Characteristics of renal Rhbg as an \( \text{NH}_4^+ \) transporter

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Submitted 2 December 2003; accepted in final form 25 August 2004

Characteristics of renal Rhbg as an \( \text{NH}_4^+ \) transporter. Am J Physiol Renal Physiol 288: F170–F181, 2005. First published September 7, 2004; doi:10.1152/ajprenal.00419.2003.—Rhbg is one of two recently cloned nonerythroid glycoproteins belonging to the Rh antigen family. Rhbg is expressed in basolateral membranes of intercalated cells of the kidney cortical collecting duct and some other cell types of the distal nephron and may function as \( \text{NH}_4^+ \) transporters. The aim of this study was to characterize the role of Rhbg in transporting \( \text{NH}_4^+ \). To do so, we expressed Rhbg in Xenopus laevis oocytes. Two-electrode voltage-clamp and \( \text{H}^+ \)-selective microelectrodes were used to measure \( \text{NH}_4^+ \) currents, current-voltage plots, and intracellular \( \text{pH} \) (pH(\text{i})). In oocytes expressing Rhbg, 5 mM \( \text{NH}_4^+ \) induced an inward current of 93 ± 7.7 nA (\( n = 20 \)) that was significantly larger than that in control oocytes of \( -29 \pm 7.1 \) nA (\( P < 0.005 \)). Whole cell conductance, at all tested potentials (–60 to +60 mV), was significantly more in oocytes expressing Rhbg compared with \( \text{H}_2\text{O} \)-injected oocytes. In Rhbg oocytes, 5 mM \( \text{NH}_4^+ \) depolarized the oocyte by 28 ± 3.6 mV and decreased pH(\text{i}) by \( 0.30 \pm 0.04 \) at a rate of \( -20 \pm 2.5 \times 10^{-4} \) pH/s. In control oocytes, 5 mM \( \text{NH}_4^+ \) depolarized V(\text{m}) by only 20 ± 5.8 mV and pH(\text{i}) decreased by \( 0.07 \pm 0.01 \) at a rate of \( -2.7 \pm 0.6 \times 10^{-4} \) pH/s. Raising bath [\( \text{NH}_4^+ \)] in increments from 1 to 20 mM elicited a proportionally larger decrease in pH(\text{i}); larger depolarization (\( \Delta V(\text{m}) \)); and a faster rate of pH(\text{i}) decrease. Bathing Rhbg oocytes in 20 mM \( \text{NH}_4^+ \) induced an inward current of 140 ± 7 nA that was not significantly different from 178 ± 23 nA induced in \( \text{H}_2\text{O} \)-injected (control) oocytes. The rate of pH(\text{i}) decrease induced by increasing external [\( \text{NH}_4^+ \)] was significantly faster in Rhbg than in \( \text{H}_2\text{O} \)-injected oocytes at all external \( \text{NH}_4^+ \) concentrations. In oocytes expressing Rhbg, net \( \text{NH}_4^+ \) influx (estimated from \( \text{NH}_4^+ \)-induced \( \text{H}^+ \) influx) as a function of external [\( \text{NH}_4^+ \)] saturated at higher [\( \text{NH}_4^+ \)] with a \( V(\text{m}) \) of ~30.8 and an apparent \( K(\text{m}) \) of 2.3 mM (\( R^2 = 0.99 \)). These data strongly suggest that Rhbg is a specific electrogenic transporter of \( \text{NH}_4^+ \):

ammonium; acid-base homeostasis; ammonium-hydrogen exchanger

RENA L EXCRETION OF AMMONIUM (\( \text{NH}_4^+ \)) is critical for acid-base homeostasis, normally accounting for at least two-thirds of daily net acid excretion (19). \( \text{NH}_4^+ \) excretion can undergo significant upregulation during chronic acid loads and appears to be regulated at several steps, including both ammoniagenesis in the proximal tubule (18) and transport in virtually all segments of the nephron (2, 9, 10, 12, 19).

Ammonium produced in the proximal tubule is secreted predominantly into the luminal fluid (13, 14). This occurs by both \( \text{NH}_3 \) diffusion into the more acid luminal fluid and by \( \text{NH}_4^+ \) transport in exchange for sodium on the sodium-hydrogen exchanger (17, 19). Despite these mechanisms for proximal tubule secretion of ammonium, a significant amount of synthesized ammonia (>20%) is released across the basolateral membrane of the proximal tubule or escapes from the lumen of the proximal tubule into the basolateral interstitial compartment and ultimately reaches renal venous blood.

Ammonium secreted into the lumen of the proximal tubule does not simply continue into the urine. In the loop of Henle and thick ascending limb, total ammonia appears to undergo a recycling and a countercurrent concentration (10, 19). Total ammonia may be secreted into the descending limb of Henle but is then reabsorbed in the thick ascending limb. Ammonia lost in the loop of Henle is transported back into the collecting duct. Intersitial total ammonia concentrations rise from the outer medullary region to the highest concentration in the deep papilla. The main driving force for this medullary concentration of ammonia appears to be reabsorption of total ammonia in the thick ascending limb. The thick ascending limb has been found to have unique \( \text{NH}_3/\text{NH}_4^+ \) transport characteristics. First, the apical membrane of the thick ascending limb has a low permeability to \( \text{NH}_3 \); in contrast, \( \text{NH}_4^+ \) is reabsorbed from the lumen into the cell via substitution for potassium on both the Na-K-2Cl cotransporter and the apical membrane potassium channel (see Refs. 2, 9).

With ammonium removal in the loop of Henle and thick ascending limb, total ammonia delivery to the distal convoluted tubule is lower than to the end of the proximal tubule. But total ammonia is secreted along the length of the collecting duct. Most, if not all, of this secretion has been thought to occur by nonionic diffusion of \( \text{NH}_3 \) along the length of the collecting duct from the progressively increasing concentrations of ammonia in the medullary interstitium (11). Acid secretion along the length of the collecting duct (for example, by H-K-ATPase) keeps luminal concentrations of \( \text{NH}_3 \) low, maintaining a \( \text{NH}_3 \) gradient from interstitium to lumen for \( \text{NH}_3 \). Although nonionic diffusion of \( \text{NH}_3 \) across the apical membrane of collecting duct cells appears to be the rate-limiting step, other transporters in the collecting duct may transport \( \text{NH}_4^+ \) (15, 19).

In the collecting duct and other segments of the nephron, there appears to be net transport of \( \text{NH}_4^+ \) as outlined above. However, no \( \text{NH}_4^+ \)-specific transporters have been previously identified anywhere in the kidney. Given the importance of transporting this ion, it seems likely that the kidney would have such mechanisms. Rhbg and Rhcg may very well be these
membrane proteins that play a significant role in transporting NH$_4^+$ in the kidney.

