Basolateral ammonium transport by the mouse inner medullary collecting duct cell (mIMCD-3)

Mary E. Handlogten, Seong-Pyo Hong, Connie M. Westhoff, and I. David Weiner. Basolateral ammonium transport by the mouse inner medullary collecting duct cell (mIMCD-3). Am J Physiol Renal Physiol 287: F628–F638, 2004. First published May 18, 2004; 10.1152/ajprenal.00363.2003.—The renal collecting duct is the primary site for the ammonia secretion necessary for acid-base homeostasis. Recent studies have identified the presence of putative ammonia transporters in the collecting duct, but whether the collecting duct has transporter-mediated ammonia transport is unknown. The purpose of this study was to examine basolateral ammonium transport in the mouse collecting duct cell (mIMCD-3). To examine mIMCD-3 basolateral ammonium transport, we used cells grown to confluence on permeable support membranes and quantified basolateral uptake of the radiolabeled ammonia analog [14C]methylammonia ([14C]MA).

mIMCD-3 cell basolateral MA transport exhibited both diffusive and transporter-mediated components. Transporter-mediated uptake was not altered by inhibitors of Na+/H+exchange, Na+/K+-ATPase, or KCC proteins, by extracellular sodium or potassium removal or by varying membrane potential, suggesting the presence of a novel, electroneutral ammonia-MA transport mechanism. Increasing the outwardly directed transmembrane H+ gradient increased transport activity by increasing Vmax. Finally, mIMCD-3 cells express mRNA and protein for the putative ammonia transporter Rh B-glycoprotein (RhBG), and they exhibit basolateral RhBG immunoreactivity. We conclude that mIMCD-3 cells express a basolateral electroneutral NH4+/H+ exchange activity that may be mediated by RhBG.

Renal ammonia production and transport are critically important in the maintenance of acid-base homeostasis. Ammonia is produced in the proximal tubule, actively secreted into the luminal fluid, actively reabsorbed into the renal interstitium in the loop of Henle, and then transported from the renal interstitium and secreted into the luminal fluid by the connecting segment and the collecting duct (14, 21, 28). 

Excretion of ammonia into the urine results in equimolar bicarbonate production and return to the systemic circulation via the renal vein (14, 21, 28). Conditions associated with increased renal bicarbonate production, such as metabolic acidosis, are associated with increased renal ammonia production and transport (14, 21, 28). Accordingly, understanding the characteristics and mechanisms of renal ammonia transport is important.

Ammonia transport mechanisms in the proximal tubule and the loop of Henle have been extensively studied. Ammonia secretion in the proximal tubule occurs primarily by ammonium (NH4+) secretion by NHE3, the apical Na+/H+ exchanger (23, 37, 38). A lesser contribution occurs via a Ba2+-sensitive transporter, most likely an apical K+ channel (23, 48). In the thick ascending limb of the loop of Henle, the apical Na+/K+-2Cl– cotransporter BSC2 transports NH4+ (4, 8, 20). This occurs as NH4+ substitutes for K+ at the K+ transport site. Other proteins, including an apical K+/NH4+ antiporter and an amiloride-sensitive NH4+ conductance, also contribute to ammonia reabsorption (3, 8, 19). Thus multiple proteins contribute to ammonia transport in the proximal tubule and the loop of Henle.

Recently, a new family of putative ammonia transporters has been identified, Rh-associated glycoprotein (RhAG), a member of the Rh multimeric complex in erythrocytes, transports the NH4+ analog methylammonia, enables ammonia to efflux when expressed in Saccharomyces cerevisiae, and complements growth defects in S. cerevisiae deficient in endogenous NH4+ transporters (33, 66). Rh B-glycoprotein (RhBG) and Rh C-glycoprotein (RhCG) are homologous proteins widely expressed in ammonia-transporting tissues, including kidney, liver, brain, and skin (31, 32, 57, 64). More specifically, RhBG and RhCG are expressed in specific plasma membranes in the renal connecting segment and collecting duct, leading to the suggestion that they may contribute to transepithelial ammonia secretion in these segments (57, 63, 64).

However, previous studies examining the characteristics of transepithelial ammonia transport in the collecting duct suggested that ammonia transporters were not present in the collecting duct (16, 17, 29). These studies demonstrated that transepithelial ammonia secretion was unrelated to active sodium reabsorption, was stimulated by luminal acidification, and that there was either no or only very minimal transepithelial NH4+ permeability (16, 17, 24, 29). One interpretation was that transepithelial ammonia secretion was mediated by passive NH4+ diffusion. Another interpretation, however, is that trans-
port might be mediated by one or more proteins that effect net NH₃ transport.

Because RhBG and RHCG, the putative ammonia transporters, are expressed in the connecting segment and collecting duct (15, 43, 57), determining whether ammonia transport has the characteristics of protein-mediated transport or is mediated solely by passive NH₃ diffusion is important. The current studies examine this question by using the mIMCD-3 mouse collecting duct cell line. The radiolabeled ammonia analog, [¹⁴C]methylammonia ([¹⁴C]MA), was used as an ammonia surrogate for transport studies. We then examined whether [¹⁴C]MA uptake was inhibitable, whether it was electroneutral or electrogenic, whether it was mediated by known or likely ammonia transporters, and whether transport might be mediated via NH₄⁺/H⁺ exchange. Finally, we examined whether mIMCD-3 cells express the renal putative ammonia transporter RhBG, mRNA, and protein.

