Haploinsufficiency of the ammonia transporter Rhcg predisposes to chronic acidosis. Rhcg is critical for apical and basolateral ammonia transport in the mouse collecting duct

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Abstract: Ammonia secretion by the collecting duct (CD) is critical for acid-base homeostasis and, when defective, causes distal renal tubular acidosis (dRTA). The Rhesus protein RhCG mediates NH(3) transport as evident from cell-free, cellular models, and Rhcg-null mice. Here we investigated in a Rhcg mouse model, the metabolic effects of Rhcg haploinsufficiency, the role of Rhcg in basolateral NH(3) transport, and mechanisms of adaptation to the lack of Rhcg. Both Rhcg(+/+) and Rhcg(+-) mice were able to handle an acute acid load, whereas Rhcg(-/-) mice developed severe metabolic acidosis with reduced ammonuria and high mortality. However, chronic acid loading revealed that Rhcg(+-) mice did not fully recover, showing lower blood HCO(3)(-) concentration and more alkaline urine. Micropuncture studies demonstrated that transepithelial NH(3) permeability was reduced by 80% and 40%, respectively, in CDs from Rhcg(-/-) and (+-) mice compared to controls. Basolateral membrane permeability to NH(3) was reduced in CDs from Rhcg(-/-) mice consistent with basolateral Rhcg localization. Rhcg(-/-) responded to acid-loading with normal expression of enzymes and transporters involved in proximal tubular ammoniagenesis but reduced abundance of the NKCC2 transporter responsible for medullary accumulation of ammonium. Consequently, tissue ammonium content was decreased. This data demonstrate a role for apical and basolateral Rhcg in transepithelial NH(3) transport, uncover an incomplete dRTA phenotype in Rhcg(+/-) mice. Haploinsufficiency or reduced expression of RhCG may underlie human forms of (in)complete dRTA.

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Haploinsufficiency of the Ammonia Transporter Rhcg Predisposes to Chronic Acidosis

Rhcg IS CRITICAL FOR APICAL AND BASOLATERAL AMMONIA TRANSPORT IN THE MOUSE COLLECTING DUCT

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Background: Rhesus proteins transport NH₃ and/or NH₄⁺ in heterologous expression systems.

Results: Heterozygous Rhcg mice develop delayed metabolic acidosis, whereas homozygous KO mice display severe metabolic acidosis. RhCG functions as an NH₄⁺ transporter on apical and basolateral membranes.

Conclusion: Rhcg is an NH₄⁺ but not NH₃ transporter.

Significance: Loss or reduced expression of RhCG may underlie inherited or acquired forms of human acidosis.

Ammonia secretion by the collecting duct (CD) is critical for acid-base homeostasis and, when defective, causes distal renal tubular acidosis (dRTA). The Rhesus protein RhCG mediates NH₄⁺ transport as evident from cell-free and cellular models as well as from Rhcg-null mice. Here, we investigated in a Rhcg mouse model the metabolic effects of Rhcg haploinsufficiency, the role of Rhcg in basolateral NH₄⁺ transport, and the mechanisms of adaptation to the lack of Rhcg. Both Rhcg⁺/+ and Rhcg⁺/− mice were able to handle an acute acid load, whereas Rhcg⁻/− mice developed severe metabolic acidosis with reduced ammonuria and high mortality. However, chronic acid loading revealed that Rhcg⁻/− mice did not fully recover, showing lower blood HCO₃⁻ concentration and more alkaline urine. Micropertusion studies demonstrated that transepithelial NH₄⁺ permeability was reduced by 80 and 40%, respectively, in CDs from Rhcg⁻/− and Rhcg⁺/− mice compared with controls. Basolateral membrane permeability to NH₄⁺ was reduced in CDs from Rhcg⁻/− mice consistent with basolateral Rhcg localization. Rhcg⁻/− responded to acid loading with normal expression of enzymes and transporters involved in proximal tubular ammoniagenesis but reduced abundance of the NKCC2 transporter responsible for medullary accumulation of ammonium. Consequently, tissue ammonium content was decreased. These data demonstrate a role for apical and basolateral Rhcg in transepithelial NH₄⁺ transport and uncover an incomplete dRTA phenotype in Rhcg⁺/− mice. Haploinsufficiency or reduced expression of RhCG may underlie human forms of (in)complete dRTA.

Ammonium (NH₄⁺) is the main component of urinary acid excretion. Renal synthesis and excretion of NH₄⁺ rise in response to an acid load, allowing kidneys to regenerate bicarbonate and increase net acid excretion (1, 2). Impaired renal acid excretion characterizes type I distal renal tubular acidosis (dRTA) with low urinary ammonium and inappropriately alkaline urinary pH (3).

Ammonium is formed in the proximal tubule from the metabolism of glutamine and added to the luminal fluid. It is reabsorbed into the medullary interstitium in the thick ascending limb creating a cortical-papillary NH₄⁺/NH₃⁺ gradient (4, 5). The final step of NH₃/NH₄⁺ excretion is achieved by the CD (6). The high tissue concentration of NH₃/NH₄⁺ and the pH gradient between interstitium and urine provide the driving forces for NH₄⁺ excretion into urine. NH₄⁺ secretion results from the trapping of NH₃ in the tubular lumen after being titrated by H⁺ ions stemming from active secretion by V-type H⁺-ATPases (7).

