Expression of RhCG, a New Putative NH$_3$/NH$_4^+$ Transporter, along the Rat Nephron

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Abstract. Two non-erythroid members of the erythrocyte Rhesus (Rh) protein family, RhBG and RhCG, have been recently cloned in the kidney. These proteins share homologies with specific NH$_3$/NH$_4^+$ transporters (Mep/Amt) in primitive organisms and plants. When expressed in a Mep-deficient yeast, RhCG can function as a bidirectional NH$_3$/NH$_4^+$ transporter. The aim of this study was to determine the intrarenal and intracellular location of RhCG in rat kidney. RT-PCR on microdissected rat nephron segments demonstrated expression of mRNAs encoding RhCG in distal convoluted tubules, connecting ducts, and cortical and outer medullary collecting ducts but not in proximal tubules and thick ascending limbs of Henle’s loop. Immunolocalization studies performed on rat kidney sections with rabbit anti-human RhCG 31 to 48 antibody showed labeling of the apical pole of tubular cells within the cortex, the outer medulla, and the upper portion of the inner medulla. All cells within connecting tubules had identical apical staining. In cortical collecting ducts, a subpopulation of cells that has either apical staining (α-intercalated cells) or diffuse staining (β-intercalated cells) for the β1 subunit of the H$^+$/ATPase, was heavily stained at their apical pole with the RhCG antibody while principal cells identified as H$^+$/ATPase negative cells showed a faint apical staining for RhCG that was much less intense than in adjacent intercalated cells. In the outer medulla and the upper portion of the inner medulla, RhCG labeling was restricted to a subpopulation of cells within the collecting duct that apically express the β1 subunit of the H$^+$/ATPase, indicating that RhCG expression in medullary collecting ducts is restricted to intercalated cells. No labeling was seen in glomeruli, proximal tubules, and limbs of Henle’s loop. Immunoblotting of apical membrane fractions from rat kidney cortex with the rabbit anti-human RhCG 31 to 48 antibody revealed a doublet band at approximately 65 kD.

The kidney has to face a daily net acid load, mainly generated by protein metabolism, of approximately 1 mmol/kg per d and maintains acid-base balance through bicarbonate absorption and regulation of acid excretion. NH$_4^+$ is the major component of net acid excretion, and its excretion adapts appropriately to challenge acid-base disorders such as metabolic alkalosis or metabolic acidosis (1). Furthermore, distal renal tubular acidosis, a disorder leading to chronic metabolic acidosis, is defined as synthesis of NH$_4^+$ in this nephron segment. Virtually all the NH$_4^+$ is synthesized and secreted by proximal tubule cells (3). It is believed to be mostly absorbed in the loop of Henle, concentrated in the medullary interstitium by countercurrent multiplication (4), and then secreted into the collecting duct lumen (5).

Transepithelial transport of NH$_4^+$ has been described to occur through various Na$^+$/K$^+$ exchangers, the Na$^+$/H$^+$ exchangers, the Na$^+$/K$^+$/2Cl$^-$ cotransporters, and the (Na$^+$/K$^+$/2H$^+$)-ATPase (see reference 4 for review), but no specific NH$_3$/NH$_4^+$ transporter has been yet reported in mammals. By contrast, in lower organisms and plants, ammonium transporters (Amt, synonym ≤Mep≥ for Methylammonium ammonium permease) constitute a superfamily of transmembrane proteins with multiple members differing by their primary structure, number of transmembrane segments, and kinetic of NH$_3$/NH$_4^+$ transport (6–8). A marginal homology between erythrocyte Rh (Rhesus) proteins, particularly Rh-associated glycoprotein (RhAG), and Mep/Amt protein family, has raised the possibility that RhAG may be a mammalian equivalent of Amt (9).
RhAG is not expressed in the kidney; it could not therefore be involved in renal NH$_4^+$ excretion. However, two non-erythroid RhAG-homologues, RhBG and RhCG (synonym RhGK), have been cloned recently in the kidney (10–12). These proteins, like RhAG, share homologies with the Mep/Amt protein superfamily. Moreover, when expressed in a Mep-deficient yeast, RhCG (RhGK) specifically complements growth defect at low external NH$_4^+$ concentration, confers resistance to toxic concentration of methylamine, and increases NH$_4^+$ efflux after cell loading (12). These results suggest that RhGK could represent a new bidirectional kidney NH$_4^+$/NH$_3$ transporter. RhBG and RhCG are found to be both abundantly expressed in kidney epithelial cells. However, their localization, assessed by RNA in situ hybridization in the mouse kidney, appears to be different. Indeed, RhBG transcript is found to be expressed in proximal tubules and thick ascending limbs of Henle (10), whereas expression of RhCG transcripts is confined to collecting ducts (11). No studies have rendered a complete description of the RhCG protein distribution along the rat nephron. We therefore investigated RhCG expression from glomeruli down to inner medullary collecting duct cells by specific nephron segments RT-PCR and immunohistochemically by using a polyclonal antibody raised against a 18-amino acid external peptide of the RhCG protein.

