The ammonia transporter RhCG modulates urinary acidification by interacting with the vacuolar proton-ATPases in renal intercalated cells

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Ammonium, stemming from renal ammoniagenesis, is a major urinary proton buffer and is excreted along the collecting duct. This process depends on the concomitant secretion of ammonia by the ammonia channel RhCG and of protons by the vacuolar-type proton-ATPase pump. Thus, urinary ammonium content and urinary acidification are tightly linked. However, mice lacking Rhcg excrete more alkaline urine despite lower urinary ammonium, suggesting an unexpected role of Rhcg in urinary acidification. RhCG and the B1 and B2 proton-ATPase subunits could be co-immunoprecipitated from kidney. In *ex vivo* microperfused cortical collecting ducts (CCD) proton-ATPase activity was drastically reduced in the absence of Rhcg. Conversely, overexpression of RhCG in HEK293 cells resulted in higher proton secretion rates and increased B1 proton-ATPase mRNA expression. However, in kidneys from *Rhcg*$^{-/-}$ mice the expression of only B1 and B2 subunits was altered. Immunolocalization of proton-ATPase subunits together with immuno-gold detection of the A proton-ATPase subunit showed similar localization and density of staining in kidneys from *Rhcg*$^{+/+}$ and *Rhcg*$^{-/-}$mice. In order to test for a reciprocal effect of intercalated cell proton-ATPases on Rhcg activity, we assessed Rhcg and proton-ATPase activities in microperfused CCD from *Atp6v1b1*$^{-/-}$ mice and showed reduced proton-ATPase activity without altering Rhcg activity. Thus, RhCG and proton-ATPase are located within the same cellular protein complex. RhCG may modulate proton-ATPase function and urinary acidification, whereas proton-ATPase activity does not affect RhCG function. This mechanism may help to coordinate ammonia and proton secretion beyond physicochemical driving forces.

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The renal collecting duct excretes acid into urine by parallel secretion of protons (H^+) and ammonia (NH_3) by vacuolar H^+-ATPases (H^+-ATPase) and the ammonia transporter RhCG.[1–3] Renal acid excretion is critical for maintaining normal acid-base homeostasis and is increased during acidosis or following a systemic acid-load.[2]

Highly conserved through evolution from bacteria to humans, RhCG belongs to the Rh (Rhesus) glycoprotein family and has been characterized by *in vitro* and *ex vivo* studies as a channel selectively permeable to NH_3 but not to ammonium (NH_4^+).[4–6] In the kidney, RhCG expression specifically spreads from the late distal convoluted tubule to the inner medullary collecting duct, on both basolateral and apical poles of most epithelial cells (with the exception of selective apical expression in non–type-A intercalated cells).[7–11] We recently showed that RhCG is responsible for a major part of NH_3 transported through apical and basolateral membranes of the collecting duct resulting in a drastic reduction of transepithelial NH_3 transport in collecting ducts from *Rhcg*$^{-/-}$ mice.[11,12] As a consequence of this defect, chronically acid-loaded *Rhcg*$^{-/-}$ mice cannot eliminate NH_4^+ into urine and develop a severe incomplete distal renal tubular acidosis characterized by low blood pH and HCO_3^- concentration.[11,12] Similarly, mice with selective deletion of Rhcg from only the collecting duct or intercalated cells show reduced urinary ammonium excretion.[13,14]

While RhCG is responsible for luminal NH_3 secretion, the renal H^+-ATPase is critical to actively secrete H^+ into urine.[15,16] H^+-ATPases are ubiquitous multi-subunit proteins composed of a cytosolic V_1 catalytic domain, responsible for ATP hydrolysis and a membrane-associated V_0 domain mediating H^+ translocation.[16,17] In the collecting duct, H^+-ATPases are localized at the luminal side of type-A intercalated cells and basolateral side of non–type-A intercalated cells. The V_1 domain contains 8 subunits A–H, including the

tissue-specific subunit isoforms B1 and B2. The B1 isoform is highly enriched in intercalated cells of kidney and its genetic mutation or deletion cause a form of distal renal tubular acidosis.[18–20] The V_0 domain contains 6 different subunits, a, d, c, c', c", and e, including a1, a2, a3, and a4. Also, the a4 isoform is highly expressed in kidney[21–22] and mutations in humans and its deletion in mice are associated with renal tubular acidosis.[23–25]

Urinary acidification and ammonium content are closely linked.[26] Secretion of NH_3 and H^+ by RhCG and H^+-ATPases may be coordinated, as protonation of NH_3 and subsequent trapping of NH_4^+ are critical for the efficient excretion of NH_4^+.[26–29] The partial overlap of both proteins in type-A intercalated cells may allow even for a direct functional coupling or interaction.[30] We have recently shown in 2 differently constructed mouse models lacking Rhcg that urinary pH is more alkaline during stimulation of urinary acidification and NH_4^+ excretion.[11,12] Because *Rhcg*$^{-/-}$ mice have highly reduced NH_3 secretion, the lack of NH_3 buffering capacity would be expected to result in a rather more acidic urinary pH, as shown in another mouse model with intact Rhcg-mediated NH_3 secretion but reduced medullary NH_4^+ accumulation.[31] Thus, we hypothesized that the absence of Rhcg may reduce proton secretion by H^+-ATPases.