The mechanisms mediating NH₃/NH₄⁺ transport across cell membranes into urine have only recently become uncovered. Members of the Rhesus protein family have been identified as pathways for NH₃/NH₄⁺ transport in yeast, plants, fish, and mammals (2, 5, 8). In the kidney CD, RhCG and RhBG are expressed (9, 10). Mice lacking Rhbg show either no phenotype or only a very mild reduction in urinary ammonium excretion (11, 12). In contrast, cell-specific or complete Rhcg deficiency

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causes a massive reduction in urinary ammonium excretion in three different mouse models (13–15). Microperfusion experiments using CDs from Rhcg-deficient mice demonstrated that RhCG is critical for the apical exit of NH₄⁺ into urine (13). Thus far, a phenotype of dRTA has only been reported in Rhcg⁻/⁻ mice, whereas potential changes caused by haploinsufficiency in Rhcg have not been investigated. This issue is relevant because heterozygous abnormalities in Rhcg might be more frequent and may affect the renal capacity to cope with an acid load leading to incomplete dRTA. Furthermore, Rhcg⁺/⁻ mice may also serve as a model to examine the consequences of reduced RhCG expression that may occur during kidney disease. Finally, RhCG localization has been controversial for years. Rhcg has been localized by some groups only at the apical side of CD cells, whereas others have found RhCG on both the apical and basolateral membranes (9, 10, 16, 17). The functionality of basolateral RhCG protein remains unknown.

Here, we used a novel Rhcg mouse model to provide the first evidence that haploinsufficiency in Rhcg impairs the handling of a chronic acid load in Rhcg⁺/⁻ mice, which develop an incomplete metabolic acidosis. Microperfusion studies provide the functional basis of the defect and show that RhCG is absolutely required for apical and partially for basolateral NH₃ transport. Moreover, loss of Rhcg is associated with a profound down-regulation of NKCC2 and reduced medullary accumulation of ammonium impairing the gradient necessary for the final excretory step. These data provide new insights into the complex role of Rhcg and suggest that congenital or acquired defects in RhCG protein expression may be associated with incomplete dRTA.

EXPERIMENTAL PROCEDURES

Animals—Rhcg⁺/⁻ mice were purchased from the Texas Institute of Genomic Medicine (Houston TX). Mice were generated by replacing exon 1 by a vector carrying a LacZ/Neo cassette (Fig. 1A). Mice were genotyped by PCR directly on a 3-μl 25 mM NaOH tail digestion product. Genomic DNA was amplified using primer pairs specific for exon 1 forward (AGACCCCAACTGGAAGCTTAA), wild type reverse (CAAACGAAAATCCTCCCATGTCAG), and knock-out reverse (ATGGGCTGACCCTCTCCTGCTTTC). The products were separated by electrophoresis in 1% agarose gel (mutant product, 522 bp; wild type product, 376 bp). Mice were generated by mating Rhcg⁺/⁻ mice, and mice were bred in the Entres Vivants Exempts d’Organismes Pathogènes Spécifiques Animal Facility. For acid-loading experiments, mice in metabolic cages were given 0.2 M HCl added to powdered standard food. All experiments were performed according to Swiss Animal Welfare laws approved by the local veterinary authority (Veterinäramt Zürich).

In Vivo Experiments—All experiments were performed using age- and sex-matched Rhcg wild type (Rhcg⁺/⁺), Rhcg knock-out (Rhcg⁻/⁻), and Rhcg heterozygote (Rhcg⁺/⁻) littermate mice (3–4 month-old) that were housed in metabolic cages (Techniplast, Switzerland). Mice were given deionized water ad libitum and were fed with a standard powdered laboratory chow (Kliba, Augst, Switzerland). Mice were allowed to adapt to metabolic cages for 3 days, and a first retro-orbital blood sample was taken for blood gas analysis under base line. Then two 24-h urine samples were collected under light mineral oil in the urine collector to determine daily urinary parameters. Mice were then allowed to recover for 2 weeks before giving an HCI-containing diet (0.2 M HCl added to powdered standard food) in normal cages. Food, water intake, and urine excretion were monitored following the same procedures as under base-line conditions. Urine collections were performed on the 1st and 2nd day of acid loading and then on the 6th and 7th day. Retro-orbital blood samples were taken on the 2nd and 7th days of HCI diet.

Analytic Procedures—Blood pH, pCO₂, and electrolytes were measured with a pH/blood-gas analyzer (ABL 77 Radiometer). Urinary Na⁺ and K⁺ concentrations were measured by flame photometry (IL943, Instruments Laboratory); titratable acids were measured using a DL 50 titrator (Mettler Toledo) (18, 19) and creatinine by a modified kinetic Jaffe colorimetric method (20). Urinary pH and bicarbonate were measured with a pH/blood-gas analyzer (ABL 725, Radiometer). Urinary NH₄⁺ was measured with the Berthelot protocol (21).