**Materials and Methods**

**Animals**

Experiments were performed on male Sprague Dawley rats (Ilfa-Credo, L’Arbresle, France) weighing 200 to 300 g. All animals had access to a standard laboratory diet and water ad libidum. Animals were anesthetized with intraperitoneal injection of sodium pentobarbital at 5 mg/100 g body wt. 

**Isolation of Nephron Segments and RNA Extraction**

The left kidney was perfused with microdissection solution (see composition below) containing 0.16% (wt/vol) collagenase (Serva, Heidelberg, Germany). The microdissected solution was prepared from sterile Hanks solution (Eurobio, France) supplemented with 1 mM acetate, 1 mM lactate, 1 mM glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), and RNase-free bovine serum albumin (BSA, 1 mg/ml), at pH 7.4. Thin pyramids were cut from the kidney, incubated for 20 min at 30°C in 0.12% collagenase solution, and then thoroughly rinsed in ice-cold solution.

The glomeruli and the successive nephron segments (proximal convoluted and straight tubules [PCT and PST]; medullary and cortical thick ascending limb of Henle’s loop [MTAL and CTAL]; distal convoluted tubules and connecting ducts [DCT and CNT]; cortical and outer medullary collecting ducts [CCD and OMCD]) were isolated under stereomicroscopic observation in microdissected solution at 0 to 4°C as described previously (13).

Pools of 20 to 50 microdissected tubules were transferred on a microscopic slide and photographed for subsequent tubular length measurement. RNAs were extracted according to the technique previously described (14) and briefly summarized below. Pools of tubules were transferred with 10 μl of microdissection solution in 400 μl of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.1 M β-mercaptoethanol, and 0.5% sarcosyl) containing 20 μg of yeast RNA used as carrier. After phenol-chloroform extraction and isopropyl alcohol precipitation, the RNA pellets were dried under vacuum and dissolved in RNA dilution buffer (10 mM Tris [pH 7.6], 1 mM ethylenediamine tetra-acetic acid, 2 mM dithiothreitol, and 40 U/ml Rnasein; Promega, Madison, WI) at a dilution corresponding to 1 mm of tubular length or 1 glomerulus/3 μl of dilution buffer.

**RT-PCR Analyses of mRNAs Encoding RhCG**

The presence of the mRNAs encoding RhCG along the nephron was determined by RT-PCR. Primers were designed from a rat EST (Genbank BF545324) showing homologies with the 3’ coding and uncoding regions of human RhCG mRNA (Genbank AF193809). Within the coding region, the deduced amino acid sequence of the rat EST displayed 64% identity with the corresponding region of human RhCG protein (Figure 1). The sense (5’-GGTACAAGCCCGACTG-GACCAAGA-3’) and antisense (5’-GAGGTAGAATGTTTGT-GAGCGAGGTC-3’) primers corresponded to bases 78 to 101 and 287 to 312 of the rat EST, respectively.