**METHODS**

mIMCD-3 cells. mIMCD-3 cells were obtained from American Type Culture Collection (Manassas, VA) and used between passages 20 and 40. mIMCD-3 cells were grown to confluence in 10% FCS containing DMEM:F-12 media on permeable support membranes (Costar Transwell filters). FCS was then decreased to 0.1% for 48 h to induce expression of specialized collecting duct proteins (11, 51).

Measurement of [¹⁴C]MA transport activity. We measured transporter activity as [¹⁴C]MA uptake from the basolateral media using standard techniques. Briefly, cells were rinsed with radiotracer-free uptake media, followed by exposure to uptake media. [¹⁴C]MA (0.275 μCi/ml, 5 μM, unless otherwise specified) was added only to the peritubular solution. At the conclusion of uptake, cells were rinsed rapidly with ice-cold, radiotracer-free uptake media. Soluble radioactivity was extracted by precipitating proteins with 10% TCA; cell protein was then solubilized in 0.2% SDS/0.2 N NaOH and quantified using a BCA assay. Uptake of [¹⁴C]MA is expressed as picomoles per milligram protein per 3 minutes unless specified otherwise. Appearance of [¹⁴C]MA in the luminal fluid was measured in all experiments and was always <1% of peritubular [¹⁴C]MA.

Uptake media, unless otherwise detailed, contained (in mM) 130 NaCl, 5 KCl, 10 HEPES, 10 choline chloride, 5 glucose, and 1.2 CaCl₂ and was titrated to pH 7.5. Equimolar choline substituted for sodium or potassium in Na⁺-, or K⁺-free solutions. NH₄Cl or methylammonium chloride substituted for choline chloride when used.

Methylammonia transport modeling. To determine the relative contributions of diffusive and transporter-mediated transport to total methylammonia uptake, we modeled uptake using the equation (12)

\[
J_{\text{total}} = (J_{\text{diffusive}} \cdot [\text{MA}]) + (J_{\text{transporter}} \cdot ([\text{MA}] / ([\text{MA}] + K_m))
\]

where \(J_{\text{total}}\) is total uptake, \(J_{\text{diffusive}}\) is the diffusive rate coefficient, [MA] is extracellular methylammonia concentration, \(J_{\text{transporter}}\) is the transporter-mediated rate coefficient, and \(K_m\) is the affinity for transporter-mediated methylammonia transport. \(J_{\text{diffusive}}\), \(J_{\text{transporter}}\), and \(K_m\) were calculated using least-squares minimization (Quattro Pro, version 9, Corel). Because \(J_{\text{total}}\) varied by three orders of magnitude as a function of methylammonia concentration, we used log-transformed data.

To determine the methylammonia concentration at which diffusive and transporter-mediated uptake were equal, we used the following approach. Diffusive uptake can be calculated as \(J_{\text{diffusive}}\cdot[\text{MA}]\) and transporter-mediated uptake can be calculated as \(J_{\text{transporter}}\cdot([\text{MA}] / ([\text{MA}] + K_m))\). If they are equal then

\[
J_{\text{diffusive}} \cdot [\text{MA}]_{\text{equal}} = J_{\text{transporter}} \cdot ([\text{MA}] / ([\text{MA}] + K_m))
\]

where [MA]_{equal} is the methylammonia concentration at which transporter-mediated uptake equaled diffusive uptake. Mathematically rearranging this formula results in

\[
[\text{MA}]_{\text{equal}} = (J_{\text{transporter}}/J_{\text{diffusive}}) - K_m
\]

To determine the relative contribution of transporter-mediated methylammonia uptake to total uptake, we rearranged Eq. 1 to obtain

\[
\%\text{Transporter} = J_{\text{transporter}} \cdot ([\text{MA}] / ([\text{MA}] + K_m)) \cdot 100
\]

**Competitive inhibition of methylammonia transport by ammonia.** To determine whether ammonia competitively inhibits transporter-mediated methylammonia uptake, we used Dixon plot analysis. Briefly, we measured uptake of either 5 or 10 μM [¹⁴C]MA from the peritubular media in the presence of graded concentrations of extracellular ammonia. Because uptake can be diffusive or inhibitable, transporter-mediated [¹⁴C]MA uptake by mIMCD-3 cells, we determined the diffusive component of transport (\(J_{\text{diffusive}}\), using the equation (12)

\[
J_{\text{total}} = J_{\text{diffusive}} + J_{\text{transporter}} \cdot ([\text{Amm}] / ([\text{Amm}] + K_s))
\]

where \(J_{\text{total}}\) is the total uptake in the absence of inhibitor, \(J_{\text{diffusive}}\) is the diffusive, noninhibitable uptake, \(J_{\text{transporter}}\) is the noninhibitable, transporter-mediated uptake, transport activity, \([\text{Amm}]\) is ammonia concentration, and \(K_s\) is the calculated concentration of the inhibitor that results in 50% inhibition of transporter-mediated uptake. \(J_{\text{diffusive}}\), \(J_{\text{transporter}}\), and \(K_s\) were determined using least-squares minimization (Quattro Pro, version 9, Corel). We then calculated the transporter-mediated component of [¹⁴C]MA uptake at each ammonia concentration by subtracting the diffusive component, \(J_{\text{diffusive}}\), from total uptake. A Dixon plot (1/V vs. [I]) was used to determine the \(K_i\) for ammonia to inhibit transporter-mediated methylammonia uptake and to determine whether ammonia was a competitive or noncompetitive inhibitor of transporter-mediated [¹⁴C]MA uptake.