Immunoblotting—Crude membrane proteins or cytosolic fractions were obtained from kidneys homogenized in 250 mM sucrose, 10 mM Tris-HCl, pH 7.5, and in the presence of protease inhibitors. Forty micrograms of crude membrane proteins or cytosolic proteins were solubilized in loading buffer containing DTT and separated on 8–10% polyacrylamide gels. For immunoblotting, the proteins were transferred electrophoretically to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA). After blocking with 5% milk powder in Tris-buffered saline, 0.1% Tween 20 for 60 min, the blots were incubated with primary antibodies overnight at 4°C as follows: phosphate-dependent glutaminase (PDG), which recognizes both the rat (KGA) and human (GAC) kidney-type isoforms of PDG forming the mature PDG protein (66 and 68 kDa; a kind gift from N. Curthoys, Colorado State University; diluted 1:5000) (22); rabbit polyclonal anti-PEPCK (Cayman Chemical, Ann Arbor, MI; diluted 1:5000); rabbit polyclonal anti-NKCC2 (kind gift from Johannes Loffing, Institute of Anatomy, University of Zurich; diluted 1:5000); rabbit polyclonal anti-NHE3 (StressMarq Biosciences Inc., Victoria, British Columbia, Canada); rabbit polyclonal anti-pendrin (Pineda Antibody Service, Berlin, Germany, diluted 1:5000) (23); and mouse monoclonal anti-β-actin antibody (Sigma; diluted 1:5000). After washing and blocking with 5% milk powder for 60 min, membranes were then incubated for 2 h at room temperature with secondary goat anti-rabbit or donkey anti-mouse antibodies (diluted 1:5000) linked to alkaline phosphatase (Promega, Madison, WI). The protein signal was detected with the appropriate substrate (Millipore Corp, Bedford, MA) using the las-4000 image analyzer system (Fujifilm Life Science). All images were analyzed using the software Advanced Image Data Analyzer AIDA (Raytest, Straubenhardt, Germany) to calculate the protein of interest/β-actin ratio.

Immunostaining and Immunogold—Immunohistochemistry and immunogold staining were performed on frozen sections, and specificity was demonstrated on Rhcg⁺/⁻ tissues. Mouse kidneys were fixed by perfusion retrograde through the aorta.
with 3% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. The tissue was either trimmed into small blocks, further fixed by immersion in 1% paraformaldehyde, infiltrated with 2.3 M sucrose for 30 min, and frozen in liquid nitrogen or prepared for routine paraffin embedding.

For electron microscopy, 70–90-nm cryosections were obtained at −100°C with an FCS Reichert Ultracut S cryoultramicrotome as described previously (24), and 2-μm paraffin sections were obtained with a Leica RM2165 microtome. For LM immunolabeling, the sections were incubated with a rabbit polyclonal antibody against RhCG (a kind gift from Dr. Yves Colin, INSERM, Paris, France (16, 25)) at room temperature for 1 h after preincubation in PBS containing 0.05 M glycine and 1% bovine serum albumin. The sections were subsequently incubated with peroxidase-conjugated secondary antibody (Dako); the peroxidase was visualized with diaminobenzidine, and the sections were counter-stained with Maier’s stain for 2 min. The sections were examined in a Leica DMR microscope equipped with a Leica DFC320 camera. Images were transferred by a Leica TFC Twain 6.1.0 program and processed using Adobe Photoshop 8.0. For EM immunolabeling, the sections were incubated with the primary antibody at 4°C overnight followed by incubation at room temperature for 1 h with 10-nm gold particles coupled to anti-rabbit IgG (BioCell, Cardiff, UK). The cryosections were embedded in methylcellulose containing 0.3% uranyl acetate and studied under a Philips CM100 electron microscope. For controls, sections were incubated with secondary antibodies alone or with nonspecific IgG.

**RNA Extraction and Reverse Transcription**—Snap-frozen kidneys (five kidneys for each condition) were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) supplemented with β-mercaptoethanol to a final concentration of 1%. Total RNA was extracted from 200-μl aliquots of each homogenized sample using the RNeasy mini kit (Qiagen, Basel, Switzerland) according to the manufacturer’s instructions. Quality and concentration of the isolated RNA preparations were analyzed on the ND-1000 spectrophotometer (Nano-Drop Technologies). Total RNA samples were stored at −80°C. Each RNA sample was diluted to 100 ng/μl, and 3 μl was used as a template for reverse transcription using the TaqMan reverse transcription (0.4 units/l), the multiscribe reverse transcriptase enzyme (Microsynth, Balgach, Switzerland). The primers were designed to target the deleted sequence in Rhcg−/− animals (accession number NM_019799) with 5’-ATGCAGGGATGTCTCATTAT-3’ (238–258 bp) as the left primer located within exon 1 and 5’-TGGAAAGGTCTCATAATGAGCAG-3’ (373–392 bp) as the right primer located within exon 2, and 5’-TTAC-TATCGCTAACCAGGTCGACGTCCAG-3’ as the probe (292–317 bp). Real time PCRs were performed using TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA). Briefly, 3 μl of cDNA, 0.8 μl of each primer (25 μM), 0.4 μl of labeled probe (5 μM), 5 μl of RNase-free water, and 10 μl of TaqMan Universal PCR master mix reached 20 μl of final reaction volume. Reaction conditions were denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s with auto ramp time. All reactions were run in triplicate. For analyzing the data, the threshold was set to 0.2 as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of the genes of interest was calculated in relation to hypoxanthine-guanine phosphoribosyltransferase (accession number NM_013556, forward primer, 5’-TTATCAGACTGAAGCTACTGTGA-GATC-3’ (442–471), reverse primer, 5’-TTACCAGTGTCAA-TATATCTCACAATC-3’ (539–568), and probe, 5’-TGAGAGATCTATGCTCACAATATACTTTATGTC-3’ (481–515)). Relative expression ratios were calculated as $C_t^{(test\ gene)} - C_t^{(reference\ gene)}$, where $C_t$ represents the cycle number at the threshold 0.02; and HPRT is hypoxanthine-guanine phosphoribosyltransferase.