RT was carried out for 45 min at 46°C in a final volume of 50 μl in the presence of mRNA corresponding to 0.5 mm of nephron segment or one glomerulus, antisense primer (6.25 pmol) and Moloney Murine Leukemia virus reverse transcriptase (200 U). RT was also carried out in the absence of reverse transcriptase as negative control. PCR was subsequently carried out in a final volume of 100 μl after addition of the sense primer (6.25 pmol), (α-32P)dCTP (5 μCi/sample; 6000 Ci/mmol-1), and Taq polymerase (1.25 U). Samples were submitted to 31 cycles of three temperatures steps: 95°C for 30 s; 60°C for 30 s; and 72°C for 1 min. During the last cycle, the elongation step lasted 5 min. Aliquots of 16 μl of each RT-PCR sample were electrophoresed through 2% agarose slab gels. The gels were fixed in 10% acetic acid, dried under vacuum at 70°C, and submitted to PhosphorImager (Molecular Dynamics) for determining the intensity of the signal. For each animal and each experiment, the signal intensity (arbitrary units) of the different samples was expressed as percent of the signal detected in the CCD. Results are presented as mean ± SEM from different animals.

**Anti-RhCG Antibody**

Rabbit polyclonal anti-RhCG antibody was raised against the synthetic peptide (Neosystem, Strasbourg, France) CRYDFEAADAHW-WSERTHK corresponding to amino acids 31 to 48 predicted from the human RhCG (RHGK) sequence (11,12) with an added N-termi-
nal extra cysteine, as described previously (12). The free carboxyl
group at the C terminal was not amidated. Note that the asparagine 48
was predicted to be a glycosylation site (11). The human RhCG
polypeptide 31 to 48 and the corresponding mouse amino acid se-
cquence share almost 70% identity, suggesting that crossreactivity to
other species may exist. The peptide was coupled to Keyhole limpet
hemocyanin through the M-maleimido benzoic acid N-hydroxysuc-
icinimide ester protocol (15), and the conjugate (0.3 mg/ml) was
eumulsified with complete Freund adjuvant and injected subcutane-
ously (0.5 ml) to New Zealand rabbits at 3-wk intervals. Progress of
immunization was monitored by ELISA performed with peptide-
coated 96-well plates and Western blot of ovalbumin-coupled peptide.
Immunopurification of antiserum was performed using the Pierce
sulfolink procedure; 3.5 mg of peptide per ml of gel peptide were
actually coupled on the gel adhering to manufacturer instructions.
Serum (4.6 ml) was loaded onto the 2-ml column after dilution with
one volume of phosphate-buffered saline (PBS). Column was then
rinsed with PBS, and immunoglobins were eluted with 0.1 M
glycine buffer (pH 2.8). pH of collected fractions was immediately
raised to 7.2 with 1 M Tris base. Specificity of the anti-RhCG peptide
antibody was previously demonstrated using immunoblot against re-
combinant RhCG in yeast (12). The specificity was further confirmed
by indirect immunofluorescence labeling of stable HEK293-RhCGK
transfectants (Figure 2A), but not of parental HEK293 cells (Figure
2B), with the rabbit anti-RhCG antibody, as described (16).

Immunolabeling

After clamping the abdominal aorta above and below the renal
arteries and cutting open the inferior vena cava, the rat kidneys were
perfused with cold 4% paraformaldehyde freshly prepared in Dulbec-
co’s modified Eagle’s-Ham’s F-12 medium. After removing the kid-
neys, coronal sections were cut, postfixed for 4 h at 4°C in 4% paraformaldehyde, and embedded in paraffin. Subsequently, 4-µm
sections of the paraffin block were deparaffinized with toluene,
washed in ethanol, and rehydrated in water.

For RhCG immunolabeling, slides were first placed in plastic tank
filled with 1 mM EDTA, pH 8.0, and heated for 40 min at 96 to 98°C

Figure 2. Indirect immunofluorescence staining of HEK293-RhCG
transfectants and parental HEK293 cells with the rabbit anti-RhCG
antibody. $4 \times 10^4$ stable HEK293-RhCG transfectants and parental
HEK293 cells washed in phosphate-buffered saline (PBS) were se-
quentially fixed with 4% paraformaldehyde for 20 min, permeabilized
with 0.5% Triton X-100 and 1% SDS as described (16), and incubated
with the purified anti-Nter RhCG (1/50) for 1 h at 22°C. After three
washes in PBS containing bovine serum albumin (BSA), the cells were
stained with Alexa Fluor 488-conjugated anti-rabbit IgG. After
washes in PBS, stable HEK293-RhCG transfectants (panel A) and
parental HEK293 cells (panel B) were mounted in Prolong (Molecu-
lar-Probes, Interchim, Asnières, France) and the membrane fluores-
cence was observed with a Nikon TE 300 equipped with a mercury
lamp and a 60XA/1.4 oil objective lens.

