The rhesus protein RhCG: a new perspective in ammonium transport and distal urinary acidification

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Urinary acidification is a complex process requiring the coordinated action of enzymes and transport proteins and resulting in the removal of acid and the regeneration of bicarbonate. Proton secretion is mediated by luminal H^+-ATPases and requires the parallel movement of NH_3, and its protonation to NH_4^+, to provide sufficient buffering. It has been long assumed that ammonia secretion is a passive process occurring by means of simple diffusion driven by the urinary trapping of ammonium. However, new data indicate that mammalian cells possess specific membrane proteins from the family of rhesus proteins involved in ammonia/μm permeability. Rhesus proteins were first identified in yeast and later also in plants, algae, and mammals. In rodents, RhBG and RhCG are expressed in the collecting duct, whereas in humans only RhCG was detected. Their expression increases with maturation of the kidney and accelerates after birth in parallel with other acid–base transport proteins. Deletion of RhBG in mice had no effect on renal ammonium excretion, whereas RhCG deficiency reduces renal ammonium secretion strongly, causes metabolic acidosis in acid-challenged mice, and impairs restoration of normal acid–base status. Microperfusion experiments or functional reconstitution in liposomes demonstrates that ammonia is the most likely substrate of RhCG. Similarly, crystal structures of human RhCG and the homologous bacterial AmtB protein suggest that these proteins may form gas channels.

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The kidneys excrete ~70 mmol of acids per day from the body. Only a minute fraction is excreted as free protons, but most acids are in the form of ammonium (about 2/3) and titratable acids (about 1/3), such as phosphate. The importance of renal acid elimination is underlined by a variety of syndromes of acquired or inherited forms of renal tubular acidosis.[1,2] Chronic metabolic acidosis represents a major morbidity and mortality risk factor and may even accelerate deterioration of renal function in patients with early stages of renal disease.[3,4]

MECHANISMS OF DISTAL ACID EXCRETION

Type A intercalated cells (A-ICs) in the collecting duct system (late distal convoluted tubule to the initial third of the inner medullary collecting duct) mediate the removal of acids (protons and ammonium), as well as the *de novo* generation of bicarbonate.[2] Cytosolic carbonic anhydrase II hydrates CO_2 to form H^+ and HCO_3^-, which in turn is released into the interstitium involving the basolateral and A-IC-specific chloride/bicarbonate exchanger AE1.[5,6] H^+-ATPases localized at the luminal pole of A-IC excrete protons,[7] thereby acidifying urine. However, H^+-ATPases can establish a maximal pH gradient of about 2–2.5 units pH between the intracellular compartment (~pH 7.2) and urine, thus limiting removal of hydrogen ions. The daily amount of acids removed is about 1 mEq per kg body weight (about 70 mEq in a healthy adult person). The excretion of this amount of acid in an unbuffered solution would thus require several hundred liters of urine (1 l of unbuffered urine, pH 4.5, containing maximally 30 μM protons). Titratable acids (mainly phosphate, to a lesser extent citrate, and creatinine) can help buffer protons (about 1/3 of the daily acid load). A major fraction of protons, however, is buffered by ammonia after parallel secretion into urine (approximately 2/3 of the daily acid load). Ammonia secretion occurs along the entire length of the collecting duct system, but increases substantially in the later parts.[8,9]

In 1945, Robert Pitts[10,11] had described in two seminal papers the role of ammonium in renal acid secretion and postulated that ammonium secretion is a passive process driven by the ammonia concentration gradient, the diffusion of ammonia across the luminal membrane, and the

