ca absorption. On the NCa diet, UCa excretion was significantly higher in the TK−/− than the female WT mice in both strains. In 129Sv mice, the difference was also present when UCa was normalized to creatinine or Na excretion to take into account possible interanimal differences in urine sampling and/or food intake. In C57Bl/6 mice, when normalized to UCr excretion, Ca excretion also tended to be higher ($P = 0.069$) in TK−/− mice than in WT mice. Plasma Ca, creatinine, and protein concentrations did not differ between TK−/− and WT mice. Plasma 1,25-dihydroxyvitamin D and PTH levels and cAMP and DPyr excretions were also the same in both genotypes. Plasma phosphate concentration and urinary phosphate excretion, measured in C57Bl/6 mice, did not differ between genotypes.

On the LCa diet, UCa excretion was lower than on the NCa diet (Table 1 and Figure 2). In both strains, the UCa/UCr ratio remained higher in female TK−/− mice than in WT mice. This difference persisted when UCa excretion was expressed as a ratio to UNa excretion. Unlike the WT mice, which remained normocalcemic, the female 129Sv TK−/− mice exhibited significant hypocalcemia. Plasma Ca concentration decreased by approximately 0.35 mmol/L ($P < 0.05$; Table 1 and Figure 2). However, in C57Bl/6 mice, plasma Ca concentration remained normal in TK−/− mice. In both strains, plasma PTH concentration tended to be higher on the LCa diet in both WT and TK−/− mice, although the effect remained below the threshold of statistical significance. However, in both genotypes, urinary cAMP excretion was higher on the LCa diet than on the NCa diet (Tables 1 and 2). There was no difference between genotypes for PTH levels or urinary cAMP excretions. Urinary DPyr excretion was also higher on the LCa diet than on the NCa diet in both WT and TK−/− mice. Finally, in the WT 129Sv and C57Bl/6 mice, plasma 1,25-dihydroxyvitamin D concentration was higher on the LCa diet than on the NCa diet. This increase was also observed in the C57Bl/6 TK−/− mice but not in 129Sv TK−/− mice.

Male C57Bl/6 TK−/− and WT mice were also studied, as presented in Table 3. Compared with WT littermates, TK−/− mice exhibited significant hypercalciuria. Plasma Ca, protein, creatinine, PTH, and phosphate concentrations did not differ between genotypes, whereas 1,25 dihydroxyvitamin D levels were significantly higher in TK−/− mice.

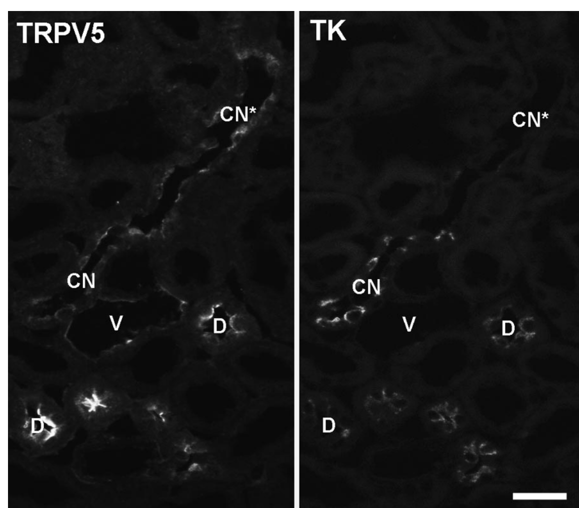

Figure 1. Distribution of TRPV5 and tissue kallikrein (TK) in the kidney of a wild-type (WT) mouse. Consecutive cryosections immunostained with an affinity-purified guinea pig anti-rabbit TRPV5 antibody and a rabbit anti-rat TK antiserum, followed by Cy3-conjugated goat/anti–guinea pig and goat/anti-rabbit IgG, respectively. TRPV5 and TK immunostaining are co-localized in late distal convoluted tubules (D) and early and middle portions of connecting tubules (CN). While TK immunostaining has vanished, TRPV5 is also detectable in the late portion of the connecting tubule (CN*); weak immunostaining in cortical radial veins (V) is due to unspecific binding of goat/anti–guinea pig IgG to endothelial cells. Bar = approximately 50 μm.

