Impaired Renal NaCl Absorption in Mice Lacking the ROMK Potassium Channel, a Model for Type II Bartter’s Syndrome*

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ROMK is an apical K+ channel expressed in the thick ascending limb of Henle (TALH) and throughout the distal nephron of the kidney. Null mutations in the ROMK gene cause type II Bartter’s syndrome, in which abnormalities of electrolyte, acid-base, and fluid-volume homeostasis occur because of defective NaCl reabsorption in the TALH. To understand better the pathogenesis of type II Bartter’s syndrome, we developed a mouse lacking ROMK and examined its phenotype. Young null mutants had hydrenephrosis, were severely dehydrated, and ~95% died before 3 weeks of age. ROMK-deficient mice that survived beyond weaning grew to adulthood; however, they had metabolic acidosis, elevated blood concentrations of Na+, and Cl−, reduced blood pressure, polydipsia, polyuria, and poor urinary concentrating ability. Whole kidney glomerular filtration rate was sharply reduced, apparently as a result of hydrenephrosis, and fractional excretion of electrolytes was elevated. Micropuncture analysis revealed that the single nephron glomerular filtration rate was relatively normal, absorption of NaCl in the TALH was reduced but not eliminated, and tubuloglomerular feedback was severely impaired. These data show that the loss of ROMK in the mouse causes perturbations of electrolyte, acid-base, and fluid-volume homeostasis, reduced absorption of NaCl in the TALH, and impaired tubuloglomerular feedback.

Barter’s syndrome, a hypokalemic alkalosis with dehydration, hypotension, and severe polyuria which develops before birth or during infancy (1), is caused by null mutations in any of four genes encoding proteins involved in NaCl absorption in the renal thick ascending limb of Henle (TALH). These are the NKCC2 Na+/K+2Cl⁻ cotransporter (2), the ROMK potassium channel (3), the CLC-KB chloride channel (4), and barttin (5), a β-subunit of the chloride channel (6). Na+ and Cl−, in a 1:2 ratio, are absorbed across the apical membrane of TALH cells by the coupled activities of NKCC2 and ROMK and extruded via the basolateral Na⁺,K⁺-ATPase and chloride channel (6, 7); additional Na+ is absorbed via the paracellular pathway. Although NKCC2 directly mediates uptake of Na+, K+, and Cl−, the activity of ROMK is critical because the K+ concentration in the luminal fluid is much lower than that of Na+ and Cl−. Thus, the continuous electroneutral uptake of Na+, K+, and Cl− requires that K+ be recycled to the lumen of the tubule. Apical K+ secretion via ROMK replenishes luminal K+ and also contributes, in concert with basolateral Cl− efflux via CLC-KB/barttin (5, 6), to the transcellular electrical potential that is the driving force for Na+ absorption via the paracellular pathway (8).

The different types of Bartter’s syndrome, caused by null mutations in NKCC2, ROMK, CLC-KB, and barttin, are referred to as types I–IV, respectively. The syndrome is thus heterogeneous, consistent with the variety in genetic mechanisms, and the physiological phenotypes overlap to some degree with those of Gitelman’s syndrome, a milder hypokalemic alkalosis caused by null mutations in the thiazide-sensitive NaCl cotransporter of the distal convoluted tubule (9, 10). Detailed analysis of the physiological functions and relative importance of the transporters involved in each type of Bartter’s syndrome would be facilitated by the development of knockout mouse models. A mouse model for Bartter’s syndrome type I, involving NKCC2, has already been developed (11); null mutants exhibit severe hydrenephrosis, dehydration, polydipsia, polyuria, and an inability to concentrate the urine, and they usually die before weaning.

There are multiple N-terminal variants of ROMK (gene locus Kcnj1) (12–14). One or more of these variants is expressed in the TALH, distal convoluted tubule, connecting tubule, collecting duct, and macula densa (15, 16), consistent with functions in K+ recycling to facilitate Na+ reabsorption in the TALH, K+ secretion in the distal nephron, and tubuloglomerular feedback (17, 18). The broad distribution of ROMK in the renal nephron, in contrast to the restricted distribution of NKCC2, and the possibility that other apical K+ channels (17) or K+-independent modes of NaCl transport for NKCC2 (19) might provide some compensation for its absence, suggested that the loss of ROMK might lead to a different phenotype than that of the Nkcc2 knockout.

In this study we have developed a mouse model for Bartter’s syndrome type II, involving ROMK. ROMK-deficient mice have hydrenephrosis, polydipsia, polyuria, extracellular fluid vol-

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The abbreviations used are: TALH, thick ascending limb of Henle; NKCC2, isoform 2 of the Na+/H+2Cl⁻ cotransporter; SNGFR, single nephron glomerular filtration rate; TGF, tubuloglomerular feedback. NKCC2 and Nkcc2 refer to human and mouse genes, respectively, encoding NKCC2. ROMK and Romk are human and mouse genes, respectively, encoding ROMK. Romk+/-, Romk+/-, and Romk+/- refer to wild-type, heterozygous, and homozygous mutant mice, respectively.
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ume depletion, and a urinary concentrating defect. This phenotype is similar to that of the Nkcc2 knockout, although it is not as severe, suggesting that renal Na⁺ handling is perturbed less by the loss of ROMK than by the loss of Nkcc2. To examine this possibility we performed micropuncture analysis of a single nephron function. These experiments revealed that tubuloglomerular feedback is severely impaired and that Na⁺ reabsorption is significantly reduced but not eliminated in the TALH of the Romk knockout. The latter observation supports the hypothesis that ROMK plays an essential role in K⁺ recycling in the TALH, which is required for maximum Na⁺ reabsorption via Nkcc2, and also shows that a significant amount of Na⁺ reabsorption does take place in its absence.