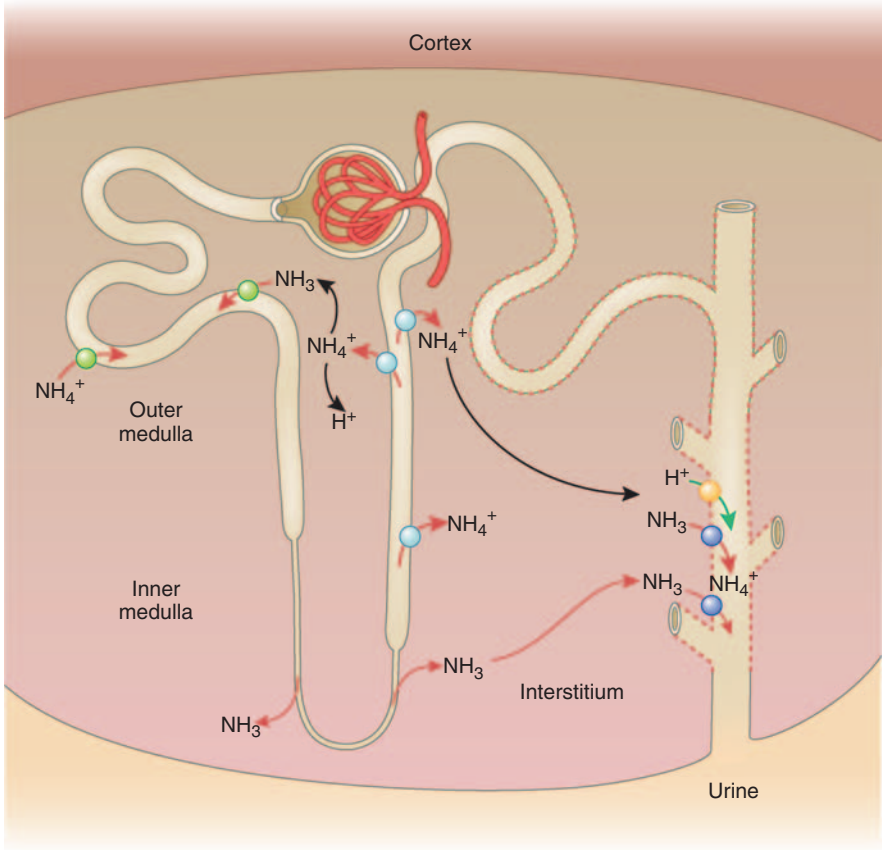

Figure 1 | Urinary ammonium generation and transport along the nephron. Ammonium is generated in the cells of the proximal tubule from metabolism of glutamine and secreted into primary urine. At the level of the thin descending and ascending limb of the loop of Henle, low amounts of NH_3 are absorbed into the interstitium, generating a cortico-papillary gradient (shaded in red). Massive absorption of NH_4^+ occurs in the thick ascending limb of Henle via the furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter NKCC2 accumulating high interstitial concentrations of NH_4^+. Finally, NH_3 is secreted into urine along the collecting duct, protonated, trapped as NH_4^+, and excreted with urine. Colored circles indicate carrier-mediated transport of NH_4^+ or NH_3, red and green dotted lines indicate expression of RhCG in intercalated (red) or principal cells (green) (for details see text).

subsequent trapping of ammonium in urine after protonation. This hypothesis remained textbook knowledge until recently. Further work demonstrated that ammoniagenesis occurs from metabolism of glutamine in the proximal tubule regenerating the bicarbonate lost while buffering protons stemming from metabolism.[12–14] Ammonium is then secreted into urine at the level of the proximal tubule, is mostly actively reabsorbed in the thick ascending limb, and finally accumulates in the interstitium with a high cortico-medullary gradient (Figure 1).[8] This interstitial high concentration, together with a pH gradient (from inside the cells of the collecting duct into urine), provides the driving force for ammonia transport by intercalated cells. Ammonia secretion in the collecting duct is mediated by intercalated cells, but principal cells may also contribute although their permeability is lower.[15]

NH_3/NH_4^+ uptake from interstitium by intercalated cells may be mediated by several pathways, including the $Na^+/K^+/2Cl$-cotransporter NKCC1, the Na^+/K^+-ATPase, and might involve also the RhCG protein (for a review, see Weiner and Hamm[9]). Preliminary data from our group show reduced basolateral NH_3 permeability in RhCG KO mice. The final step of ammonia secretion into urine had been studied in great detail by Knepper and colleagues[8] using microperfusion experiments and demonstrating that it involved high apical ammonia permeability. Whether this is an active process or involves transport proteins has remained elusive.

