Ammonium transport in the colonic crypt cell line, T84: role for Rhesus glycoproteins and NKCC1

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Worrell RT, Merk L, Matthews JB. Ammonium transport in the colonic crypt cell line, T84: role for rhesus glycoproteins and NKCC1. Am J Physiol Gastrointest Liver Physiol 294: G429–G440, 2008. First published November 21, 2007; doi:10.1152/ajpgi.00251.2006.—Although colonic lumen NH4+ levels are high, 15–44 mM normal range in humans, relatively few studies have addressed the transport mechanisms for NH4+. More extensive studies have elucidated the transport of NH4+ in the kidney collecting duct, which involves a number of transporter processes also present in the distal colon. Similar to NH4+ secretion in the renal collecting duct, we show that the distal colon secretory model, T84 cell line, has the capacity to secrete NH4+ and maintain an apical-to-basolateral NH4+ gradient. NH4+ transport in the secretory direction was supported by basolateral NH4+ loading on NKCC1, Na+/K+-ATPase, and the NH4+ transporter, RhBG. NH4+ was transported on NKCC1 in T84 cells nearly as well as K+ as determined by bumetanide-sensitive 86Rb-uptake. 86Rb-uptake and ouabain-sensitive current measurement indicated that NH4+ is transported by Na+/K+-ATPase in these cells to an equal extent as K+. T84 cells expressed mRNA for the basolateral NH4+ transporter RhBG and the apical NH4+ transporter RhCG. Net NH4+ transport in the secretory direction determined by 14C-methylammonium (MA) uptake and flux occurred in T84 cells suggesting functional RhG protein activity. The occurrence of NH4+ transport in the secretory direction within a colonic crypt cell model likely serves to minimize net absorption of NH4+ because of surface cell NH4+ absorption. These findings suggest that we rethink the present limited understanding of NH4+ handling by the distal colon as being due solely to passive absorption.

RhBG; RhCG; colon; hyperammonemia

The observation that portal vein ammonium (NH3 + NH4+) concentration (∼350 μM) is substantially higher than systemic levels (∼45 μM) is prima facie evidence that NH4+ production and/or absorption occurs in the gastrointestinal tract (7, 41). In particular, the colon is an ammonium-rich organ because of bacterial fermentation of nonabsorbed dietary nutrients in the luminal compartment, leading to concentrations typically around 14–20 mM but as high as 100 mM (41). It is well accepted that the colonic mucosa absorbs ammonium, that is, that the direction of net colonic movement of NH3/NH4+ (Jnet, representing the difference between mucosal-to-serosal and serosal-to-mucosal fluxes, Jms − Jsm) is in the absorptive direction. However, the extent to which the colonic mucosa can generate a significant secretory flux of NH3/NH4+ (i.e., a nonzero Jsm) against the larger absorptive vector Jms is unknown. Moreover, the possibility that transepithelial secretion and absorption of NH3/NH4+ might occur in different cell types (as it does for Na+ and Cl−) has not been addressed previously. However, it is intuitively appealing to consider that a subset of colonic epithelial cells might actively extrude NH4+ so as to temper the otherwise unopposed interstitial accumulation of this toxic substance. Indeed, there are a number of examples in nature where epithelia faced with a high external NH4+ concentration are able to actively excrete NH4+ against an uphill gradient (38, 40, 43, 47).

Although there are some data that address absorptive NH3/NH4+ flux (Jms), the cell type and pathways remain incompletely defined. It is controversial whether absorption occurs by the primary movement of NH3 or NH4+. Early studies supported a NH3 diffusion model on the basis of the pH sensitivity of ammonium movement (3, 4, 55); however, this does not appear to be the case for ammonium movement across mammalian ileum (28, 29). Recent studies indicate that colonic crypts, gastric glands, and renal cells have a low apical NH4+ permeability (2, 18, 42, 44). Entry of NH4+ in the absorptive direction has been postulated to occur via apical Ba2+-sensitive K+ channels in the surface epithelium (10, 21) or perhaps via aquaporin-1 (33).

In contrast, there is almost no information concerning NH4+ secretory flux (Jms) in intestine. NH4+ secretion in other epithelia occurs via its transport on known K+ transporters owing to the similar hydrated radius of K+ and NH4+. In most models, the basolateral loading step for NH4+ uses the K+ site on Na+-K+-ATPase and/or the Na+/K+-2Cl− cotransporter NKCC1. Apical ammonium exit pathways vary from system to system. Passive diffusion of NH3+ across the apical membrane in combination with H+ secretion by H+-ATPase results in an “ion trapping” mechanism of secretion in fresh water fish gill (51) and possibly in mammalian inner medullar collecting duct (IMCD) (45). In contrast, in the case of salt water fish gill (51) and the desert locust, Schistocerca gregaria, rectum (43), apical secretion of NH4+ occurs by replacing H+ on an apical Na+/H+-exchanger (NHE). In the giant mudskipper, Periophthalmus schlosseri, both ion trapping and NHE are used to maintain NH4+ gradients up to 100 mM (37, 38). In the green crab, Carcinus maenas, NH4+ transport across the apical membrane occurs by first vesicular ion trapping of NH4+ and then microtubule-dependent vesicular release (40, 47). An alternative model for mammalian IMCD involves NH4+ exit on the H+ site of colonic-type H+-K+-ATPase, with K+ recycling occurring on ROMK1 K+ channel (30). A common feature of the above systems is physiological exposure to a...
significant NH$_4^+$ concentration ([NH$_4^+$]) (20–100 mM). Human colonic epithelia exposure is within this elevated range (41). Apical NHEs and colonic-type H$^+$-K$^+$-ATPase are present in the apical membrane of colonic epithelia (22). Likewise, Na$^+$-K$^+$-ATPase and NKCC1 are present. Given a similar environment and the fact that many of the same basolateral and apical membrane transport pathways are present in colonic cells circumstantially, this suggests the colon may, indeed, actively secrete ammonia as do these other tissues.

