Apical ammonium inhibition of cAMP-stimulated secretion in T84 cells is bicarbonate dependent

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Worrell, Roger T., Alison Best, Oscar R. Crawford, Jie Xu, Manoочer Soleimani, and Jeffrey B. Matthews. Apical ammonium inhibition of cAMP-stimulated secretion in T84 cells is bicarbonate dependent. Am J Physiol Gastrointest Liver Physiol 289: G768–G778, 2005.- Normal human colonic luminal (NH4+) concentration ([NH4+]) ranges from 3–10 mM. However, the base of the colonic epithelium is poorly understood. We elucidate here the effects of apical NH4+ on cAMP-stimulated Cl− secretion in colonic T84 cells. In HEPES-buffered solutions, 10 mM apical NH4+ had no significant effect on cAMP-stimulated current. In contrast, 10 mM apical NH4+ reduced current within 5 min to 61 ± 4% in the presence of 25 mM HCO3−. Current inhibition was not simply due to an increase in extracellular K+−like cations, in that the current magnitude was 95 ± 5% with 10 mM apical K+ and 46 ± 3% with 10 mM apical NH4+ relative to that with 5 mM apical K+. We previously demonstrated that inhibition of Cl− secretion by basolateral NH4+ occurs in HCO3−-free conditions and exhibits anomalous mole fraction behavior. In contrast, apical NH4+ inhibition of current in HCO3− buffer did not show anomalous mole fraction behavior and followed the absolute [NH4+] in K+−NH4+ mixtures, where K+−concentration + [NH4+] = 10 mM. The apical NH4+ inhibitory effect was not prevented by 100 μM methazolamide, suggesting no role for apical carbonic anhydrase. However, apical NH4+ inhibition of current was prevented by 10 min of pretreatment of the apical surface with 500 μM DIDS, 100 μM 4,4′-dinitrostilbene-2,2′-disulfonic acid (DNDS), or 25 mM niflumic acid, suggesting a role for NH4+ action through an apical anion exchanger. mRNA and protein for the apical anion exchangers SLC26A3 [downregulated in adenoma (DRA)] and SLC26A6 [putative anion transporter (PAT1)] were detected in T84 cells by RT-PCR and Northern and Western protein for the apical anion exchanger. mRNA and protein for the apical anion exchangers SLC26A3 [downregulated in adenoma (DRA)] and SLC26A6 [putative anion transporter (PAT1)] were detected in T84 cells by RT-PCR and Northern and Western.

AMMONIUM (NH4+) CONCENTRATION ([NH4+]) IN THE HUMAN COLON RANGES FROM 15 TO 100 mM (41). NORMAL ARTERIAL PLASMA LEVELS OF NH4+ ARE RELATIVELY LOW, I.E., 45 μM (10, 41). THE ELEVATION OF PORTAL VEIN NH4+ LEVELS WITH RESPECT TO ARTERIAL PLASMA (~350 μM) INDICATES NET ABSORPTION OF NH4+ ACROSS THE COLONIC EPITHELIUM (41). ALTHOUGH THE NH4+ LEVEL AT OR NEAR THE BASE OF THE COLONIC EPITHELIUM IS UNKNOWN, IT IS LIKELY TO BE SIGNIFICANTLY HIGHER (~3–10 mM) THAN IN THE PORTAL VEIN BECAUSE OF THE ~10-FOLD DISSOLUTION OF “COLONIC CRYPT” BLOOD BY THE TIME IT REACHES THE PORTAL VEIN. HOWEVER, LITTLE IS KNOWN ABOUT NH4+ TRANSPORT OR NH4+ EFFECTS ON COLONIC ION TRANSPORT. EXCESS IN 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, IT IS POSSIBLE THAT SOME LEVEL OF BODY NH4+ CONTROL CAN BE ACCOMPLISHED VIA CHANGES IN COLONIC FUNCTION.