RESULTS

Rhcg and B1 and B2 subunits of H^+-ATPase are part of 1 protein complex in native kidney

To assess whether RhCG and H^+-ATPase were located within the same cellular protein complex in intercalated cells, we performed co-immunoprecipitation of RhCG and B1 and B2 H^+-ATPase subunits from native rat kidney medulla (Figure 1). Immunoprecipitation with antibodies against RhCG or B1 (Figure 1a and b) or RhCG or B2 (Figure 1c and d) after chemical crosslinking yielded protein complexes that contained both proteins, whereas immunoprecipitation with control IgG did not detect RhCG or the B subunits. Thus, RhCG, B1, and B2 containing H^+-ATPases are part of the same protein complex in kidney.

We further confirmed partial colocalization of Rhcg and B1 subunits at the apical pole of intercalated cells in mouse kidney by immunohistochemistry (Figure 1e).

Rhcg$^{-/-}$ mice show a reduced capacity to excrete H^+ from kidney cortical collecting duct cells

We recently showed in *ex vivo* microperfused collecting ducts from *Rhcg*$^{-/-}$ mice a reduced NH_3 transepithelial flux due to decrease in apical and basolateral NH_3 permeabilities.[11,12] Here, using the same approach, we assessed H^+ transport activity in microperfused cortical collecting ducts (CCDs) from *Rhcg*$^{+/+}$ and *Rhcg*$^{-/-}$ mice challenged for 2 days with an HCl load in food to maximize transport activities. Luminal NH_4Cl prepulses were performed and H^+ transport activity was assessed from the steep intracellular pH (pH_i) recovery rates after maximal intracellular acidification when NH_4Cl was removed from the lumen.[32,33] The initial steep rate of alkalinization reflects H^+ extrusion, mostly by H^+-ATPases[33] (Figure 2a and b). We also observed in wild-type (WT) mice an early, almost immediate but slow pH_i recovery that was not observed in knockout mice, suggesting that it is related to the function of Rhcg. As the initial pH_i values were slightly different in CCDs from *Rhcg*$^{-/-}$ and *Rhcg*$^{+/+}$ mice, we also measured intracellular buffering power and calculated H^+ fluxes (JH^+) across the membrane to directly compare transport rates. Similar to pH_i recovery rates, JH^+ was greatly reduced in CCDs from *Rhcg*$^{-/-}$ by 89% (*Rhcg*$^{+/+}$: 32.6 ± 12.8 pmol/mm/min vs. *Rhcg*$^{-/-}$: 3.6 ± 0.63 pmol/mm/min, $P \leq 0.0001$) (Figure 2c). Thus, the more alkaline urine observed in *Rhcg*$^{-/-}$ during acid-loading results from impaired proton secretion along the collecting duct.

The absence of Rhcg alters B1 and B2-H^+-ATPase subunits expression

Next, we investigated whether the reduced H^+ flux observed in microperfused CCDs from *Rhcg*$^{-/-}$ mice could be linked to altered expression of H^+-ATPase subunits. At the mRNA level, we could not detect any variation of the intercalated cell-enriched B1 H^+-ATPase isoform (Supplementary Figure S1A), and the more ubiquitous B2 (Supplementary Figure S1B) and a4 (Supplementary Figure S1C) H^+-ATPase isoforms between *Rhcg*$^{+/+}$ and *Rhcg*$^{-/-}$ mice. In membrane fractions from medullary kidney tissue to enrich the proportion of H^+-ATPases originating from intercalated cells, the amount of the intercalated cell-specific B1 isoform (Figure 3a) was increased, whereas the B2 isoform (Figure 3b) was decreased in kidney tissue from *Rhcg*$^{-/-}$ mice ($P \leq 0.01$). However, the protein abundance of the ubiquitous E2 (Figure 3c) and a1 isoforms (Figure 3c), as well as the kidney-enriched a4 (Figure 3d) subunits of the H^+-ATPase, was not different between the 2 genotypes.

Rhcg does not regulate H^+-ATPase localization

Reduced H^+-ATPase activity may result from altered subcellular localization of H^+-ATPases. Immunostaining for the B1, B2, E, and a4 H^+-ATPase subunits showed no apparent difference in subcellular localization of the 3 proteins in type-A intercalated cells in *Rhcg*$^{+/+}$ and *Rhcg*$^{-/-}$ kidneys (Figure 4). Electron microscopy with immunogold staining for the ubiquitous A subunit similarly detected no difference in the subcellular localization of this H^+-ATPase subunit (Figure 5). The absence of Rhcg did not affect the immunoreactivity for the A subunit in type-A intercalated cells of *Rhcg*$^{-/-}$ mice. H^+-ATPase staining was observed mostly in intercalated cells and was associated with the luminal membrane as well as with intracellular vesicles, as described previously.[16] Thus, the absence of Rhcg is not associated with a redistribution of H^+-ATPases in type-A intercalated cells.

The overexpression of RhCG in HEK293 cells increases H^+ secretion rates

To test if the expression of RhCG was associated with stimulation of H^+-ATPase activity, we used HEK293 cells,