RHESUS PROTEINS: NOVEL AMMONIUM TRANSPORT PROTEINS?

The discovery of Marini *et al.*[16] that the mammalian homologs (RhAG and RhGK/RhCG) of the yeast methyl-ammonia permeases ammonium transporters could also mediate transport of ammonia/um opened the possibility that these molecules might participate in renal ammonium elimination. Similar molecules were also found in plants, algae, and fish. Expression of rhesus proteins RhAG, RhBG, and RhCG in various heterologous cell models induced ammonia/ammonium transport. However, the mode of transport and exact substrate (NH_3 or NH_4^+), as well as the coupling to other ions (counter- or cotransport of protons) and stoichiometry, have remained controversial

(see below and for a review, Weiner and Hamm[9]). Moreover, deletion of the algae Rh1 protein suggested even the possibility that rhesus proteins might be involved in CO_2 permeability of biological membranes.[17] In fish, four homologous proteins, fRhag, fRhbg, Rhcg1, and Rhcg2, are expressed in gills, transport ammonia in heterologous expression system, and are thought to mediate active ammonia excretion.[18,19] In mammals, members of the rhesus protein family are expressed in various organs and distinct cells. RhAG is mainly detected in erythrocytes, RhBG in the liver, kidney, and ovary, and RhCG in the kidney, liver, brain, skeletal muscle, prostate, and pancreas.[9] However, recent data suggest that species differences may exist. RhBG protein was detected in mouse and rat kidney, but not in human kidney.[20]

RHESUS PROTEINS IN MAMMALIAN KIDNEY

In the kidney, RhBG and RhCG have been localized exclusively to the distal tubule, connecting tubule, and cortical and medullary collecting duct.[9,20,21] Small differences in the localization have been reported from different laboratories using different antibodies and different species. RhBG was detected in mouse and rat kidney on the basolateral side of various cell types. RhBG is found in the distal convoluted tubule in intercalated cells (and possibly also in distal convoluted tubule cells), in the connecting tubule in all cell types, in the cortical collecting duct in A-ICs and principal cells in mouse, whereas in rat expression might be restricted to A-ICs. In the outer medullary collecting duct and inner medullary collecting duct, only A-ICs express RhBG.[21,22] The localization of RhCG has been reported in mouse, rat, and human kidney. However, the exact distribution is controversial. Quentin *et al.*[21] described only apical staining for RhCG in rat kidney, whereas the laboratory of D. Weiner has reported both apical and basolateral staining for RhCG in human, rat, and mouse kidney (for references, see detailed review[9]). In human kidney, RhCG localization has been reported for basolateral and apical membranes, but RhBG could not be detected.[9,20] Our own results support the notion of RhCG localization at both poles of cells in mouse and human kidney. RhCG is found in the late distal convoluted tubule, in the connecting tubule and cortical collecting duct, and in the outer stripe of the outer medulla in all cell types (possibly excluding non-A-ICs), whereas in the late outer medullary in collecting duct and inner medullary collecting duct only A-ICs are stained.[20,22–24]

Ammonia excretion along the collecting duct varies in the different subsegments.[8] During acidosis, the cortical collecting duct becomes a major site of ammonia secretion,[25] coinciding with the strongest staining for RhCG. Staining by RhCG is only weak in the inner stripe of the outer medulla and inner medulla, segments that secrete considerable amounts of ammonia.[8] One explanation might be that RhCG is required in the portions of the collecting duct (cortex) in which the ammonium gradient from interstitium to lumen is less steep and RhCG facilitates NH_3 transport, whereas in the medullary regions the gradient is steeper and ammonium excretion is less dependent on transport pathways.