NH3 AND NH4+ EXIST IN AN EQUILIBRIUM. NH3 IS A WEAK BASE, WITH AN ACID-BASE IONIZATION CONSTANT (pKa) OF 9.2, AND, WITH SOME NOTABLE EXCEPTIONS (21, 42), CAN DIFFUSE ACROSS THE PLASMA MEMBRANE. HOWEVER, AT PHYSIOLOGICAL pH, ~98% OF NH3 EXISTS IN THE IONIZED FORM, I.E., NH4+, WHICH IS NOT FREELY PERMEABLE ACROSS THE PLASMA MEMBRANE. AS WITH OTHER IONS, CELL PERMEABILITY TO NH4+ REQUIRES CHANNELS, COTRANSPORTERS, OR PUMPS WITHIN THE MEMBRANE. NH4+ IS SIMILAR IN MOLECULAR SIZE TO K+ AND HAS BEEN FOUND TO SUBSTITUTE FOR K+ ON A NUMBER OF ION CHANNELS, COTRANSPORTERS, AND PUMPS.

Previous studies in rat and human colon demonstrated that luminal NH4+ can inhibit Na+ and Cl− absorption, an effect that involves interaction of NH4+ with an apical Na+/H+ exchanger (6, 7). Luminal NH4+ inhibition of forskolin-activated short-circuit current (Isc) has been reported in rat and human colon by Mayol et al. (32), but little effect was observed with basolateral NH4+. However, the extent to which K+ secretion may have contributed to the measured current is unclear. In contrast, using Cl− flux measurements, Cermak et al. (6) found little effect of luminal NH4+ on Cl− secretion in rat colon.

The T84 secretory colonic cell line was used to show that NH4+ can affect cAMP- and cGMP-dependent Cl−, but not carbachol-induced (Ca2+, secretion) (31). In T84 cells, K+ secretion is virtually absent, presumably because of the lack of an appropriate apical K+ channel. In these cells, stimulated electrogenic secretion can be attributed almost entirely to Cl− secretion. Under HCO3−-free conditions, there was a sidedness to the NH4+ effect on cAMP-stimulated Cl− secretion in these cells, with application to the basolateral side having an inhibition constant (Ki) of 5 mM and application to the apical side a Ki of 50 mM (37). This suggested that the NH4+ effect on Cl− secretory rate occurred by affecting the basolateral membrane transport processes. In addition, NH4+ was found not to alter apical Cl− conductance (CFTR) but, rather, to affect basolateral K+ conductance (16).

Although some reports have shown that the apical membrane of colonic crypts is relatively impermeable to NH3 and

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NH$_4^+$ (14, 42), this does not appear to be the case with cultured cells. In T84 cells, NH$_4^+$ application on the apical or basolateral side leads to intracellular alkalinization consistent with net NH$_3$ entry. However, with basolateral application, cell acidification quickly follows and indicates subsequent net NH$_3$ entry (16, 48). By comparing intracellular pH (pHi) changes with half-maximal inhibition of current with apical or basolateral NH$_4^+$, Hmjez et al. (16) determined that changes in pHi did not correlate with changes in Cl$^-$ secretion. Furthermore, Worrell et al. (48) demonstrated that the basolateral NH$_4^+$ inhibition of current in the absence of K$^+$ was not as significant as that in the presence of K$^+$, with maximal inhibition at a mole fraction ratio of K$^+$ to NH$_4^+$ of 0.25:1. The observed anomalous mole fraction behavior of the basolateral K$^+$/NH$_4^+$ ratio is inconsistent with basolateral NH$_4^+$ exerting an inhibitory effect through a change in pH$_i$.

Because inhibition of cAMP-activated $I_{sc}$ by luminal NH$_4^+$ was observed in rat and human colon in the presence of HCO$_3^-$ (32) and little effect of apical NH$_4^+$ was observed in T84 cells in the absence of HCO$_3^-$ (37), we sought to determine the effect of apically applied NH$_4^+$ on T84 cells in the presence of HCO$_3^-$.

Our data suggest that apical NH$_4^+$ acts on an apical anion exchanger to inhibit cAMP-stimulated Cl$^-$ current in T84 cells.

**MATERIALS AND METHODS**

**Cell culture.** T84 cells (11) obtained from the American Type Culture Collection were grown to confluence at pH 7.4 in 162-cm$^2$ flasks with 1:1 DMEM-Ham’s F-12 supplemented with 6% FBS, 15 mM HEPES, 14 mM NaHCO$_3$, 170 μM penicillin G, and 69 μM streptomycin sulfate. Amphoterin B was not included in the medium 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 PBS at a split ratio of 1:2. Cells for experimentation were plated on uncoated 12- or 24-mm Costar Transwell (3-μm pore) inserts at a seeding density of 4–5 × 10$^5$ cells/cm$^2$ and cultured for 8–14 days with feeding in the above-described medium three times per week. Cell monolayers were determined to be acceptable for use when the transepithelial resistance reached $\geq 1,200$ Ω·cm$^2$ as measured by an epithelial volt-ohm meter (EVOM; see below). All experiments were performed at 37°C.