Effect of B2 Deficiency and B1 Antagonism on Renal Ca Handling

The data for the C57Bl/6 B2-deficient mice and their WT littermates that were studied on the NCa diet are shown in Table 4. UCa excretion, plasma Ca, and 1,25-dihydroxy-vitamin D concentrations were the same for both genotypes.

In a separate set of experiments, B2−/− mice and their WT littermates were studied on the LCa diet. After 14 d of Ca restriction, UCa excretion was the same in B2−/− and WT mice (0.93 ± 0.19 *versus* 1.06 ± 0.27 μmol/24 h, WT and B2−/−, respectively). Similarly, no difference in plasma Ca

Table 1. Phenotypic characterization of female 129Sv TK−/− and WT littermates on NCa diet (0.9% wt/wt) or on LCa diet (0.01% wt/wt Ca)[a]

	0.9% Wt/Wt Ca		0.01% Wt/Wt Ca	
	WT (*n* = 19)	TK−/− (*n* = 20)	WT (*n* = 11)	TK−/− (*n* = 11)
Weight (g)	24.7 ± 0.9	23.9 ± 1	21.7 ± 0.6	21.6 ± 0.6
Food intake (g/24 h)	1.92 ± 0.36	1.89 ± 0.35	1.25 ± 0.35	1.07 ± 0.33
Urine values				
volume (ml/24 h)	1.10 ± 0.14	1.25 ± 0.14	0.90 ± 0.11	1.10 ± 0.11
creatinine excretion (μmol/24 h)	4.48 ± 0.33	4.91 ± 0.32	3.87 ± 0.22	4.00 ± 0.21
Ca excretion (μmol/24 h)	4.72 ± 0.60	6.85 ± 0.58[b]	1.00 ± 0.18	1.49 ± 0.17
UCa/UCr (mmol/mmol)	1.09 ± 0.11	1.51 ± 0.18[b]	0.25 ± 0.04	0.38 ± 0.04[b]
UNa/UCr (mmol/mmol)	36.2 ± 2	30.8 ± 1.9	37.5 ± 3.3	31.4 ± 3.2
UCa/UNa (μmol/mmol)	33.2 ± 7.6	56.6 ± 7.5[b]	7.1 ± 1.1	12.2 ± 1.1[b]
UMg/UCr (mmol/mmol)	2.83 ± 0.18	3.05 ± 0.18	4.34 ± 0.42	3.78 ± 0.40
UDPyr/UCr (nmol/mmol)	9.86 ± 0.68	9.99 ± 2.03	15.49 ± 0.63[c]	13.64 ± 0.60[c]
UcAMP/UCr (μmol/mmol)	6.16 ± 0.32	5.62 ± 1.02	8.89 ± 0.66[c]	8.69 ± 0.44[c]
Plasma values				
total Ca (mM)	1.92 ± 0.07	1.87 ± 0.1	1.85 ± 0.1	1.50 ± 0.06[b]
magnesium (mM)	0.83 ± 0.02	0.80 ± 0.02	0.88 ± 0.02	0.86 ± 0.02
protein (g/L)	45.3 ± 1.9	45.6 ± 2.8	45.2 ± 0.9	43.8 ± 1.8
creatinine (μM)	9.7 ± 2.0	9.8 ± 1.6	9.3 ± 1.2	8.5 ± 1.5
PTH (pg/ml)	6.8 ± 2.2	12.5 ± 4.2	26.7 ± 16.3	29.9 ± 14.8
1,25-dihydroxyvitamin D (pmol/L)	64 ± 12	76 ± 15	113 ± 13	72 ± 8[b]

[a]TK−/−, tissue kallikrein–deficient; WT, wild-type; UCa, urinary calcium; UCr, urinary creatinine; UNa, urinary sodium; UMg, urinary magnesium; UcAMP, urinary cAMP; PTH, parathyroid hormone.
[b]$P < 0.05$ *versus* WT on same diet.
[c]$P < 0.05$ *versus* NCa diet.

concentration was observed (2.05 ± 0.11 *versus* 2.03 ± 0.11 mM, WT and B2−/−, respectively). These data indicate that the B2−/− mice were able to adapt normally to the LCa diet.