The Rh glycoproteins, originally described in human blood cells, are mostly recognized for their immunogenic characteristics and importance in pregnancy (for a review, see Ref. 3). The erythroid Rh protein (RhAG) is associated with other glycoproteins that contain the D antigen or the C/c and E/e antigens to form the “Rh complex” (3). In addition to its antigenic property, the Rh complex is thought to contribute to the membrane stability and structure of red blood cell. However, other functions are yet to be determined.

Recently, two nonerythroid Rh glycoproteins have been cloned from the mouse (Rhcg and Rhbg) and human (RhCG and RhBG) (21, 23, 24). RhCG, also known as RhGK (24), is a 53-kDa glycoprotein with 12 transmembrane domains and cytoplasmic COOH and NH$_2$ termini. It is expressed at the membrane surface with no apparent need for heteromeric interaction with other Rh glycoproteins (8, 35). Rhbg and Rhcg are more similar to RhAG than Rh CE/D but occur later in development than erythrocyte Rh glycoproteins. Whereas Rhbg transcripts were present in fetal tissues and in embryos at gestation age of 15–17 days, Rhcg transcripts were absent in embryos at gestation age of 7–19 days. Rhcg is abundant and broadly expressed in kidney where it is localized in the apical cell membrane of the collecting duct (21, 24). Rhbg and its human analog (RhBG) also have 12 transmembrane domains and are expressed mainly in the liver, skin, and kidney (23). Although originally thought to be localized in the proximal tubule, recently Weiner et al. (36) used immunohistochemistry to localize Rhbg to the basolateral membrane of the cortical collecting duct and the connecting segment, whereas Rhcg was restricted to the apical membrane.

The original link between these membrane proteins and NH$_4^+$ transport was provided by studies in yeast mutants rendered incapable of NH$_4^+$ transport by deletions of three endogenous NH$_4^+$ transporter genes (24). Yeast, like many other fungal species as well as bacteria, efficiently scavenge NH$_4^+$, which is required as a N$_2$ source, without which growth would be greatly hampered. The yeast transporters linked to NH$_4^+$ transport are known as Mep proteins and their bacterial equivalents are Amt proteins. Studies have not yet clarified the characteristics of these related transport proteins. A recent study suggested that RhAG transports NH$_4^+$ by a H$^+$ countertransport mechanism similar to the Na/H exchanger (39). However, other investigators suggested that Amt and Rh1 (a bacterial member of this family) actually transport NH$_4^+$ rather than NH$_4^+$ and possibly CO$_2$ as well (31, 32).

Based on structural similarities to the MEP/Amt NH$_4^+$ transporters in the yeast and their sequence homology, it is suggested that Rh proteins probably function as NH$_4^+$ transporters. Although the evidence for such a function is still circumstantial, it is intriguing that this class of membrane proteins may be the elusive NH$_4^+$ transporter in the kidney and other tissues as well (16). Thus far, data indicating that Rh proteins function as NH$_4^+$ transporters are indirect. The present studies directly address the mode of NH$_4^+$ transport by Rhbg, one member of the nonerythroid Rh proteins.

METHODS

Reagents and Solutions

All salts and general reagents used to prepare the bath solutions were purchased from Sigma (St. Louis, MO) unless otherwise noted. The standard solution used to perfuse the bath was ND96 medium containing (in mM) 100 NaCl, 2 KCl, 1.8 CaCl$_2$, and buffered with 5 mM HEPES to pH 7.5. The NH$_3$/NH$_4^+$ solution contained 20 mM NH$_4$Cl (replacing NaCl) at pH 7.5. Mixing standard ND96 solution with 20 mM NH$_4$Cl solution in the proper proportions was used to prepare solutions containing varying concentrations of NH$_3$/NH$_4^+$. Na$^+$-free solutions had all Na$^+$ replaced with NMDG$^+$. For K$^+$-free solutions, KCl (2 mM) was replaced with NaCl. Osmolarity of all solutions was ~200 mosM. OR3 medium (GIBCO BRL Leibovitz media) contained glutamate, 500 units each of penicillin/streptomycin, with pH adjusted to 7.5 and osmolarity adjusted to ~200 mosM.

Cloning of Mouse Rhbg cDNA

We cloned Rhbg by amplifying the cDNA from mouse kidney. Total RNA was prepared from dissected mouse kidney cortex using the Ultraspec-II RNA Isolation System (Biotec Laboratories) as specified by the protocol. The total RNA purity was checked by electrophoresis and quantitated by absorbance. First-strand cDNA was then prepared from the total RNA by reverse transcription using the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer’s protocol. Based on the published sequences, specific primers were designed flanking the coding region for each clone. Using high-fidelity DNA polymerase (Pfu-Turbo Polymerase, Stratagene), the clone was amplified by PCR. The PCR product was extracted from the gel and cleaned with QIAquick kit (Qiagam) as directed. Cloning of the PCR product was accomplished using a Zero Blunt Topo PCR Cloning Kit (Invitrogen). Full-length cDNA of Rhbg was subcloned in an oocyte expression vector, PGH19 (courtesy of Dr. W. Boron, Yale University). Restriction digestes, using HindIII and XbaI restriction sites, were performed to cut the Rhbg insert from the TOPO vector and ligate it into PGH19 expression vector. The full sequence (GenBank accession numbers bankit530763 AY254685) differs from the published sequence of Rhbg (23) at amino acid numbers 195 with tryptophan replacing arginine.

Isolation of Oocytes

*Xenopus laevis* oocytes were used to address the specific aims of this study because they provide distinct advantages that are not available in other preparations. We harvested oocytes in stage 5/6 from female *X. laevis* as described earlier (26). Briefly, this was done by anesthetizing the frog by mild hypothermia in water containing 0.2% tricaine. A 1-cm incision was made in the abdominal wall, one lobe of the ovary was externalized, and the distal portion was cut. The wound was closed by a few stitches in the muscular plane of the peritoneum using 5–0 catgut followed by two to three stitches in the abdominal skin using 6–0 silk. The excised piece of ovary containing the oocytes was rinsed several times with Ca-free ND96 solution until the solution was clear. The tissue was then prepared from the total RNA by reverse transcription using the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer’s protocol. Based on the published sequences, specific primers were designed flanking the coding region for each clone. Using high-fidelity DNA polymerase (Pfu-Turbo Polymerase, Stratagene), the clone was amplified by PCR. The PCR product was extracted from the gel and cleaned with QIAquick kit (Qiagam) as directed. Cloning of the PCR product was accomplished using a Zero Blunt Topo PCR Cloning Kit (Invitrogen). Full-length cDNA of Rhbg was subcloned in an oocyte expression vector, PGH19 (courtesy of Dr. W. Boron, Yale University). Restriction digestes, using HindIII and XbaI restriction sites, were performed to cut the Rhbg insert from the TOPO vector and ligate it into PGH19 expression vector. The full sequence (GenBank accession numbers bankit530763 AY254685) differs from the published sequence of Rhbg (23) at amino acid numbers 195 with tryptophan replacing arginine.