ONTOGENY OF RHESUS PROTEINS IN DEVELOPING MOUSE KIDNEY

During pre- and postnatal nephrogenesis, the expression and maturation of several transport proteins implicated in the final urinary acidification is tightly regulated in order to compensate the acid-generating process of growth.[26–28] These changes are paralleled by the maturation of intercalated cells, which includes the acquisition of the subcellular localization of H^+-ATPases similar to the adult kidney and removal of non-A-ICs from the inner medulla and the inner stripe of the outer medulla.[7,26,29] These features correlate with the fact that mutations in *ATP6V0A4* and *ATP6V1B1* genes, encoding the intercalated cell-specific V_0 a4 and V_1 B1 subunits, respectively,[7] have been associated with early-onset cases of distal renal tubular acidosis (dRTA), suggesting that the segmental distribution of intercalated cell-specific isoforms of H^+-ATPases is acquired at birth or during early infancy. Jouret *et al.*[27] showed that the intercalated cell-specific a4 and B1 subunits were induced from E15.5 in the mouse developing kidney, following the onset of expression of the forkhead transcription factor, Foxi1. From E15.5, Foxi1 mRNA was detected in intercalated cells, where it co-distributed with B1 in late nephrogenesis. Our preliminary investigations in mouse kidney[30] reveal that both RhBG and RhCG show an early (E13.5) and progressive increase in the renal expression, followed by a strong induction after birth (Figure 2). A similar expression pattern was detected for the intercalated cell markers (a4 and B1 subunits of H^+-ATPase, AE1, pendrin) and the intercalated cell-specific transcription factor Foxi1. By *in situ* hybridization, RhCG was detected at E14.5 in kidney tubules. From E17.5 on, RhBG and RhCG are expressed in the distal convoluted and connecting tubules and collecting ducts. The adult kidney displayed a strong signal in the connecting tubule and cortical collecting ducts and in some cells lining the outer-medullary and inner-medullary collecting ducts. Immunostaining failed to identify RhBG at the embryonic stages analyzed, whereas RhCG expression was observed in developing collecting ducts at E17.5. After birth, RhBG and RhCG are expressed in cortex and medulla, in which they show distinct basolateral (RhBG) and basolateral and apical (RhCG) reactivity in intercalated cells, like in adult kidneys. Similar observations were made in rat kidney, with the difference that RhBG was detectable before birth and that RhCG was first detected in basolateral membranes and only later also in apical membranes.[31] To date, no detailed information is available on the mechanisms of transcriptional regulation of RhCG in the mammalian kidney.

IMPAIRED RENAL AMMONIUM EXCRETION IN RHCG KO MICE

RhBG-deficient mice were reported in 2005 by Chambrey *et al.*[32] These mice did not show altered urinary ammonium excretion under basal conditions or after an acid load. Basolateral NH_3/NH_4^+ permeabilities, as well as transepithe-