**Measurement of Renal Ammonia Content**—The renal tissue ammonia content was measured by an enzymatic technique (ammonia assay kit, Sigma) as described previously (26). Mice were anesthetized, and the kidneys were removed and immediately frozen in liquid nitrogen. They were then sliced frozen to yield a column of tissue, which extended from the cortex to the tip of the papilla. Sections were cut along the corticomedullary axis to yield three slices as follows: cortex, outer medulla, and inner medulla. Two kidneys from the same animal were pooled for each sample. Tissue slices were then homogenized in 300 μl of ice-cold 7% trichloroacetic acid, and the solution was centrifuged. The supernatant was drawn off, and the pH of a 250-μl sample was adjusted to near neutral by the addition of 12 μl of 10 mM Na2HPO4 in 9 N NaOH. A 200-μl sample of buffered supernatant was then analyzed for ammonium. The pellet was resuspended in 1 N NaOH, shaken overnight, and analyzed for total protein using the Bio-Rad protein assay.

**Microperfusion Studies on Isolated Tubules**—Mice were anesthetized with 50 mg/kg pentobarbital sodium or xylazine/ketamine intraperitoneally. Both kidneys were cooled in situ with control bath solution (see below) for 1 min and then removed and cut into thin coronal slices for tubule dissection. CCDs were dissected from the cortex at 10°C in the control solution. In vitro microperfusion of single CCD segments, intracellular pH, and transepithelial NH3 permeability measurements were performed as described previously (13).

**Intracellular pH Measurement**—The isolated tubule was transferred to the bath chamber on the stage of an inverted microscope (Axiovert 200, Carl Zeiss, Germany) in the control
solution containing (in mM) 138 NaCl, 1.5 CaCl₂, 1.2 MgSO₄, 2 K₂HPO₄, 10 HEPES, 5.5 glucose, 5 alanine, pH 7.40, and then was mounted on concentric pipettes and perfused in vitro with Na⁺-free, ammonium-free solution, where N-methyl-d-gluta-
mine (NMDG⁺) replaced Na⁺. All solutions were equili-
brated with 100% O₂ passed through a 3 x KOH CO₂ trap. Once the solutions were gassed and the pH checked, they were placed in a reservoir and were continuously bubbled with 100% O₂.

The average tubule length exposed to bath fluid was limited to 300–350 μm to prevent motion of the tubule. CCOs or OMCDs were loaded with 5 μM of the fluorescent probe 2’7’-bis(2-carboxyl)-5-(and-6)-carboxyfluorescein (BCECF Invitro-
buffering capacity (pH 9.26) was loaded with 5

The tubules were first transiently acidified by peritubular
solution, where

solution was changed by a 6 mM NH₄Cl solution, pH 7.40, in the

- The intrinsic

mechanisms, Na⁺-free, HEPES-buffered solutions were used in the

- The basic approach used
to determine NH₃ permeability involved construction of a tran-
sepithelial gradient of NH₃ and measurement of the resulting
NH₃ flux from the basolateral to the luminal side as described
previously (11, 13, 30). The isolated CCD was transferred to the
bath chamber on the stage of an inverted microscope (Axiovi-
sion A1, Zeiss, Germany) and mounted on concentric glass
pipettes for microperfusion. Bath solution was delivered at a
rate of 20 ml/min and warmed to 37 °C by a water jacket imme-
diate upstream of the chamber. The perfusion rate was
adjusted by hydrostatic pressure to ~10 nl/min. The tubules
were equilibrated for 20–30 min at 37 °C before the begin-
ing of collections. To construct a transepithelial NH₃ gradient, the
perfusion (lumen) solution contained (in mM) 139 NaCl, 1
NH₄Cl, 2.5 K₂HPO₄, 2 NaHCO₃, pH 6.4; the bath solution con-
tained (in mM) 117 NaCl, 1 NH₄Cl, 2.5 K₂HPO₄, 23 NaHCO₃,
pH 7.4, and in addition, both solutions contained (in mM) 5.5
glucose, 2 CaCl₂, 1.2 MgSO₄ and 10 HEPES. The osmolarity of the solution was 295 ± 5 mosmol/kg H₂O. All solutions were
equilibrated with 95% O₂, 5% CO₂. Once the solutions were
gassed and the pH checked, they were placed in a reservoir and
continuously bubbled with 95% O₂, 5% CO₂.

The actual pH of the solutions was monitored several times during the experi-
ments, and the pH of solutions was checked at the end of the experiment to ensure that changes did not occur. Carbonic
anhydrase (Sigma) was added to the perfusate solution (1
mg/10 ml of solution). The purpose of carbonic anhydrase was
to prevent any pH disequilibrium that might arise from proton
secretion or NH₃ transport. Total ammonium concentration
was measured in 10–12-nl samples of peritubular, perfused,
and collected fluids using an NH₃ diagnostic kit (Sigma) and
the flow-through microfluorometer Nanoflow apparatus (World
Precision Instruments, UK) (31).

Calculations of Transepithelial NH₃ Permeability—Assuming
an absence of osmotic or hydrostatic pressure gradients
across the epithelium and therefore an absence of net fluid
transport, the passive transepithelial transport of total ammno-
ium (Am) may be described by Equation 1,

\[ J_{\text{Am}} = \frac{P_{\text{NH}_3}}{V} \times A_s \times \Delta C_{\text{NH}_3} \]  
(Eq. 1)

where \(P_{\text{NH}_3}\) is diffusive permeability of NH₃ (cm/s), \(A_s\) is tubule
luminal surface area (cm²), and \(\Delta C_{\text{NH}_3}\) is the transepithelial
concentration difference for NH₃ (mm). To calculate the perme-
ability to NH₃, Equation 1 is rearranged as Equation 2,

\[ P_{\text{NH}_3} = \frac{J_{\text{Am}}(A_s \times \Delta C_{\text{NH}_3})}{V} \]  
(Eq. 2)