**EXPERIMENTAL PROCEDURES**

**Generation of Mutant Mice**—A phage library prepared using genomic DNA from a 129/SvJ mouse was screened with a Romk cDNA probe. Clones containing the Romk gene were isolated and analyzed by restriction endonuclease mapping. Two restriction fragments containing sequences from the large core exon were inserted into the MJK-KO targeting vector (20), which allows a positive-negative selection strategy. A 3.8-kb BglII fragment terminating with codon 181 was inserted between the 5′ and the neomycin resistance gene and the 5′-end of the herpes simplex virus-thymidine kinase gene, and a 2.8 kb BglII fragment beginning with codon 182 was inserted between the 5′-end of the neomycin resistance gene and vector sequences. The targeting construct was linearized and electroporated into embryonic stem cells derived from 129/SvJ mice, which were then cultured in the presence of G418 and gancyclovir as described previously (20). DNA was isolated from cells that survived the selection procedure and analyzed by Southern blot analysis using a 2-3 kb HindIII-BglII fragment from the region just 5′ to fragments used to prepare the targeting construct. Blastocyst-mediated transgenesis was performed, chimeric mice were bred with Black Swiss mice, and a colony carrying the null allele was established using a three primer set. A 275-bp fragment from the wild-type gene targeting vector (20), which allows a positive-negative selection strategy, was hybridized, and washed as described previously (21), using rat ROMK cDNA probes corresponding to anticodons 240–254 and codons 372–385 from the mutant gene. Blots were probed, hybridized, and washed as described previously (21), using rat ROMK cDNA probes complementary to sequences in the 5′-untranslated region of the neomycin resistance gene.

**Northern Blot Analysis**—Total RNA was isolated from pooled kidneys of 8-day-old Romk+/- mice and Romk-/- mice and from individual adult and kidney cortex subadjacent to the capsule (to correlate with those nephrons handled by micropuncture). A grid of 75 intersections was visualized on the light microscopic image using a camera lucida drawing of the grid, and the numbers of positive intersections lying over all the tissue. In addition, a subjective appraisal of the degree of mitochondrial organization at the base of the tubular cells was made, with 1+ connoting no organization and 6+ being the highly organized and parallel alignment of mitochondria in a perpendicular position relative to the basement membrane. Means and S.E. for genotypes were generated using the statistical package of SAS 6.1. Differences were considered significant when p < 0.05.

**Blood Pressure and Fluid Handling in Adult Mice**—Adult Romk-/- mice of either sex (n = 4) and matched Romk+/- mice (n = 6) were housed individually in conventional shoebox cages for the duration of the experiment, and they were provided a diet of normal rodent chow. Blood pressure measurements were made by tail-cuff using a Visitech Systems BP-2000 blood pressure analyzer (Apex, NC) after 4 days of acclimation to the instrument. Urine concentrating ability was evaluated by measuring osmolality in urine samples obtained by bladder massage before and after 6 h of water deprivation. For the following 3 days, mice were given distilled water to drink, and water intake, body weight, and blood pressure were monitored daily. For the next 3 days, animals were provided with both water and isotonic saline to drink, and measurements were repeated. At the end of the tail-cuff protocol, animals resumed drinking water only and were made available for clearance and micropuncture experiments.

**Clearance and Micropuncture Experiments**—Experiments were performed on adult Romk-/- mice of either sex (n = 5) and matched Romk+/- mice (n = 5). Water clearances were performed in all of the mice, and micropuncture measurements were made in three mutant and three wild-type mice. Animals were surgically prepared according to conventional techniques modified for use in the mouse as described previously (23). Mice were anesthetized with separate intraperitoneal injections of ketamine (50 μg/μl of body weight) and thiobutabarbital (Inactin, Research Biochemicals International, Natick, MA; 100 μg/μl of body weight) and placed on a thermally controlled surgical table. After tracheostomy, the right femoral artery and vein were cannulated with polyethylene tubing hand-drawn to a fine tip over a flame (OD 0.3–0.5 mm). The arterial catheter was connected to a COBE CDXIII fixed dome pressure transducer (COBE Cardiovascular, Arvada, CO) for measurement of arterial blood pressure, and the venous catheter was connected to a syringe pump for infusion. The bladder was also cannulated with flared PE-10 tubing for the collection of urine. Blood pressure and heart rate were monitored throughout the experiment using a PowerLab data acquisition system (AD Instruments, Boston) with a sampling rate of 200 samples/s. Body temperature was maintained at 37.5 °C, and animals were provided with a steady stream of 100% O₂ to breathe. For micropuncture experiments, the kidney was exposed, a small incision was made, carefully dissected free of adherent fat and connective tissue, placed in a Lucite cup and covered with mineral oil. A 4-μl of body weight bolus infusion of isotonic saline containing 0.75 g of fluorescein isothiocyanate-inulin/100 ml (Sigma), 2.25 g of bovine serum albumin/100 ml, and 1.0 g of glucose/100 ml was then administered, followed by a maintenance infusion of the same solution at 0.2 μl/min of body weight. After a 30–45 min equilibration period, micropuncture collections and/or urine collections were begun. For micropuncture collections, surface convolutions of the same nephron were identified by injecting a small volume of saline containing 0.25% Fast Green dye (Sigma) into a random proximal segment. Late proximal puncture sites were identified as the last surface segment to fill with green dye before it disappeared into the loop of Henle. In a small population of nephrons (10–20%), an early distal puncture site could be identified when the green dye returned to the kidney surface. During two consecutive clearance periods lasting 30–60 min, at least five timed proximal collections were made, and usually two to three paired distal collections were made. In those nephrons having both proximal and distal collection sites, the distal tubule collection was performed before the proximal collection. Blood samples (5–10 μl) were taken in harneparized tubes before and after each clearance period. Sharpened glass micropipettes used for dye injection were 2–3 μm in diameter, and those used for fluid collection were 6–7 μm. At the end of each experiment, tubular fluid samples were transferred individually to 1-μl constant bore micropipettes for determination of volume and inulin concentration as described previously (24). The tubular fluid chloride concentration was determined by electrometric titration (25). Blood electrolytes were analyzed by Stop-flow Pressure Measurements—Separate Romk-/- (n = 3) and wild-type mice (n = 5) of either sex and weighing 20–30 g were prepared and proximal tubule segments identified for micropuncture as
ROMK Gene Targeting