**Measurement of transepithelial current.** The quality of high-resistance monolayer formation was monitored using an EVOM (World Precision Instruments) as described previously (47, 48). Transepithelial potential (mV) and transepithelial resistance (kΩ/cm$^2$) was measured with the EVOM. Transepithelial current ($\mu$A·cm$^2$) was calculated by Ohm's law and is referred to as open-circuit current to clearly distinguish it from $I_{sc}$. Although the open-circuit current technique tends to underestimate the amount of current that would be obtained in $I_{sc}$ measurements, EVOM measurements proved consistent and reliable for detecting changes in transepithelial voltage, resistance, and current (47, 48). Moreover, measurements in the open-circuit mode represent the normal condition (e.g., not short circuited) of native epithelia. EVOM measurements do not provide for an adequate sampling rate to monitor the secretory response to carbacbol; thus the conventional $I_{sc}$ technique was used.

Transepithelial measurements were carried out in a base solution consisting of (in mM) 135 NaCl, 5 KCl, 0.5 CaCl$_2$, 2 MgCl$_2$, and 10 Na-HEPES, pH 7.4, for HCO$_3^-$-free conditions. Measurements in the presence of HCO$_3^-$ were carried out in the following solution (in mM): 110 NaCl, 5 KCl, 0.5 CaCl$_2$, 2 MgCl$_2$, 10 Na-HEPES, and 25 mM HCO$_3^-$, pH 7.4. For measurements in the presence of HCO$_3^-$, cells were kept in a custom-made water bath chamber constantly bubbled with 5% CO$_2$, K$^+$- and/or NH$_4^+$-glucanate was added to the respective K$^+$-free solutions, and solution pH was adjusted to pH 7.4 as necessary. For experiments involving nominally free Cl$^-$ solution (glucanate salts), short 3 M KCl agar bridges were fashioned from cut 100-μl pipette tips to minimize electrode junction potentials. As a control, K$^+$-glucanate was substituted at equal-molar concentration for NH$_4^+$-glucanate, such that Cl$^-$ and glucanate concentrations and osmolality were held constant. cAMP-dependent Cl$^-$ secretion was stimulated by 10 μM basolateral forskolin.

**RT-PCR of SLC26A3 and SLC26A6.** RT-PCR was performed on RNA isolated from T84 cells to determine the expression of SLC26A3 [downregulated in adenoma (DRA)] and SLC26A6 [putative anion transporter 1 (PAT1)], two well-known anion exchangers in small and large intestine. For SLC26A3, the following oligonucleotide primers were designed and used for RT-PCR: ATG CCT TCA CTG TGT CTC TCT GGT CTT GCC (175 nt, sense) and AAT ATG CAC CAG TTC CTC CCC TGT ACC GC (2699 nt, antisense; GenBank accession no. NM_134426). These primers encode the full-length DRA cDNA. PCR cycle conditions were as follows: 94°C for 1 min; 35 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 3 min; and 68°C for 3 min. For SLC26A6, the following oligonucleotide primers were designed and used for RT-PCR: ATG CCT TCA CTG TGT CTC TCT GGT CTT GCC (sense) and AAT ATG CAC CAG TTC CTC CCC TGT ACC GC (antisense). These primers encode the full-length PAT1 cDNA. PCR cycle conditions were as follows: 94°C for 1 min; 35 cycles at 94°C for 30 s, 65°C for 30 s, and 68°C for 3 min; and 68°C for 3 min. Both primer sets would distinguish between a DNA and an mRNA product.

**Northern blots.** T84 total RNA (30 μg/lane) was run on a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane. RNA was covalently bound to the nylon membrane by ultraviolet cross-linking. cDNA probes were labeled with [32P]dCTP using High Prime (Roche). For SLC26A6 (PAT1), a 2.52-kb PCR fragment encoded by mouse PAT1-specific primers [ATG CCT TCA CTG TGT CTC TCT GGT CTT GCC (sense) and AAT ATG CAC CAG TTC CTC CCC TGT ACC GC (antisense)] was purified and used as a specific probe for Northern blot hybridization. For SLC26A3 (DRA), a 2.33-kb PCR fragment end coded by the primers ATG ATT GAA CCC TTT GGG TGT ACC GC (antisense) and TACG CAG TAT GAC TTT ACC GC (sense) was used as a specific probe. Both Northern blot hybridization and RT-PCR of SLC26A3 and SLC26A6.