The net rate of transport \(J_{\text{Am}}\) is calculated as shown in Equation 3,

\[ J_{\text{Am}} = ([\text{Am}]_l - [\text{Am}]_p) \times V/L \]  
(Eq. 3)
Incomplete dRTA in Rhcg-targeted Mice

FIGURE 1. Rhcg gene targeting and deletion. A, Rhcg gene knock-out was achieved by replacement of exon 1 by a LacZ/neomycin cassette (Texas Institute of Genomic Medicine, Houston, TX). B, RT-qPCR with primers placed in exon 1 of Rhcg demonstrated lower mRNA in kidneys from Rhcg −/− mice and absence of a detectable PCR product in kidneys from Rhcg +/+ mice (n = 5 mice). C and D, Rhcg immunodetection in kidney sections from wild type (C) and Rhcg −/− (D) mice. * indicates significantly different p < 0.05.

where [Am] is the concentration of total ammonium in the perfusate; [Am]o is the concentration of total ammonium in the collected fluid; V is the collection rate (nl/min), as measured in precalibrated constriction pipettes, and L is the perfused tubule length (mm). A, may be calculated as Lmd, where d (mm) is the inner tubeule diameter. The total ammonium concentration ([Am]) is equal to the sum of the concentrations of the two species NH₃ and NH₄⁺ and is the quantity actually measured by the microfluorimetric assay. The equilibrium between the two species is defined by the Henderson-Hasselbalch Equation 4,

\[
\text{pH} = \text{p}K_a + \log\left(\frac{[\text{NH}_3]}{[\text{NH}_4^+]}\right) \tag{Eq. 4}
\]

The pK_a equals 9.03 at physiological pH and temperature. Knowing the values for pH and [Am], the values for [NH₃] and [NH₄⁺] may be determined simultaneously.

Statistics—Data are expressed as means ± S.E. Statistical comparisons were tested by analysis of variance and Student’s t test using the Graphpad Prism software (GraphPad). p values < 0.05 were considered as statistically significant.

RESULTS

Deletion of Rhcg in Mice—Mice lacking Rhcg were generated using gene trap technology in a mixed genetic background (129SvEvBrd, C57/BL6) (Fig. 1A) and were purchased from the Texas Institute of Genomic Medicine (32). Rhcg mRNA was undetectable in renal tissue from Rhcg knock-out (Rhcg −/−) mice and reduced by ~50% in heterozygous mice (Rhcg +/−) mice (Fig. 1B). RhCG protein was completely absent from the kidney of Rhcg −/− mice as confirmed by immunochemistry (Fig. 1C).

Heterozygous Mice Develop Metabolic Acidosis While on Long Term Acid Load—We first assessed acid-base status under basal conditions and during an acid load in Rhcg littermates. Throughout the study, food intake was similar in all three genotypes. At base line, no difference in acid-base and electrolyte levels was observed (Tables 1 and 2).

The effects of both acute (2 days) and chronic (7 days) HCl load were tested (Tables 1–3 and Fig. 2). On the 2nd day of the HCl load, blood pH and HCO₃− concentration were decreased in all genotypes, as compared with base line (Table 1, and Fig. 2, D and E). Blood pH and HCO₃− concentrations were significantly lower in Rhcg −/− mice but similar in Rhcg +/− and Rhcg +/+ mice. Urinary ammonium excretion rate increased significantly on the 1st day of acid loading in Rhcg +/+ and Rhcg +/− mice but much less in Rhcg −/− mice (Tables 2 and 3 and Fig. 2A). Urinary pH decreased in Rhcg +/+ and Rhcg +/− mice as compared with base-line values (Table 2 and Fig. 2B) but to a lesser extent in Rhcg −/− mice. Urinary titratable acid excretion was unaltered in all three genotypes during the acute acid load. Long term HCl loading resulted in the death of most Rhcg −/− mice, which poorly excreted ammonium and exhibited a very severe metabolic acidosis. These animals showed a higher loss of body weight after the acute HCl load presumably due to dehydration (Table 3).

In contrast to the Rhcg +/− mice, which adapted and nearly normalized their blood pH and HCO₃− concentration, Rhcg +/+ remained acidic at day 7 of the HCl load, even though both genotypes maintained a high NH₄⁺ excretion. At the end of the chronic acid load, Rhcg −/− mice showed less increase in their titratable acid excretion and had a more alkaline urine pH (Tables 1 and 2 and Fig. 2, B and C). Thus, both Rhcg −/− and Rhcg +/− exhibited renal acid handling defects.

Absence of RhCG Abolishes Medullary Ammonium Accumulation—To assess the cortico-papillary gradient of NH₃/NH₄⁺ in kidneys from Rhcg mice, we measured ammonium content in

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TABLE 1

| Basal status | 2 days HCl | 7 days HCl |
|--------------|------------|------------|
| pH | 7.38 ± 0.02 | 7.38 ± 0.02 | 7.28 ± 0.04 |
| pCO₂ (mm Hg) | 34.0 ± 1.3 | 34.0 ± 1.3 | 32.7 ± 0.9 |
| HCO₃⁻ (mM) | 19.0 ± 1.0 | 19.0 ± 1.0 | 18.3 ± 2.1 |
| pO₂ | 74.7 ± 9.7 | 74.7 ± 9.7 | 63.9 ± 8.3 |
| Na⁺ (mM) | 143.9 ± 1.5 | 146.4 ± 0.9 | 140.6 ± 0.4 |
| Cl⁻ (mM) | 117.5 ± 1.9 | 112.1 ± 1.7 | 111.7 ± 1.5 |
| Urea (mM) | 1.23 ± 0.02 | 1.25 ± 0.01 | 1.29 ± 0.03 |
| Glucose (mM) | 10.6 ± 0.7 | 10.9 ± 0.5 | 10.8 ± 0.8 |
| Hb (g/dL) | 15.0 ± 1.1 | 15.7 ± 1.0 | 16.3 ± 0.5 |