Preparation of cRNA

Plasmid containing the appropriate template DNA was purified by Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). The plasmid was then digested with an appropriate restriction enzyme as specified by the protocol. The total RNA purity was checked by electrophoresis and quantitated by absorbance. First-strand cDNA was then prepared from the total RNA by reverse transcription using the Superscript First-Strand Synthesis System for RT-PCR (Life Technologies) according to the manufacturer’s protocol. Based on the published sequences, specific primers were designed flanking the coding region for each clone. Using high-fidelity DNA polymerase (Pfu-Turbo Polymerase, Stratagene), the clone was amplified by PCR. The PCR product was extracted from the gel and cleaned with QIAquick kit (Qiagam) as directed. Cloning of the PCR product was accomplished using a Zero Blunt Topo PCR Cloning Kit (Invitrogen). Full-length cDNA of Rhbg was subcloned in an oocyte expression vector, PGH19 (courtesy of Dr. W. Boron, Yale University). Restriction digestes, using HindIII and XbaI restriction sites, were performed to cut the Rhbg insert from the TOPO vector and ligate it into PGH19 expression vector. The full sequence (GenBank accession numbers bankit530763 AY254685) differs from the published sequence of Rhbg (23) at amino acid numbers 195 with tryptophan replacing arginine.
extraction and ethanol precipitation. cDNA was transcribed in vitro with either T3, T7, or the appropriate promoter polymerase. The in vitro synthesis of capped RNA (cRNA) transcripts was then accomplished using mCAPTM RNA Capping Kit (Stratagene). The concentration of cRNA was determined by UV absorbance and its quality was assessed by formaldehyde/MOPS/1% agarose gel electrophoresis.

**Injection of Oocytes**

Oocytes in OR3 medium were visualized with a dissecting microscope and injected with 50 nl of cRNA for Rhbg (0.05 μg/μl, for a total of 2.5 μg of RNA). Control oocytes are injected with 50 nl of sterile H2O. The sterile pipettes have tip diameters of 20–30 μm. They are backfilled with paraffin oil and are connected to a Nanoject motor-driven pipette (Drummond Scientific). Injected oocytes are used 3–5 days after injection with cRNA.

**Electrophysiological Measurements in Frog Oocytes**

**Measurements of intracellular pH.** The pH microelectrodes were of the liquid ion exchanger type and the resin (H+ ionsphere 1, *cocktail B*) was obtained from Fluka (Buchs, Switzerland). Single-barrelled microelectrodes were manufactured as described earlier (29). Briefly, aluminosilicate glass tubings (1.2-mm OD × 0.86-mm ID; Frederick Haer, Brunswick, MD) were pulled to a tip diameter <0.2 μm and dried in an oven at 200°C for 2 h. The electrodes were vapor silanized with bis(dimethylamino)-dimethyl silane in a closed vessel (300 ml) as described by Siebens and Boron (30). The exchanger was then introduced into the tip of the electrodes by means of a very fine glass capillary. pH electrodes were backfilled with a buffer solution containing 0.04 M KH2PO4, 0.023 M NaOH, 0.15 M NaCl, pH 7.0 (1). The electrodes were fitted with a holder with an Ag-AgCl wire attached to a high-impedance probe of a WPI FD-223 electrometer and calibrated. The pH electrodes were calibrated in standard solutions of pH 6 and 8. All the electrodes used in this study had a slope of >58 mV/pH.

Ling-Gerard (voltage) microelectrodes were pulled from 1.5-mm (OD) borosilicate fiber capillaries and filled with 3 M KCl. Their resistances were in the range of 8–16 MΩ and their tip potential was <4 mV. Both electrodes then impaled the oocyte. The membrane potential (Vmem) was obtained by measuring the voltage difference between the voltage microelectrode and a free flowing 3 M KCl Ag:AgCl reference electrode in the bath. The gross potential of the pH microelectrode was measured between the pH microelectrode and the microelectrode was measured between the voltage microelectrode and a free flowing 3 M KCl Ag;AgCl reference electrode in the bath. The pure intracellular pH (pHi) voltage was obtained by subtracting electronically Vmem from the gross potential of the pH electrode. Two-electrode voltage clamp. Two-electrode voltage clamp (OC-725, Warner Instruments, Hamden, CT) was used to measure whole cell currents. For these experiments, electrodes were pulled from borosilicate glass capillaries (OD 1.5 mm, Frederick Haer) using a vertical puller (model 700C, David Kopf Instruments). Electrodes were filled with 3 M KCl solution and had resistances of 1–4 MΩ. Bath electrodes were also filled with 3 M KCl and were directly immersed in the chamber. In most cases, oocytes were clamped at −60 mV and long-term readings of current were sampled at a rate of one per second. For current-voltage (I-V) relationships, oocytes were clamped at −60 mV and stepped from −60 to +60 mV in 20-mV steps (sampled at 10 times per second for a period of ~1 s at each step). Slope conductance was calculated from the slope of the I-V line at specified clamp potentials. Inward flow of cations is defined by convention as inward current (negative current).

The oocyte, visualized with a dissecting microscope, was held on a nylon mesh in a special chamber, through which solutions flow continuously at a rate of 3–5 ml/min. Incoming solution passed through a water-jacketed stainless-steel tube (22°C). Solutions (6 possible) were switched by a combination of a six-way and a four-way valves system that was activated pneumatically. Complete exchange of the solutions in the chamber occurred in 6–8 s, which was verified by placing a pH microelectrode near the outflow port and timing the change in pH on running solutions of different pH. The rate of change of solutions in the chamber was much faster than the usually slow pH changes that occur in the oocyte. Initial rates of change of pH (dpH/dt) were determined by fitting pH vs. time to a linear regression line. In all protocols, values are reported as means ± SE. Statistical significance was judged from Student’s t-test, unless otherwise noted.

**RESULTS**

**Transport of NH3/NH4+ in H2O-Injected Oocytes**

To investigate the transport characteristics of Rhbg, we relied on measurements in oocytes expressing Rhbg. In these experiments, NH3/NH4+ transport was assessed from measurements of pHi, by ion-selective microelectrodes and whole cell currents using two-electrode voltage clamp. In oocytes, NH3/ NH4+ transport is characterized by changes that are different from those in most mammalian cells. Namely, the oocyte membrane is apparently very permeable to NH4+ and less so to NH3 (6, 27). Therefore, in our experiments, we first characterized the NH3/NH4+-induced changes in H2O-injected oocytes. As can be seen in Fig. 1, exposing the oocyte to 20 mM NH4Cl caused a pH decrease (segment ab) and substantial depolarization of the cell. Both changes are due to significant net NH4+ influx that was faster than NH3 influx via diffusion and consequently masked any NH3-induced pH change (an expected increase).