**Western blots.** CFTR immunoprecipitation, and immunostaining. Whole cell lysates were made from T84 cells grown on permeable supports using a lysis buffer composed of (in mM) 50 HEPES, 150 NaCl, 1 EGTA, 1 EDTA, 0.1 Na-orthovanadate, 10 tetrasodium pyrophosphate, and 100 NaF with 10% glycerol, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche). One hundred milligrams of total protein per lane of cell lysate were run on a 6% polyacrylamide gel and transferred to a nitrocellulose membrane. For DRA, the blot was probed with a rabbit anti-DRA antibody raised against mouse slc26a3 (13). For PAT1, the blot was probed with a rabbit anti-PAT1 antibody raised against human SLC26A6 (44). Protein was detected by rabbit secondary antibody (KPL) and enhanced chemiluminescence (Amersham).

**CFTR** was immunoprecipitated from T84 cell lysate obtained from six 4.7-cm$^2$ Transwell inserts using the Seize Classic Mammalian Immunoprecipitation Kit (Pierce) and M3A7 mouse monoclonal anti-CFTR antibody (Upstate). The amount of total protein in the lysates was determined and equalized between treatment groups with lysis buffer before immunoprecipitation. The immunoprecipitate was split into two equal fractions for gel separation and detection of DRA and PAT1. DRA and PAT1 were detected in the CFTR immunoprecipitate blots as described above. Cells were stimulated with 10 μM forskolin for 5 min and then treated with 10 μM apical NH$_4^+$ for 10 min before lysis with M-PER reagent (Pierce) or left untreated.

For immunostaining, T84 cells on 1-cm$^2$ Transwell inserts were treated with 10 μM forskolin for 5 min and then washed three times.
in PBS and fixed for 20 min in 3% paraformaldehyde. Monolayers were permeabilized with 0.1% Triton X-100 for 4 min and then treated for 10 min with 1% SDS in K\(^+\)-free PBS for antigen retrieval. Cells were blocked with 10% goat serum in PBS for 1 h. Rabbit anti-DRA or anti-PAT1 antibody was applied in conjunction with mouse anti-CFTR (M3A7, Upstate) for 1 h at room temperature. After they were washed three times in PBS, the monolayer and support were mounted on glass slides using Vectashield hard set with 4',6-diamidino-2-phenylindole and imaged using a confocal microscope (model 510, Zeiss).

**Apical \(^{36}\text{Cl}\) uptake.** DIDS-sensitive apical \(^{36}\text{Cl}\) uptake was used to assess apical anion-exchange activity. Apical \(^{36}\text{Cl}\) uptake was similar to previously reported \(^{36}\text{Cl}\) basolateral uptake (48). T84 cell monolayers were grown on 1-cm\(^2\) Transwell inserts. Cells were incubated in the presence or absence of 200 \(\mu\text{M}\) apical DIDS for 5 min, and 10 \(\mu\text{M}\) basolateral forskolin was added to stimulate \(\text{Cl}\) secretion. After 5 min, the apical solution was changed to 10 mM K\(^+\) or 10 mM NH\(_4\)\(^+\) in the presence or absence of apical DIDS. At 5 min after apical ion substitution, the apical solution was replaced with an identical solution containing 1 \(\mu\text{Ci/ml}\) \(^{36}\text{Cl}\). After 10 min the inserts were sequentially dunked into three reservoirs containing >500 ml ice-cold 0.1 M MgCl\(_2\) and 10 mM Tris-Cl, pH 7.4, to end uptake. The inserts were then cut from the supports and placed in scintillation vials with scintillation cocktail for determination of radioactivity. DIDS-sensitive uptake was determined as the difference between uptake in the presence and uptake in the absence of DIDS.

**Reagents.** Forskolin and carbachol were obtained from Calbiochem and FBS and PenStrep from Gibco BRL. All other chemicals, which were of the highest grade, were obtained from Sigma.

**Significance tests.** Values are means ± SE. Significance was estimated using Student’s \(t\)-test and two-way ANOVA when indicated, with \(P < 0.05\) indicating a significant difference.

