Supporting Information

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SI Materials and Methods

Physiological Studies. All experiments were performed by using age- and sex-matched $TK^{+/+}$ and $TK^{-/-}$ littermates mice (3- to 5-mo-old). In all physiological studies, mice were pair-fed. Mice were given deionized water ad libitum and pair-fed with standard laboratory chow containing 0.3% of sodium (Institut National de la Recherche Agronomique) and were housed at constant room temperature (24 ± 1 °C) with 12-h light/dark cycle. For urine collection, mice were housed in metabolic cages (Techniplast). They were first allowed to adapt for 3–5 d to the cages. At steady state, urine collection was performed daily under mineral oil in the urine collector for electrolyte measurements. Urine creatinine (modified kinetic Jaffé colorimetric method) and phosphate were measured with a Konelab 20i auto-analyzer (Thermo Electron). Plasma creatinine concentration was measured by ion-exchange chromotography; urinary Na^+ and K^+ concentrations were measured by flame photometry (IL943; Instruments Laboratory). Urinary pH and bicarbonate were measured with a pH/blood-gas analyzer (ABL 725; Radiometer). Urinary NH_4^+ and titratable acid were measured by titration with a DL 55 titrator (Mettler Toledo).

For acid loading experiments, mice were given 0.28 M NH_4Cl in their drinking water for 2, or 6 d. It was verified every day that the volume of drinking water ingested was identical in all mice. Blood gases analyses were performed by retro-orbitary punction, and pH, PCO_2, PO_2, Na^+, and Cl^- were measured with an ABL 77 pH/blood-gas analyzer (Radiometer) before acid loading experiments (day 0) and after 2 or 6 d of ingestion of NH_4Cl. Blood bicarbonate concentration was calculated from the measured values by using the Henderson–Hasselbalch equation.

In an other set of experiments, designed to study the ability of mice to adapt to a high K^+ diet, $TK^{-/-}$ and $TK^{+/+}$ mice were equilibrated for few days on the standard diet and then switched to the 2% K^+-containing diet. Twenty-four-hour urine and blood were collected before the switch and on the first day of the high K^+ diet period for urinary aldosterone measurement by RIA (DPC Dade Behring). Blood collection by tail incision on anesthetized mice by peritoneal injection of a mixture (0.1 mL/g body weight) of ketamine (Imalgene; 10%) and xylazine (Rompun; Bayer; 5%) was performed for K^+ measurement with an ABL 77 pH/blood-gas analyzer (Radiometer).

The object of the next sets of experiments was to maximize feeding-dependent influences on plasma K^+ level (1), $TK^{-/-}$ and $TK^{+/+}$ mice were supplied with their daily ration, which they were allowed to eat over a 4-h period (between 10:00 AM and 2:00 PM). Blood samples were taken at 1 h before and at 4 h and 8 h after the beginning of the feeding period. Blood K^+ concentration was measured as described above. For convenience, the 12:12 light/dark cycle was inverted. Mice were allowed to adapt to a once-a-day feeding over a week.

In experiments designed to determine whether renal kallikrein synthesis and excretion in response to high K^+ diet depend on aldosterone, $AS^{-/-}$ and $AS^{+/+}$ mice were housed in metabolic cages and acclimatized for a few days on the standard diet and then switched to the 2% K^+-containing diet for an additional 2 d. Twenty-four-hour urine was collected the day of the switch and at day 1 and 2 of the high K^+ diet period. Urinary kallikrein activity was quantified by an enzymatic assay with the chromogenic tripeptide D-valyl-leucyl-arginine-p-nitroanilide (S 2266), as described (2). Briefly, hydrolysis of S 2266 was measured with a microplate reader by incubation of 0.02 mL of urine for 15 min at 37 °C with 0.01 mM S 2266 in 200 μL of 20 mM Tris·HCl buffer (pH 9.0) containing 1 mM EDTA. Results are expressed in the change in optical density units per min of incubation, for the total urine collected (ΔODU/min).