Figure 3. Expression of RhCG mRNAs along the rat nephron. mR-
NAs from one glomerulus or 0.5 mm of different segments of the
nephron were reverse transcribed and the cDNA were amplified by
PCR using primers specific for RhCG. The DNA fragments were
separated on 2% agarose gels and visualized using a PhosphoImager.
Below the histogram, two representative gels from micro-
dissected from two different animals are shown. In each experiment,
values were expressed as percent of the CCD value. Mean ± SEM
from several animals are indicated as number in circles. G, glomer-
ulus; PCT and PST, proximal convoluted and straight tubule; MTAL
and CTAL, medullary and cortical thick ascending limbs of Henle’s
loop; DCT, distal convoluted tubule; CNT, connecting tubule; CCD
and OMCD, cortical and outer medullary collecting duct, respectivly.
NT, not tested.
Figure 4. Sections of rat kidney double-stained for RhCG (green) and the β1 subunit of the H⁺-ATPase (red). The localization of RhCG relative to the H⁺-ATPase in the cortical labyrinth (A), a medullary ray (B), the inner stripe of the outer medulla (C), and the inner medulla (D) is shown. (A) Apical RhCG staining is detected in connecting tubules. When comparing the merged image, note that RhCG appears to be expressed in all cells (intercalated and connecting tubule cells) within connecting tubules. Proximal tubules and glomerulus showed no detectable staining with the RhCG antibody. (B) In collecting ducts, cells that strongly stained for the H⁺-ATPase (intercalated cells) have
in a water bath. This step unmasked immunostaining on paraformaldehyde-fixed paraffin sections, as determined in preliminary experiments. Sections were then rinsed in Tris-buffered saline (TBS), pH 7.6, for 10 min and preincubated for 20 min with background-reducing buffer (Dako Corp., Copenhagen, Denmark) to block non-specific staining. The affinity-purified anti-RhCG antibody diluted 1:100 in background-reducing buffer was applied for 1 h at room temperature. After three washes, sections were incubated sequentially with a 1:200 dilution (in background-reducing buffer) of goat biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), Cy2-conjugated streptavidin (Amersham Pharmacia Biotech) diluted 1:1000 in TBS, each for 30 min at room temperature, with three TBS washes in between. After washing, sections were mounted and observed using a Leica TCS SP confocal laser microscope equipped with an Ar-Kr laser (excitation, 488 nm; detection, 502 to 601 nm).

RhCG labeling in rat kidney was also performed by using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA), according to the manufacturer’s instructions. Peroxidase activity was revealed with 3-amino-9-ethylcarbazol (AEC, Dako Corp.). Sections were examined with a Zeiss photomicroscope.

In control experiments, a 1:100 dilution of the affinity-purified anti-RhCG antibody was preabsorbed with the immunizing peptide (100 μg/ml), and the resulting medium was used as a negative control. Controls using preimmune serum were also negative.

Rat kidney sections were also double-labeled with the rabbit polyclonal antibody against the β1 subunit of the H+-ATPase and the anti-human RhCG antibody. As heating greatly increased H+-ATPase staining, sections heated as before were first stained for the H+-ATPase using a three-layer immunofluorescence labeling procedure; sections were incubated sequentially with rabbit antibody against the 56-kD β1 subunit of the H+-ATPase at a dilution of 1:200 (kindly provided by Dr. Dennis Brown), goat biotinylated anti-rabbit IgG (diluted 1:500), and Cy5-conjugated streptavidin (Amersham Pharmacia Biotech) diluted 1:1000. Because the two primary antibodies used for double labeling study are raised in rabbit, microwave oven heating of sections was performed before RhCG labeling to avoid the detection of bound antibody during the second round of three-layer labeling as described previously by Lan et al. (17). As determined in preliminary experiments, microwave oven heating of sections for 2.5 min (500 W) in 0.01 M sodium citrate buffer, pH 6, was needed to block antibody crossreactivity. This procedure has also been shown not to alterate subsequent RhCG immunostaining. Sections were then incubated overnight at 4°C with affinity-purified rabbit anti-human RhCG peptide polyclonal antibody at a dilution of 1:100 followed by goat biotinylated anti-rabbit IgG (diluted 1:400) and Cy2-conjugated streptavidin (diluted 1:1000) for 30 min each. Sections were observed using a Leica TCS SP confocal laser microscope. Cy2 was excited at 488 nm and detected at 498 to 550 nm. Cy5 was then excited on the exact same field at 647 nm and detected at 663 to 758 nm. To check that there was no crossreactivity in double stained sections, anti-RhCG antibody was omitted or replaced by normal rabbit IgG (Dako Corp.) during the second round of labeling.