**Intracellular acid loading.** Cells were acid-loaded using a 20-min preincubation with NH₄Cl (10 mM), followed by washout of NH₄Cl. We used a sodium-free peritubular solution beginning with the NH₄Cl addition and continuing throughout the remainder of the experiment to prevent intracellular pH recovery via basolateral Na⁺/H⁺ exchange activity (50). Control cells were treated identically, except for substitution of choline chloride for NH₄Cl during the 20-min preincubation.

**Antibodies.** We used polyclonal anti-peptide antibodies to RhBG generated in our laboratory and characterized previously (57, 63, 64).

**Membrane protein preparation.** For protein extraction, culture dishes were washed twice with PBS containing 0.5 mM PMSF. mIMCD-3 cells were collected in PBS containing 0.5 mM PMSF and 1 mM EDTA, 1 mM PMSF, 2 μM aprotinin, 2 μM leupeptin, 2 μM pepstatin, pH 7.8) for 15 min. The cells were homogenized, had 10X salts added, and were then centrifuged at 1,000 g for 1 min at 4°C. The collected supernatants were centrifuged at 1,500 g for 5 min, followed by centrifugation of the supernatants at 21,000 g for 30 min at 4°C. The remaining pellet was resuspended in swelling buffer (10 mM Tris, 1 mM EDTA, 1 mM PMSF, 2 μM aprotinin, 2 μM leupeptin, pH 7.8) for 15 min. The cells were homogenized, had 10X salts added, and were then centrifuged at 1,000 g for 1 min at 4°C. The collected supernatants were centrifuged at 1,500 g for 5 min, followed by centrifugation of the supernatants at 21,000 g for 30 min at 4°C. The remaining pellet was resuspended in a mixture of swelling buffer and 10X salts (300 mM NaCl, 20 mM MgCl₂, 10 mM Tris, pH 7.8). Protein concentration was measured by the bicinchoninic acid method with BSA as a standard. The remainder was stored at −20°C until used.

**Immunoblotting procedure.** Immunoblotting was performed as we recently described in detail (57, 63). Briefly, 20 μg of protein/lane were separated on 10% SDS-PAGE ReadyGels (Bio-Rad, Hercules, CA), transferred electrophoretically to nitrocellulose membranes, blocked, incubated with primary antibody, washed, incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Promega, Madison, WI), and sites of antibody-antigen reaction were visualized using enhanced chemiluminescence (Super-
Signal West Pico Substrate, Pierce, Rockford, IL) and a Kodak Image Station 440CF digital imaging system.

Fluorescent microscopy. Confocal laser-scanning microscopy was used to identify RhBG immunoreactivity. Briefly, confluent mIMCD-3 cells on permeable support membranes were fixed by incubation for 1 h with 4% paraformaldehyde in PBS. Tissues were then treated with graded ethanol, rinsed with PBS, blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS, and incubated at 4°C overnight with primary antibody diluted 1:1,000 in PBS. Cells were washed with PBS, incubated with secondary antibody (FITC-labeled goat anti-rabbit IgG, 1:100 dilution, Sigma), and rinsed with PBS. The filter was then excised and mounted onto microscope slides and cover-slipped using Fluoromount (Southern Biotechnology Associates, Birmingham, AL). We then visualized the cell using an Axiovert 100M Laser Scanning Confocal Microscope (Carl Zeiss, Thornwood, NY) and LSM 510 Software (version 2.8, Carl Zeiss).

Real-time RT-PCR. We performed real-time RT-PCR as we previously described in detail (63). Briefly, total RNA was extracted using RNeasy MidiKit (Qiagen, Valencia, CA) and stored in a −70°C freezer until used. The forward primer for RhBG was 5′-GCC-TGCC-AGA-GTG-TGT-TTC-CA-3′, the reverse primer was 5′-GAG-CTG-ATA-GGC-GCC-CTG-AGA-3′, and the fluorescent probe was 6FAM-TGG-CAC-TCC-GCT-GAC-CCT-TGG-TAMRA. RNA was reverse transcribed using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers. Real-time RT-PCR was performed with an ABI Prism GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA), and results were analyzed using GeneAmp 5700 SDS software (version 1.3, PerkinElmer Applied Biosystems).

Chemicals. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise detailed. [(Dihydroindenyl)oxy] alkanoic acid (DIOA) was obtained from Alexis (Newington, NH), [14C]methylammonium chloride was obtained from ICN (Irvine, CA).

Statistics. All experiments were performed in at least three separate mIMCD-3 preparations. Statistical significance was determined using a paired t-test. In some cases, analysis of variance was used and is specifically noted in the text (Quattro Pro, version 9.0, Corel). In all cases, n refers to the number of separate mIMCD-3 preparations.

RESULTS

Methylammonia uptake by mIMCD-3 cells. Examining the characteristics of collecting duct epithelial cell ammonia transport requires the ability to assess transport across specific membranes. To examine basolateral ammonia uptake mechanisms, we used mIMCD-3 cells grown on semipermeable support membranes and quantified uptake from the peritubular media of the ammonia analog methylammonia, which is available as the radiolabeled compound [14C]MA. There was rapid peritubular methylammonia uptake; uptake was linear during the initial 3 min. Figure 1 shows a representative experiment.