FIG. 1. Romk gene targeting strategy and genotype analysis. A, targeting strategy. Top, restriction map of the region of the wild-type gene used to prepare the targeting construct and outside probe. Most of the 391-amino acid ROMK sequence is encoded by a large core exon. Middle, targeting construct in which a 3.8-kb BglII fragment terminating with codon 181 was inserted between the 3′-end of the neomycin resistance gene (Neo) and the 5′-end of the herpes simplex virus-thymidine kinase (TK) gene, and a 2.8-kb BglII fragment beginning with codon 182 was inserted between the 5′-end of the neomycin resistance gene and vector sequences. Bottom, targeted Romk allele. Restriction enzyme sites: B, BglII; S, StuI; H, HindIII. B, Southern blot analysis of tail DNA (first lane) from a mouse in which germ line transmission of the null allele had occurred and from wild-type (second lane) and targeted (third lane) embryonic stem cells. The samples were digested with StuI and hybridized with the outside probe (a 2-kb HindIII, BglII fragment), which identifies a 14.5-kb fragment in the wild-type allele and 10.5-kb fragment in the mutant allele. C, PCR analysis of tail DNA from offspring of a heterozygous mating which identifies 385- and 275-bp fragments from the mutant and wild-type genes, respectively. Offspring of all three genotypes were observed. D, Northern blot analysis of total RNA from kidneys of 8-day-old and adult null mutant and wild-type mice hybridized with cDNA probes corresponding to core exon sequences 5′ and 3′ to the neomycin resistance insertion point.

described above. Early proximal portions of the identified tubules were blocked with wax, and a micropipette attached to a nanoliter infusion pump was inserted into the last superficial proximal segment for loop of Henle perfusion. Another micropipette, attached to a Servo-null pressure device (World Precision Instruments, Sarasota, FL), was then inserted into an early proximal segment recognizable from the widening of the tubular lumen. When stop-flow pressure stabilized, the loop of Henle perfusion rate was altered from 0 to 40 nl/min, and maximal responses in stop-flow pressures were recorded. Perfusion fluid contained (in mM) 136 NaCl, 4 NaHCO₃, 4 KCl, 2 CaCl₂, 7.5 urea, and 1 mg/ml Fast Green. In a few tubules (see under "Results"), 36 mM NaCl in the perfusion fluid was replaced with 36 mM KCl to test the effect of perfusion with high K⁺ (100 mM Na⁺, 40 mM K⁺).

Statistics—Statistical analysis was performed by analysis of variance using a single factor design or a mixed factorial design with repeated measures on the second factor. Where necessary, individual comparisons of group means were accomplished using individual contrasts. Data are expressed as means ± S.E., and differences are regarded as significant at p < 0.05.

RESULTS

Generation of Romk Null Mutant Mice and Gross Phenotype—The Romk gene was disrupted in embryonic stem cells by insertion of the neomycin resistance gene into the large core exon (Fig. 1A). Targeted cells were identified by Southern blot analysis (Fig. 1B) and used to generate a mutant line carrying the null allele. As shown by PCR analysis of tail DNA (Fig. 1C), breeding of heterozygous mutant mice resulted in the birth of live pups of all three genotypes. Romk⁺/+ homozygous, and Romk⁻/⁻ mice were born in a normal Mendelian ratio (26.8% +/+ , 49.8% +/⁻, and 23.6% −/− among the first 500 pups), demonstrating that ROMK is not required for survival of the embryo. Northern blot analysis of kidney mRNA from wild-type and Romk⁻/⁻ mice showed that insertion of the neomycin resistance gene had virtually eliminated expression of Romk mRNA (Fig. 1D), although trace levels of an mRNA that was larger than the wild-type mRNA was detected with the 3′-probe.

Null mutants exhibited growth retardation, and by 1 week of age most of them could be identified because of poor turgor and wrinkled skin, probably reflecting fluid volume depletion. Mortality of Romk⁻/⁻ mice was high (Fig. 2), with 85% dying by 12 days of age, and only 5% surviving to weaning at 21 days. Daily subcutaneous injections of either indomethacin, which was reported to improve the survival rate of NKCC2-deficient mice (11), or isotonic saline, which rescued mice lacking the mineralocorticoid receptor (26), did not improve the survival rate of null mutants. Romk⁻/⁻ mice that survived to weaning exhibited no excess mortality and grew well. Adult mutants were slightly smaller than wild-type mice but appeared healthy. Both male and female null mutants were fertile.

Histological Analysis of Kidneys—Grossly, mutant kidneys from young (7–9 days) and adult mice showed moderate to severe dilation of the renal pelvis, indicative of hydronephrosis (Fig. 3, A and B). Histologically, it was readily apparent at the macro level that the renal cortex was considerably thinned, and an enormously dilated renal pelvis surrounded the renal papilla in mutant kidneys, which made it difficult to visualize
from both young and adult null mutants were usually histologically indistinguishable from those of wild-type mice, on occasion the glomerular tuft was dwarfed by a ballooning of the urinary space. The glomerular basement membrane in mutant mice was examined for thickening and for calcium deposits using electron microscopy, but neither was encountered. Distal and collecting tubules were often dilated in both young and adult Romk−/− mice. The volume density of proximal tubule cells was significantly decreased in the knockout compared with the wild-type, and at the same time the volume density of distal tubules was significantly increased. The ratio of distal to proximal tubules was significantly increased in mutant mice (Table I), indicating that a compensatory increase had occurred in the relative length of the distal tubule in the knockout. A loss of organization of mitochondria within the basal portion of individual proximal tubule cells was found in the mutant mice. With electron microscopy (data not shown), proximal tubule cells from mutant animals showed less organelar order; specifically, there were fewer basal membrane infoldings and mitochondrial interdigitation in knockout mice than in the wild-type mice. This was not true of the distal tubule, however, where the level of subcellular organization appeared to be similar in wild-type and null mice. The amount of lipid debris in the proximal tubule was significantly greater in the knockout.

7–9-Day-Old Romk−/− Mice Are Acidotic and Volume-depleted—Prior to weaning, pups were evaluated for blood and urine chemistries (Table II). Wild-type and heterozygous pups were indistinguishable based on the measured variables. Compared with Romk+/+ mice, most of the null mutants had markedly reduced body weight and failed to thrive. Wet kidney weight did not differ between wild-type and mutant mice, so the kidney weight–body weight ratio was significantly greater in Romk−/− mice. There were no significant differences in blood pCO₂ or pO₂ or between wild-type and mutant mice; however, pH and HCO₃⁻ were significantly reduced, and hematocrit and plasma Na⁺ and Cl⁻ concentrations were significantly elevated in the mutants, consistent with metabolic acidosis, volume depletion, and dehydration.