**SDS-PAGE and Western Blotting**

Apical membrane fractions were isolated from whole rat renal cortex using a Ca²⁺ aggregation method described previously (18).
Protease inhibitors were added to all buffers. Proteins were solubilized and separated by 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech) and analyzed by Western blotting. Nitrocellulose membranes were first incubated in 5% nonfat dry milk in PBS, pH 7.4, for 1 h at room temperature to block nonspecific binding of antibody and were then incubated overnight at 4°C with affinity-purified anti-RhCG antibody diluted 1:100 in PBS containing 1% nonfat dry milk. After four 5-min washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:3000 dilution of goat biotinylated anti-rabbit IgG and then Cy2-conjugated streptavidin. In panels A and C, Cy5 staining (red) of the H⁺-ATPase is normally detected, whereas in panels B and D, no specific Cy2 staining (green) is detected, indicating no crossreactivity between the two rounds of labeling. Scale bar, 15 μm.

**Figure 6.** Control for blocking by microwave heating treatment of crossreactivity in double-labeling experiments. The first round of staining is performed as described in Materials and Methods. Briefly, after a first antigen retrieval-heating step, sections were sequentially incubated with rabbit anti H⁺-ATPase, goat biotinylated anti-rabbit IgG, and Cy5-conjugated streptavidin. To avoid the detection of bound antibody during the second round of labeling, microwave oven heating treatment of sections was then performed as described in Materials and Methods. To check that there was no crossreactivity, in double-labeling experiments, replacement of the primary antibody during the second round of labeling by antibody diluant (panels A and B), or nonspecific rabbit Ig at protein concentration equivalent to that of diluted anti-RhCG antibody (panels C and D) was used. Sections were then incubated with goat biotinylated anti-rabbit IgG and then Cy2-conjugated streptavidin. In panels A and C, Cy5 staining (red) of the H⁺-ATPase is normally detected, whereas in panels B and D, no specific Cy2 staining (green) is detected, indicating no crossreactivity between the two rounds of labeling. Scale bar, 15 μm.

Protease inhibitors were added to all buffers. Proteins were solubilized and separated by 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Hybond ECL nitrocellulose (Amersham Pharmacia Biotech) and analyzed by Western blotting. Nitrocellulose membranes were first incubated in 5% nonfat dry milk in PBS, pH 7.4, for 1 h at room temperature to block nonspecific binding of antibody and were then incubated overnight at 4°C with affinity-purified anti-RhCG antibody diluted 1:100 in PBS containing 1% nonfat dry milk. After four 5-min washes in PBS containing 0.1% Tween-20, membranes were incubated with 1:3000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) in PBS containing 5% nonfat dry milk for 2 h at room temperature. Blots were washed as above, and luminol-enhanced chemiluminescence (NEN Life Science Products, Inc. Boston, MA) was used to visualize bound antibodies on Polaroid film. Photographs of immunoblots were numerized with NIH image software. For peptide inhibition experiments, the anti-RhCG antibody was preincubated with the specific immunizing peptide (100 μg/ml) before immunoblotting cortical apical membrane fractions.