Competitive inhibition of transport. Diffusive uptake of a compound can be differentiated from transporter-mediated uptake by determining the relationship between uptake and extracellular concentration (12). The magnitude of diffusive uptake is directly proportional to the extracellular concentration, whereas transporter-mediated uptake demonstrates saturation kinetics. Accordingly, we determined the relationship between basolateral methylammonia uptake and extracellular methylammonia concentration. Figure 2A shows results of a representative experiment. mIMCD-3 cell peritubular methylammonia uptake was curvilinear with respect to extracellular methylammonia concentration. As shown in Fig. 2A, this uptake demonstrated characteristics of both diffusive and transporter-mediated uptake. The Km for methylammonia for the transporter-mediated component averaged 4.6 ± 0.2 mM (n = 3 preparations). At methylammonia concentrations below 7.0 ± 1.8 mM (n = 3), the transporter-mediated component of methylammonia uptake exceeded the diffusive component. Figure 2B shows the proportion of total uptake that was modeled as transporter-mediated uptake, using the data from Fig. 2A. These results indicate the mIMCD-3 cell possesses two methylammonia transport mechanisms, an inhibitable, transporter-mediated component and a noninhibitable, diffusive component. The lower the methylammonia concentration, the relatively greater role of transporter-mediated uptake in total uptake.

Ammonia and methylammonia compete for transport by the same transporter in a large number of systems. Accordingly, we examined whether ammonia inhibited mIMCD-3 basolateral methylammonia transport activity. In the first set of experiments, we observed that extracellular ammonia decreased [14C]MA uptake. Results of a representative experiment are shown in Fig. 3A. Next, we determined whether ammonia was a competitive or noncompetitive inhibitor of [14C]MA uptake by examining the effect of ammonia to inhibit transporter-mediated [14C]MA uptake in the presence of differing concentrations of peritubular [14C]MA. Dixon plot analysis showed that ammonia acted as a competitive antagonist of [14C]MA uptake with a mean Ki of 2.1 ± 0.6 mM (n = 4). Figure 3B shows a representative experiment. Thus ammonia competitively inhibits mIMCD-3 basolateral methylammonia transport activity.
mediate mIMCD-3 basolateral [14C]MA uptake. In all experiments, we calculated the transporter-mediated uptake activity averaged 20.0 ± 1.5 pmol [14C]MA·mg protein−1·3 min−1, respectively (n = 5). None of these inhibitors altered inhibitable basolateral [14C]MA transport activity significantly (P = NS by ANOVA). Figure 4A summarizes these results. mIMCD-3 basolateral [14C]MA uptake activity is unlikely to be mediated by basolateral Na+-K+-ATPase, the Na+-K+-2Cl− cotransporter, or K+ channels.

Potassium chloride cotransporter (KCC) proteins are another family of transporters that can transport ammonia (10). To examine the possibility that one of the KCC proteins might mediate mIMCD-3 basolateral methylammonia transport, we examined the effect of the KCC inhibitor DIOA (58). Inhibitable methylammonia transport activity averaged 27.1 ± 3.3 pmol [14C]MA·mg protein−1·3 min−1 under basal conditions and 32.4 ± 1.3 pmol [14C]MA·mg protein−1·3 min−1 in the presence of DIOA, 50 μM (n = 3). DIOA did not alter inhibitable transport activity significantly (P = NS by paired t-test, n = 3). Figure 4B summarizes these results. KCC

In the studies described below, we examined the characteristics of mIMCD-3 basolateral methylammonia transport further. In all experiments, we calculated the transporter-mediated component of [14C]MA uptake as that inhibited by an excess of unlabeled methylammonia (10 or 20 mM) added in parallel studies.

Effect of K+ transport inhibitors on methylammonia transport. NH4+ and K+ have nearly identical biophysical properties when present in aqueous solutions. As a result, many K+ transporters also transport NH4+ (14, 21, 28). Because the mIMCD-3 cell has many K+ transporters, including Na+-K+-ATPase, the Na+-K+-2Cl− cotransporter, and K+ channels (13, 27, 47, 61), we examined whether K+ transporters might mediate mIMCD-3 basolateral [14C]MA uptake.

First, we examined the effect of inhibiting Na+-K+-ATPase, the Na+-K+-2Cl− cotransporter, and K+ channels using ouabain (10 μM), furosemide (100 μM), and Ba2+ (2 mM), respectively. In the absence of these compounds, inhibitable transport activity averaged 20.0 ± 1.5 pmol [14C]MA·mg protein−1·3 min−1 (n = 3). In the presence of ouabain, furosemide or Ba2+, transport activity averaged 27.3 ± 3.1, 24.3 ± 3.0, and 22.2 ± 2.8 pmol [14C]MA·mg protein−1·3 min−1, respectively (n = 5). None of these inhibitors altered inhibitable basolateral [14C]MA transport activity significantly (P = NS by ANOVA). Figure 4A summarizes these results. mIMCD-3 basolateral [14C]MA uptake activity is unlikely to be mediated by basolateral Na+-K+-ATPase, the Na+-K+-2Cl− cotransporter, or K+ channels.

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To further examine the possibility that [14C]MA transport activity might be mediated by a K+ transporter, we tested whether extracellular K+ would competitively inhibit [14C]MA transport activity. To do so, we compared transport activity in the presence of 5, 60, and 120 mM extracellular K+. To avoid possible effects of changing extracellular sodium, we maintained extracellular sodium at 12.5 mM and used choline as a cation substitute to maintain constant osmolality. Figure 5 summarizes the results. Inhibitable transport activity averaged 30.9 ± 3.2, 31.1 ± 1.1, and 35.0 ± 4.4 pmol [14C]MA·mg protein−1·3 min−1 in the presence of 5, 60, and 120 mM extracellular potassium, respectively (n = 3 for each). Increasing extracellular potassium did not alter inhibitable transport activity significantly (P = NS by ANOVA). These results provide further evidence that methylammonia uptake is distinct from the substitution of ammonium (NH4+) or methylammonium (CH3NH2+) at the potassium binding site of a potassium-transporting protein.