Adult Romk−/− Mice Have Low Blood Pressure, Polydipsia, and Reduced Urinary Concentrating Ability—A small percentage of null mutant mice survived to weaning and grew to adulthood. Tail-cuff evaluation of blood pressure (Fig. 4, left) showed that Romk−/− mice had significantly lower blood pressure than wild-type mice: 87 ± 7 versus 105 ± 5 mm Hg, respectively (p < 0.001). When animals were provided with a choice between water and isotonic saline to drink, blood pressure remained significantly lower in the mutant animals: 91 ± 6 versus 101 ± 4 mm Hg (p < 0.01). Intra-arterial measurements of mean arterial pressure (Fig. 4, right) in the anesthetized mice used in the renal studies discussed below also revealed a significantly lower blood pressure in null mutants: 74 ± 3 versus 95 ± 3 mm Hg (p < 0.001).

Fluid consumption was monitored during the 6-day blood pressure protocol, and the results are presented in Fig. 5. Romk−/− mice drank about three times as much water as wild-type mice when water only was provided (days 1–3). When both isotonic saline and water were provided, wild-type and null mutant mice drank similar amounts of saline (4.6 ± 0.4 or 4.1 ± 1.5 ml/day, respectively); wild-type mice lowered their water consumption to 2.9 ± 0.2 ml/day, whereas null mutants maintained a constant level of water intake (12.4 ± 1.0 ml/day). To evaluate the urine concentrating ability of Romk−/− mice, we measured urine osmolality before and after 6 h of water deprivation (Fig. 6). As expected, wild-type mice had relatively concentrated urine under base-line conditions, and osmolality

thin loops in the mutant mice. This was accompanied by a loss of organization and structure within the medulla of the kidney. Unlike in wild-type mice (Fig. 3 C), abundant droplets of lipidic material were seen within the proximal tubule cells of adult Romk−/− mice (Fig. 3D). This debris, possibly lysosomal, sometimes had a myelin character. Similar structures were found as casts in the tubular lumen (Fig. 3E). These structures may represent the lysis or loss of apical membranes of compromised cells but did not contain calcium crystalline deposits (27). In the proximal tubule mainly, but also in the distal tubule, mitochondria of adult Romk−/− mice contained significantly more dense, native intramitochondrial granules (Fig. 3F) than those of wild-type mice, often accompanied by an increase in overall electron density of the cell.

The results of morphometric analyses of the renal cortex are presented in Table I. There were no differences in volume density of the glomeruli between wild-type and Romk−/− mice of any age, although in young mutant mice there were often dark bodies within the glomeruli (perhaps apoptotic debris). This difference was not found in adults. Although glomeruli

FIG. 3. Histological analysis of kidneys from Romk+/+ and Romk−/− mice. A and B, light microscopic montages of sections of wild-type and knockout kidneys from 8-day-old (top) and adult animals (bottom). Renal pelvises are grossly dilated in the knockout mice, and cortex is thinned, relatively more in the adult than in the 8-day-old mouse. Bar = 3 mm. C and D, light microscopic images of subcortical kidney from wild-type (C) and knockout (D) mice. Large lipidic inclusions (arrows) were especially numerous in the cytoplasm of the proximal tubular cells of both 7–9-day and adult knockout mice. Bar = 10 μm. E, electron micrograph showing the lipid and myelin-like debris often found in the distal tubular lumen of adult knockout mice. Bar = 1 μm. F, electron micrograph of mitochondria showing increased numbers of native intramitochondrial granules (arrow), a change most prominent in proximal tubular cells of adult knockout mice. Bar = 1 μm.
formed in wild-type (583 ± 28 mosm/liter), and in response to water restriction there was a small but significant increase in urine osmolality (732 ± 50 mosm/liter). Thus, despite an impaired urinary concentrating ability, Romk−/− mice were able to concentrate their urine to almost double the osmolality of their plasma.

**Adult Romk−/− Mice Have Mild Hypernatremia and Hyper-**

| Vd glomeruli | Vd PT | Vd DT | Distal: proximal tubule ratio | PT mitochondrial organization | DT mitochondrial organization | Vd lipid debris |
|--------------|-------|-------|-----------------------------|-----------------------------|-----------------------------|---------------|
| 7.4 ± 1.9    | 64.5 ± 3.56 | 28.0 ± 2.6 | 0.47 ± 0.07 | 3.36 ± 0.24 | 2.24 ± 0.26 | 0.22 ± 0.05 |

**Table I**

*Fig. 4. Blood pressure measurements from Romk+/+ and Romk−/− mice. Left, tail-cuff blood pressure measurements were performed in wild-type (open bars, n = 6) and knockout (closed bars, n = 4) mice over a period of 6 days. During the first 3 days, mice were given water to drink, and during the following 3 days (days 4–6), mice were given both water and saline to choose from. For each day, a mean pressure was obtained from each animal by taking the average of 10 consecutive measurement cycles. Right, mean arterial pressure in wild-type (n = 5) and knockout (n = 5) mice determined by intra-arterial cannulation under anesthesia. *p < 0.05 compared with corresponding wild-types.*