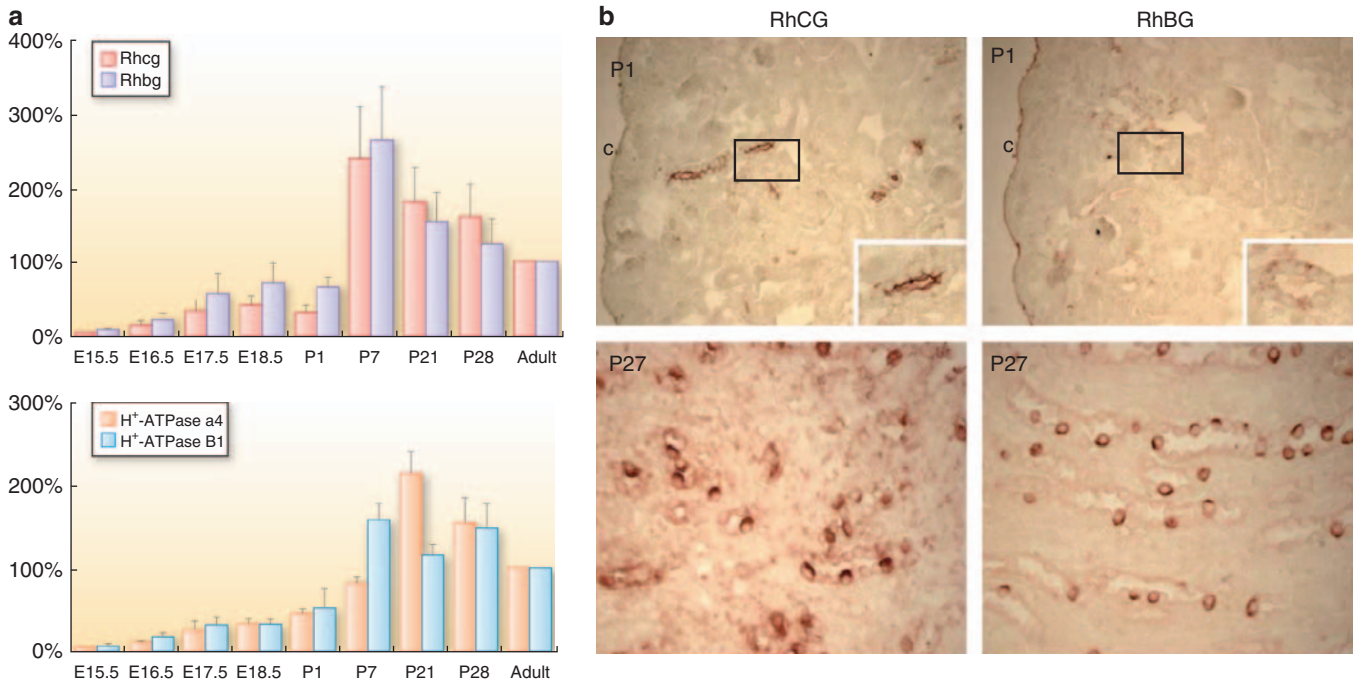

Figure 2 | Developmental expression of rhesus proteins RhBG and RhCG in mouse kidney. (**a**) Real-time pPCR analyses showed an early (E13.5) and progressive increase in the renal expression of both RhCG and RhBG, followed by a strong induction after birth (P7). A similar expression pattern was detected for the intercalated cell (IC) markers (a4 and B1 subunits of V-ATPase) and the IC-specific transcription factor Foxi1 (not shown). Relative mRNA expression levels are shown (in %) normalized against expression levels in adult kidney. (**b**) After birth, staining for RhCG is detected in the cortex and medulla, whereas RhBG is barely detected (postnatal day 1, P1). There is a massive induction of both isoforms after P7, matching the qPCR profiles. After P21, distinct apical (RhCG) and basolateral (RhBG) reactivity is observed in intercalated cells. Original magnification: **a**, ×100; **b**, ×400. Modified from Jouret *et al.*[27]

lial ammonia fluxes were similar in wildtype and RhBG KO mice. Thus, presently the physiological significance of RhBG remains unknown. The fact that RhBG does not have a major role in renal ammonium handling could be explained by the basolateral expression of RhCG or the existence of alternate basolateral entry pathways for ammonium.

In contrast, genetic ablation of RhCG in at least three different mouse models demonstrates a critical role for this protein in urinary ammonium excretion.[33–35] Two mouse models of complete RhCG deficiency[33,35] show only mildly reduced ammonium excretion under basal conditions and normal blood acid–base parameters. However, acid-loading mice with HCl or NH_4Cl in food induced more severe metabolic acidosis and KO mice had a very significant defect in their maximal capacity to increase urinary ammonium excretion as compared with wild-type mice. The excretion of titratable acidity is not affected by the loss of RhCG. Lower urinary ammonium excretion is most likely not due to the effect of reduced ammoniagenesis on the level of the proximal tubule, as the expression of ammoniagenic enzymes, and the concentration of blood glutamine were similar in all mice. At the cellular level, microperfusion experiments in the cortical and outer medullary collecting duct from acid-loaded mice demonstrated that the NH_3, but not the NH_4^+ permeability of the apical membrane was reduced by about 60%. Similarly, when we measured total transepithelial NH_3 permeability, we found a 60% reduction.[33] To further test the possible significance of basolaterally localized RhCG, we also performed experiments assessing the basolateral NH_3 and NH_4^+ permeabilities and found a reduction of the basolateral NH_3 permeability by about 40%, suggesting that RhCG contributes to basolateral NH_3 fluxes, but that additional pathways exist. Thus, RhCG is critical for urinary ammonium excretion and is required for collecting duct NH_3 secretion. A mouse model with partial deletion of RhCG only in the cortical and medullary collecting duct, but not in the connecting tubule (due to the choice of Cre-deleted mice under the control of the Ksp-cadherin promoter that is not expressed in the connecting tubule) shows similar features to the total KO mouse models with reduced urinary ammonium excretion under basal conditions and during an HCl acid load.[34] However, the reduction is less severe than in total KO mice. Partial KO mice had normal urinary pH, whereas total KO mice displayed more alkaline urine under all conditions.[33] These discrepancies may be explained by the important contribution of the late distal tubule and particularly the connecting tubule to overall renal acid excretion.[36] Taken together, these data indicate that RhCG is required for normal ammonium excretion, that on the levels of the apical and basolateral membranes RhCG is involved in mediating NH_3 fluxes, and that free diffusion, as postulated by Pitts, does not account for the majority of NH_3 excretion. What mediates the remaining NH_3 fluxes—free diffusion or membrane proteins—remains to be established.