Effect of varying membrane potential. Because mIMCD-3 cells appear to express a novel ammonia-sensitive methylammonia transport activity, we designed the next set of experiments to determine further characteristics of this transport activity. First, we examined whether transport activity was regulated by membrane potential. We used the K+ ionophore valinomycin (10 μM) and varied extracellular K+ at 1, 5, 25, and 100 mM to clamp membrane potential over a range. Extracellular sodium was maintained constant at 20 mM to avoid possible effects of extracellular sodium changes on transport activity. Figure 6 summarizes these results. As shown, membrane depolarization, as occurred with increasing extracellular potassium in the presence of valinomycin, tended to increase inhibitable transport activity, but these changes were not statistically significant (P = NS by ANOVA, n = 5). Of note, if methylammonium (CH3NH2+) were the only molecular species transported, then depolarization should decrease transport activity, not increase it. Thus these results indicate that mIMCD-3 cells express an electroneutral basolateral [14C]MA transport activity.

H+-gradient stimulated transport. A recently identified ammonia-transporting protein, RhAG, appears to function as an electroneutral, ammonia-inhibitable methylammonium+/H+ exchanger (66). To determine whether mIMCD-3 basolateral [14C]MA transport activity might have similar characteristics, we examined the effect of acute intracellular acidification on transport activity. If mIMCD-3 methylammonia transport is mediated by methylammonium+/H+ exchange, then increasing the intracellular H+ concentration should increase methylammonia uptake.

We first increased intracellular H+ concentration using the standard ammonium chloride prepulse technique (46). Figure 7 shows representative results. Increasing intracellular H+ concentration significantly increased saturable, transporter-mediated uptake (Jact) to 400 ± 25% of that observed in control cells (P < 0.01, n = 3). The K1/2 for methylammonia increased.
slightly from 3.5 ± 0.3 to 5.5 ± 0.1 mM (P < 0.05, n = 3). This indicates a slightly decreased affinity for methylammonia, which cannot explain the increased methylammonia transport activity observed.

The identification that an ammonium chloride prepulse increases mIMCD-3 methylammonia transport activity suggests that increasing intracellular H\(^+\) concentration stimulates transport activity. To confirm this interpretation, we examined the effect of an alternative technique to alter intracellular pH.

When the K\(^+\)/H\(^+\) exchanger nigericin is added to cells, it inserts into the plasma membrane and results in equilibration of intracellular proton and potassium with extracellular proton and potassium such that the net chemical gradients for proton and potassium transport counterbalance (55). For example, when used in solutions that have an extracellular K\(^+\) concentration similar to intracellular K\(^+\) concentration, intracellular pH is fixed to extracellular pH (55). When used with solutions that have a K\(^+\) concentration less than intracellular K\(^+\) concentration, the intracellular proton concentration proportionally increases. When used with solutions that have parallel changes in extracellular H\(^+\) and K\(^+\) concentration, the equilibrium for proton transport via the K\(^+\)/H\(^+\) exchanger nigericin does not change, resulting in constant intracellular pH, while allowing changes in extracellular pH. The combination of extracellular pH and extracellular potassium concentrations used and their resultant intracellular pH are shown in Table 1.

Using this technique, we were able to examine the effects of altering intracellular at constant extracellular pH. As shown in Table 2 and Figure 8, intracellular acidification, whether at extracellular pH 7.4 or 7.7, stimulated inhibitable transport activity significantly (P < 0.05 and P < 0.02, respectively, n = 3). Thus intracellular acidification, whether induced using the NH\(_4\)Cl prepulse technique or the modified nigericin-K\(^+\) technique, increases mIMCD-3 basolateral methylammonia transport activity.

An increase in saturable transport with intracellular acidification could reflect increased methylammonium\(^+\)/H\(^+\) exchange activity due to an increased transmembrane H\(^+\) gradient.

### Table 1. Independent control of intracellular and extracellular pH using the K\(^+\)/H\(^+\) exchanger nigericin and variations in extracellular pH and potassium concentration

<table>
<thead>
<tr>
<th>Extracellular [K(^+)], mM</th>
<th>Extracellular pH</th>
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<tr>
<td>120</td>
<td>7.4</td>
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<tr>
<td>60</td>
<td>7.4</td>
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<tr>
<td>30</td>
<td>7.1</td>
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To allow separate changes in extracellular and intracellular pH, cells were incubated with solutions containing nigericin (10 \(\mu\)g/ml) and varying potassium concentrations ([K\(^+\)]) and varying extracellular pH. The combinations of extracellular pH and extracellular [K\(^+\)] used, and their resultant intracellular pH, are shown. Extracellular sodium was maintained constant at 20 mM in these solutions; changes in extracellular potassium were balanced by equimolar changes in choline chloride.