**Table II**

**Blood and urine data from 7–9-day-old Romk+/+ and Romk−/− pups**

| BW (g) | KW (g) | KW/BW | Hct (%) | pCO2 (mm Hg) | pO2 (mm Hg) | pH | PHCO3− (mM) | PNa+ (mM) | PK+ (mM) | PCL− (mM) | UNa+ (mM) | UK+ (mM) | Uosm (mosm/liter) |
|--------|--------|--------|---------|--------------|-------------|----|------------|-----------|---------|----------|-----------|---------|---------------|
| 5.9 ± 0.6 | 0.08 ± 0.01 | 1.26 ± 0.07 | 37 ± 1 | 51.5 ± 2.7 | 43.8 ± 5.3 | 7.38 ± 0.01 | 30.0 ± 1.6 | 131 ± 2 | 6.4 ± 0.3 | 98 ± 2 | 35 ± 8 | 55 ± 19 | 685 ± 134 |
| 5.0 ± 0.3 | 0.08 ± 0.01 | 1.34 ± 0.02 | 36 ± 1 | 50.6 ± 1.8 | 47.0 ± 5.1 | 7.40 ± 0.01 | 30.3 ± 1.0 | 132 ± 1 | 6.3 ± 0.4 | 98 ± 1 | 26 ± 6 | 58 ± 7 | 582 ± 39 |
| 3.3 ± 0.6* | 0.08 ± 0.01 | 2.36 ± 0.19* | 47 ± 2* | 53.3 ± 3.7 | 36.6 ± 3.8 | 7.26 ± 0.04* | 24.7 ± 1.1* | 170 ± 6* | 7.2 ± 0.4 | 119 ± 5* | 41 ± 19 | 55 ± 11 | 566 ± 57 |

**Fig. 5. Fluid intake in Romk+/+ and Romk−/− mice. Water (dark bars) and saline (light gray bars) consumption was monitored for 6 days in wild-type (n = 6) and knockout (n = 4) mice. During the first 3 days (days 1–3) mice were given only water to drink. During the following 3 days (days 4–6) mice were given both water and saline to choose from, and each was monitored separately; the total height of the dark and light gray bars combined indicates the total fluid intake. *p < 0.05 compared with total fluid consumption in wild-types.**
degree of hydronephrosis in these kidneys was severe, and much of the additional weight was the result of accumulated fluid within the kidney. The significant reduction in mean arterial pressure mentioned previously was not accompanied by a difference in heart rate. Hematocrit did not differ significantly between the two groups, but plasma Na⁺, Cl⁻, and osmolality were significantly elevated in null mutants, again characteristic of a dehydrated state. The mutants had a metabolic acidosis, with reduced plasma pH and bicarbonate concentration. It should be noted that the PO₂ values listed in Table III were high because the mice were provided with 100% oxygen to breathe.

Whole kidney clearance data revealed polyuria and marked disturbances of renal function in the Romk⁻/⁻ mice (Table III). Consistent with the polydipsia (increased water intake) noted above, urine flow in the mutant mice was increased nearly 3-fold compared with the wild-type. Also consistent with data in the awake animals, urine osmolality was low in Romk⁻/⁻ mice, representing an unusually dilute urine given the high plasma osmolality. The whole kidney glomerular filtration rate was reduced in null mutants, to ~10–15% of that in wild-type mice. The urine electrolyte excretion rate in null mutants was not significantly different from that of wild-type mice (except that chloride excretion was mildly increased), but because of the low glomerular filtration rate in the mutant animals, fractional excretions of Na⁺, K⁺, Cl⁻, and solutes were significantly elevated compared with those of wild-type mice.

The Single Nephron Glomerular Filtration Rate (SNGFR) Is Relatively Normal in Romk⁻/⁻ Kidneys, and NaCl Absorption in the Thick Ascending Limb Is Reduced But Not Eliminated—Results from free flow micropuncture experiments are presented in Table IV. Unlike glomerular filtration rate values obtained from whole kidney studies, the SNGFR was not substantially compromised in Romk⁻/⁻ kidneys, suggesting that the impairment at the whole kidney level was largely caused by a reduced number of functioning nephrons. Although the mean value for SNGFR was slightly lower in Romk⁻/⁻ mice, the differences were not significant, whether measured from proximal or distal tubular sites. Both absolute and fractional fluid reabsorption in the loop segment (calculated from late proximal and early distal differences) were significantly reduced in the null mutant. It should be noted that the observed deficit in fluid absorption would likely occur in the descending portion of the loop of Henle, which is permeable to water and mediates isotonic absorption of NaCl, rather than in the TALH, which is impermeable to water. However, because the late proximal fluid flow rate was also significantly lower in the mutant mice, as a result of increased fractional fluid reabsorption in the proximal convoluted tubule and the small reduction in SNGFR, the rate of fluid delivery to the early distal tubule was not significantly different between mutant and wild-type mice. The proximal Cl⁻ concentration was significantly greater in Romk⁻/⁻ mice, as would be expected given the elevated plasma electrolyte concentrations and osmolality. The distal tubular Cl⁻ concentration was also significantly higher in the mutant than in wild-type mice, but it was nevertheless sharply reduced relative to that of the proximal collection. These data demonstrate that NaCl absorption is impaired in the Romk⁻/⁻ TALH but that substantial absorptive capacity remains. Calculations of Cl⁻ absorption in the loop segment between the two puncture sites reveal that the Romk⁻/⁻ loop segment absorbs ~60% as much Cl⁻ as that of wild-type mice (see Table IV).

Tubuloglomerular Feedback (TGF) Is Impaired in Romk⁻/⁻ Mice—To investigate whether TGF was impaired in Romk⁻/⁻ mice, we first compared measurements of SNGFR obtained from proximal and distal collection sites of the same nephron (Fig. 7). Proximal collections are made while flow to the macula densa is interrupted, whereas distal collections are made with intact flow. Thus, analysis of the distal:proximal SNGFR ratio can be viewed as an index of the prevailing level of feedback activation under a given set of conditions. It should be noted that the mean values presented in Fig. 7 do not strictly reflect the values presented in Table IV because several proximal measurements included in the data in Table IV do not have corresponding distal measurements. Only those nephrons from which both proximal and distal measurements were obtained are included in Fig. 7. In wild-type mice, distal values for SNGFR were consistently lower than the corresponding values from proximal tubules, with a distal:proximal ratio of 91 ± 5% and consistent with activation of a TGF response. In contrast, Romk⁻/⁻ mice showed no consistent differences between the distal and proximal SNGFR measurements, with a distal:proximal ratio of 101 ± 4%.