Perhaps one of the most significant recent discoveries involving NH$_3$/NH$_4^+$ permeability is that certain mammalian nonerythroid cells express members of the SLC42 solute carrier family (14, 32, 48). The SLC42 family is comprised of the human Rhesus-associated glycoproteins (RhG), which are homologous to the yeast Mep family of proteins (15). These proteins transport both ammonia, as well as methylammonium (MA). It is notable that the Mep transporters can concentrate MA by ~1,000-fold (9, 39). Not surprisingly, RhGs are found predominately in tissues that are exposed to elevated NH$_4^+$ levels such as liver, kidney, and stomach (24, 27, 48, 49, 50). RhGs have been detected in the gut, including proximal colon (13). Interestingly, two of the family members show polarized expression; RhBG is expressed in the basolateral membrane, and RhCG is apically expressed (48). Thus each is poised to be a potential player in the vectorial transport of NH$_3$/NH$_4^+$ (32). The actual mode of NH$_3$/NH$_4^+$ transport by the SLC42 family is not clear. Transport has been shown to occur in an electrogenic manner (31, 34) as electroneutral transport by acting as an NH$_4^+$-H$^+$ exchanger (11, 12, 25, 26) and as an NH$_3$ “gas channel” (17, 20, 23). Part of the confusion may derive from the results showing that the affinity for NH$_4^+$ is affected by NH$_3$ (1). Regardless, the functional significance of RhG proteins in gut has not yet been elucidated.

As a first step in understanding the possible contribution of an NH$_3$/NH$_4^+$ secretory vector ($J_{sm}$) to ammonium handling by the colonic mucosa, we used the colonic cell line T84 as a model of crypt enterocytes to define the relative contributions of NKCC1 and RhGs in basolateral uptake and transepithelial movement of NH$_3$/NH$_4^+$. Our data suggest that colonic transport of ammonium includes a secretory vector that is nonzero permeability of ammonium includes a secretory vector that is nonzero.

**MATERIALS AND METHODS**

**Cell culture.** T84 cells (8) obtained from Jim McRoberts (University of California, Los Angeles) were grown to confluence at pH 7.4 in 162-cm$^2$ flasks with DMEM-Hams’s F-12, 1:1 mix supplemented with 6% fetal bovine serum, 15 mM HEPES, 14 mM NaHCO$_3$, 170 μM penicillin G, and 69 μM streptomycin sulfate. Amphotericin B was not included in the media to avoid potential complications due to its ionophoretic activity. Cells were maintained in culture with weekly passage by trypsinization in Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline at a split ratio of 1:2. Cells for experimentation were plated on uncoated 12- or 24-mm Costar Transwell (0.4-μm pore size) inserts at a seeding density of 4–5 × 10$^4$ cells/cm$^2$ and cultured 8–14 days with feeding in the above media three times per week. Cell monolayers were determined to be of acceptable use when the transepithelial resistance reached >1,200 Ω·cm$^2$ as measured by an electrotvolthometer (EVOM) (World Precision Instruments) (see below). All experiments were performed at 37°C.

**EPithelial monolayer integrity.** The quality of high resistance monolayer formation was monitored using an EVOM as described previously (52, 54). The T84 cell line used in these experiments typically had a transepithelial resistance of >2,000 Ω·cm$^2$. Both transepithelial potential (mV) and transepithelial resistance (kΩ) were measured with this instrument. Transepithelial current was calculated by Ohm’s law and expressed as μA/cm$^2$. EVOM measurements proved consistent and reliable for detecting changes in transepithelial voltage, resistance, and current (52–54). Moreover, measurements in the open-circuit mode represent the normal condition (e.g., not short circuited) of native epithelia.

**Real-time RT-PCR of RhBG and RhCG.** Total RNA was extracted from T84 cells with TRizol Reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed by using 50 μg/ml oligo(dT) 20 primer by using SuperScript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Amplification reactions were performed with 1× SybrGreen PCR master mix (Applied Biosystems) or with a gene-specific fluorescent-labeled probe, gene-specific primers, and 200-ng sample cDNA in a 50-μl final volume. Real-time RT-PCR was performed on a DNA Engine Opticon 2 detector (DYAD). Thermal conditions (50°C for 2 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min) were used for all primer sets. Emitted fluorescence was measured during the annealing/extension phase; amplification plots were generated by using the Opticon Monitor Analysis software. Standard curves were generated for each primer set (Table 1) by using a known concentration of plasmid DNA; the amount of mRNA was then extrapolated and normalized to GAPDH for comparison.

Plasmids were made by obtaining a portion of the gene by performing RT-PCR on cDNA from T84 cells that were positive for the gene. The RT-PCR product was then cloned into a TA cloning vector. Real-time RT-PCR was performed to confirm that the plasmid contained the insert. A standard curve was then generated from known concentrations of plasmid DNA for each primer set. Well behaved standard curves for each primer set were obtained, $r^2$ ≥ 0.993.

**$^{14}$C-MA uptake and flux.** T84 cell monolayers were grown on 1-cm$^2$ Transwell inserts. Uptake and flux measurements were carried out in the following base solution (in mM): 135 NaCl, 3 KCl, 0.5 CaCl$_2$, 2 MgCl$_2$, 10 Na-HEPES at a pH of 7.4. Apical media volume was 0.5 ml and basolateral media volume 1 ml. For apical uptake and apical-to-basolateral flux ($J_{ab}$) MA studies, apical volume was reduced to 250 μl to conserve $^{14}$C-MA tracer. Cells were incubated in cold MA for 5–10 min before uptake/flux initiation. Use of tracers,