In Vitro Microperfusion of Mice CCDs. Kidneys were removed and cut into 1–2 mm coronal slices that were transferred into a chilled dissection medium containing the following: 118 mM NaCl, 25 mM $NaHCO_3$, 2.0 mM K_2HPO_4, 1.2 mM $MgSO_4$, 2.0 mM calcium lactate, 1.0 mM sodium citrate, 5.5 mM glucose, and 12 mM creatinine, pH 7.4, and gassed with 95% O_2–5% CO_2. CCD segments were isolated from cortico-medullary rays under a dissecting microscope with a sharpened forceps. Because CCDs are highly heterogeneous, relatively short segments (0.45–0.6 mm) were dissected to maximize the reproducibility of the isolation procedure. In vitro microperfusion was performed as described by Burg (3): Isolated CCDs were rapidly transferred to a 1.2-mL temperature- and environmentally controlled chamber, mounted on an inverted microscope, and then perfused and bathed initially at room temperature with dissection solution. The specimen chamber was continuously suffused with 95% O_2–5% CO_2 to maintain pH at 7.4. Once secure, the inner perfusion pipette was advanced, and the tubule was opened with a slight positive pressure. The opposite end of the tubule was then pulled into a holding collection pipette. In the holding collection pipette, 2–3 cm of water-saturated mineral oil contributed to maintain the tubule open at a low flow rate of perfusion. The perfusing and collecting end of the segment was sealed into a guard pipette by using Dow-Corning 200 dielectric fluid. The tubules were then warmed to 37 °C and equilibrated for 20 min while the collection rate was adjusted to a rate of 1–4 nL/min. The length of each segment was measured by using an eyepiece micrometer. Because CCDs from mice are frequently unstable and collapse rapidly, measurements were conducted during the first 90 min of perfusion. Usually, collections from 4 periods of 15 min were performed in which 25–30 nL of fluid were collected. The volume of the collections was determined under water-saturated mineral oil with calibrated volumetric pipettes. Twenty nanoliters were required for [Na^+], [K^+], and [creatinine] measurements. Transepithelial voltage (Vte) was measured continuously between Ag-AgCl electrodes connected to 0.15 M NaCl-agar bridges inserted in the perfusion pipette and bathing solutions. The initial value after rewarming of the tubule was noted. Values of each period were averaged.

Measurements of Na^+, K^+, and Creatinine Concentrations with HPLCy. Cation determination was performed as described (4). The Dionex-500 system (Dionex DX-500) consisted of an AS50 autosampler, a GP50 gradient pump, an ED40 electrochemical detector (Na^+ and K^+), and an AD20 UV absorbance detector 220 nm (creatinine). The signal-to-noise ratio of the conductivity measurement was enhanced by using a cation self-regenerating suppressor (CSRS-ultra, 4 mm) that was set in the autosuppression recycle mode. The HPLC column consisted in a Dionex IonPac CS12 column (4 × 250 mm), equipped with a guard column CG12A (4 × 50 mm). Mobile phase consisted in 18 mM methanesulfonic acid. Tubular fluid, perfusion solution, and standard solutions were sampled under mineral oil with a calibrated pipette (≈20 nL), and transferred in a vial containing 39 μL of mobile phase of the HPLC with $LiNO_3$ as internal standard. Peaks of each measured analyte (Na^+, K^+, creatinine) were adjusted with the value of the Li^+ internal standard, to limit the variations due to automatic injection. In each run of experiment, perfusion and bath solutions were tested in four or five replicates, and the reproducibility of the measure was evaluated: CV < 0.10 for K^+ determination, and CV < 0.05 for creatinine

determination. In addition, a calibration curve for each analyte was tested: correlation coefficients >0.98.

Measurement of Fluid Absorption. Creatinine was used as the volume marker and, therefore, was added to the perfusion solutions (both perfusate and bath) at a concentration of 12 mM. The rate of fluid absorption (*Jv*) was calculated as Jv = (Vperf – Vcoll)/L, with Vperf = Crcoll/Crperf × Vcoll.

Crcoll and Crperf are the concentrations of creatinine in the collected fluid and perfusate, respectively. Vcoll is the collection rate at the end of the tubule. L is the length of the tubule.

Calculation of the Rate of Absorption of Na and K. For each collection, Na^+ flux (JNa) and K^+ flux (JK) where calculated and reported to the length of the tubule:

$$JNa = [([Na]perf \times Vperf) - ([Na]coll \times Vcoll)]/L$$

$$JK = [([K]perf \times Vperf) - ([K]coll \times Vcoll)]/L$$

Therefore, positive values indicate net absorption, whereas negative values indicate net secretion of the ion. For each tubule, the mean of the four collection periods was used.