A subset of B2−/− mice were treated with the B1 antagonist SSR240612 while on the NCa diet. B1-antagonist treatment of the B2−/− mice had no effect on their UCa excretion or other parameters (Table 4).

Kallikrein Gene Expression in the Kidney

As shown in Figure 3, in 129Sv WT mice, renal TK mRNA levels were twice as high on the LCa diet than on the NCa diet ($P < 0.001$). As expected, no kallikrein mRNA was detected in TK−/− mice.

Gene Expression of Ca Transporters in the Distal Nephron

To investigate the effect of the absence of TK on the expression of several renal distal Ca transporters genes, we performed quantitative real-time PCR on TRPV5, TRPV6, NCX1, PMCA1b, Calbindin-D_{9k}, and Calbindin-D_{28k} mRNA using total kidney RNA from 129Sv TK−/− and WT mice that were subjected to either the NCa or the LCa diet (Table 5). In both genotypes, Calbindin-D_{9k} gene expression was significantly higher on the LCa diet than on the NCa diet. No effect of the diet was observed for TRPV5 gene expression. Calbindin-D_{28k} mRNA was downregulated on the LCa diet. None of these Ca transporter mRNA levels differed in TK−/− and WT mice. Similar results were observed in C57Bl/6 WT and TK−/− mice on the NCa diet (data not shown).

Discussion

The intrarenal factors that are involved in the control of tubular Ca reabsorption are largely unknown. Here we report data obtained with genetically deficient mice, suggesting that renal kallikrein is involved in the control of tubular Ca reabsorption.

The genetic background of the mice can strongly influence the phenotype linked to gene-inactivating mutations by means of counterregulation processes (22). We observed this gene-interaction effect in the kallikrein-deficient mice for cardiac morphology and function (13,14). Our study documents differences in UCa excretion between the C57Bl/6 and 129Sv mouse strains. Despite their higher food intake, the C57Bl/6 mice had lower UCa excretion, suggesting lower intestinal Ca absorption. Similarly, the change in plasma Ca concentration that occurs in 129Sv TK−/− mice on a LCa diet is not observed in C57Bl/6 TK−/−. However, despite these interstrain differences in Ca metabolism, the effect of kallikrein gene inactivation on UCa excretion was reproducible in both strains. This suggests that kallikrein may play an important role in the renal Ca handling.

While on a 0.9% wt/wt Ca diet, both male and female TK−/− mice exhibited significant hypercalciuria. 129Sv and C57Bl/6 TK−/− mice excreted approximately 50% more UCa