Consistent with electrogenic ammonium influx, exposing oocytes to 20 mM NH4Cl also resulted in an inward current of 178 ± 23 nA (n = 9) as shown in Fig. 1B. All changes were completely reversed on removal of bath ammonium, with pH recovery more slowly (segment bc) than either Vmem or I. In previous studies, we documented that 20 mM NH4Cl caused a decrease in pH of 0.28 ± 0.05 (n = 20) and depolarized the cell near to 0 mV (28). Other studies reported similar changes in pHCl and Vmem (5–7).

To assess ammonium transport by Rhbg, we compared the ammonium-induced pH decrease and current changes in H2O-injected oocytes to those expressing Rhbg. If Rhbg transports ammonium in an electrogenic fashion, then expressing Rhbg would result in increased ammonium transport and the following predictions can be made. 1) The magnitude of ammonium-induced inward current should be more in Rhbg-expressing oocytes demonstrating increased ammonium transport. 2) The whole cell conductance would be greater and the I-V relationships would be steeper. 3) The ammonium-induced pH decrease should also be faster in oocytes expressing Rhbg. This is expected to occur irrespective of whether NH4+ transport is electrogenic or not.

In the following experiments, we used these criteria to characterize ammonium transport by Rhbg.

**Effect of NH4Cl in Oocytes Expressing Rhbg**

**Effect on current.** The first prediction assumes that Rhbg transports ammonium in an electrogenic manner and therefore ammonium influx in Rhbg oocytes could be detected as an enhanced inward current compared with that in H2O-injected oocytes. To investigate this possibility, we conducted the experiments depicted in Fig. 2A. In these experiments, we exposed the oocytes to increasing concentrations of bath am-
monium and measured the resulting inward current. Figure 2A shows superimposed tracings from a H2O-injected oocyte and an Rhbg-expressing oocyte. As can be seen, lower concentration of bath ammonium (5 mM) elicited almost no inward current in H2O-injected oocytes but a significant current in Rhbg oocytes. Similarly, 10 mM ammonium caused a bigger effect on current (I) in Rhbg-expressed oocytes than in H2O-injected oocytes. Interestingly though, 20 mM ammonium seemed to induce an inward current that was not significantly different from that in H2O-injected oocytes, suggesting that ammonium transport saturates at higher concentrations of bath ammonium.

In similar experiments, it was consistently clear that the difference in magnitude of the ammonium-induced inward current was most significant at lower concentrations of ammonium and therefore, 5 mM ammonium was subsequently used in most experiments. As shown in Fig. 2B, the change in inward current (ΔI) induced by 5 mM bath NH4Cl was −93 ± 7.7 nA (n = 20) in oocytes expressing Rhbg but only −29 ± 7.1 nA (n = 7) in H2O-injected oocytes (P < 0.005).

I-V Relationship for Rhbg and H2O-Injected Oocytes in the Presence of NH4Cl

To investigate further whether Rhbg transports ammonium, we measured the whole cell currents in relation to clamped potentials between −60 and +60 mV. Figure 3A shows the I-V relationship of H2O-injected and Rhbg-expressing oocytes in the absence and presence of 5 mM NH4Cl in the bath. In the absence of bath NH4Cl, the I-V curves of H2O-injected oocytes were not significantly different from those of Rhbg-expressing oocytes (bottom tracing). In the presence of 5 mM NH4Cl, there was enhanced transport in H2O-injected oocytes (middle tracing). In oocytes expressing Rhbg, exposure to 5 mM NH4Cl increased whole cell conductance at all potentials significantly more than in H2O-injected oocytes in 5 mM NH4Cl (top tracing). Figure 3B shows the net change in current at different voltages due to the presence of 5 mM NH4Cl. In this case, the plots are the difference between the presence of 5 mM NH4Cl and the absence of NH4Cl in both Rhbg-injected and H2O-injected oocytes. This figure also shows that the reversal potential was +2 mV in Rhbg-injected oocytes and was slightly more negative (−2 mV) in H2O-injected oocytes. These data clearly demonstrate enhanced electrogenic transport due to ammonium, which is more significant in oocytes expressing Rhbg than in H2O-injected oocytes.

pHi Changes Induced by NH4Cl in Rhbg Oocytes

The experiments presented above indicated that expression of Rhbg enhanced NH4+ transport. Moreover, ammonium transport by Rhbg was electrogenic and significantly evident at low concentrations of bath ammonium (5 mM). To character-
that caused by 20 mM NH₄Cl in H₂O-injected oocytes (0.29 ± 0.05) and the rate of acidification was even faster than the
-9.2 ± 1.1 × 10⁻⁴ pH/s observed in H₂O-injected oocytes. By comparison, exposure to 5 mM NH₄Cl in H₂O-injected
oocytes caused a sustained depolarization of 20 ± 5.8 mV and
pHᵢ decreased only by 0.07 ± 0.01 at a rate of -2.7 ± 0.6 × 10⁻⁴ pH/s (n = 6). These changes in Vₗ and pHᵢ indicate that
Rhbg expression enhanced NH₄⁺ transport as observed in the
voltage-clamp experiments. These data do not clearly indicate
any significant effect of Rhbg expression on NH₃ transport (in
which case pHᵢ would increase) but rather confirm that only
NH₄⁺ transport was enhanced.

Ammonium-Induced pHᵢ Changes at Constant NH₃

To characterize ammonium transport via Rhbg further, we
measured the NH₄⁺-induced pHᵢ changes when external NH₃
was kept constant. As shown in Fig. 5, exposing oocytes that
express Rhbg to 5 mM ammonium at bath pH (pHᵢᵣ) of 7.5
caused pHᵢ to decrease (segment ab) from 7.16 ± 0.04 to
6.88 ± 0.06 (n = 5) and depolarized the cell from -50 ± 3.2
mV to a peak value of -22 ± 2.3 mV (n = 6). These changes
are very similar to those observed earlier (see Fig. 4 above).
When pHᵢ reached a stable value, external NH₄Cl was in-
creased to 10 mM at a bath pH of 7.21. Under these conditions
(assuming a pKa of NH₃/NH₄⁺ of 9.25), bath [NH₄⁺] will
increase from 4.91 mM (at 5 mM NH₄Cl, pH 7.5) to 9.91 mM,
whereas [NH₃] will remain constant at ~0.09 mM. Raising
external [NH₄⁺] at constant [NH₃] in this manner caused a
small further decrease in pHᵢ (segment bc) by 0.05 ± 0.04
(n = 5) accompanied by an additional peak depolarization of
3 ± 0.6 mV. Removal of external NH₄Cl resulted in complete
recovery of pHᵢ and Vₗ (segment cd).