Table 1. Primer sets used for PCR

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primers to Create Plasmid (5’-3’)</th>
<th>Primers for Real-Time PCR (5’-3’)</th>
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<tbody>
<tr>
<td>Human RhBG</td>
<td>For – GACGCCGCTTACCACAGATCT</td>
<td>For – GCCCTGAGAAGTGGTTCTCA</td>
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<tr>
<td></td>
<td>Rev – AAAAAAGGCTGTTGCCTCTCA</td>
<td>Rev – GACCTGATGCATGGCTCGTA</td>
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<tr>
<td>Human RhCG</td>
<td>For – CACCTTCTTCTTGAGAAGT</td>
<td>For – GACCTGATGCATGGCTCGTA</td>
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<tr>
<td></td>
<td>Rev – GACCTCCTTGGAGGCAACC</td>
<td>Rev – CGATGAGACCTTGAAGGCTGCG</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>For – GAGTGAGATGGCTGAGCTC</td>
<td>Same</td>
</tr>
<tr>
<td></td>
<td>Rev – GAGATGATGGATGGATGATC</td>
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For, forward; Rev, reverse.
which are transported as well as diffusible through the cell membrane (exist in both charge and uncharged form), present a unique situation to classical ion tracer experiments. It is noteworthy that the steady-state condition may not be obtained during this incubation time; thus uptake and flux measurements may represent an overestimate of true values. However, experiments shown are comparable to those published by Handlogten et al. (11, 12) and thus support the functional presence of RhGs in T84 cells. Long-term measurements were carried out in culture media with cells kept in the tissue culture incubator between time points. Under these conditions “steady-state” does exist. Short- and long-term fluxes are consistent, thus supporting the physiological relevance of the methods used. For consistency with initial studies and those involving NKCC1-mediated NH₄⁺ transport, study of ammonium transport by RhGs was done under conditions of active cAMP-dependent Cl⁻ secretion (stimulated by basolateral 10 μM forskolin, 5 min). As subsequently demonstrated by data in Fig. 8, A and B, RhG function is not altered by forskolin. When bumetanide or ouabain was used, drugs were applied 5 min before initiation of uptake and flux.

J_{Na} and basolateral-to-apical flux (J_{ba}) are used to indicate absorptive (J_{abs}) and secretory (J_{sec}) flux as would occur in intact colon. J_{net} is used to indicate the combined effect of each vector, i.e., J_{na} – J_{ba} (When plotted together, J_{ba} is assigned a negative value.). ¹⁴C-MA (1 or 0.5 μCi/ml for the long-term uptake and flux) was used. Radioactivity in an aliquot of initial loading media was determined to establish specific activity for each experiment. At the end of the flux period (typically 2 min) the entire volume of transmedia was collected and radioactivity determined to calculate transepithelial flux. Uptake was ended by sequentially dunking insertions into three reservoirs of >500 ml ice-cold 0.1 M MgCl₂, 10 mM Tris·HCl (pH 7.4). Inserts were then cut from the supports and placed in scintillation vials with scintillation cocktail to determine total cellular radioactivity. For uptake calculations, the radioactivity of the cellular fraction and transmedia fraction were combined. Previously, we determined that correcting for protein content by protein measurement on individual inserts did not lead to a significant decrease in uptake. Indeed, ¹⁴C-MA uptake was found to be reduced in the presence of 10 mM NH₄⁺. Inclusion of 10 mM NH₄⁺ reduced apical ¹⁴C-MA uptake ~36% to 25 ± 1 pmol·min⁻¹·cm⁻² MA. Basolateral ¹⁴C-MA uptake was reduced ~44% to 217 ± 16 pmol·min⁻¹·cm⁻² MA by 10 mM NH₄⁺. These data together indicate the functional presence of RhG proteins at both the apical and basolateral membrane of polarized T84 monolayers. In addition, the data suggest that basolateral loading of NH₄⁺ exceeds apical loading (presumably by RhBG and RhCG, respectively, although this has not been directly determined in T84 cells). Transepithelial flux with symmetrical 1 mM cold MA reveals a net secretory direction of flux in T84 cells (Fig 2B). The apical-to-basolateral flux (J_{ba}) of MA was ~134 ± 7 pmol·min⁻¹·cm⁻² MA, whereas the basolateral-to-apical flux (J_{ba}) was 241 ± 7 pmol·min⁻¹·cm⁻² MA. Inclusion of 10 mM NH₄⁺ reduced J_{ba} by ~22% to ~104 ± 6 pmol·min⁻¹·cm⁻² MA, J_{ba} by 31% to 167 ± 6 pmol·min⁻¹·cm⁻² MA, and J_{ba} by ~41% to 63 ± 12 pmol·min⁻¹·cm⁻² MA.

To determine if net secretion occurs over a longer time period under culture conditions, long-term flux experiments were carried out with T84 cell monolayers over a 60-min...
period in complete culture media. Results are qualitatively similar to short term (2-min flux period). Calculated rates (Fig. 2C) were $257 \pm 11$ for basolateral and $107 \pm 5$ pmol·min$^{-1}$·cm$^{-2}$ MA for apical uptake. $J_{ba}$ was $113 \pm 8$, and $J_{ab} = 44 \pm 3$ pmol·min$^{-1}$·cm$^{-2}$ MA, resulting in a secretory direction flux ($J_{net}$) of $69 \pm 7$ pmol·min$^{-1}$·cm$^{-2}$ (Fig. 2D). The lower values for long-term flux rates may be due to the different bathing conditions (culture media vs. minimal media) or possibly could represent the effect of closer to steady-state intracellular MA concentration ([MA]) in the long-term experiments. Nonetheless, qualitatively the data, in whole, support an appreciable secretory direction flux due to RhG activity.

Specificity of $^{14}$C-MA as a tracer for RhG-mediated uptake and flux. $^{14}$C-MA is known to be transported by the RhGs; thus their activity can be assessed by $[^{14}$C$]$-MA uptake and flux. A: uptake of $[^{14}$C$]$-MA when applied to the apical or basolateral side of T84 cell monolayers. The magnitude of uptake is significantly greater when applied basolaterally. MA uptake from either side is attenuated by the addition of $NH_4^+$ characteristic of RhG activity. B: transepithelial flux of MA in T84 cells. Tracer $[^{14}$C$]$-MA was added to either the apical or basolateral side to determine unidirectional flux in either direction. The combined results demonstrate net secretion of MA. Transepithelial MA flux was attenuated by the addition of $NH_4^+$ characteristic of RhG activity. In A and B, symmetrical 1 mM cold MA was used with 1 µCi/ml $^{14}$C-MA applied to either the apical or the basolateral side. Uptake and flux period was 2 min. C: long-term MA uptake over a 60-min period obtained in cells bathed in culture media and kept in tissue culture incubator. D: long-term MA flux over a 60-min period for cells bathed in culture media and kept in tissue culture incubator. The combined results are qualitatively similar to those in A and B. In C and D, symmetrical 1 mM cold MA was used with 0.5 µCi/ml $^{14}$C-MA applied to either the apical or the basolateral side; $n = 6$ for all groups. $J_{ba}$, apical-to-basolateral flux; $J_{ab}$, basolateral-to-apical flux; $J_{net}$, combined effect of each vector.