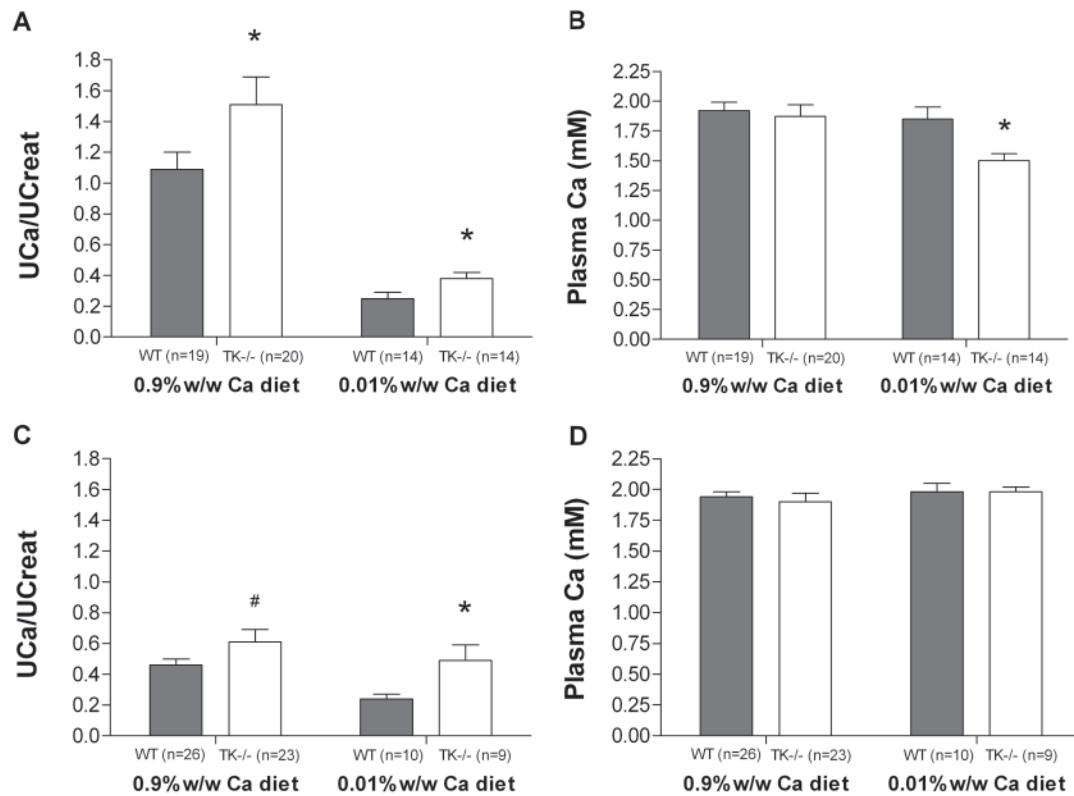

Figure 2. Urinary Ca excretion (129Sv mice [A]; C57Bl/6 mice [C]) and total plasma Ca concentration (129Sv mice [B]; C57Bl/6 mice [D]) of WT (▩) and TK−/− (□) mice that were fed the normal Ca (NCa) diet (0.9% wt/wt Ca) or the low Ca (LCa) diet (0.01 wt/wt %). $^*P < 0.05$ *versus* WT mice on the same diet; $^\#P = 0.069$ *versus* WT on the same diet.

than did 129Sv and C57Bl/6 WT mice, respectively, whereas for both strains, UCa excretion was within the ranges previously reported for WT mice (23,24). Hypercalciuria in TK−/− mice is observed in the absence of any difference in plasma Ca or creatinine concentration, suggesting that the Ca filtered loads are similar in WT and TK−/− mice.

At steady state, an increase in UCa excretion can be the consequence of three distinct primary disorders: An increase in intestinal Ca absorption, an increase in net bone resorption, and, finally, a decrease in renal tubular Ca reabsorption. As stated by Bushinsky *et al.* (25), each condition is responsible for a recognizable phenotype. A convenient way to distinguish between these disorders is to challenge the animals with a very low Ca diet for several days. Hypercalciuria that depends on increased intestinal Ca absorption can be expected to disappear under these conditions. However, when the primary mechanism is a decreased ability of the renal tubule to reabsorb the appropriate amount of filtered Ca, the renal loss of Ca can be expected to induce either persistent hypercalciuria, when the loss of Ca can be compensated for by an increase in bone Ca release, or a significant decrease in blood Ca concentration. For example, genetically hypercalciuric rats, which have defective tubular Ca reabsorption (26), display higher UCa excretion than normal rats when fed an NCa diet. Moreover, on a LCa diet, the blood Ca concentration in these animals decreases significantly and UCa excretion decreases but remains higher than that in normal rats that are fed the same diet (27). The difference in UCa excretion observed under these conditions is assumed to correspond to a higher bone Ca release in hypercalciuric rats. Indeed, when hypercalciuric rats that are fed a LCa diet are treated with alendronate, a potent bone resorption inhibitor, their UCa excretion falls to the same level as in the normal rats (25).