By comparison, exposing H₂O-injected oocytes to 5 mM
NH₄Cl (at pH 7.5) in the bath (Fig. 6) caused a smaller
decrease in pHᵢ, (segment ab) from 7.21 ± 0.03 to 7.15 ± 0.02
(n = 6) and the depolarization of the cell was also less (ΔVₗᵢ = 
14 ± 3 mV, n = 6). In several instances, 5 mM did not even
elicit a change in pHᵢ in H₂O-injected oocytes. Subsequent
increase in bath [NH₄⁺] at constant [NH₃] (ammonium in-
creased from 4.91 to 9.91 mM) caused a further decrease in pHᵢ of
0.05 ± 0.004 and depolarized the oocyte further by 7 ± 2.6
mV (n = 6). All changes in pHᵢ and Vₗ were fully reversed
when NH₄Cl was removed from the bath (segment cd). The
results of these experiments indicate that 1) NH₄⁺-induced pHᵢ
decrease at 5 mM was more pronounced in Rhbg oocytes than
in H₂O-injected oocytes. 2) Raising external [NH₄⁺] (from 4.91
to 9.91) at constant [NH₃] did not significantly reduce pHᵢ or
change Vₗ in Rhbg oocytes. This may be due to significant
accumulation of intracellular [NH₄⁺] on initial exposure to 5
mM NH₄⁺. In this case (relatively high intracellular NH₄⁺), the
increase in bath [NH₄⁺] from 4.9 to 9.91 mM was not high
enough to significantly raise the out-to-in gradient of NH₄⁺. 3) In
H₂O-injected oocytes, there was little transport of NH₄⁺ at
low concentrations.

NH₄⁺-Induced Dose-Dependent pHᵢ Changes in Rhbg
and H₂O-Injected Oocytes

NH₄⁺ influx through Rhbg can best be assessed by measure-
ment of NH₄⁺-induced H⁺ flux, because direct measurement of
intracellular NH₄⁺ is not possible. To this end, defining the

Fig. 3. Current-voltage (I-V) relationship for H₂O-injected oocytes and oocytes expressing Rhbg in the presence and absence of 5 mM NH₄⁺ in the bath. A: in the absence of external NH₄⁺, whole cell recordings indicate similar I-V relationships for oocytes expressing Rhbg and H₂O-injected oocytes. In the presence of 5 mM NH₄⁺, the I-V curve in H₂O-injected oocytes indicates increased conductance (at all tested potentials) that was even more pronounced in oocytes expressing Rhbg. B: net effect of 5 mM NH₄⁺ on whole cell recordings. The 2 curves are obtained from A by subtracting the respective plots of H₂O-injected or Rhbg-expressing oocytes from that in the absence of external NH₄⁺. The figure clearly shows enhanced electrogenic transport of NH₄⁺ in oocytes expressing Rhbg.

To characterize these observations further, we measured the pHᵢ changes induced by 5 mM ammonium in oocytes expressing Rhbg. As shown in Fig. 4, exposing Rhbg oocytes to 5 mM NH₄Cl caused a rapid decrease in pHᵢ (segment abc) and the cell
depolarized significantly. In most experiments, the change in
Vₗ showed a transient fast depolarization (28 ± 3.6 mV) followed by a slow partial recovery (segment a'b'c'). The final
depolarization averaged 20 ± 3.5 mV (n = 6). All changes
were fully reversed when NH₄Cl was removed from the bath.
PΔHᵢ increased slowly (segment cd) and Vₗᵢ recovered with a
transient hyperpolarization that mirrored the effect of exposure
to ammonium (segment c'd'e'). The transient changes in Vₗᵢ on
exposure to NH₄Cl were only observed in oocytes expressing Rhbg and were not evident in H₂O-injected oocytes. In six experiments on oocytes expressing Rhbg, 5 mM NH₄Cl caused
pHᵢ to decrease by 0.3 ± 0.04 pH units and the initial rate of acidification was -20 ± 2.5 × 10⁻⁴ pH/s. The decrease in pHᵢ caused by 5 mM NH₄Cl in Rhbg oocytes was comparable to
relationship between NH$_4^+$ influx (as estimated from NH$_4^+$-induced H$^+$ gain) and the transmembrane [NH$_4^+$] gradient is essential to identify the mechanism of NH$_4^+$ transport by Rhbg. To do so, we measured the NH$_4^+$-induced pH$_i$ changes in response to varying concentrations of NH$_4^+$ in the bath. As shown in Fig. 7, raising bath NH$_4^+$ concentration in increments from 1 to 20 mM elicited a proportionally faster rate of pH$_i$ decrease, a larger decrease in pH$_i$ ($\Delta$pH$_i$), and larger depolarization ($\Delta V_m$). The rate of pH$_i$ decrease can be used to assess NH$_4^+$-induced H$^+$ gain because H$^+$ influx can be calculated as the product of the rate of pH$_i$ decrease (dpH$_i$/dt) and the intracellular buffering power ($B_{\text{int}}$). A plot of dpH$_i$/dt vs. external [NH$_4^+$] in Rhbg oocytes was compared with that of H$_2$O-injected oocytes and is shown in Fig. 8A. As can be seen in this figure, the NH$_4^+$-induced H$^+$ flux (as indicated by dpH$_i$/dt) was significantly higher in oocytes expressing Rhbg than in H$_2$O-injected oocytes at all values of external NH$_4^+$. The proportional increase in NH$_4^+$-induced H$^+$ influx was not linear but saturates at higher NH$_4^+$ concentrations in the bath. Moreover, the background NH$_4^+$ influx in H$_2$O-injected oocytes shows a reduced pattern of response to external NH$_4^+$. In fact, the plot of rate vs. concentration in H$_2$O-injected oocytes

Fig. 4. NH$_4^+$ effects on pH$_i$ and $V_m$ in oocytes expressing Rhbg. In Rhbg oocytes, 5 mM NH$_4^+$ in the bath caused transient peak depolarization of the cell (28 ± 3.6 mV) with a partial recovery of $V_m$ and decreased pH$_i$ by 0.3 ± 0.04 (segment abc) at a rate of $-20 \pm 2.5 \times 10^{-4}$ pH/s. The transient cell depolarization was typical of oocytes expressing Rhbg and were never observed in H$_2$O-injected oocytes where depolarization was sustained (see Fig. 1). These changes were significantly larger than in control oocytes. All changes were reversed on removal of external NH$_4^+$ (segments cd and c’d’e’).

Fig. 5. pH$_i$ and $V_m$ changes induced by raising external [NH$_4^+$] at constant NH$_3$ in oocytes expressing Rhbg. Five millimolar NH$_4^+$ in the bath depolarized the oocyte by 23 mV and caused substantial and fast decrease in pH$_i$ at a rate of $-18 \times 10^{-4}$ pH/s (segment ab) as observed earlier. Subsequent exposure to 10 mM NH$_4^+$ at constant external NH$_3$ (pH$_e$ = 7.21) decreased pH$_i$ and depolarized the oocyte only slightly (segment bc). Changes were reversed on removal of bath NH$_4^+$ (segment cd).
Fig. 6. pH and $V_m$ changes induced by raising external $[\text{NH}_4^+]$ at constant $\text{NH}_3$ in $\text{H}_2\text{O}$-injected oocytes. Five millimolar $\text{NH}_4^+$ depolarized $\text{H}_2\text{O}$-injected oocytes by only $14 \pm 3.3$ mV and pH decreased by $0.07 \pm 0.01$ at a rate of $-2.7 \times 10^{-4}$ pH/s (segment ab). These changes are substantially smaller than in Rbgb oocytes. Increasing external $[\text{NH}_4^+]$ at constant $\text{NH}_3$ decreased pH, and depolarized the cell further (segment bc). These changes were reversed on removal of bath $\text{NH}_4^+$ (segment cd).