### Table 2. Effect of variations in extracellular and intracellular pH on mIMCD-3 basolateral transporter-mediated transport activity

<table>
<thead>
<tr>
<th>Intracellular pH</th>
<th>Extracellular pH</th>
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<tbody>
<tr>
<td>7.4</td>
<td>7.4</td>
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<td>7.1</td>
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Intracellular and extracellular pH were fixed using nigericin (10 \(\mu\)g/ml) as described in Table 1. A 5-min equilibrium was allowed before measurements were begun. \(^{14}\)C-labeled methylammonia uptake was measured in the absence and presence of excess unlabeled methylammonia, and the inhibitable component of transport activity was determined. Results are reported in pmol methylammonium/mg protein \(\cdot\)3 min \(^{-1}\). 1\(^{14}\)P < 0.05 vs. extracellular pH 7.4, intracellular pH 7.4. 1\(^{14}\)P < 0.01 vs. extracellular pH 7.4, intracellular pH 7.1. 1\(^{14}\)P < 0.001 vs. extracellular pH 7.4, intracellular pH 7.1.
ent, or it could reflect effects of cytosolic H⁺ to increase transport activity independently of H⁺ transport. For example, cytosolic H⁺ increases the \( V_{\text{max}} \) of the Na\(^+\)/H\(^+\) exchanger NHE1 through allosteric mechanisms (6). To differentiate between these possibilities, we examined the effect of changing extracellular pH without altering intracellular pH. The solutions used are those described in Table 1, and the results are summarized in Table 2 and Fig. 8. Increasing extracellular pH from 7.4 to 7.7 at constant intracellular pH, either 7.4 or 7.1, stimulated inhibitable methylammonia transport activity significantly (\( P < 0.001 \) and \( P < 0.01 \), respectively, \( n = 3 \)). Thus increasing the intracellular-to-extracellular H⁺ gradient, whether by increasing intracellular H⁺ concentration at a constant extracellular pH or decreasing extracellular H⁺ concentration at a constant intracellular H⁺ concentration, increases basolateral methylammonia transport activity. These results suggest that the mIMCD-3 has ammonia-sensitive, electroneutral, Na\(^+\)- and K\(^+\)-independent basolateral methylammonium\(^-\)/H\(^+\) exchange activity.

**Cation dependence of transport activity.** Electroneutral mIMCD-3 methylammonia transport activity could also reflect methylammonium (CH\(_3\)NH\(_3\)) uptake in exchange for intracellular cations other than H\(^+\). Because the cations with greatest intracellular concentration are sodium and potassium, we examined the possibility of methylammonia uptake via either sodium-methylammonia or potassium-methylammonia exchange activity. If methylammonia uptake occurs via either methylammonium-sodium or methylammonium-potassium exchange, then acute removal of extracellular sodium or potassium should stimulate sodium or potassium exit, respectively, via this transporter, thereby increasing methylammonia uptake. The next set of studies examined this possibility. Inhibitable transport activity averaged 20.5 ± 2.7 pmol [\(^{14}\)C]MA·mg protein\(^{-1}\)·min\(^{-1}\) in the presence of extracellular sodium and potassium and 23.2 ± 4.7 and 19.0 ± 2.3 pmol [\(^{14}\)C]MA·mg protein\(^{-1}\)·min\(^{-1}\) in the acute absence of extracellular sodium or potassium, respectively (\( n = 3 \)). Removing extracellular sodium or potassium did not alter transport activity significantly (\( P = \text{NS} \) by ANOVA and by paired t-test, \( n = 3 \)).

Figure 9 summarizes these results. mIMCD-3 basolateral electroneutral ammonia-sensitive methylammonia transport is unlikely to be mediated by methylammonium-sodium or methylammonium-potassium exchange. Moreover, the lack of effect of extracellular potassium removal provides further evidence that the transport activity is both electroneutral and is not mediated by potassium transporters.

**Expression of other ammonia transporters.** Recent studies demonstrate basolateral expression of the putative ammonia transporter RhBG in collecting duct cells (43, 57, 64). To examine the possibility that RhBG might contribute to mIMCD-3 basolateral \([^{14}\text{C}]\text{MA}\) transport activity, we determined whether mIMCD-3 cells express RhBG. Real-time RT-PCR confirmed expression of RhBG mRNA (not shown). Amplification of mIMCD-3 mRNA with RhBG-specific primers and fluorescent probes identified amplification of RhBG mRNA. No amplification was observed when reverse transcription was not performed, ensuring that the amplification observed after reverse transcription was performed reflected amplification of mRNA, not genomic cDNA.

To examine RhBG protein expression, we performed both immunoblot analysis and immunohistochemical localization. Immunoblot analysis confirmed expression of a ~52-kDa protein identical in apparent molecular mass to mouse renal RhBG protein (57). Preincubating the antibody with the immunizing peptide prevented immunoreactivity, confirming the specificity of protein recognition. Confocal laser-scanning fluorescent microscopy identified basolateral RhBG immunoreactivity. Figure 10 summarizes these results. Although mIMCD-3 cells also expressed both RhCG mRNA and protein (data not shown), confocal laser-scanning fluorescent microscopy identified that mIMCD-3 cells express apical RhCG immunoreac-

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**Fig. 8.** Effect of altering intracellular-to-extracellular H⁺ gradient on methylammonia transport activity. Intracellular (pHi) and extracellular pH were varied independently as described in Table 1. Increasing the intracellular-to-extracellular proton gradient by inducing intracellular acidification at constant extracellular pH or by inducing extracellular alkalization at constant intracellular pH significantly stimulated methylammonia transport activity (*\( P < 0.05 \) vs. extracellular pH 7.4, intracellular 7.4; **\( P < 0.001 \) vs. extracellular pH 7.4, intracellular pH 7.4; ***\( P < 0.02 \) vs. extracellular pH 7.7, intracellular pH 7.4 and \( P < 0.01 \) vs. extracellular pH 7.4, intracellular pH 7.1; \( n = 3 \)).