In our experiments, as expected, UCa excretion was lower in both TK−/− and WT mice on the LCa than on the NCa diet. At the new steady state, however, UCa excretion remained higher in TK−/− than in WT mice; urinary DPyr excretion similarly increased in TK−/− and WT mice, indicating an appropriate increase in bone resorption on the LCa diet, in both genotypes. This increase in bone resorption and Ca release is sufficient to maintain a normal plasma concentration of Ca in WT mice, which have renal tubules with a normal ability to reabsorb Ca. It is also sufficient to maintain a normal plasma Ca value in C57Bl/6 TK−/− mice but not in 129Sv TK−/− mice. Some striking interstrain differences in the response of calciotropic hormones can be observed: Plasma PTH and 1,25 dihydroxyvitamin D concentrations appropriately increase in C57Bl/6 TK−/− mice but not in 129Sv TK−/− mice. Indeed in 129Sv TK−/−, the LCa diet induces a similar increase in PTH concentration as in WT mice despite the occurrence of hypocalcemia in TK−/− mice. In addition, no change in 1,25 dihydroxyvitamin D is observed in 129Sv TK−/− mice on a LCa diet. Conversely, in C57Bl/6 mice, plasma PTH and 1,25 dihydroxyvitamin D concentrations both increase to the same extent

Table 2. Phenotypic characterization of female C57Bl6/J TK−/− and WT littermates on NCa diet (0.9% wt/wt Ca) or on LCa diet (0.01% wt/wt Ca)[a]

	0.9% wt/wt Ca		0.01% wt/wt Ca	
	WT (n = 26)	TK−/− (n = 23)	WT (n = 10)	TK−/− (n = 9)
Weight (g)	21.9 ± 0.4	22.1 ± 0.4	20.3 ± 0.4	21.9 ± 0.5[b]
Food intake (g/24 h)	3.77 ± 0.22	4.11 ± 0.12	3.48 ± 0.15	4.33 ± 0.30[b]
Urine values				
volume (ml/24 h)	1.04 ± 0.09	1.52 ± 0.26	0.82 ± 0.15	1.20 ± 0.28
creatinine excretion (μmol/24 h)	2.89 ± 0.19	3.39 ± 0.29	2.87 ± 0.37	3.24 ± 0.34
Ca excretion (μmol/24 h)	1.36 ± 0.17	2.16 ± 0.35[b]	0.75 ± 0.15	1.41 ± 0.25[b]
UCa/UCr (mmol/mmol)	0.46 ± 0.04	0.61 ± 0.08[c]	0.24 ± 0.03	0.49 ± 0.1[b]
Na excretion (μmol/24 h)	231 ± 14	275 ± 22	230 ± 33	252 ± 34
UNa/UCr (mmol/mmol)	84.2 ± 4.4	83.5 ± 4.7	78.9 ± 3.4	77.2 ± 5.1
UCa/UNa (μmol/mmol)	6.2 ± 0.9	8.1 ± 1.1	3.1 ± 0.3	6.6 ± 1.4[b]
UPi/UCr (mmol/mmol)	61.7 ± 3.5	66.7 ± 5.9	80.6 ± 4.9	82.9 ± 7.0
UcAMP/UCr (μmol/mmol)	5.13 ± 0.43	5.40 ± 0.28	6.21 ± 0.36[d]	7.28 ± 0.74[d]
UDPyr/UCr (nmol/mmol)	14.87 ± 1.09	14.94 ± 0.59	19.18 ± 0.80[d]	19.62 ± 1.28[d]
Plasma values				
total Ca (mM)	1.94 ± 0.04	1.90 ± 0.07	1.98 ± 0.07	1.98 ± 0.04
1,25-dihydroxy vitamin D (pmol/L)	210 ± 27	262 ± 38	563 ± 65	517 ± 68
protein (g/L)	37.0 ± 0.6	35.6 ± 0.6	36.1 ± 0.5	35.8 ± 0.6
creatinine (μM)	14.4 ± 1.2	15.5 ± 1.5	9.9 ± 0.9	11.8 ± 1.7
PTH (pg/ml)	26.0 ± 7.4	13.8 ± 1.4	35.6 ± 1.4	31.2 ± 11.7
Pi (mM)	3.29 ± 0.28	3.29 ± 0.11	3.44 ± 0.12	3.02 ± 0.13[b]

[a]Pi, inorganic phosphate.
[b]$P < 0.05$ *versus* WT.
[c]$P = 0.069$ *versus* WT.
[d]$P < 0.01$ *versus* NCa diet.