Fig. 7. Concentration effects of bath $\text{NH}_4^+$ on Rbgb oocytes. Oocytes expressing Rbgb were pulsed with increased concentrations of bath $\text{NH}_4^+$ (ranging from 1 to 20 mM). The rate of pH decrease and cell depolarization ($\Delta V_m$) caused by increasing external $[\text{NH}_4^+]$ were directly proportional to the concentration of $\text{NH}_4^+$ in the bath. The numbers on the pH tracing are the rates of pH decrease in PH/s $\times 10^{-4}$.

**Ion and Substrate Specificity of Rbgb**

$K^+$ dependence. To investigate whether $\text{NH}_4^+$ transport by Rbgb was dependent on $K^+$, we performed the experiments depicted in Fig. 9, A and B. In these experiments, we measured $\text{NH}_4^+$-induced inward current in the presence and absence of external $K^+$. If $\text{NH}_4^+$ transport by Rbgb was dependent on $K^+$ (as with $\text{NH}_4^+$-$K^+$ exchangers, for example), then removal of external $K^+$ is expected to inhibit $\text{NH}_4^+$-induced currents. As shown in Fig. 9A, exposing Rbgb-expressing oocytes to 5 mM...
NH$_4^+$ caused an inward current of $-58 \pm 3.1$ nA ($n = 8$; segment ab), which was readily reversed on removal of external NH$_4^+$ (bc). At point c, K$^+$ was removed from the bath, which caused no significant change in current ($\Delta I = -1 \pm 0.6$ nA, $n = 8$, segment cd). At point d, the oocyte was again exposed to 5 mM NH$_4^+$ in the absence of K$^+$, which resulted in an inward current of $-86 \pm 7$ nA ($n = 8$; segment de). This NH$_4^+$-induced current in the absence of external K$^+$ was significantly larger than in the presence of K$^+$ ($P < 0.01$, $n = 8$). These changes were completely reversed on removal of NH$_4^+$ (segment ef) and readdition of external K$^+$ (segment fg). The results of these experiments indicate that in oocytes expressing Rhbg, removal of K$^+$ did not inhibit the NH$_4^+$-induced inward current but rather NH$_4^+$-induced current was slightly increased ($\Delta I = -28.2 \pm 7.0$ nA).

Similar experiments were conducted in H$_2$O-injected oocytes. As shown in Fig. 9B, exposing the oocyte to 5 mM NH$_4^+$ caused an inward current (ab), which reversed on removal of bath NH$_4^+$ (bc). Removal of bath K$^+$ caused a small outward deflection of current (segment cd) of $+6 \pm 1.3$ nA ($n = 7$). Exposing the oocyte to 5 mM NH$_4^+$ in the absence of K$^+$ still caused inward current (de), which readily recovered on removal of bath NH$_4^+$ (ef). In seven experiments, NH$_4^+$-induced current was $-42 \pm 2.4$ nA in the presence of K$^+$ and $-56 \pm 6$ nA in the absence of K$^+$ ($P < 0.05$). The net increase in current in the absence of K$^+$ was $-14.3 \pm 4.5$ Na$^+$. These changes were very similar to those observed in the oocytes expressing Rhbg ($P = 0.13$) and indicate that NH$_4^+$-induced changes in whole cell current were not inhibited by removal of external K$^+$.

**Potential Inhibitors of NH$_4^+$ Transport**

To further investigate potential inhibitors of NH$_4^+$ transport, we checked the effect of tetraethyl ammonium (TEA) and tetramethyl ammonium (TMA). In paired experiments on oocytes expressing Rhbg, 5 mM NH$_4^+$ in the bath induced an inward current of $-61 \pm 19$ nA ($n = 5$) and $-60 \pm 18$ nA in the presence of 1 mM TMA. TMA did not cause any change in whole cell current. In experiments on H$_2$O-injected oocytes, pretreatment of oocytes with 1 mM TMA also did not cause any significant effect on NH$_4^+$-induced inward current.

Similarly, exposing oocytes to TEA (up to 1 mM) did not cause any significant effect on NH$_4^+$-induced current (at a holding potential of $-60$ mV) in oocytes expressing Rhbg or in H$_2$O-injected oocytes. In this respect, both TEA and TMA were not effective inhibitors of NH$_4^+$ transport in oocytes.

**Effect of Methyl Ammonium**

Methyl ammonium (MA) has been used extensively as an ammonium analog to measure NH$_4^+$ uptake. In our studies, we measured whole cell currents in oocytes exposed to (MA) and/or NH$_4^+$. In oocytes expressing Rhbg (Fig. 10A), 5 mM NH$_4^+$ induced an inward current (ab) of $-83 \pm 9.6$ nA ($n = 8$), which readily reversed on removal of external NH$_4^+$ (bc), as usually observed before. MA (5 mM) also induced a significant inward current of $-63 \pm 7.6$ nA (segment cd), which also reversed on removal of MA from the bath (de). At point e, the oocyte was exposed again to 5 mM MA causing an inward current (ef). In the presence of MA, exposing the oocyte to 5 mM NH$_4^+$ caused a further increase of inward current to...
These changes suggest that the effect of MA may be additive to that of NH$_4^+$.

On the other hand, in H$_2$O-injected oocytes, MA did not elicit similar effects. In those experiments (Fig. 10B), exposing the oocyte to 5 mM NH$_4^+$ caused the usual inward current (ab), which recovered on removal of both NH$_4^+$ (segment bc). In contrast to Rhbg, MA (5 mM) in the bath did not cause any change in whole cell current (cd). Moreover, exposing the oocyte to 5 mM NH$_4^+$, in the presence of MA (5 mM), induced an inward current (segment de) of $-98 \pm 12$ nA ($n = 5$), which was not statistically different from that in the absence of MA ($P > 0.05$).