To evaluate more quantitatively the strength of the TGF response, we performed stop-flow pressure measurements during orthograde perfusion of the loop of Henle at different flow
rates. Typical TGF responses from a wild-type mouse are shown in Fig. 8A, left panel, illustrating robust and repeatable decreases in proximal stop-flow pressure in response to increasing the loop of Henle perfusion rate from 0 to 40 nl/min. In contrast, the right panel of Fig. 8A shows a response from a Romk\textsuperscript{−/−} mouse. In this particular tubule, perfusion of the loop at 40 nl/min resulted in small but repeatable decrease in stop-flow pressure. It is important to note that this tracing represents the largest response observed in Romk\textsuperscript{−/−} mice and that nephrons from these animals more typically showed little or no TGF response. Data from all of the experiments are summarized in Fig. 8B. Each thin line represents the average of two to four TGF responses obtained from a single tubule. As illustrated in the right panel of Fig. 8B, only 3 of 13 nephrons in Romk\textsuperscript{−/−} mice demonstrated even small TGF responses. In 4 of the nephrons tested, illustrated by the dotted lines, the loop of Henle perfusate composition was altered to contain 40 mM K\textsuperscript{+}; however, this maneuver had no discernible effect on the feedback responsiveness. Proximal stop-flow pressure was 31.1 ± 1.7 mm Hg during 0 flow and 30.1 ± 1.8 mm Hg during high flow. In contrast, wild-type tubules had a strong TGF response, from 41.4 ± 1.5 to 32.2 ± 1.8 mm Hg at 0 and 40 nl/min, respectively.

### DISCUSSION

To develop a mouse model that could be used to study the pathogenesis of Bartter's syndrome type II and the function of ROMK in the kidney, we disrupted the large core exon that encodes most of the protein. This was done to eliminate functional expression of all ROMK variants, which are generated by the use of alternative promoters and alternative splicing of N-terminal coding sequences present in small exons 5’ to the core exon (12, 14). Northern analysis using probes derived from sequences on both sides of the neomycin resistance gene insertion demonstrated that the mutation eliminated expression of Romk mRNA. In a separate study, patch clamp analysis showed that the mutation eliminates activity of the apical small conductance (SK) K\textsuperscript{+} channel in the TALH and cortical collecting duct, which has been attributed to ROMK, and immunocytochemical analysis showed that ROMK protein was present in the apical membranes of TALH and cortical collecting duct cells but absent in the knockout (28). Thus, analyses of the Romk gene and mRNA (Fig. 1), ROMK protein and channel activity (28), and the phenotype of ROMK-deficient mice, all confirm the null mutation.

Romk\textsuperscript{−/−} mice have a severe disease phenotype, with 95% of the mutants dying before weaning, apparently as a result of fluid volume depletion. As in the Nhce2 knockout (11), Romk null mutant kidneys were hydrenephrotic. In an attempt to improve the survival rate of Romk\textsuperscript{−/−} mice, we bred surviving null mutant males with heterozygous females from litters in which there were surviving null mutants. Our rationale was that it might be possible to develop an inbred line in which the more favorable alleles for genes affecting survival, carried in the original mixed background of 129/SvJ and Black Swiss mice, might be selected. After three generations of such inbreeding the survival of null mutants improved to ~20–25%, but in later rounds of inbreeding the survival rate in our colony

### TABLE III

|                  | +/+ (n = 5) | −/− (n = 5) |
|------------------|------------|-------------|
| BW (g)           | 31.1 ± 2.9 | 27.4 ± 3.6  |
| KW (g)           | 0.46 ± 0.02| 0.77 ± 0.18*|
| MAP (mm Hg)      | 94.9 ± 2.9 | 74 ± 3*     |
| HR (bpm)         | 483 ± 17   | 484 ± 20    |
| Hct (%)          | 52.8 ± 0.8 | 49.1 ± 2.8  |
| pCO\textsubscript{2} (mm Hg) | 53.0 ± 2.4 | 58.6 ± 3.8  |
| pO\textsubscript{2} (mm Hg) | 460.6 ± 14.7 | 397.9 ± 66.7 |
| pH               | 7.258 ± 0.016 | 6.972 ± 0.139*|
| PHCO\textsubscript{2} (mat) | 22.9 ± 1.1 | 13.0 ± 3.9* |
| FN\textsubscript{a} (mat) | 161.0 ± 1.7 | 170.2 ± 10.5* |
| FK\textsubscript{c} (mat) | 5.0 ± 0.5  | 5.6 ± 0.8   |
| PCL (mat)        | 123.7 ± 4.3 | 138.0 ± 10.3* |
| Posm (mosm/liter)| 330 ± 7    | 384 ± 28*   |
| BE (meq/liter)   | -5.2 ± 2.7 | -9.75 ± 0.95* |
| Urine flow (µl/min) | 1.59 ± 0.25 | 4.68 ± 0.70* |
| GFR (µl/min)     | 422.6 ± 30.2 | 542.2 ± 12.4* |
| Uosm (mosm/liter)| 1386.3 ± 84.8 | 476.7 ± 49.2* |
| UNa\textsuperscript{e} (µeq/min) | 0.221 ± 0.056 | 0.320 ± 0.063 |
| UK\textsuperscript{E} (µeq/min) | 0.464 ± 0.081 | 0.383 ± 0.090 |
| UC\textsuperscript{L} E (µeq/min) | 0.187 ± 0.026 | 0.450 ± 0.127* |
| Uosm\textsuperscript{E} (µosm/min) | 2.156 ± 0.321 | 2.184 ± 0.290 |
| FE Na\textsuperscript{e} (%) | 0.36 ± 0.11 | 4.14 ± 0.77* |
| FE K\textsuperscript{+} (%) | 26.25 ± 5.74 | 158.19 ± 31.60* |
| FE CL\textsuperscript{−} (%) | 0.38 ± 0.05 | 8.07 ± 0.74* |
| FE osm (%)       | 1.23 ± 0.12 | 13.08 ± 3.81* |