Weight (g) | ND
Weight loss in % by weight under basal status | ND
Food intake (g/24 h/body weight) | 0.31 ± 0.06

| Basal status | 2 days HCl | 7 days HCl |
|--------------|------------|------------|
| Weight (g) | 26.4 ± 0.6 | 27.6 ± 1.0 | 24.9 ± 0.5 |
| Weight loss in % of body weight under basal status | ND
| Food intake (g/24 h/body weight) | 0.26 ± 0.02

**p < 0.05 versus base-line period in same genotype is shown.
ND, not determined.

**p < 0.05 versus Rhcg⁻/⁻ mice during the same period is shown.

**p < 0.05 versus base-line period in same genotype is shown.

TABLE 3

| Basal status | 6 days of HCl | 7 days of HCl |
|--------------|---------------|---------------|
| Weight (g) | ND
| Weight loss in % of body weight under basal status | ND
| Food intake (g/24 h/body weight) | ND

| Basal status | 6 days of HCl | 7 days of HCl |
|--------------|---------------|---------------|
| Volume (mL/24 h) | 1.9 ± 0.2 | 2.0 ± 0.1 | 2.0 ± 0.1 |
| Creatinine excretion (µmol/24 h) | 7.6 ± 0.3 | 6.2 ± 0.6 | 7.8 ± 0.4 |
| Urinary pH | 6.13 ± 0.04 | 6.18 ± 0.06 | 6.03 ± 0.06 |
| UNH₄/UCr (mEq/mmol) | 4.6 ± 0.1 | 5.1 ± 0.7 | 4.1 ± 1.0 |
| UTA/UCr (mEq/mmol) | 15.4 ± 1.2 | 14.4 ± 1.0 | 13.6 ± 1.1 |
| UP/UCr (mEq/mmol) | 16.1 ± 1.7 | 20.3 ± 1.0 | 17.7 ± 1.4 |
| Urea (mg/dL) | 266.3 ± 14.8 | 279.3 ± 14.8 | 280.8 ± 15.0 |
| UNA/UCr (mEq/mmol) | 20.0 ± 1.4 | 19.4 ± 0.8 | 17.7 ± 1.3 |
| UNP/UCr (mEq/mmol) | 16.8 ± 2.0 | 10.0 ± 2.0 | 15.5 ± 2.0 |
| UC1/UCr (mEq/mmol) | 32.8 ± 2.0 | 30.6 ± 1.7 | 32.6 ± 2.3 |
| UK/UCr (mEq/mmol) | 60.1 ± 3.9 | 60.2 ± 4.6 | 63.1 ± 4.9 |

**p < 0.05 versus Rhcg⁻/⁻ mice during the same period is shown.

The cortex and outer and inner medulla after 4 days of HCl treatment. There was no difference between Rhcg⁻/⁻ and Rhcg⁻/⁻ mice. However, the inner medulla ammonium content was strongly reduced to 39% in Rhcg⁻/⁻ mice (Fig. 3). Thus, the absence of Rhcg impairs the ability to concentrate ammonium in the interstitium of the inner medulla.

RhCG Is Located at the Apical and Basolateral Sides of Cells Along the Distal Neprhon—RhCG has been localized to most cells of the CD, including type A intercalated cells as well as principal cells (9, 17). However, the subcellular localization of RhCG has remained controversial because some groups reported both apical and basolateral staining (10, 17), whereas...
others detected only apical staining for RhCG (9) based on different immunohistochemical methods. We confirmed a strong labeling of both apical and basolateral poles of CD cells in mouse kidneys (Fig. 4 and Table 4). This staining was absent from Rhcg−/− kidneys demonstrating its specificity. In the kidney cortex, RhCG was localized in the distal convoluted tubule, connecting tubule, and CCD (Fig. 4A). In the distal convoluted tubule cells, RhCG was mainly present at the apical side (Fig. 4A). In the connecting tubule, CCD and OMCD, both intercalated and principal cells, were stained. Principal cells and some intercalated cells exhibited RhCG staining at both the apical and basolateral sides (Fig. 4, A–C). However, in some cells
RhCG was strictly localized at the apical pole (see arrowheads in Fig. 4, A, B, and D). These cells have been previously identified as non-A/non-B type intercalated cells (33, 34). Finally, in the inner medulla, only strong apical and faint basolateral staining were found in intercalated cells (Fig. 4 and Table 4). Immunogold electron microscopy demonstrated RhCG associated with the apical membrane as well as the basolateral interdigitations of segment specific and intercalated cells (Fig. 4, E and F). No specific immunogold labeling was found in kidneys from Rhcg/−/− mice (data not shown). Thus, RhCG protein is expressed on both apical and basolateral membranes in mouse segment-specific principal and intercalated CD cells.