**DISCUSSION**

The present study demonstrates that Rhbg transports NH$_4^+$, based on changes in $V_m$, pH$_i$, and whole cell currents in response to direct exposure of oocytes to NH$_4$Cl. In the absence of direct measurement of intracellular NH$_4^+$, the NH$_4^+$-induced pH$_i$ acidification, membrane depolarization, and inward current are the most reliable and expected indexes of NH$_4^+$ influx for several reasons. First, influx of the positively charged NH$_4^+$ across the membrane would depolarize the cell and decrease pH$_i$ as intracellular NH$_4^+$ dissociates to release H$^+$. Net NH$_4^+$ influx is further confirmed by measurement of an inward current proportional to NH$_4^+$ concentration in the bath. These parameters ($V_m$, pH$_i$, and $I$) were used in other studies to investigate NH$_4^+$ transport in oocytes and were directly attributed to net NH$_4^+$ influx (6, 7). Second, these measurements allow identification of properties not easily detected by other means. For example, current measurements, whole cell conductance, and $I$-$V$ plots can identify the electrogenic transport characteristics of these pathways. pH$_i$ measurements, coupled to measurements of $I$, can distinguish between NH$_3$ and NH$_4^+$ transport. In the first case, NH$_3$ transport by Rhbg, as was suggested in some studies (33, 34), would result in enhanced pH$_i$ increase, rather than pH$_i$ decrease observed in our studies.
Moreover, in the case of NH₃ transport by Rhbg, exposure to NH₄Cl would not result in an increased inward current as observed in oocytes expressing Rhbg. Third, by using these measurements, it may also be possible to distinguish whether these proteins act as channels or transporters. For example, NH₄⁺-induced influx that is not linear in relation to transmembrane concentration gradient is more characteristic of facilitated transport than of a channel.

Renal handling of total ammonia has two transport components: diffusion of lipid-soluble NH₃ and transport of charged NH₄⁺. Although NH₃ is highly permeant, its transport cannot account for all NH₄⁺ secretions in all segments of the nephron (19). Several mechanisms of NH₄⁺ transport have been described, including Na/H exchange in the proximal tubule, and Na-K-2Cl cotransport and K⁺ channels in the thick ascending loop (2, 9, 17). However, unlike most other solutes, NH₄⁺ stands out as a rare molecule whose renal transport is vital, yet no specific transporter for NH₄⁺ has yet been identified in any segment of the nephron. This observation and the fact that net NH₄⁺ transport cannot be totally accounted for by suggested transporters (such as Na/H exchange, K⁺ channels, etc.) suggest that NH₂⁻-specific transporters in the renal tissue are likely to exist. Renal Rh glycoproteins may actually be NH₂⁻ transporters in the kidney. The possibility that Rhbg and Rhcg are NH₂⁻ transporters can be postulated from several observations. 1) Both are membrane proteins that are abundant in tissues where significant NH₄⁺ transport occurs, most importantly, the kidney and liver (8, 35, 38). 2) Both proteins closely resemble known NH₂⁻ transporters in yeast (MEP proteins) and bacteria (Amt) in structure and molecular properties (21, 24, 25). 3) Limited uptake studies of MA in cells expressing RhAG and RhCG further suggest a possible role of these proteins as NH₂⁻ transporters (24). These observations, although important, provide only indirect evidence. The main goal of our studies was to examine whether Rhbg transports NH₂⁻.

In this study, we examined the transport characteristics of Rhbg. With Rhbg transport of net NH₂⁻, we would expect the following findings. First, NH₂⁻-induced pH decrease (ΔpHi) would be more pronounced than in H₂O-injected oocytes. This was clearly evident from the experiments of Figs. 1 and 4. In fact, at 5 mM NH₂⁻ in the bath (Fig. 4), the pHi decrease was more than that caused by 20 mM NH₄⁺ in H₂O-injected oocytes (Fig. 1). Second, NH₂⁻-induced H⁺ influx would increase. NH₂⁻-induced H⁺ influx would result from influx of NH₂⁻ that dissociates intracellularly to release H⁺. Therefore, NH₂⁻ influx can be indirectly estimated from the rate of NH₂⁻-induced pH decrease where H⁺ influx is calculated from the product of the buffering power (β) and the rate of pH decrease (β × ΔpHi/dt). In our experiments, the rate of NH₂⁻-induced pH decrease at 5 mM [NH₂⁻]b was 20 ± 2.5 × 10⁻⁴ pH/s, a value almost an order of magnitude more than 2.7 ± 0.6 × 10⁻⁴ pH/s in H₂O-injected oocytes.

Whereas these data strongly support enhanced net NH₂⁻ transport by Rhbg, two additional observations are noteworthy. First, in the experiments of Fig. 5, raising [NH₂⁻]b from 5 to 10 mM (at constant [NH₃]) did not lead to a substantial further decrease in pHi. One possibility is that transport of NH₂⁻ by Rhbg was diminished because bath pH was lowered (pHb = 7.21), suggesting that Rhbg might be sensitive to pH. A more likely cause, however, is that initial exposure of the Rhbg oocytes to 5 mM NH₂⁻ leads to significant intracellular accumulation of NH₄⁺. On shifting to 10 mM NH₄⁺, the inward gradient for [NH₄⁺] in Rhbg oocytes is not as high as that in H₂O-injected oocytes. Second, the NH₄⁺-induced depolarization in Rhbg oocytes was distinctly different from that in H₂O-injected oocytes. In the presence of Rhbg, the change in Vm has a transient profile with partial recovery, whereas in H₂O-injected oocytes, the depolarization was sustained. In oocytes expressing Rhbg, the initial rapid depolarization is due to the inward flux of NH₄⁺, the relaxation of Vm is consistent with redistribution of intracellular ions caused by the accumulation of intracellular NH₄⁺, and the accompanying NH₄⁺-induced pHi decrease. In this case, it is likely that the initial depolarization is even an underestimate with partial recovery of Vm triggered by the intracellular changes caused by the influx of NH₄⁺.

It is to be noted that two recent preliminary reports (37, 40) concluded that Rhbg functions as an electroneutral NH₂⁻/H⁺ exchanger or as an NH₃ transporter. These studies relied predominantly on uptake of radiolabeled MA as an analog of NH₂⁻. A possible drawback of such an approach is the difficulty in ascertaining that MA is truly a good substrate for NH₂⁻. For example, in experiments on oocytes, 20 mM MA did not elicit any significant changes in pHi; Vm, or I. Our data, obtained in oocytes expressing Rhbg, do not support these conclusions but rather indicate that transport of NH₂⁻ by Rhbg is electrogenic. This is evidenced by the fact that exposure of oocytes to NH₂⁻ in the bath substantially depolarized the cell (see Figs. 4 and 5). The amount of depolarization was significantly more in oocytes expressing Rhbg than in H₂O-injected oocytes. In addition, in the presence of NH₂⁻, the I-V curve in Rhbg oocytes was steeper than in H₂O-injected oocytes (Fig. 3A), indicating increased whole cell conductance at all clamp voltages. In Rhbg oocytes, the larger inward current at negative potential is indicative of a larger NH₂⁻ influx and agrees with the depolarization recorded in open-circuit experiments as discussed above. At positive potentials, the increased outward current could be due to activation of another outward current (e.g., Cl entry) or could be due to enhanced efflux of the accumulated intracellular NH₂⁻.