### TABLE IV

|                  | +/+         | −/−         | p value |
|------------------|-------------|-------------|---------|
| SNGFR (nl/min)   |             |             |         |
| Prox             | 12.5 ± 1.1 (12) | 10.1 ± 1.1 (15) | 0.21    |
| Dist             | 11.2 ± 0.7 (8)  | 9.2 ± 1.5 (5)  | 0.21    |
| Flow (nl/min)    |             |             |         |
| Prox             | 6.7 ± 0.7 (12)  | 4.4 ± 0.4 (15)  | 0.01    |
| Dist             | 2.4 ± 0.5 (8)  | 2.4 ± 0.5 (5)  | 0.69    |
| Fluid abs (nl/min) |             |             |         |
| Prox             | 5.8 ± 0.8 (12)  | 5.7 ± 0.7 (15)  | 0.93    |
| Dist             | 8.6 ± 0.6 (8)  | 6.9 ± 1.1 (5)  | 0.146   |
| % Fluid abs      |             |             |         |
| Prox             | 46.2 ± 4.4 (12) | 55.4 ± 1.4 (15) | 0.03    |
| Dist             | 76.9 ± 2.7 (8)  | 74.5 ± 2.7 (5)  | 0.57    |
| TF\textsubscript{cl} (meq/liter) |             |             |         |
| Prox             | 137.7 ± 1.0 (12) | 146.5 ± 1.7 (15) | 0.002   |
| Dist             | 44.7 ± 4.7 (7)  | 61.3 ± 6.6 (5)  | 0.03    |
| Cl\textsuperscript{−} delivery (peq/min) |             |             |         |
| Prox             | 916 ± 94 (12)  | 669 ± 66 (15)  | 0.02    |
| Dist             | 112 ± 20 (8)    | 156 ± 38 (5)   | 0.29    |
| LS fluid abs (nl/min) | 4.0 ± 0.6 (6)  | 1.4 ± 0.2 (5)  | 0.004   |
| LS CL\textsuperscript{−} abs (nl/min) | 690 ± 120 (5) | 388 ± 58 (5)  | 0.05    |
ROMK Gene Targeting

Before weaning, null mutant mice were severely dehydrated, as indicated by poor turgor, elevated hematocrit, and high blood Na$^+$ and Cl$^-$ concentrations. These data are almost identical to those obtained with NKCC2-deficient mice of similar age (11). Also like the Nkcc2 knockout, young Romk$^{-/-}$ mice were acidotic (Table II and Ref. 28), although the acidosis was less severe than in the Nkcc2 knockout. Analysis of blood from adult Romk$^{-/-}$ mice (Table III) revealed high blood Na$^+$ and Cl$^-$ concentrations, elevated plasma osmolality, and a metabolic acidosis that was worse than that observed in young mutants. This acid-base disturbance in adult Romk$^{-/-}$ mice was the opposite of that in adult NKCC2-deficient mice, which had a significant alkalosis (11). Interestingly, the adult ROMK-deficient mice examined by Lu et al. (28), in which hydropenia was sharply reduced or eliminated, had normal acid-base values, suggesting that the metabolic acidosis observed in adult Romk$^{-/-}$ mice in our study may be secondary to renal insufficiency rather than to altered Na$^+$ or K$^+$ handling. These data indicate that there are both major similarities and dissimilarities between type I and type II Bartter’s syndrome in mice.

As in both human Bartter’s syndrome and murine type I Bartter’s syndrome, adult Romk$^{-/-}$ mice exhibited polydipsia and polyuria and had a urinary concentrating defect. These aspects of the phenotype and the significant reduction in blood pressure are consistent with extracellular volume depletion caused by a deficit in renal NaCl and water handling. Although many aspects of the gross phenotype were similar in mouse and human, Romk$^{-/-}$ mice did not exhibit hypokalemic alkalosis, as reported in most cases of human Bartter’s syndrome. This and other apparent differences between the two species may stem in part from criteria used to diagnose human Bartter’s syndrome, which has traditionally included a clinical finding of hypokalemic alkalosis. There are recent indications that Bartter’s syndrome in humans might be more variable than recognized previously. Children with NKCC2 mutations have been identified who initially lacked characteristic hypokalemia, were acidotic rather than alkalotic, and had both hypernatremia and hyperchloremia (29), as observed in Romk$^{-/-}$ mice. Before the genetic analysis had been performed, some of these children were tentatively diagnosed with distal renal tubular acidosis or nephrogenic diabetes insipidus. In a recent study of Bartter’s patients with mutations in the NKCC2, ROMK, and CLC-KB genes, initially identified by the occurrence of hypokalemia and renal salt wasting, significant differences in phenotypic characteristics were noted (30). Hypokalemia in patients with ROMK mutations was not as severe as in those with NKCC2 mutations, and during infancy transient hyperkalemia and metabolic acidosis were observed in some of the ROMK patients (30). The results of these clinical findings and our experiments in the mouse suggest that the patient population used in clinical screening for mutations in the four genes known to cause Bartter’s syndrome should be broadened to include patients with metabolic disturbances that are not normally considered as diagnostic of the disease syndrome. Such studies may reveal greater phenotypic variability than is currently recognized.

The high plasma Na$^+$ and Cl$^-$ concentrations in both young and adult null mutants indicate that the absence of ROMK causes a greater loss of water than of NaCl. When given a choice between water and isotonic saline to drink, wild-type mice prefer saline, whereas the knockouts had a greater preference for water. The urinary concentrating defect in Romk$^{-/-}$ mice, which is responsible for the water loss, was not sharply diminished. However, during the period when the survival rate had increased, breeding pairs were provided for a separate colony, which was subsequently used in the studies of ROMK channel activity and immunolocalization (28). After multiple additional rounds of inbreeding, survival of null mutants in that colony had stabilized at the higher rate (~25%) and some of the surviving mutants had little, if any, evidence of hydronephrosis (28). These results suggest that multiple genes, not necessarily involved in NaCl handling, may be involved in modulating both the survival rate and the degree of hydronephrosis and consequent renal insufficiency.

It has been noted that hydronephrosis occurs occasionally in human Bartter’s syndrome (see the Discussion in Ref. 11), but it is not a consistent feature. The occurrence of hydronephrosis in the mouse model is a confounding factor that could potentially limit its usefulness as a model of the human disease. However, with further breeding, as indicated by the results of Lu et al. (28), and testing of different genetic backgrounds, it may be possible to develop a line that more faithfully recapitulates human type II Bartter’s syndrome, with sharply increased survival and absence of significant hydronephrosis.