**Fig. 9.** Effect of extracellular sodium or potassium removal on basolateral methylammonia transport activity. Basolateral methylammonia transport activity measured after acute change in either control ("normal") or sodium-free ("no sodium") or potassium-free ("no potassium") solutions. Neither sodium nor potassium removal altered inhibitable basolateral methylammonia transport activity significantly (\( P = \text{NS} \) by paired t-test and ANOVA, \( n = 3 \)).
brane. Thus mIMCD-3 cells express a basolateral NH$_4$ transport activity. Finally, mIMCD-3 cells express the ammonia ionic, hydrophilic form, NH$_4$CH$_3$NH$_2$, diffuse across many lipid membranes, whereas cat-nondiffusive basolateral methylammonia transport. Moreover, duct cell mIMCD-3 exhibits both diffusive and inhibitable, transport by renal collecting duct cells. The mouse collecting mIMCD-3 basolateral $[^{14}\text{C}]$MA transport activity.

This study provides the first detailed examination of the cellular mechanisms underlying basolateral methylammonia transport by renal collecting duct cells. The mouse collecting duct cell mIMCD-3 exhibits both diffusive and inhibitable, nondiffusive basolateral methylammonia transport. Moreover, the transporter-mediated activity predominates at low extracellular methylammonia concentrations, is not mediated by K$^+$ transporters that might have affinity for NH$_3^+$, is electoneutral, is stimulated by increasing intracellular-to-extracellular H$^+$ gradients, and is unrelated to sodium or potassium. Thus the transport activity can be characterized as an NH$_3^+/H^+$ exchange activity. Finally, mIMCD-3 cells express the ammonia transporter family member RhBG at their basolateral membrane. Thus mIMCD-3 cells express a basolateral NH$_3^+/H^+$ exchange activity, and this activity may be mediated by basolateral RhBG.

The first major finding of this study is that the mouse collecting duct cell line mIMCD-3 transports methylammonia across the basolateral membrane via both diffusive and transporter-mediated mechanisms. Ammonia and methylammonia exist in aqueous solution in two molecular forms, a neutral, lipophilic form, NH$_3$ and CH$_3$NH$_2$, respectively, and a cationic, hydrophilic form, NH$_3^+$ and CH$_3$NH$_2^+$, respectively. Small, uncharged lipophilic molecules, such as NH$_3$ and CH$_3$NH$_2$, diffuse across many lipid membranes, whereas cationic, hydrophilic molecules, such as NH$_3^+$ and CH$_3$NH$_2^+$, in general, do not. Diffusive NH$_3$ and CH$_3$NH$_2$ transport likely explains the intracellular alkalization that occurs when collecting duct cells are exposed to extracellular ammonia (26, 62, 65) or methylammonia (Handlogten ME and Weiner ID, unpublished observations). Importantly, basolateral NH$_3$ and CH$_3$NH$_2$ permeability is finite in both the CCD (68) and the IMCD (current study). This suggests that both diffusive and inhibitable, transporter-mediated components of transport may contribute to net ammonia transport. Moreover, the transporter-mediated component appears to mediate the majority of transport at low extracellular concentrations.

Although a variety of potassium- and sodium-transporting proteins can transport ammonia, they are unlikely to mediate mIMCD-3 ammonia-sensitive methylammonia transport. Extracellular potassium neither competitively inhibits methylammonia transport, nor do potassium transporter inhibitors alter transport activity. Thus, although K$^+$ and NH$_4^+$ have nearly identical biophysical characteristics in aqueous solutions and a number of potassium-transporting proteins are expressed in collecting duct cells, the mIMCD-3 cell basolateral ammonia-sensitive methylammonia transport observed is unlikely to be mediated by a potassium transporter. Finally, basolateral Na$^+/H^+$ exchange activity is unlikely to mediate methylammonia transport. The only NHE isoform known to transport NH$_3^+$, NHE3, is not expressed in either the mIMCD-3 cell (50) or the collecting duct in vivo (1, 49), and acute peritubular sodium removal did not alter methylammonia transport activity, as would be expected if uptake occurred by methylammonium-sodium exchange. However, it is also important to note that there was a tendency for more prolonged extracellular sodium removal, as used in the control studies for the NH$_4$Cl prepulse acid-loading studies, to increase transport activity. This most likely represents intracellular acidification, due to reversal and/or inhibition of basolateral Na$^+/H^+$ exchange, which then stimulated methylammonia transport activity.

Some of the current findings differ slightly from findings in the rat IMCD, in which basolateral Na$^+/K^+$-ATPase contributed to basolateral NH$_3^+$ uptake (60), and in a study involving mIMCD-3 cells where NH$_3^+$ competitively inhibited both ouabain- and bumetanide-sensitive $^{86}$Rb$^+$ uptake (61). The explanation for the observation in the current study that neither ouabain nor furosemide inhibited methylammonia uptake is unclear. One possibility is that methylammonium cannot, whereas NH$_3^+$ can, substitute for potassium at the potassium-binding site of Na$^+/K^+$-ATPase and the Na$^+/K^+$.2Cl$^-$ co-transporter.