Table 3. Phenotypic characterization of male C57Bl6/J TK−/− and WT littermates on NCa diet (0.9% wt/wt Ca)

	0.9% wt/wt Ca	
	WT (n = 15)	TK−/− (n = 14)
Weight (g)	24.9 ± 0.4	24.0 ± 0.5
Food intake (g/24 h)	4.16 ± 0.15	4.18 ± 0.25
Urine values		
volume (ml/24 h)	1.27 ± 0.17	1.71 ± 0.28
creatinine excretion (μmol/24 h)	4.08 ± 0.18	4.60 ± 0.29
Ca excretion (μmol/24 h)	1.56 ± 0.16	2.54 ± 0.44[a]
UCa/UCr (mmol/mmol)	0.38 ± 0.03	0.52 ± 0.08
Na excretion (μmol/24 h)	322 ± 19	311 ± 26
UNa/UCr (mmol/mmol)	78.9 ± 2.7	67.3 ± 3.2[a]
UCa/UNa (μmol/mmol)	5.0 ± 0.4	8.7 ± 1.5[a]
UPi/UCr (mmol/mmol)	49.9 ± 2.3	44.1 ± 2.3
UcAMP/UCr (μmol/mmol)	7.18 ± 0.35	6.78 ± 0.45
UDPyr/UCr (nmol/mmol)	13.41 ± 1.03	11.63 ± 0.58
Plasma values		
total Ca (mM)	2.20 ± 0.08	2.03 ± 0.1
protein (g/L)	36.7 ± 0.6	36.2 ± 0.7
creatinine (μM)	16.6 ± 1.4	13.5 ± 1.3
1,25-dihydroxy vitamin D (pmol/L)	120 ± 12	203 ± 25[b]
PTH (pg/ml)	15.1 ± 1.4	18.3 ± 6.1
Pi (mM)	3.06 ± 0.18	2.99 ± 0.08

[a]$P < 0.05$ *versus* WT.
[b]$P < 0.01$ *versus* WT.

in TK−/− and WT mice. These significant interstrain differences in the response to LCa diet may have consequences for the ability to maintain normal plasma Ca concentration in the face of impaired renal calcium reabsorption.

Nevertheless, irrespective of the strain, hypercalciuria persists on an LCa diet in TK−/− mice. As the filtered load of Ca is identical in WT and TK−/− mice in the C57Bl/6 strain and even lower in TK−/− than in WT mice in the 129Sv strain, these data demonstrate that TK−/− mice have a default in RTCa absorption. On the NCa diet, an excessive Ca absorption from the gut may contribute to the hypercalciuria in the TK−/− mice because bone resorption does not seem to be increased, as compared with WT mice, as attested by DPyr measurements. In the male TK−/− mice, 1,25-dihydroxyvitamin D concentration is higher than in male WT mice. This difference in 1,25-dihydroxyvitamin D concentration is likely an adaptive consequence of the renal leak of Ca, allowing a higher intestinal Ca absorption to occur to match the renal loss. In the female TK−/− mice, we did not observed a significant increase in 1,25-dihydroxyvitamin D concentration in the TK−/− mice, but a trend toward higher values was noted. Presently, we do not have an explanation for this difference between genders.