Increasing $[MA]$ increases, there develops in parallel a nonsaturable component of MA uptake, representing linear diffusive entry, particularly at concentrations above ~5 mM MA. To determine the time course of MA uptake and flux, as well as the sampling rate at which RhG activity could be more accurately accessed, 3 mM [MA] was used. This concentration was chosen to provide an extra safety margin in sampling times. The time course of MA uptake (Fig. 3B) and transcellular flux (Fig. 3C) indicate little effect of bumetanide on MA uptake or flux at time points less than ~3 min. However, at longer time points (>5 min), a more significant bumetanide-sensitive component for MA uptake is revealed. Since these data were obtained at 3 mM MA, use of 1 mM MA in combination with a 2-min sample period is more than adequate to access specific RhG function. Transepithelial flux of MA is less sensitive to bumetanide treatment, even at extended flux durations. There was no discernable $K^+$ competition (measured by Dixon plot with 1 and 3 mM MA and 0–10 mM K$^+$) for the total (Fig. 3D), as well as bumetanide-sensitive (Fig. 3E) component of MA uptake, as would be expected if MA were carried on the $K^+$ site of NKCC1 or the pump, suggesting that this small effect of bumetanide on MA uptake is indirect. The greater impact of bumetanide at longer
time periods and a slightly greater effect on uptake vs. trans-
epithelial flux are supportive of an indirect effect as well.

Increasing extracellular pH has been shown to increase MA
uptake in mIMCD-3 cells (12). Figure 4A demonstrates a
similar increase in MA uptake with increased basolateral ex-
tracellular pH and a decrease with basolateral extracellular
acidification. Total MA uptake increased by approximately
threefold from 1,349 ± 49 to 4,379 ± 16 pmol·min⁻¹·cm⁻²
on increasing outside (extracellular) pH (pHₒ) to 9.0, whereas
MA uptake decreased by ~77% to 315 ± 16 pmol·min⁻¹·cm⁻²
at pHₒ 5. Transcellular flux of MA (Fig. 4B) in the secretory
direction also increased with an increase in basolateral pHₒ. A
pHₒ increase from 7.4 to 9.0 increased secretory direction MA
flux ~3.6-fold from 148 ± 3 to 534 ± 24 pmol·min⁻¹·cm⁻².
Acidifying pHₒ to 5.5 produced an ~54% decrease in MA
uptake (68 ± 11 pmol·min⁻¹·cm⁻²). Change in basolateral
extracellular pH did not produce any significant increase in a
bumetanide-sensitive component of MA uptake, which further
supports lack of MA transport on NKCC1 and Na⁺-K⁺-
ATPase.

Fig. 3. MA uptake and flux in T84 cells. A: dose response for MA uptake in the absence and presence of basolateral 100 μM bumetanide. Total (●), bumetanide-insensitive (◇), and bumetanide-sensitive (■) uptakes are shown. Uptake period was 2 min. Uptake represents combined uptake due to RhBG transporter activity and linear diffusive uptake (≥5 mM MA). B: time course of MA uptake with 3 mM symmetrical cold MA. Total (●), bumetanide-insensitive (◇), and bumetanide-sensitive (■) uptakes are shown. A significant bumetanide-sensitive component of MA uptake is observed only at uptake times exceeding 3–4 min. C: time course of completed transepithelial MA flux in the secretory direction. Total (●), bumetanide-insensitive (◇), and bumetanide-sensitive (■) fluxes are shown. 3 mM symmetrical MA was used. A slight bumetanide-sensitive component of MA flux is observed only at time points exceeding 5 min, and n = 4 for each data point. *P < 0.05 D: Dixon plot of total basolateral MA uptake at varying K⁺ concentration [K⁺] with 1 mM (●) or 3 mM (◇) MA demonstrating lack of competitive inhibition. E: Dixon plot of bumetanide-sensitive basolateral MA uptake at varying [K⁺] with 1 mM (●) or 3 mM (◇) MA demonstrating no clear relation and lack of competitive inhibition. *P < 0.05.
Apical pH increase from 7.4 to 9.0 increased MA \( J_{ab} \) approximately eightfold from 54 \( \pm \) 3 to 437 \( \pm \) 15 pmol\( \cdot \text{min}^{-1} \cdot \text{cm}^{-2} \). Acidifying pHo to 5.5 produced an \( \sim \) 54\% decrease in MA uptake (25 \( \pm \) 2 pmol\( \cdot \text{min}^{-1} \cdot \text{cm}^{-2} \)). These data combined demonstrate that apical media (luminal) alkalinization has a more significant effect on MA absorption than does basolateral alkalinization on MA secretion (approximately eightfold vs. \( \sim \) 3.6-fold increase).

It has been established that \( \text{NH}_4^+ \) will compete with MA uptake on RhG proteins (11, 12). Thus we used a transepithelial \( \text{NH}_4^+ \) gradient to ascertain the degree to which basolateral MA (and thus \( \text{NH}_4^+ \)) loading and secretory direction \( \text{NH}_4^+ \) transport could occur. The rationale is that apical solution \( \text{NH}_4^+ \) might retard the exit of MA across the apical membrane (presumably RhCG), or, if apical \( \text{NH}_4^+ \) were to enter and remain in the cell at significant levels, a trans effect on basolateral transport (presumably RhBG) might be revealed. In this case \(^{14}\text{C}\)-MA is being used as a tracer for \( \text{NH}_4^+ \) transport to determine whether \( \text{NH}_4^+ \) secretion can occur against a substantial gradient. A caveat is that MA will only sample a subset of the available secretory mechanisms since it is not transported by NKCC1 or, to our knowledge, by other known \( \text{K}^+/\text{NH}_4^+ \) transporters. Initial experiments with 1 mM MA and 2-min uptake showed an \( \sim \) 50\% decrease in basolateral MA uptake from 1,514 \( \pm \) 41 to 773 \( \pm \) 29 pmol\( \cdot \text{min}^{-1} \cdot \text{cm}^{-2} \) with the inclusion of 20 mM basolateral \( \text{NH}_4^+ \) (thus confirming effective \( \text{NH}_4^+ \) competition), whereas inclusion of 20 mM \( \text{NH}_4^+ \) on the apical side produced no significant decrease in basolateral MA uptake (1,578 \( \pm \) 33 pmol\( \cdot \text{min}^{-1} \cdot \text{cm}^{-2} \)). Figure 5 shows that apical \( \text{NH}_4^+ \) concentrations as high as 100 mM have no effect on either basolateral MA loading or on the secretory direction flux of MA. These data indirectly indicate that \( \text{NH}_4^+ \) transport in the secretory direction driven by basolateral RhG (presumably RhBG) can occur against a significant \( \text{NH}_4^+ \) gradient, one within the physiological range of luminal \([\text{NH}_4^+]_o\).