Also, RhAG KO mice have been generated and show greatly reduced ammonia and methyl-ammonia fluxes in red blood cells,[37] resembling patients with inherited disorders of the red blood cells rhesus complex.[38]

In zebrafish, knock-down experiments with morpholinos reduced expression of fRhag, fRhbg, or fRhcg and ammonia secretion across gills, further supporting a role of rhesus proteins in ammonia transport in other species.[39]

IS RHCG A GAS CHANNEL?

The functional data from the isolated collecting duct indicate that RhCG is required for NH_3 fluxes, but the question remains whether RhCG is a channel or transporter for NH_3 or NH_4^+. Several lines of evidence from functional experiments and structural data suggest that RhCG and related rhesus proteins function as gas channels. First, functional data from heterologous expression systems have yielded controversial results whether RhCG mediates NH_3 uniport or NH_4^+/H^+ antiport. Heterologously expressed RhCG in mammalian cell lines, as well as in Xenopus oocytes, provided evidence that both NH_3 and NH_4^+ interact with the protein.[40,41] Experiments in Xenopus oocytes expressing several aquaporine water channels and rhesus family members demonstrated NH_3 permeability in the rhesus, but not in aquaporine family members.[42] The *in vitro* microperfusion experiments in the cortical collecting duct and outer medullary collecting duct from wild-type and RhCG-deficient mice are consistent with NH_3 fluxes, but do not rule out other transport modes. Second, the related RhAG protein mediates NH_3 fluxes in human and in mouse red blood cells.[38,43] Ablation of the green alga Rh1 protein affects CO_2 permeability, hinting at a role of rhesus protein in gas permeation.[17] Third, reconstitution of human RhCG in liposomes demonstrates NH_3, but not NH_4^+ fluxes.[44,45] Finally, crystal structures from the *E. coli* homolog AmtB, as well as from human RhCG, have become available at high resolution.[44,46–48] The data show a vestibule gated by phenylalanines and a pore region lined by histidine residues, which would exclude charged molecules such as NH_4^+ and allow only the passage of a neutral NH_3. Collectively, these data demonstrate that RhCG or other family members are not only subunits of the permeation pathway but also form the pore of the transporter/channel and mediate the passage of the gas NH_3.

REGULATED AMMONIUM EXCRETION

According to Overton's rule,[49,50] gases such as CO_2 or NH_3 are thought to move easily across biological membranes via diffusion with a few exceptions, including the apical membrane of the thick ascending limb of the loop of Henle.[51] Apparently, the apical membrane of collecting duct cells depends also on the presence of RhCG for its high NH_3 permeability during states of acidosis. One major role of RhCG is to mediate NH_3 fluxes. Another important role may be inferred from the observation that RhCG expression is strongly regulated during conditions associated with high urinary ammonium excretion, such as metabolic acidosis. Weiner and colleagues[52,53] demonstrated that RhCG protein abundance increases and that more RhCG protein is shifted to the apical membrane. In contrast to a membrane freely permeable to NH_3, the presence of specific transport proteins, such as RhCG, may provide the kidney with the ability to regulate and rapidly adapt urinary ammonium excretion by controlling its transport rates. Clearly, understanding the acute and chronic regulation of ammonium transport and the role of RhCG will require further experiments.