A decrease in RTCa reabsorption can be induced by several physiologic situations, such as volemic expansion or metabolic

Table 4. Phenotypic characterization of C57Bl/6 B2−/− and WT littermates and B2−/− mice that were treated with a B1 receptor antagonist on NCa diet (0.9% wt/wt Ca)

	0.9% wt/wt Ca		
	WT (n = 10)	B2−/− (n = 10)	B2−/− (n = 12)
Treatment	Vehicle	Vehicle	B1 antagonist SSR240612
Weight (g)	22.1 ± 0.5	20.9 ± 0.6	23.0 ± 0.5
Food (g/24 h)	2.99 ± 0.23	3.44 ± 0.31	3.73 ± 0.25
Urine values			
volume (ml/24 h)	0.94 ± 0.17	0.90 ± 0.18	1.26 ± 0.13
creatinine excretion (μmol/24 h)	2.68 ± 0.27	2.51 ± 0.27	3.20 ± 0.24
Ca excretion (μmol/24 h)	1.46 ± 0.39	1.48 ± 0.37	1.75 ± 0.35
UCa/UCr (mmol/mmol)	0.50 ± 0.08	0.53 ± 0.11	0.53 ± 0.08
UNa/UCr (mmol/mmol)	72.0 ± 8.9	86.8 ± 8.7	92.8 ± 5.3
UCa/UNa (μmol/mmol)	7.21 ± 0.93	6.63 ± 0.80	7.12 ± 0.50
Plasma values			
total plasma Ca (mM)	1.82 ± 0.04	1.78 ± 0.05	1.86 ± 0.04
1,25-dihydroxyvitamin D (pmol/L)	217 ± 33	265 ± 41	281 ± 31

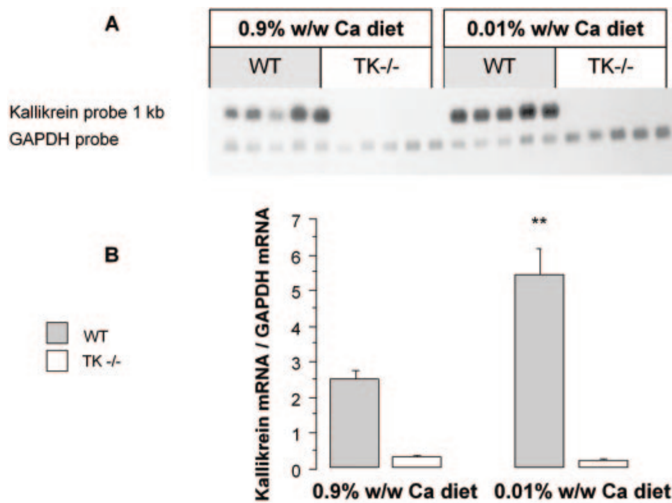

Figure 3. Effect of a LCa diet on TK gene expression in the kidney. (A) Representative Northern blot using a TK probe on whole-kidney mRNA of TK−/− and WT mice that were subjected to NCa or LCa diet. (B) Summary (mean ± SEM) of sample analysis for TK gene expression in WT and TK−/− mice on a NCa and a LCa diet (n = 14 per group). For each sample, kallikrein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA abundance was quantified by densitometric analysis. The ratio (Kallikrein mRNA/GAPDH mRNA) was calculated. ** $P < 0.01$ *versus* NCa diet.

acidosis (28). Moreover, the UCa:UNa ratio was higher in TK−/− than in WT mice, suggesting that the defect in renal tubular Ca absorption occurs independent of any change in tubular Na transport. Similarly, under baseline conditions, TK−/− mice have similar plasma PTH concentration as WT mice and display similar tubular responsiveness to PTH, as shown by their urinary cAMP excretion levels. Finally, the lower tubular Ca reabsorption in the TK−/− mice could not be ascribed to higher plasma Ca or Mg concentration. In addition, in independent experiments, plasma total CO_2 concentration was measured in both C57Bl/6 WT and TK−/− mice and found to be the same, ruling out the possibility that TK−/− mice could have metabolic acidosis (unpublished results). Therefore, in the absence of any known cause of reduced renal tubular Ca reabsorption, the defect in tubular Ca transport is probably related to the loss of a direct TK effect on this transport. This hypothesis that TK may play a physiologic role in the control of intrarenal Ca transport is strengthened by the fact that the renal expression of the TK gene markedly increases under LCa diet. However, the mechanism of this increased expression remains unknown.