Transepithelial transport of \( \text{NH}_4^+ \) in T84 cells. Although \(^{14}\text{C}\)-MA uptake and flux is a useful tool to investigate the contribution of the RhG proteins to secretory direction move-
ment of NH$_4^+$, it does not allow one to determine the contribution of Na$^+$-K$^+$-ATPase or NKCC1 to NH$_4^+$ secretion. Unfortunately, there is no convenient radioisotope to directly measure NH$_4^+$ transport. We have previously shown that NH$_4^+$ competes with K$^+$ on Na$^+$-K$^+$-ATPase as well as NKCC1 in T84 cells and that both transporters will transport (load) NH$_4^+$ into T84 cells (54) and thus may contribute to NH$_4^+$ secretion.

Initial studies were designed to determine the relative magnitude of NH$_4^+$ flux in T84 cell monolayers under asymmetric conditions and were performed in the presence of forskolin to maximize NKCC1 activity. Unidirectional flux of NH$_4^+$ was determined by the inclusion of 10 mM NH$_4^+$ on one side of the cell monolayer and sampling the trans-side media. NH$_4^+$ flux in the secretory direction ($J_{ab}$) exceeded flux in the absorptive direction ($J_{ba}$) (Fig. 6), resulting in a calculated secretory direction for the net movement of NH$_4^+$. Flux rates were $-6.7 \pm 1.9$ for $J_{ab}$, $38.3 \pm 8.1$ for $J_{ba}$, and $30.8 \pm 7.8$ nmol·min$^{-1}$·cm$^{-2}$NH$_4^+$ for the calculated net flux. These data add further support to the MA uptake and flux experiments indicating that T84 cells are capable of net NH$_4^+$ secretion.

To determine whether transepithelial potential (apical negative because of Cl$^-$ secretion with forskolin stimulation) affected NH$_4^+$ secretion, flux studies were performed in an Ussing chamber system (4-ml symmetrical volume) either under open-circuit or short-circuit (transepithelial voltage = 0 mV) conditions. Asymmetric 0.1 mM NH$_4^+$ gradients were established and 0.1 ml transside bathing media sampled at 10-min intervals for 60 min. Calculated secretory rate for the initial 10-min period was not significantly different between open-circuit and short-circuit mode ($29 \pm 6$ and $24 \pm 2$ nmol·min$^{-1}$·cm$^{-2}$, respectively). Similarly secretory rate did not differ over the full 60-min period between open-circuit and short-circuit modes ($30 \pm 3$ and $26 \pm 2$ nmol·min$^{-1}$·cm$^{-2}$, respectively). These data indicate that transepithelial NH$_4^+$ movement is electroneutral (supportive of electroneutral movement on RhGs as well as NKCC1). With present techniques it is difficult to distinguish between paracellular NH$_4^+$ movement and cellular membrane NH$_3$ movement, although the short-circuit current experiments suggest little contribution of paracellular NH$_4^+$ movement to the overall transepithelial flux.

To determine the degree to which NKCC1 and Na$^+$-K$^+$-ATPase contribute to the secretory direction flux, cells were treated with basolateral bumetanide or ouabain for 5 min before the flux period. Both bumetanide and ouabain decreased $J_{ba}$ NH$_4^+$ flux (Fig. 7A). Bumetanide decreased the unidirectional secretory direction NH$_4^+$ flux by $\sim44\%$ from 73 ± 10 to 40 ± 5 nmol·min$^{-1}$·cm$^{-2}$, whereas pump inhibition reduced NH$_4^+$ transport by $\sim33\%$ to 48 ± 6 nmol·min$^{-1}$·cm$^{-2}$, suggesting that NKCC1 and, to some extent, pump activity contribute to the secretory flux of NH$_4^+$. In a separate experiment, the effect of basolateral bumetanide on the unidirectional absorptive flux of NH$_4^+$ was assessed. Whereas inhibition of NKCC1 caused a decrease in secretory direction NH$_4^+$ flux, absorptive NH$_4^+$ flux was enhanced by NKCC1 inhibition. $J_{ab}$ for NH$_4^+$ was increased approximately twofold from $-1.6 \pm 0.2$ in control to $-3.2 \pm 0.5$ nmol·min$^{-1}$·cm$^{-2}$ in bumetanide-treated cells. These data suggest that NKCC1 may play a key role in scavenging NH$_4^+$ traversing the cell from the lumen, thus limiting NH$_4^+$ absorption. To further lend support for NKCC1 limiting NH$_4^+$ absorption, an additional experiment was designed under gradient conditions with NH$_4^+$ initially present on both sides of the monolayer (Fig. 7B). Depicted is the change in basolateral media [NH$_4^+$] in cells exposed to 10 mM apical NH$_4^+$ and 3 mM basolateral NH$_4^+$.
in the absence and presence of basolateral bumetanide. Under control conditions, there was no significant increase in basolateral $\text{NH}_4^+$ (which would represent net absorption), or rather the monolayer is capable of preventing diffusive $\text{NH}_3$ entry at the apical membrane from being delivered to the basolateral medium. However, in the presence of basolateral bumetanide, basolateral $\text{NH}_4^+$ levels increase by $0.42 \pm 0.02$ mM (14%) in 10 min and by $0.55 \pm 0.04$ mM (18%) by 20 min. The diminished increase in basolateral $\text{NH}_4^+$ with time suggests the possibility that other mechanisms of limiting absorptive $\text{NH}_4^+$ flux are acting in a compensatory manner.