Intracellular pH Measurements on Isolated Microperfused Tubules. The isolated CCDs were transferred, one at a time, to the bath chamber on the stage of an inverted microscope (Axiovert 100; Carl Zeiss) in the control solution containing the following: 138 mM NaCl, 1.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM K_2HPO_4, 10 mM Hepes, 5.5 mM glucose, 5 mM alanine, (pH 7.4) and then, was mounted on concentric pipettes and perfused in vitro with Na^+-free, ammonium-free solution where *N*-methyl-D-Glutamine$^+$ ($NMDG^+$) replaced Na^+. All solutions were equilibrated with 100% O_2 passed through a 3 M KOH CO_2 trap. The average tubule length exposed to bath fluid was limited to 300–350 μm to prevent motion of the tubule.

Peanut lectin has been widely used as a marker for intercalated cells in rabbit. To identify PCs and ICs, we labeled intercalated cells by adding fluorescent peanut lectin (PNA; Vector Labs) to the luminal perfusate for 5 min and observed which cells were fluorescent. In our previous paper (5), we have shown that ENaC activity in mouse isolated CCD was exclusively detected in peanut lectin negative cells, indicating that PCs in mouse are peanut lectin negative like in rabbit. We also recently showed that the activity of pendrin, the apical Cl^-/HCO_3^- exchanger of B-intercalated cells, was strictly restricted to peanut lectin positive cells (4). However, we also observed that some of the peanut lectin-positive cells (≈50%) are devoid of apical Cl^-/HCO_3^- activity, suggesting that these cells might be A-IC. Accordingly, we observed that the pattern of peanut lectin-positive cells strictly overlapped with the cells exhibiting a much greater uptake of BCECF from the perfusion solution, which is a characteristic of both types of IC (6).

pHi in CCD cells was assessed with imaging-based, dual excitation-wavelength fluorescence microscopy by using the fluorescent probe 2',7'-bis-(2-Carboxyethyl)-5- (and-6)- carboxyfluorescein (BCECF; Molecular Probes). Tubules were loaded for ≈20 min at room temperature with 5×10^{-6} mol/L of the acetoxymethyl ester of BCECF. The loading solution was then washed out by the initiation of bath flow, and the tubule was equilibrated with dye-free, bath solution for 5–10 min. Bath solution was delivered at a rate of 20 mL/min and warmed to 37 ± 0.5 °C by water jacket immediately upstream to the chamber. After this temperature equilibration in control solution, tubules were first transiently acidified by peritubular Na^+ removal (sodium-free, ammonium-free solution) (10 min duration), isoosmotically replaced by $NMDG^+$ to avoid exit of NH_4^+ by basolateral Na^+-coupled transport. This maneuver was done in the absence of luminal Na^+. During the fluorescence recording, perfusion solution was delivered to the perfusion pipette via a chamber under an inert gas (N_2) pressure (≈1 bar) connected through a manual six-way valve. With this system, opening of the valve instantaneously activates flow of one solution. The majority of the fluid delivered to the pipette exits the rear of the pipette system through a drain port at 4 mL/min. This method results in a smooth and complete exchange of the luminal solution in <3–4 s as measured by the time necessary for appearance of colored dye at the perfusion pipette tip. After the fluorescence signal stabilization, luminal fluid at the rate of 4 mL/min in the draining, was instantaneously replaced by a Na^+-free solution containing 10 mM NH_4Cl (and 118 mM NMDG-Cl) for 2 min that elicited a rapid intracellular alkalinization, followed by a progressive acidification; then, luminal fluid was replaced by the sodium-free, ammonium-free solution that elicited a rapid intracellular acidification. After the nadir intracellular pH had been reached, the initial rate of intracellular alkalinization was measured. Fluorescence monitoring and calibration were performed with a video imaging system as described (7).

Intracellular dye was excited alternatively at 440 and 490 nm with a 100 W halogen lamp and a computer-controlled chopper assembly. Emitted light was collected through a dichroic mirror, passed through a 530-nm filter and focused onto a charge-coupled device camera (ICCD 2525F; Videoscope International) connected to a computer. The measured light intensities were digitized with 8-bit precision (256 gray level scale) for further analysis. For each tubule, four to five intercalated cells and four to five principal cells were analyzed; the mean gray level for each excitation wavelength was calculated with the Starwise Fluo software (Imstar).

Intracellular dye was calibrated at the end of each experiment by using the high [K^+]-nigericin technique. Tubules were perfused and bathed with a Hepes-buffered, 95 mM K^+ solution containing 10 μM of the K^+/H^+ exchanger nigericin. Four different calibration solutions, titrated to pH 6.5, 6.9, 7.2, or 7.5 were used.