**Total and Apical Membrane Permeabilities for NH₃ Are Reduced in CDs from Rhcg+/− Mice**—We assessed total transepithelial permeability for NH₃ in in vitro microperfused cortical CDs from Rhcg mice after a 2-day HCl diet, a condition causing a strong difference in urinary ammonium excretion between Rhcg+/+ and Rhcg−/− mice. Imposing a bath-to-lumen NH₃ gradient in the nominal absence of an NH₃ gradient generated a measurable NH₃ secretory flux, which was significantly lower in CCDs from Rhcg+/+ and Rhcg−/− mice versus Rhcg+/−/− mice. These differences were due to a decrease in transepithelial permeability to NH₃ by 54 and 83%, respectively (Table 5 and Fig. 5). Thus, one Rhcg allele was not sufficient to sustain normal transepithelial permeability to NH₃ in the mouse collecting duct.

Next, we tested whether the apical permeability to NH₃ was affected in CDs from Rhcg+/− mice. Therefore, we measured the effects of an inwardly directed gradient on pH₄ on CCDs isolated from Rhcg+/+ and Rhcg+/− mice (13). Fig. 6A depicts the typical time course of pH₄ changes when NH₃/NH₄⁺ was added to the lumen tubule. The initial rate of cellular alkalinization is proportional to the rate of apical NH₃ entry as described previously (13, 35, 36). To directly compare transport rates, we measured intracellular buffering power and calculated the amount of H⁺ used to titrate NH₃ transported across the membrane. Fig. 6B depicts the calculated rate of NH₃ transported into CDs from Rhcg+/+ and Rhcg+/− mice, which was drastically reduced in Rhcg−/− tissue.

**Basolateral RhCG Accounts for Peritubular Membrane Permeability to NH₃**—Because RhCG is expressed at both sides of CD cells, transport pathways for NH₃ have been proposed at the basolateral side of CD cells, including the Na⁺−K⁺−2Cl⁻ cotransporter NKCC1 and the Na/K-ATPase where NH₃ would substitute for K⁺ (37, 38). Therefore, we performed experiments in the nominal absence of sodium to block the activity of both transport pathways. Peritubular NH₃/NH₄⁺ prepulses were performed on cortical CDs from Rhcg+/+, Rhcg+/−, and Rhcg−/− mice submitted to HCl loading for 2 days. As summarized in Fig. 7, when NH₃/NH₄⁺ (6 mM) was applied to the basolateral side, the calculated rate of NH₃ transport into CD cells was unchanged in Rhcg+/− tissue but strongly reduced in Rhcg−/− tissue. Thus, RhCG sustains both apical and basolateral transport of NH₃ in CD cells, in agreement with our immunohistochemical results.
Incomplete dRTA in Rhcg-targeted Mice

**TABLE 4**

Summary of RhCG localization along the mouse nephron

| Tubule type | Cell type  | Localization       | Staining intensity |
|-------------|------------|--------------------|--------------------|
| Cortex      | DCT        | Apical             | High               |
|             |            | Basolateral        | Weak               |
| CNT         | Principal cells | Plasma membrane | High               |
|             | Intercalated cells | Plasma membrane/apical | High               |
| CDD         | Principal cells | Plasma membrane | High               |
|             | Intercalated cells | Plasma membrane/apical | High               |
| Outer medulla | OMCD        | Principal cells | No staining         |
| Inner medulla | IMCD        | Principal cells | Plasma membrane/apical | Weak/high |

**TABLE 5**

CCD *in vitro* microperfusion data from Rhcg+/+, Rhcg+-/-, and Rhcg-/- mice

Values are mean ± S.E.; n means no. of mice.

| Rhcg+/+ (n = 7) | Rhcg+-/- (n = 9) | Rhcg-/- (n = 5) |
|-----------------|------------------|----------------|
| Tubule length, mm | 0.38 ± 0.04 | 0.36 ± 0.05 | 0.034 ± 0.08 |
| Tubule diameter, μm | 54.98 ± 4.55 | 47.92 ± 4.24 | 44.31 ± 8.88 |
| Collection rate, nl/min·mm⁻¹ | 4.61 ± 0.22 | 4.37 ± 0.29 | 3.96 ± 0.42 |
| Perfusate pH | 6.44 ± 0.04 | 6.37 ± 0.04 | 6.50 ± 0.04 |
| Bath pH | 7.42 ± 0.03 | 7.37 ± 0.03 | 7.38 ± 0.03 |
| Total ammonia perfusate, mm | 1.41 ± 0.08 | 1.40 ± 0.08 | 1.42 ± 0.13 |
| [NH₄] perfusate, μM | 3.74 ± 0.37 | 3.30 ± 0.32 | 3.16 ± 0.25 |
| Total ammonia bath, mm | 1.41 ± 0.08 | 1.40 ± 0.08 | 1.42 ± 0.13 |
| [NH₄] bath, μM | 34.38 ± 3.26 | 32.57 ± 3.72 | 31.39 ± 3.71 |
| Total ammonia collected, mm | 3.88 ± 0.42 | 2.47 ± 0.36 | 1.48 ± 0.35 |
| [NH₄] collected, μM | 10.37 ± 1.45 | 5.90 ± 1.24* | 4.25 ± 0.93* |
| Total ammonia flux, pmol/mm·min⁻¹ | 32.98 ± 3.32* | 17.84 ± 4.56* | 2.51 ± 3.51* |
| NH₃ permeability, mm/s | 0.13 ± 0.02 | 0.08 ± 0.02* | 0.02 ± 0.02* |

* p < 0.05 versus control mice.

with structural data and reconstituted RhCG suggesting that the protein forms a NH₃-permeable channel (39, 40).