**cAMP-stimulates ammonium but not MA transport.** Because cAMP activates transepithelial $\text{Cl}^-$ secretion and NKCC1 in T84 cells, we examined the effect of forskolin on MA uptake and flux. Our previous studies demonstrated that basolateral ammonium dampens cAMP-mediated $\text{Cl}^-$ secretion in T84 cells (16, 35, 54). Since little is known regarding the regulation of RhG ammonium transport, uptake and flux measurements were determined in unstimulated T84 cells. As shown in Fig. 8, $A$ and $B$, forskolin stimulation did not significantly alter MA transport. Basolateral MA uptake was $3,394 \pm 255$ pmol·min$^{-1}$·cm$^{-2}$ under control conditions and $3,024 \pm 214$ pmol·min$^{-1}$·cm$^{-2}$ in the presence of forskolin. Basolateral-to-apical flux was $312 \pm 89$ pmol·min$^{-1}$·cm$^{-2}$ for control and $260 \pm 21$ pmol·min$^{-1}$·cm$^{-2}$ in the presence of forskolin. Apical MA uptake was $1,535 \pm 109$ pmol·min$^{-1}$·cm$^{-2}$ under control conditions and $1,503 \pm 125$ pmol·min$^{-1}$·cm$^{-2}$ with forskolin. Under control conditions apical-to-basolateral MA flux was $157 \pm 40$ and $187 \pm 8$ pmol·min$^{-1}$·cm$^{-2}$ with forskolin. In contrast to the lack of effect of cAMP stimulation on MA transport, secretory direction $\text{NH}_4^+$ flux was increased ~24% in the presence of forskolin (Fig. 8C). Basolateral-to-apical $\text{NH}_4^+$ flux was $17 \pm 1$ nmol·min$^{-1}$·cm$^{-2}$ under control conditions and increased to $21 \pm 2$ nmol·min$^{-1}$·cm$^{-2}$ with forskolin. cAMP stimulation of $\text{NH}_4^+$ movement in the secretory direction is consistent with our previous data (54) and supportive of NKCC1 participation in basolateral $\text{NH}_4^+$ loading (see below). Consistent with NKCC1 activity limiting the transepithelial absorptive vector, forskolin stimulation results in decreased $\text{NH}_4^+$ $J_{\text{ab}}$ flux (Fig. 8D). $J_{\text{ab}}$ for $\text{NH}_4^+$ was $5.8 \pm 0.5$ nmol·min$^{-1}$·cm$^{-2}$ under control condition and decreased ~44% to $3.3 \pm 0.8$ nmol·min$^{-1}$·cm$^{-2}$ with forskolin stimulation. As with other classic secretory or absorptive processes the magnitude variability in $\text{NH}_4^+$ flux across experiments may, in part, relate to variability in the magnitude of forskolin response in T84 cells between cell platings and passages. However, despite the magnitude variability, the relative effect is consistent across different groups.

**T84 monolayers maintain an applied ammonium gradient.** The ability of T84 cells to maintain basolateral [NH$_4^+$] when exposed to a high apical-to-basolateral NH$_4$+ gradient is shown in Fig. 9. Cell monolayers were exposed to various initial apical [NH$_4^+$] ranging from 0 to 30 mM, whereas basolateral [NH$_4^+$] was initially 3.11 ± 0.08 mM. Over a period of 30

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**Fig. 8.** cAMP effect on MA and $\text{NH}_4^+$ transport. $A$: basolateral MA uptake and $J_{\text{ba}}$ flux in the absence or presence of 10 $\mu$M forskolin (FSK). $B$: apical MA uptake and $J_{\text{ab}}$ flux in the absence or presence of 10 $\mu$M forskolin. For $A$ and $B$, symmetrical 1 mM MA and a 2-min sample time were used. $C$: $J_{\text{ba}}$ flux of $\text{NH}_4^+$ in the absence or presence of 10 $\mu$M forskolin. Initial condition was 0 mM apical and 10 mM basolateral $\text{NH}_4^+$. $D$: $J_{\text{ab}}$ flux of $\text{NH}_4^+$ in the absence or presence of 10 $\mu$M forskolin. Initial condition was 10 mM apical and 0 mM basolateral $\text{NH}_4^+$. For $C$ and $D$, a 5-min sample time was used, and $n = 6$ for each bar. NS, no statistical significance and $*P < 0.05$ for control vs. forskolin-treated groups.
min, cells in the control group (no added NH₄⁺) did not produce significant amounts of NH₄⁺. During the initial 10 min, basolateral media [NH₄⁺] decreased in all groups. However, at apical NH₄⁺ concentrations in excess of 10 mM, basolateral NH₄⁺ increased by the 20-min time point. Basolateral [NH₄⁺] continued to decrease when the initial apical [NH₄⁺] was at or below that of basolateral NH₄⁺ and was relatively unchanged with an initial apical (10 mM)-to-basolateral (3 mM) gradient. Thus despite increased apical membrane permeability to NH₃, T84 cells can maintain at least a 10:3 NH₄⁺ gradient, indicating an ability to transport NH₄⁺ in the secretory direction to minimize back diffusion of NH₃.

The passive diffusion of NH₃ from the apical media into and across T84 cells is supported by data obtained at differing apical pH. Figure 10 shows the basolateral media [NH₄⁺] in cells exposed to 10 mM apical NH₄⁺ at apical media pH of 5.5, 7.4, and 9.0. Initial basolateral media [NH₄⁺] was 2.88 ± 0.02 mM. At an apical media pH of 5.5, 7.4, and 9.0, basolateral [NH₄⁺] decreased to 2.69 ± 0.03 and 2.78 ± 0.01 mM in the first 10 min and then rose to levels not significantly different than the starting values. However, with an apical media pH of 9.0, at which more NH₃ is present, basolateral [NH₄⁺] increased to 3.00 ± 0.05 mM in 10 min and to 3.45 ± 0.09 by 20 min.