Recent experiments from our group using microperfusion of isolated collecting duct segments indicate that the genetic ablation of RhCG is associated with a strong reduction in H^+-ATPase activity in A-ICs.[35] However, at light microscopy level the localization of several H^+-ATPase subunits appeared normal. It remains speculative at this time whether RhCG may have an additional role as regulator of H^+-ATPase activity or whether deprotonation of NH_4^+ by RhCG may provide a major source of protons for the H^+-ATPase. Nevertheless, it may explain why RhCG-deficient mice have more alkaline urine.

ROLE OF RHCG IN RENAL DISEASE?

Various genes have been identified that cause dRTA in humans or rodent models, including the AE1 exchanger, the a4 and B1 subunits of the H^+-ATPase, carbonic anhydrase II, or proteins involved in collecting duct sodium reabsorption and its regulation by aldosterone.[1,5] It is tempting to speculate that RhCG may be another candidate gene for dRTA in humans, in those patients in whom no mutations in any of these known genes have been identified to date. Direct sequencing of patients with recessive forms of dRTA yielded no evidence for RhCG mutations so far. Arguably, such patients may be difficult to detect, as testing for an incomplete distal RTA remains challenging and is not a standard procedure in most centers.

Of interest, our recent data suggest that also heterozygous mice lacking only one allele of RhCG develop a form of incomplete dRTA.[35] Similarly, in a rat model of cyclosporine-induced renal tubular acidosis, reduced expression of RhCG was reported.[54] Thus, reduced levels of RhCG expression (such as in haploinsufficiency of RhCG) and/or activity due to either genetic inactivation or dysregulation may be involved in specific syndromes of complete or incomplete dRTA.

SUMMARY AND PERSPECTIVES

The discovery that the rhesus proteins RhAG, RhBG, and RhCG are involved in mediating cellular NH_3/NH_4^+ transport has opened a new field of investigations into the role of these interesting proteins. In the kidney, RhCG appears to be an important molecule in ammonium excretion and urinary acidification along the collecting duct. Our picture of how the collecting duct excretes ammonium has to be revised with these new findings (Figure 3). However, many open questions