![Graph showing stop-flow pressure and tubuloglomerular feedback responses in Romk$^{-/+}$ and Romk$^{-/-}$ mice.](image-url)
as severe as that in mice lacking NKCC2 (11). Under normal conditions, urine osmolality in adult Romk /− mice was ~580 mosm and increased to ~730 mosm (almost double that of plasma) after 6 h of water restriction. In contrast, NKCC2-deficient mice exhibited no significant ability to concentrate the urine, even when treated with vasopressin (11). Part of this difference in phenotypes may be because Na+ absorption in the TALH, which is essential for the urinary concentrating mechanism (31), is eliminated in the Nkcc2 knockout but is partially functional in Romk /− mice. The substantial Na+ absorption capability remaining in the cortical TALH of adult Romk /− mice (discussed below) suggests that the severity of the concentrating defect is dependent on factors besides the deficit in Na+ absorption. Juxtamedullary nephrons, with their loops of Henle and associated vasa recta penetrating deep into the inner medulla, are essential for maximum urinary concentrating ability but are differentially lost in hydronephrosis. Therefore, it seems possible that the urinary concentrating defect in adult Romk /− mice may be caused in part by hydronephrosis.

A major objective, which cannot be studied in humans with type II Bartter’s syndrome, was to assess the overall effect of the Romk null mutation on single nephron function, with particular emphasis on the TALH, in which ROMK is expressed. Micropuncture studies, which allow an assessment of cortical nephron function, showed that the SNGFR was relatively normal in the knockout, but fluid absorption in the loop segment was reduced in Romk /− nephrons. The reason for this is unclear because the portion of this segment that reabsorbs fluid (i.e. pars recta and thin descending limb of the loop of Henle) does not contain ROMK (15); however, when mapping the surface convolutions of ROMK-deficient nephrons we noted that the dye tended to reappear more quickly after disappearing into the loop of Henle, suggesting that the loop segment was substantially shorter in the mutant. Measurements of distal Cl− concentrations yielded the unexpected observation that a substantial capacity for NaCl absorption was present in the mutant TALH. Although distal Cl− concentrations in the knockout were significantly elevated relative to those of the wild-type, consistent with an absorptive defect, the concentrations were quite hypotonic relative to those observed in the proximal collections, and calculated Cl− reabsorption in the loop segment of Romk /− mice was ~60% of that occurring in wild-type mice.

There are a number of possible explanations for this finding. Although the low conductance K+ channel is admitted in the Romk /− TALH (28), K+ secretion via other channels (17) or paracellular flux of K+ (32, 33) could allow some level of K+-dependent NKCC2 activity. Na+/NH4+/2Cl− cotransport activity by NKCC2, which is increased by in vitro conditions that simulate metabolic acidosis (34), coupled with NH4 recycling and Na+/H+ exchange via NHE3 could mediate K+-independent NaCl uptake in the TALH. A third possibility is that the observed NaCl absorption in the mutant might involve a recently identified K+-independent variant of NKCC2. Plata et al. (19) have shown that a splice variant of NKCC2, in which the last 383 amino acids of the hydrophilic C-terminal region are replaced by an alternative 55-amino acid sequence, mediates K+-independent NaCl uptake when expressed in oocytes. Although the K+-independent variant appears to be expressed at low levels relative to the K+-dependent variant (35), there is evidence for K+-independent NaCl uptake in medullary thick ascending limbs of both rabbits (36) and mice (37). Further studies will be needed to determine the mechanisms by which NaCl is absorbed in the thick ascending limb of Romk /− mice and whether this alternative absorptive mechanism is the result of a compensatory adaptation that occurs in response to the absorptive defect or is also present in mice with functional ROMK.

There is strong evidence that the signal for TGF is initiated by an increase in the activity of the apical Na+−K+−2Cl− cotransporter of macula densa cells (38), and additional evidence suggests that the splice variant of NKCC2 expressed in this portion of the TALH has a high affinity for the transported ions (39). Although the activity of the cotransporter, and therefore the TGF signal, is generally dependent upon the luminal concentration of Cl− within normal physiological ranges, alterations in Na+ and K+ availability can also influence the rate of transport (40, 41). The luminal recycling of K+ would appear to preclude K+ as a rate-limiting component of the TGF signaling process; however, recent investigations have suggested that interruption of either K+ recycling or transport, by blocking with barium or provision of a K+-free solution to the macula densa, can interfere with the TGF response (42, 43). The presence of ROMK on the apical membrane of macula densa cells (16) supports the notion that K+ entering the cell via NKCC2 exits via ROMK. The results of the present study, showing that TGF is either eliminated or sharply reduced in Romk /− tubules, suggests that ROMK activity in the apical membrane of the macula densa cell is necessary for a maximum and consistent TGF response. On the other hand, because some of the tested nephrons from Romk /− mice clearly showed intact, albeit attenuated, TGF responses (see Fig. 8), our findings indicate that ROMK channel activity is not a necessary component for the initiation of the TGF signal. Whether the activity of ROMK can act directly as a modulator of the TGF response, as suggested by Vallon et al. (43), remains to be determined.

The results of our experiments and those of Hebert and colleagues (28) reveal that in the mouse, type II (ROMK) Bartter’s syndrome is phenotypically less severe than that of type I (NKCC2) Bartter’s syndrome (11). Although there are major similarities between the two phenotypes, including hydronephrosis, polydipsia, polyuria, and dehydration, Na+ reabsorption in the TALH of Romk /− mice and the urinary concentrating mechanism are only partially impaired, and acid-base disturbances in adult Romk /− mice are different from those of Nkcc2 mutants. Breeding studies suggest that the severity of the Romk /− phenotype may be strongly dependent on genetic background (28). These findings, and the recent observation of substantial phenotypic variability in children with mutations that cause Bartter’s syndrome (29, 30), serve to emphasize the importance of identifying patients for each of the four known genetic variants of Bartter’s syndrome in a manner that is not strongly biased by phenotype and then performing comprehensive clinical studies of their disease phenotypes. Such studies of Bartter’s syndrome patients and experiments with the corresponding mouse models should lead to a comprehensive understanding of the pathophysiology of Bartter’s syndrome and the underlying mechanisms.

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