The ability of T84 cells to actively minimize net NH₄⁺ absorption when exposed to an apical-to-basolateral NH₄⁺ gradient for an extended period was further tested with cells in culture medium. Cell monolayers were maintained in complete culture medium on the basolateral side with a measured value of 4.06 ± 0.03 mM NH₄⁺ and serum-free culture medium containing 20 mM NH₄⁺ at pH 6.0 on the apical side. Apical pH 6.0 was used instead of pH 5.5 due to the buffering capacity of culture media. As shown in Fig. 11, basolateral [NH₄⁺] decreased to 2.51 ± 0.03 mM by 10 min with only a slight increase (2.66 ± 0.04 mM) at 60 min.

**DISCUSSION**

Excess systemic NH₄⁺ levels can lead to hyperammonemia-associated encephalopathy, which can be life threatening. Although the kidney is responsible for carrying out the bulk of body NH₄⁺ homeostasis, the possibility exists that some level of body NH₄⁺ control can be accomplished via changes in colonic function. Our data demonstrate the following: 1) T84 cells express mRNA for two known NH₄⁺ transporters RhBG and RhCG and display transport characteristics consistent with functional RhG proteins; 2) transepithelial MA flux in T84 cells is greater in the secretory than absorptive direction; 3) transepithelial transport of NH₄⁺ in T84 cells is greater in the secretory than absorptive direction and is sensitive to cAMP stimulation and to inhibitors of NKCC1 and Na⁺-K⁺-ATPase; and 4) T84 cells are able to maintain an apical-to-basolateral NH₄⁺ gradient despite having a greater apical membrane NH₃ permeability than native colonic crypts (2, 16, 18, 42, 44). Taken together, these data provide evidence that model colonic crypt epithelia secrete NH₄⁺ by a mechanism that involves both K⁺ transport and novel RhG protein-mediated pathways. The long-standing premise, based on greater [NH₄⁺] in portal vein vs. systemic circulation, has been that NH₄⁺ absorption occurs along the length of the intestine. No consideration has been given for an opposing NH₄⁺ secretory vector, which is surprising considering that secretory epithelia in the intestine share similar transport mechanisms with renal and gill epithelium, which do effect NH₄⁺ secretion. If confirmed in natural tissue, these findings indicate that active secretion by colonic crypts could oppose the larger absorptive NH₄⁺ vector to limit net NH₄⁺ delivery to the portal circulation.
RhBG and RhCG isoforms of the Rhesus glycoprotein family have been identified along the GI tract in mouse (13). Although limited RhBG and RhCG expression was observed in the upper portion of proximal colon crypts compared with surface cells in mouse tissue, both isoforms are present in T84 cells (human), which otherwise functionally resemble cells of crypt origin. Whether expression patterns of RhBG and RhCG differ along the length of the colon or along the entire crypt surface axis in mouse or human tissue remains to be determined as does distribution among the various cell types present within the colonic crypt. MA uptake in to T84 cells was observed from the basolateral side, thus suggesting the functional presence of RhBG. MA uptake was partially inhibited by the addition of basolateral NH₄⁺, suggestive of RhBG function similar to reported results for RhBG in renal mIMCD-3 cells (12). In mIMCD-3 cells basolateral MA transport exhibits both diffusive and carrier-mediated components with the diffusive component becoming predominant at [MA] exceeding 10 mM. The dose response relation for MA uptake observed in T84 cells shows similar properties with an almost linear relation between 5 and 15 mM MA. Handlogten et al. (12) reported that MA uptake via RhBG in mIMCD-3 cells is not affected by the NKCC1 inhibitor bumetanide. In their study, excess NH₄⁺ was used to distinguish between transporter-mediated and diffusive entry of MA. Since T84 cells have abundant NKCC1 activity (which, as discussed below, is capable of NH₄⁺ transport), such a maneuver becomes somewhat problematic; therefore, we operationally defined transporter-uptake by performing assays at <5 mM MA and <3-min uptake periods. Consistent with RhBG in mIMCD-3 cells, transporter mediated uptake of MA (e.g., at <5 mM MA and <3-min uptake periods) is insensitive to bumetanide. With increased concentration of MA as well as with increased uptake times (at a greater fraction of diffusive MA entry), total MA uptake showed some sensitivity to bumetanide. This could be due to an indirect effect of bumetanide itself on diffusive MA entry or, alternatively, an indirect effect of NKCC1 inhibition on diffusive MA entry. The later possibility is perhaps more likely, given the abundant capacity for NKCC1 activity in T84 cells. It should be noted, however, that bumetanide had little impact on the transepithelial flux of MA, perhaps due to the apical exit of MA being rate limiting (suggested by less apical MA uptake vs. basolateral MA uptake).

Uptake of MA by either RhBG or RhCG expressed in Xenopus oocytes has been shown to be stimulated by extracellular alkalosis (26), similarly RhCG activity in mIMCD-3 cells is enhanced by extracellular alkalinization (11). Both RhBG and RhCG have been identified in mouse intestine, showing basolateral and apical immunostaining, respectively (48). In mIMCD-3 cells, functional distinction between RhBG and RhCG is primarily based on basal activity, pH sensitivity, and relative affinity (11). In T84 cells, both basolateral MA uptake (RhBG) and basolateral-to-apical flux of MA is enhanced by elevated basolateral pH and inhibited by basolateral solution acidification.

MA uptake into T84 cells was also observed from the apical side, thus supporting the functional presence of an RhG protein, likely RhCG. Uptake was partially inhibited by the addition of apical NH₄⁺. Although conditions were not optimized for maximal competitive inhibition, since apical NH₄⁺ will change intracellular pH (pHₗ) (16, 54), the data are suggestive of RhG activity as reported results for RhCG in renal mIMCD-3 cells (11). Apical MA uptake in T84 cells with changes in apical solution pH are consistent with published data for RhCG (11, 26), thus supporting the functional presence of apical RhCG in T84 cells.