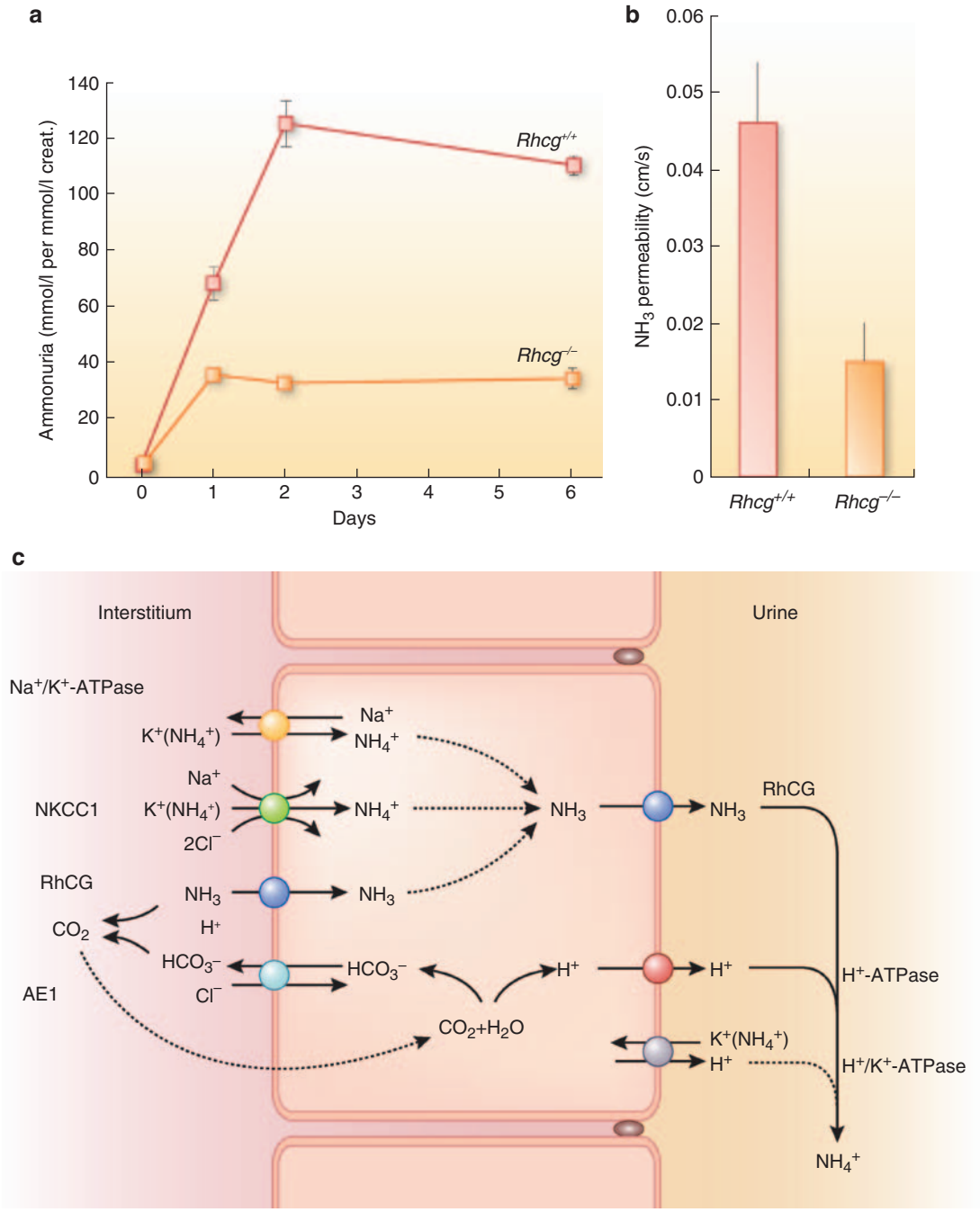

Figure 3 | Role of RhCG in urinary ammonium excretion. (**a**) Urinary ammonium/creatinine ratios from wild-type and RhCG-deficient mice over a period of 6 days of HCl loading. (**b**) Microperfusion experiments in isolated cortical collecting ducts demonstrated reduced transepithelial NH_3 permeability measured as NH_3 fluxes from bath to lumen (taken from Biver *et al.*[33]). (**c**) Novel model for the transepithelial transport of ammonium and ammonia across acid-secretory type A intercalated cells. These cells generate bicarbonate from CO_2 using carbonic anhydrase II (CAII); the newly formed HCO_3^- is released back to blood by basolateral chloride/bicarbonate exchangers, including AE1, whereas the protons are actively secreted into urine mainly by H^+-ATPase and to a lesser extent by H^+/K^+-ATPases. Ammonium uptake from the interstitium into the cells may be mediated by a variety of transporters, including the NKCC1 Na^+/K^+-$2Cl^-$-cotransporter, the Na^+/K^+-ATPase (substituting for K^+), or basolateral RhCG. Secretion of NH_3 into urine requires RhCG on the apical membrane; the proton released by this process may be secreted by H^+-ATPases. In the lumen of the collecting duct, NH_4^+ is trapped because of the low urinary pH.

remain, such as the exact transport mechanism, the acute and long-term regulation of expression and activity, its functional and physical interaction with other proteins, or its role in other extrarenal organs. Obviously, NH_3 can be excreted to some extent even in the complete absence of RhCG. What mediates NH_3 permeability—free diffusion or a transporter/channel—will be interesting to test. The role of RhCG in renal diseases of inborn or acquired forms of impaired

urinary acid excretion is to be unraveled. The fact that RhCG appears to be highly regulated makes it likely that its regulation and dysregulation may contribute to specific forms of renal tubular acidosis. The role of RhCG in other tissues such as epididymis, lung, or liver has not been examined in much detail and may link this protein to important functions, such as ammonium detoxification or male fertility.

DISCLOSURE

All the authors declared no competing interests.

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