**Results**

**Expression of RhCG mRNAs along the Rat Nephron**

Figure 3 illustrates two representative RT-PCR experiments performed to localize RhCG mRNAs in the glomerulus and different nephron segments of rats. Using dilution of RNAs corresponding to one glomerulus or 0.5 mm of tubule, the presence of RhCG mRNAs was consistently observed in DCT, CNT, CCD, and OMCD. In contrast, RhCG mRNAs were not detectable in glomerulus (G), PCT, PST, MTAL, and CTAL.
Figure 7. Immunoperoxidase staining of RhCG in rat kidney sections. (A) Low-power view of the cortex showing apical staining of connecting tubules. Note the presence of one or two heavily stained intercalated cells (arrows) in some segments that could correspond to later parts of the distal convoluted tubule. (B) View of the cortex showing apical staining of intercalated cells in a cortical collecting duct (arrows). (C) Low-power view of the inner stripe of the outer medulla showing apical staining of intercalated cells in medullary collecting ducts. (D) Detail of inner stripe of the outer medulla showing apical labeling of intercalated cells in a collecting duct. G, glomerulus; PT, proximal tubule; CNT, connecting tubule; TAL, thick ascending limb; CD, collecting duct. Scale bars: 25 μm for A, B, and C; 15 μm for D.
In the distal nephron, the expression level of RhCG mRNAs was highest in the CNT, intermediate in CCD and OMCD, and lower in DCT.

**Immunolocalization of RhCG in the Rat Kidney**

The tubular and plasma membrane location of RhCG in rat kidney was first investigated by indirect immunofluorescence on paraformaldehyde-fixed paraffin kidney sections. Staining for RhCG was restricted to some tubular structures within the cortex, outer medulla and upper portion of the inner medulla. In the cortical labyrinth, anti-RhCG antibody labeled the apical pole of CNT (Figure 4A). In the medullary rays of the cortex, a subpopulation of cells within the CCD was heavily stained at their apical pole (Figure 4B). The remaining cells were stained only weakly or not at all. In the inner stripe of the outer medulla, RhCG staining was restricted to the apical pole of a subpopulation of CCD (Figure 4C). The number of stained cells fell sharply in the inner medulla (Figure 4D). In the initial portion of the inner medulla, the number of stained cells gradually decreased, and none was found in the terminal portion of the inner medulla (data not shown). There was no evidence of RhCG staining in the proximal tubule or in the thin or thick limbs of Henle’s loop. Specificity of the labeling was demonstrated by the absence of signal when anti-RhCG antibodies were incubated before application to sections with 100 μg/ml of the peptide that was used as an immunogen (Figure 5). Note that background fluorescence of the proximal tubules was also seen in the presence of immunizing peptide.

In the kidney, the β1-subunit of the 

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**Figure 8.** Immunoblot analysis of RhCG peptide in three independent preparations of rat renal cortical apical membranes. Each lane was loaded with 20 μg of membrane protein. Affinity-purified anti-human RhCG antibody detects predominant 65-kD and less abundant 80-kD and 50-kD polypeptides in rat renal cortical apical membrane preparations (lanes 1 and 2). For peptide inhibition experiment shown in lanes 3 and 4, the anti-RhCG antibody was preincubated or not with an excess of the immunizing peptide (100 μg/ml) before application to nitrocellulose. Only band detected at 65 KD is competed by the immunizing peptide (lane 4). Arrow indicates RhCG polypeptides; asterisks indicate nonspecific reactivities not competed by immunizing peptide. In lane 3, the RhCG band appears as a doublet band of 65 kD. Left: molecular mass expressed as 10^−5 × Mr.
ected predominant 65-kD and less abundant 80-kD and 50-kD polypeptides (Figure 8, lanes 1 to 3). The 65-kD band was not detected when the antibody was preincubated with an excess of immunizing peptide before immunoblotting apical membrane proteins (lane 4).

**Discussion**

The purpose of this study was to approach the physiologic role of RhCG by describing the tubular and subcellular distribution of the protein in the kidney. Our strategy included the detection of RhCG mRNA by RT-PCR on microdissected nephron segments and the detection of the RhCG protein using a specific antibody. The results presented here demonstrate that RhCG is an apical plasma membrane protein, expressed in the CNT and the intercalated cells of the CD.

The polyclonal antibody against RhCG was obtained by immunizing rabbits with a synthetic peptide corresponding to amino acids 31 to 48 of the human RhCG sequence as described previously (12). Among the known Rh proteins, the sequence of this peptide is unique to RhCG. Furthermore, a comparison of this sequence to the GenBank database using BLAST analysis revealed no significant overlap with other known eukaryotic proteins. Moreover, EST sequences corresponding to the rat RhCG exhibit about 89% identity with human RhCG. This suggests that the resulting antibody is likely to be specific for RhCG and might detect both human and rat RhCG proteins. Furthermore, control experiments using pre-immune serum or purified antibody that was preabsorbed with the immunizing peptide showed no labeling on tissue sections.