The current study shows that mIMCD-3 cells have a basolateral proton gradient-stimulated methylammonia transport activity that is competitively inhibited by ammonia. This transport activity most likely reflects endogenous NH$_3^+/H^+$ exchange activity in which methylammonium (CH$_3$NH$_2^+$) substitutes for NH$_3^+$. Similar findings have been observed for the erythrocyte ammonia transporter protein RhAG (66). This
transport mode is functionally equivalent to facilitated NH₃ transport. Differentiating between NH₄⁺/H⁺ exchange and facilitated NH₃ transport is difficult. However, if the mode of transport is NH₄⁺/H⁺ exchange, the affinities of the relevant molecular species, NH₄⁺ and CH₃NH₃⁺, are relatively similar, 2.1 and 4.6 mM, respectively. If the mode of transport is facilitated NH₃ diffusion, then the affinities of the relevant molecular species, NH₃ and CH₃NH₂, would be ~37 and 2.9 µM, respectively, or would differ by >10-fold. At present, we are unable to distinguish definitively between NH₄⁺/H⁺ exchange and facilitated NH₃ diffusion. More important, however, is that both transport modalities result in identical net transport.

The ammonia-methylammonia transport activity identified in the current study has several characteristics that are consistent with its contribution to collecting duct transepithelial ammonia transport. First, the NH₄⁺/H⁺ exchange activity identified is functionally equivalent to facilitated NH₃ transport, and in vitro microperfusion studies show that collecting duct transepithelial ammonia secretion is predominantly, if not entirely, electroneutral, and is stimulated by increases in transmembrane potential (16–18, 24, 52). Second, mIMCD-3 basolateral ammonia-methylammonia transport is electroneutral, as is collecting duct transepithelial ammonia transport (16–18, 24, 52). Finally, the turtle urinary bladder, an amphibian collecting duct model system, secretes ammonia via a serosal (peritubular) methylammonia-inhibitable process and it secretes methylammonia via a serosal ammonia-sensitive process (7, 54). Thus a basolateral ammonia-methylammonia transporter appears to contribute to transepithelial turtle urinary bladder ammonia transport. These multiple lines of evidence are consistent with the basolateral electroneutral NH₄⁺/H⁺ exchange activity observed in the mIMCD-3 cell mediating an important role in collecting duct ammonia secretion.

The current study used the mIMCD-3 cell as a cultured collecting duct cell model system. The mIMCD-3 cell was initially derived as an osmotically tolerant collecting duct cell line from mice transgenic for the early region of simian virus SV40 (large T antigen) (44). Subsequent studies have shown that the mIMCD-3 cell possesses multiple characteristics of collecting duct cells, including expression of NHE1 and NHE2 (50); ATP-sensitive K⁺ channels (47); Na⁺-K⁺-ATPase (61); BSC2, the basolateral Na⁺-K⁺-2Cl⁻ cotransporter (13, 27); both gastric and colonic H⁺-K⁺-ATPase (41); and H⁺-ATPase (2, 5). Thus this cell line is well suited to serve as a model system for examining collecting duct ammonia and methylammonia transport mechanisms.

The ammonia transporter mechanism observed in the mIMCD-3 cell has several functional characteristics similar to that observed with a recently identified ammonia transporter family of proteins. Bacterial ammonia transporter family proteins appear to mediate sodium- and potassium-independent, ammonia-sensitive methylammonia transport (34–36, 40, 56, 59). The mammalian ammonia transporter family member RhAG also mediates sodium- and potassium-independent, ammonia-sensitive methylammonium-proton exchange activity (66). Moreover, the apparent affinity of RhAG for ammonia and methylammonia is similar to that observed in the current study (66).

RhBG, a mammalian member of the ammonia transporter family of proteins (9, 25, 31, 32), is a possible candidate to mediate mIMCD-3 basolateral ammonia-sensitive methylammonia transport. RhBG protein is expressed in the renal connecting segment and collecting duct (43, 57), critical sites for transepithelial ammonia secretion, and basolateral RhBG immunoreactivity is present throughout the collecting duct, including the IMCD (43, 57). Finally, two preliminary reports identify that the ammonia transporter family member RhBG transports both ammonia and methylammonia when expressed in Xenopus laevis oocytes (39, 67). Thus RhBG is a likely candidate to mediate mIMCD-3 ammonia-methylammonia basolateral transport.

One possible limitation of the current study is that transport was measured using methylammonia, not ammonia. Directly measuring transmembrane ammonia transport is difficult. Radiolabeled ammonium (¹⁵NH₄⁺) is not commercially available and, even if it were available, could be quantified only by mass spectrometry. Intracellular NH₃- or NH₄⁺-sensitive electrodes and fluorescent NH₃- or NH₄⁺-sensitive dyes are not available. Intracellular voltage and pH measurements can indirectly assess electrogenic NH₄⁺ transport but might not detect electroneutral net NH₃ transport. Thus they would not detect the mIMCD-3 transport activity observed in the current study. As a result, direct NH₃ transport measurements are difficult and generally not practical. Instead, methylammonia is widely used as an ammonia surrogate. Numerous studies show that methylammonia is transported through the same pathways as ammonia (22, 30, 36, 42, 45, 53). This basic technique, using a substitute radionuclide to characterize transport, is similar to the widely used technique used to examine potassium transport, where ⁸⁶Rb⁺ is commonly used as a potassium surrogate. Thus, although the current study uses methylammonia as an ammonia surrogate, the results are likely to be highly relevant for understanding the molecular mechanisms of collecting duct ammonia transport.

In summary, mIMCD-3 cells express a novel basolateral ammonia-sensitive methylammonia transport activity that is unrelated to known potassium or sodium transporters, is electroneutral, and is stimulated by increases in transmembrane proton gradients. These results functionally identify that mIMCD-3 cells possess a basolateral, ammonia-inhibitable methylammonium/H⁺ exchange activity that is likely to be important in collecting duct ammonia secretion. Moreover, mIMCD-3 cells also express basolateral RhBG immunoreactivity, raising the possibility that RhBG may mediate this methylammonia transport activity.

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