More directly, the NH₂⁻-induced current in Rhbg oocytes was significantly more than in H₂O-injected oocytes (see Fig. 2B). It is important to note that at relatively high concentrations of NH₂⁻ in the bath (20 mM), the inward current induced by NH₂⁻ saturates. One partial explanation could be that the negative holding potential (~60 mV) for a prolonged period of time before exposure to 20 mM NH₂⁻ in the bath resulted in significant accumulation of intracellular NH₂⁻ compared with H₂O-injected oocytes. In such a condition, the concentration gradient would be less steep on switching bath NH₂⁻ from 10 to 20 mM.

Our data do not support the notion that Rhbg functions as an NH₃ transporter. Some studies in yeast have suggested that certain members of the Rh family may actually transport NH₃ and not NH₂⁻ and that they may be acting as NH₃ sensors (31, 33). In this case, enhanced NH₃ transport by Rhbg expression would have resulted in more pronounced initial alkalization due to NH₃ influx (where NH₃ entering the cell would react with H⁺ to form NH₂⁻). This was not observed in any of our experiments but rather changes consistent with increased NH₂⁻ transport were evident: a decrease in pHi, depolarization, and more inward current.
Our data do not support the idea that Rhbg functions as an NH$_4^+$/H$^+$ exchanger. A 1:1 electroneutral configuration was proposed for RhAG expressed in oocytes (39). If Rhbg were such an electroneutral NH$_4^+$-H$^+$ exchanger as suggested for RhAG, then the following would be expected. 1) No enhanced effect of NH$_4^+$ on pH$_i$ would be evident in oocytes expressing Rhbg, because the exchanger would effectively operate as an NH$_3$ transporter. At best, a transient NH$_3$-induced cell alkalization would be observed. 2) Exposure to NH$_4^+$ would elicit no electrical responses (I or V_m) different from in H$_2$O-injected oocytes. 3) No difference in J-V relationship in the presence of NH$_4^+$ would be evident between Rhbg and H$_2$O-injected oocytes. In contrast to these predictions, the results from our experiments clearly show that all changes were more pronounced in oocytes expressing Rhbg. Although, conceivably, Rhbg may behave differently in different cell types, our data from three different measurements (pH$_i$, voltage, and conductance) suggest an electrogenic transport mechanism.

### Ion and Substrate Specificity

In our study, we investigated several compounds that could potentially affect NH$_4^+$ transport by Rhbg. Among these, quaternary amines such as TMA and TEA have been previously considered as competitive inhibitors of NH$_4^+$ transport in X. laevis oocytes (6a). In our experiments, neither TMA nor TEA, up to 1 mM concentration, had any effect on NH$_4^+$-induced current in oocytes expressing Rhbg. Moreover, none of these compounds induced any change in current on their own, suggesting that they are minimally transported in their charged form.

In the experiments to investigate the involvement of K$^+$ in Rhbg transport of NH$_4^+$ (Fig. 9, A and B), the following results were demonstrated. 1) Removal of K$^+$ did not abolish the NH$_4^+$-induced current. In relevance to Rhbg, this indicates that NH$_4^+$ transport by Rhbg is not coupled to K$^+$ transport. 2) On the other hand, NH$_4^+$-induced current in the presence of bath K$^+$ was slightly but significantly less than in the absence of K$^+$. This was observed in H$_2$O-injected and Rhbg-expressing oocytes and suggests that K$^+$ may be competing with NH$_4^+$ transport. This is not unusual, given the similar molar size of K$^+$ and NH$_4^+$. However, this is unlikely a characteristic of Rhbg because the effect of K$^+$ (or its absence) on current was very similar in H$_2$O-injected and Rhbg oocytes. The K$^+$-inhibitable NH$_4^+$-induced current in H$_2$O-injected oocytes (-14.3 ± 4.5 nA) was not statistically different from -28.2 ± 7.0 nA observed in oocytes expressing Rhbg (P = 0.13). These findings do not support a significant role of K$^+$ in affecting the electrogenic NH$_4^+$ transport by Rhbg.

In a final set of experiments, we investigated transport of MA in oocytes expressing Rhbg. Our data show that MA (5 mM) was readily transported by Rhbg but not in H$_2$O-injected oocytes. Moreover, NH$_4^+$ transport was not affected by the presence of MA in H$_2$O-injected oocytes but was significantly augmented in oocytes expressing Rhbg. This suggests an additive effect and may indicate that Rhbg can transport MA.

Other studies, including Na$^+$ dependence, role of intracellular and extracellular pH, as well as other potential inhibitors, are being investigated.

### Is Rhbg a Channel or a Transporter?

Our data indicate that Rhbg transports NH$_4^+$. It is very difficult to rule out the possibility that expressing Rhbg activates a native transporter or a channel in oocytes rather than expressing an exogenous transporter. However, several observations support the exogenous transporter hypothesis. First, data from yeast studies indicate that Rhbg and Rhcg act as NH$_4^+$ transporting mechanisms. Second, our studies on transport of MA indicate substrate specificity of Rhbg (Fig. 10B). Finally, the mode of transport of NH$_4^+$ in oocytes expressing Rhbg is significantly different from that in H$_2$O-injected oocytes, arguing against activation of a native transporter. Nevertheless, in the absence of specific inhibitors of Rhbg, this possibility cannot be totally excluded pending further studies.

In its simplest form, transport via a channel exhibits a linear relationship between flux of an ion and the concentration gradient across the membrane. Our data indicate that the relationship between NH$_4^+$ influx by Rhbg and the imposed NH$_4^+$ gradient follows saturation kinetics according to a Michaelis-Menten relationship. The V$_{max}$ in this case was 30.8 and K$_m$ was 2.3 mM ($R^2 = 0.99$). This observation is consistent with Rhbg behaving as a transporter rather than a channel. In summary, our data support the following conclusions. 1) Rhbg transports NH$_4^+$. 2) Transport of NH$_4^+$ is electrogenic. 3) Rhbg is unlikely to be a NH$_4^+$/H$^+$ exchanger. 4) Rhbg is unlikely to be an NH$_3$ transporter. 5) Rhbg is more likely an electrogenic transporter than an ion channel.

Excretion of NH$_2^-$ represents a principal mechanism of renal excretion of acid equivalents. Rhbg and Rhcg are likely to play a significant role in renal NH$_4^+$ handling, although the physiology at the nephron level remains to be explored further.

### NOTE ADDED IN PROOF

While this manuscript was in review, a recent publication by Shahram et al. (Science 305: 1587–1594, 2004) reported resolving the crystallographic structure of bacterial Amt-B, a protein related to the Rh glycoprotein family. The authors propose a mechanism of recruitment of NH$_4^+$ by a vestibular structure of the protein and passage of neutral NH$_3$ through a 20-A long hydrophobic pore. As such, the protein provides a unique path as a gas channel. The electrogenic characteristics of NH$_3$/NH$_4^+$ transport by some members of the Rh family, including our data on Rhbg, cannot be fully explained by this model. Applying the structural model of Amt-B to Rhbg should help to resolve this issue.

### GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-62295–01A1, National American Heart Association Grant 0050547N, and American Heart Association Southern Affiliate Grant 0255258B.

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