Immunoblot analyses of rat cortical apical membranes using affinity purified anti-RhCG rabbit polyclonal antibody revealed an apparent Mr of 65 kD for the rat renal RhCG. Specificity of the 65-kD polypeptide was demonstrated by the ability of the immunizing peptide to block the reaction. Human RhCG and mouse Rhcg cDNAs encode proteins of 53 kD and 55 kD, respectively. The observed molecular weight (65 kD) is closest to previously reported Mr of the glycosylated human RhCG (11). Indeed, immunoblots of membrane proteins isolated from HEK293 cells stably transfected with RhCG probed with a RhCG C-tail polyclonal antiserum showed a broad band at 58 kD that was shifted to a 46-kD band after N-glycanase treatment (11). The 65-kD polypeptide is therefore likely to be the glycosylated form of the RhCG protein, although this was not examined in this study.

As expected from previous Northern blot hybridization and in situ hybridization studies in both human and mouse tissues (11,12), RhCG was found in the kidney using both RT-PCR and immunohistochemistry. Using RT-PCR on rat microdissected tubules, RhCG was found in DCT, CNT, CCD, and OMCD. In rat kidney as opposed to rabbit kidney there is no clearcut boundary between the DCT (as defined by the exclusive presence of DCT cells) and CNT (defined by the presence of connecting and intercalated cells) (21), the observed expression of RhCG mRNAs in DCT could therefore have been accounted for by their expression in DCT cells or alternatively by the presence of connecting and/or intercalated cells in the DCT segment. Indeed, immunohistochemistry revealed that RhCG was present in CNT and CD but not in DCT cells. These results are consistent with the findings of in situ hybridization studies showing labeling of CD (11). Immunohistochemistry also revealed that RhCG protein is expressed in the plasma membrane of renal epithelial cells and demonstrated that this expression is polarized as RhCG protein appeared to be restricted to apical, not to basolateral, membrane of the cells. Furthermore, double-labeling studies using an antibody specific to the β1 56-kD subunit (renal isoform) of the H⁺-ATPase and the RhCG antibody showed that intercalated cells contain most of the RhCG detectable in the CD. In contrast, in the CNT, all cells, connecting tubule cells, and intercalated cells had identical labeling for RhCG.

It has been suggested that RhCG might function as an NH₄⁺ / NH₃ transporter in the kidney (12). Final secretion of NH₄⁺ is believed to occur mostly in the CD, where it is trapped and excreted (22-25). Some pieces of evidence also exist that NH₄⁺ secretion, albeit at low rates, occurs in the superficial late distal tubule including CNT and initial CD (26). Total ammonia exists in two forms, i.e., NH₄⁺ and NH₃, the mechanism of NH₄⁺ secretion across the CNT and/or the CD epithelium could therefore involve direct transport of NH₄⁺ or passive NH₃ transport in parallel with active proton secretion. Of interest, we found RhCG in the collecting duct mostly expressed in intercalated cells, which express the proton pump and are responsible for acid-base transport regulation. This raises the question of a possible functional coupling between the H⁺-ATPase and RhCG proteins to secrete NH₄⁺, i.e., RhCG could be responsible of NH₃ permeation across the apical membrane rather than direct NH₄⁺ transport. Several data support this hypothesis. Apparent NH₄⁺ permeability of CD epithelium is very low (<10⁻⁵ cm/s) (22), and NH₄⁺ shares many pathways with other cations in the renal tubule (2). In contrast, NH₃, despite its very low lipid solubility (27), is able to readily permeate CD epithelium with apparent permeability of 10⁻² cm/s (22). Finally, the dependence of NH₄⁺ secretion on the luminal acid disequilibrium pH in the CD favors the model of NH₃ secretion combined with active H⁺ secretion (28,25). In CNT, another possible role for the apical RhCG is reabsorption of NH₃ from the luminal fluid secondary to water reabsorption during antidiuresis, as proposed by Weinstein et al. (29). In conclusion, the localization of RhCG in the connecting tubules and the intercalated cells of the CD reported here is in accordance with the hypothesis that RhCG could act as an NH₃ transporter, but further studies are needed to determine the role of RhCG in NH₃ transport.

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References