1. Clarke LL, Ganjam VK, Fichtenbaum B, Hatfield D, Garner HE (1988) Effect of feeding on renin-angiotensin-aldosterone system of the horse. *Am J Physiol* 254:R524–R530.
2. Bönner G, Marin-Grez M (1981) Measurement of kallikrein activity in urine of rats and man using a chromogenic tripeptide substrate. Validation of the amidolytic assay by means of bradykinin radioimmunoassay. *J Clin Chem Clin Biochem* 19:165–168.
3. Burg M, Grantham J, Abramow M, Orloff J (1966) Preparation and study of fragments of single rabbit nephrons. *Am J Physiol* 210:1293–1298.
4. Leviel F, et al. (2010) The Na+-dependent chloride-bicarbonate exchanger SLC4A8 mediates an electroneutral Na+ reabsorption process in the renal cortical collecting ducts of mice. *J Clin Invest* 120:1627–1635.
5. Picard N, et al. (2008) Defective ENaC processing and function in tissue kallikrein-deficient mice. *J Biol Chem* 283:4602–4611.
6. Amlal H, et al. (2010) Deletion of the anion exchanger Slc26a4 (pendrin) decreases the apical Cl-/Hco3- exchanger activity and impairs bicarbonate secretion in the kidney collecting duct. *Am J Physiol Cell Physiol* 299:C33–C41.
7. Chambrey R, et al. (2005) Genetic ablation of Rhbg in the mouse does not impair renal ammonium excretion. *Am J Physiol Renal Physiol* 289:F1281–F1290.

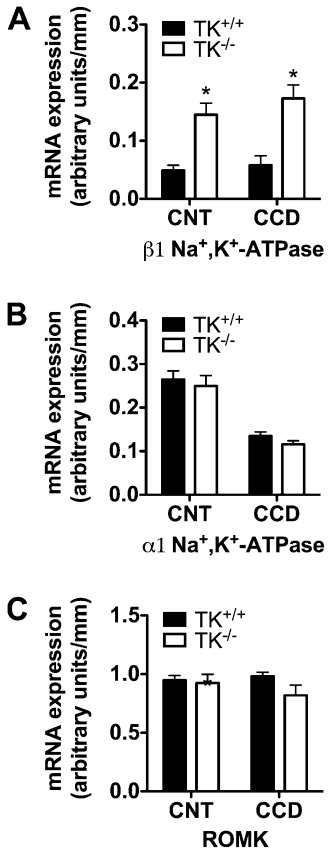

Fig. S1. Expression of the β1 (*A*) and α1 (*B*) subunits of the Na^+,K^+-ATPase and ROMK (*C*) in CNTs and CCDs from $TK^{+/+}$ (filled bar) and $TK^{-/-}$ mice (open bar). Data are means ± SE from four to six mice. Statistical significance was assessed by ANOVA followed by Bonferonni's post hoc test: *$P < 0.01$ vs. $TK^{+/+}$.

Table S1. Urinary Na^+ and K^+ excretion in $TK^{+/+}$ and $TK^{-/-}$ mice before and after 24 h administration of a high K^+ diet (2% K^+)

	Before		After	
	$TK^{+/+}$ (8)	$TK^{-/-}$ (6)	$TK^{+/+}$ (8)	$TK^{-/-}$ (6)
Urinary Na^+, nmol/min	170 ± 20	175 ± 26	129 ± 19	130 ± 21
Urinary K^+, nmol/min	123 ± 11	134 ± 26	968 ± 133*	1,103 ± 185*

Values are means ± SE (*n*), number of mice studied. Statistical significance is assessed by one-way ANOVA followed by Bonferroni's post hoc test when appropriate. *$P < 0.05$ vs. same genotype before challenge.

Table S2. Specific primers used for RT-PCR

Genes	Forward primers	Reverse primers
HKATPase (α2 subunit)	TTGGAAACTAAGAACATAGGCTTCTATT	AATGGCTATGGGTGTCTTCTCA
HKATPase (α1 subunit)	TTCGATCAGTCGTCGG	CTTTGGGGAAGCGGTC
NaKATPase (α1 subunit)	CCCAAAACGGACAAACT	GCACTACCACAATACTGAC
NaKATPase (β1 subunit)	GTAAGGCGTATGGTGAGA	ACTACAAGTCAGTTACGG
R	TGGGGTTACCGTTTTGT	CATTTGGGTGTCGTCT
Cyclophilin	ATGGCAAATGCTGGACCAAA	GCCTTCTTTCACCTTCCCAAA