Whereas the kallikrein-deficient mice exhibited hypercalciuria and displayed impaired adaptation to a LCa diet, no such abnormalities were observed in the B2−/− mice. The B2 receptor is usually considered to be the only kinin receptor constitutively synthesized in organs, including the kidney (29). However in B2−/− mice, the B1 receptor is induced and can take over functions that are normally performed by the B2 receptor, such as vasodilation and cardioprotection, and that can be suppressed in these mice by treatment with a B1 antagonist (30). The B2−/− mice therefore were treated with a B1 antagonist, but this combined genetic and pharmacologic blockade of kinin action at its two receptors did not affect renal Ca excretion. Taken together, these data suggest that the effect of kallikrein on Ca excretion is independent of kinin production.

The data available do not allow us to determine with certainty which tubular segment contains the defect in Ca transport in TK−/−. However, because hypercalciuria occurs in the absence of any difference in Na intake or, presumably, in ECF volume, the involvement of the proximal tubule is unlikely. Similarly, a defect in Ca transport located in the thick ascending limb of Henle would be expected to be associated with a defect in Mg reabsorption. Because renal tubular magnesium han-

Table 5. Effect of Ca intake on the mRNA expression of several Ca^{2+} transport proteins in the kidney of 129Sv TK−/− and WT mice[a]

	0.9% wt/wt Ca		0.01% wt/wt Ca	
	WT	TK −/−	WT	TK −/−
TRPV5 ($\times 10^3$)	71 ± 10	68 ± 7	62 ± 7	86 ± 9
Calbindin-D_{28k}	3.17 ± 0.22	3.08 ± 0.30	2.62 ± 0.28	2.36 ± 0.15
Calbindin-D_{9k}	13.20 ± 2.05	11.58 ± 1.41	21.65 ± 2.52[b]	20.58 ± 1.90[c]
TRPV6 ($\times 10^3$)	16.9 ± 1.3	13.4 ± 1.0	15.1 ± 0.9	16.7 ± 1.6
NCX1 ($\times 10^3$)	3.7 ± 0.3	3.8 ± 0.3	4.8 ± 0.8	3.6 ± 0.2
PMCA1b ($\times 10^6$)	60 ± 4	50 ± 3	55 ± 1	48 ± 2

[a]Renal mRNA levels of TRPV5, Calbindin-D_{28k}, and Calbindin-D_{9k} genes are assessed by quantitative real-time PCR and expressed as a ratio of the copy number of the target gene and the copy number of hypoxanthine-guanine phosphoribosyl transferase gene ($n = 7$ per group).
[b]$P < 0.05$ *versus* NCa diet.
[c]$P < 0.01$ *versus* NCa diet.

dling seems to be similar in TK−/− and WT mice, we suggest that the renal tubular Ca transport in TK −/− mice is perturbed in the DCT and CNT, which are also sites of TK synthesis.

The molecular events that link kallikrein to renal Ca metabolism are unknown at the present time. We did not observe any change in the expression of TRPV5, TRPV6, NCX1, PMCA1b, Calbindin-D_{9k}, or Calbindin-D_{28k} genes in TK−/− mice. However, as kallikrein is synthesized in the same tubule segment as these Ca transporters, a direct impact on a Ca transporter can be postulated. However, this effect does not seem to be mediated by kinins, despite the presence of kininogen in the distal nephron (31). A proteolytic effect on TRPV5 or some other unknown protein involved in TRPV5 regulation can be suggested but remains to be confirmed. Regulation of ion transport by limited proteolysis of transporters, involving serine proteases other than kallikrein, has been documented for Na reabsorption and epithelial Na channel (32). This hypothesis would be consistent with the well-documented observation that TK has broad enzymatic specificity *in vitro* and can hydrolyze several protein substrates besides kininogen (33–35). This study in the mouse suggests that TK is a physiologic regulator of the renal tubular Ca reabsorption from the renal tubule.

A loss-of-function polymorphism of the kallikrein gene was described in humans (36) with a marked reduction in kallikrein activity as a consequence of an amino acid mutation (R53H). It will be interesting to find out whether this polymorphism is associated with any defect in renal Ca handling in humans.

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