Compensatory Adaptations to the Loss of Rhcg—We finally examined whether Rhcg deletion affects mechanisms involved in renal ammoniagenesis and ammonium excretion. Surprisingly, immunoblot of whole kidney extracts from mice subjected to a 2-day HCl load revealed no differences in the expression of PEPC expression (Fig. 8A). In contrast, the expression of the PDG, mediating the initial step of ammoniagenesis, was decreased in Rhcg-/- kidneys as compared with both Rhcg+/+ and Rhcg+-/- kidneys (Fig. 8B). The abundance of the Na⁺/H⁺ exchanger NHE3, involved in NH₃ secretion into the proximal tubule lumen, was unchanged (Fig. 8C). Moreover, the expression of the Na⁺/K⁺/2Cl⁻ cotransporter NKCC2, the main apical NH₃ transporter in the thick ascending limb, was highly down-regulated in kidneys from Rhcg-/- and Rhcg-/- mice (Fig. 8D).

**DISCUSSION**

Renal ammonium excretion is critical for acid-base homeostasis and is achieved by a complex process involving various nephron segments (2, 5, 6). A critical role for the Rhesus protein RhCG was demonstrated in tissue-specific and complete knock-out mouse models (13–15). However, metabolic effects of Rhcg haploinsufficiency, the role of RhCG in basolateral NH₃ transport in CD cells, and the response of other pathways critical for renal ammoniagenesis and ammonium transport have remained unknown. Based on a novel Rhcg mouse model, we show that Rhcg haploinsufficiency causes incomplete metabolic acidosis in mice. Next, RhCG contributes to peritubular NH₃ uptake giving functional evidence to the basolateral localization of RhCG in CD cells.

**Rhcg-/- Mice Develop a Severe Incomplete Renal Tubular Acidosis—**Rhcg-/- develop incomplete dRTA as evident from the inability to respond to an acute oral acid load. They showed severe hyperchloremic metabolic acidosis and did not increase urinary ammonium excretion resulting in low net acid excretion. Moreover, these animals had a drastic reduction in their blood HCO₃⁻ concentration and pH. Rhcg-/- mice developed severe dehydration as indicated by high blood hemoglobin and weight loss (Tables 2 and 4). These phenotypes are more pronounced than in mice with partial deletion of Rhcg (14, 15).
The first evidence that loss of a single Rhcg allele can lead to chronic hyperchloremic metabolic acidosis.

Renal Adaptation to Rhcg Invalidation—We further examined compensatory mechanisms in Rhcg+/- and Rhcg+/− mice. The generation of the cortico-papillary gradient of ammonium is required for the subsequent uptake of NH4+ and NH4+ by intercalated cells and the secretion of NH3 into urine. Surprisingly, Rhcg−/− mice had low tissue ammonium content in the inner medulla after 4 days of acid loading. This result suggests that the absence of RhCG affects the ability of the medulla to generate or maintain a high interstitium ammonium content. Because the key enzymes of proximal tubular ammoniagenesis, PEPCK and PDG, were normally expressed in kidney tissues, NKCC2 is crucial for thick ascending limb NH4+ absorption and concentration in the medulla. Thus, our results suggest that mechanisms contributing to create a high medullary ammonium concentration are altered in Rhcg-deficient mice. Other mechanisms such as increased removal of ammonium from the interstitium with venous blood might contribute also to the low medullary ammonium content. Metabolic acidosis alters concentrations of various vasoactive substances in the kidney, including higher levels of endothelin and prostaglandins and lower concentrations of NO (41, 42), which may alter medullary blood flow and the ability of the kidneys to maintain the cortico-papillary ammonium gradient.

RhCG Mediates NH3 Transport Also at the Basolateral Side of CD Cells—Subcellular RhCG localization has remained controversial for many years (9, 10, 17). Here, we confirmed a strong labeling of both apical and basolateral poles of CD cells in mouse kidneys. These data were corroborated by our functional study on in vitro microperfused CDGs. We measured a 60% reduction in the NH3 permeability at the basolateral side of
Rhcg<sup>−/−</sup> CCD cells. This is the first evidence that Rhcg is also functional at the basolateral side of CDs cells and participates in basolateral NH₃ uptake. Previously, it has been assumed that basolateral uptake occurs mostly if not exclusively in the form of NH₄⁺ and that NH₃ plays no major role. Several pathways for NH₄⁺ uptake have been delineated from pharmacological and functional experiments that demonstrated a role for the Na⁺/K⁺/2Cl⁻ cotransporter NKCC1, the Na⁺/K⁺-ATPase, and possibly potassium channels where NH₄⁺ always would replace potassium (37, 38, 43). We performed our experiments in the absence of sodium to reduce NKCC1 and Na⁺/K⁺-ATPase activity, respectively. Thus, our experiments do not allow conclusions regarding the relative importance and contribution of these different pathways to total NH₃/NH₄⁺ uptake across the basolateral membrane, but they modify the current model of ammonium transport across the basolateral membrane.

In summary, reduced expression or complete loss of expression of RhCG affects the ability of the kidneys to excrete acidic urine and appropriately regulate acid/base homeostasis. We provide the first functional evidence for basolateral RhCG activity and show that RhCG is expressed and functional on apical and basolateral membranes of most cells lining the renal collecting duct. Thus, Rhcg haploinsufficiency or Rhcg deletion may contribute to orphan forms of inherited distal renal tubular acidosis in humans as well as to acquired forms of dRTA.

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Haploinsufficiency of the Ammonia Transporter Rhcg Predisposes to Chronic Acidosis: Rhcg IS CRITICAL FOR APICAL AND BASOLATERAL AMMONIA TRANSPORT IN THE MOUSE COLLECTING DUCT

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