The notion that NH₄⁺ secretion can be driven by NKCC and Na⁺-K⁺-ATPase with an opposing apical exit pathway is supported by work in renal cells (19, 46). In fact, Kinne et al. estimated that it is energetically possible for NKCC and Na⁺-K⁺-ATPase under physiological conditions to generate an intracellular-to-extracellular [NH₄⁺] ratio of ~2,000! As previously reported, NH₄⁺ can be effectively transported on the K⁺ site of Na⁺-K⁺-ATPase and NKCC1 in T84 cells (54). Since similar loading pathways are used by various NH₄⁺ secretory epithelia, it was logical to examine whether T84 monolayers demonstrate transepithelial ammonium transport.

Indeed, we demonstrated a role for NKCC1 and perhaps Na⁺-K⁺-ATPase in transepithelial secretory flux of NH₄⁺. Basolateral bumetanide as well as ouabain significantly decreased the movement of basolateral NH₄⁺ to the apical compartment. Although it is difficult to assess the degree to which ouabain might indirectly affect NKCC1 activity (by altering the Na⁺ gradient), these data support the involvement of additional pathways for basolateral NH₄⁺ loading (such as RhBG) as described above since bumetanide or ouabain resulted in only an ∼40 or ∼33% reduction in NH₄⁺ flux. In our study, bumetanide had little effect on transepithelial MA transport. It should be noted, as well, that Handlogten et al. (12) showed minimal MA uptake on Na⁺-K⁺-ATPase or NKCC1 in mIMCD-3 cells. Our data support a role for NKCC1 in retarding the movement of NH₄⁺ from the apical compartment to the basolateral compartment, perhaps acting as an NH₄⁺ scavenger by rapidly internalizing NH₄⁺ present in the basolateral vicinity of the crypt compartment.

Ammonium was transported by both Na⁺-K⁺-ATPase and NKCC1 [as has been described for other epithelial transport models of ammonium secretion (30, 38, 40, 43, 45, 47)], as well as RhBG and RhCG. Under asymmetric flux conditions at pH 7.4, both MA and NH₄⁺ transepithelial fluxes were greater in the secretory compared with absorptive direction. Whereas MA uptake and flux (RhBG/RhCG mediated) were unaffected by forskolin, NH₄⁺ flux was stimulated by cAMP, presumably because of increased NKCC1 activity. In vivo, luminal pH is more acidic (pH of ∼5.5 in humans); this would further favor net secretion under physiological pH conditions because relative luminal acidity favors luminal NH₄⁺ over NH₃ and thus minimizes passive backdiffusion of NH₃ through the apical membrane. The redundancy in mechanisms for basolateral ammonium loading in both kidney and intestine suggests the importance of maintaining ammonium secretory capacity. Indeed, predicted distal tubular acidosis or hyperammonemia was absent in RhBG KO mice (5, 6).

An important caveat to our study is the observation that native colonic crypt cells of certain mammals appear to exhibit low apical permeability to ammonia (NH₃) (2, 18, 42, 44). In contrast, the apical membrane of T84 cells has a more readily apparent NH₃ permeability (16). It should be noted that the higher apical NH₃ permeability in T84 cells would impair their ability to secrete NH₄⁺ against a concentration gradient and thus might underestimate the ability of intact crypts to secrete NH₄⁺ or maintain a large luminal-to-serosal NH₄⁺ gradient.
Moreover, it is unknown whether cultured crypt epithelial lines differ, in general, from native crypts with respect to apical ammonium permeability; certainly, there may be considerable species, segmental, or cellular heterogeneity.

Several models for NH₄⁺ secretion in other tissues (see introduction) include ion trapping of NH₄⁺ at the luminal surface. In particular, colonic H⁺-K⁺-ATPase along with an apical K⁺ channel have been shown to be important participants for mammalian IMCD (30). Although it is well established that native colonic mucosa contains both cH⁺-K⁺-ATPase and a ROMK1-like apical K⁺ channel (22), both appear to be absent from the T84 cell line (36). The lack of these two apical transporters (cH⁺-K⁺-ATPase and K⁺ channel) in T84 cells thus also limits their ability to secrete NH₄⁺ against a concentration gradient or maintain a large luminal-to-serosal NH₄⁺ gradient.

Due to the nature of the ammonium assay (signal-to-noise) and the NH₃ permeability of T84 apical membrane, it is difficult to measure NH₄⁺ transport against a sizeable apical [NH₄⁺]. An alternative approach would be the use of the stable nonradioactive isotope of N (15N) in tracer fluxes, although it is questionable whether this approach would enhance accuracy of transepithelial flux assays. As an indirect alternative, we determined secretory MA flux against an apical-to-basolateral NH₄⁺ gradient (Fig. 5). The rationale is that NH₄⁺ is transported by RhBG and RhCG to a greater extent than is MA; thus NH₄⁺ acts competitively against MA transport on RhGs (Fig. 2 and Ref. 12). We found that 20 mM cts NH₄⁺ inhibits uptake of MA by about half. Thus it is reasonable that trans NH₄⁺, if sensed by the secretory machinery, should also inhibit the transepithelial flux of MA. However, secretory direction MA flux was not significantly altered by the trans addition of up to 100 mM NH₄⁺. This observation suggests that NH₄⁺ secretion (in this case estimated by ¹⁴C-MA tracer) can occur against a physiologically relevant apical-to-basolateral NH₄⁺ gradient. These results underestimate the secretory capacity since MA is not transported by NKCC1 or Na⁺-K⁺-ATPase, whereas NH₄⁺ would be loaded into the cell by these transporters. T84 cells were also capable of maintaining an apical-to-basolateral NH₄⁺ gradient (Figs. 9 and 11). The initial loss of basolateral compartment NH₄⁺ may represent a combination of cellular NH₄⁺ sequestering and/or metabolism; however, this does not negate the fact that T84 cells can maintain an appreciable apical-to-basolateral gradient.

Taken together, our data suggest that ammonium transport in the secretory direction can occur in T84 model crypt epithelia and that RhG proteins, NKCC1, and, to some extent, Na⁺-K⁺-ATPase participate in basolateral loading of NH₄⁺ into the cell. We speculate that a secretory mechanism may be present in natural colonic mucosa to counter ammonium absorptive pathways and thus limit the amount of NH₄⁺ reaching the portal circulation. Further elucidation of the mechanisms involved in colonic NH₄⁺ transport and regulation may be useful in developing new targets for more effective therapeutic treatment of hyperammonemia.

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