**RESULTS**

**Apical \(\text{NH}_4^+\) effect in HEPES vs. HEPES-HCO\(_3^-\) buffer.** No significant effect on cAMP-stimulated \(\text{Cl}^-\) current in T84 cells was observed with 10 mM apical NH\(_4\)\(^+\) in HCO\(_3^-\)-free buffer (Fig. 1), consistent with the report of Prasad et al. (37). Forskolin (10 \(\mu\text{M}\) basolateral) was used to stimulate cAMP-activated \(\text{Cl}^-\) secretion. Table 1 shows transepithelial voltage, short-circuit current (\(I_{sc}\)) in T84 monolayers treated with 10 mM apical NH\(_4\)\(^+\) and 10 mM apical K\(^+\). Apical ion substitution was at time 0. Forskolin-stimulated current was normalized to the value at \(-1\) min.

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**Fig. 1.** Apical NH\(_4\)\(^+\) inhibits current only in the presence of HCO\(_3^-\). A: current in the absence and presence of 25 mM HCO\(_3^-\) with apical K\(^+\) or NH\(_4\)\(^+\). In HCO\(_3^-\)-free conditions, 10 mM apical NH\(_4\)\(^+\) has no significant effect on cAMP-stimulated Cl\(^-\) secretion in T84 cells. In the presence of HCO\(_3^-\), 10 mM apical NH\(_4\)\(^+\) produces a significant inhibition of current relative to 10 mM apical K\(^+\). Current is significantly greater in the presence of HCO\(_3^-\) than in HEPES-buffered solution. B: current relative to forskolin-stimulated current before apical ion substitution. Measurements (\(n = 4\)) were made 5 min after apical ion substitution. HEPES remained in the HCO\(_3^-\)-buffered solutions. *Significantly different from respective control. #Significantly different from HEPES control. C: relative short-circuit current (\(I_{sc}\)) in T84 monolayers treated with 10 mM apical NH\(_4\)\(^+\) and 10 mM apical K\(^+\). Apical ion substitution was at time 0. Forskolin-stimulated current was normalized to the value at \(-1\) min.
Table 1. Measured and calculated open-circuit current measurements for Fig. 1, A and B

<table>
<thead>
<tr>
<th>Conditions</th>
<th>TEP, mV</th>
<th>TER, Ω cm²</th>
<th>Initial Fsk-stimulated I, μA/cm²</th>
<th>Relative I, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>34.8±0.7</td>
<td>550±10</td>
<td>63±2</td>
<td>86±4</td>
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<tr>
<td>10 mM K⁺</td>
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<td>560±50</td>
<td>64±7</td>
<td>80±1</td>
</tr>
<tr>
<td>10 mM NH₄⁺</td>
<td>31.0±1.5</td>
<td>520±10</td>
<td>60±2</td>
<td>83±3</td>
</tr>
<tr>
<td>NHCO₃</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>42.3±2.5</td>
<td>420±20</td>
<td>101±4</td>
<td>100±4</td>
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<tr>
<td>10 mM K⁺</td>
<td>38.6±4.2</td>
<td>400±30</td>
<td>97±5</td>
<td>103±4</td>
</tr>
<tr>
<td>10 mM NH₄⁺</td>
<td>31.1±2.5*</td>
<td>500±20</td>
<td>62±3*</td>
<td>101±2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. TEP, transepithelial potential; TER, transepithelial resistance; I, current; Fsk, forskolin; Relative I is average current after apical ion substitution relative to initial forskolin-stimulated current and was calculated for each individual monolayer, such that each monolayer served as its own control and the values were averaged. *Significantly different from corresponding control. †Significantly different from corresponding 10 mM K⁺ condition. ‡Significantly different from corresponding HEPES condition.

resistance, and current with the various apical ionic conditions. For reference, the initial forskolin-stimulated current values are also shown. Relative current, expressed as a percentage of the initial forskolin-stimulated current, was calculated for each individual monolayer and then averaged. Initial forskolin-stimulated current was 86 ± 4, 80 ± 1, and 83 ± 3 μA/cm² for the respective control conditions before apical ion substitution. At 5 min after forskolin addition, the apical medium was changed to medium containing 5 mM K⁺ (control), 10 mM K⁺, or 10 mM NH₄⁺. Open-circuit current measured 5 min after the apical substitution was 63 ± 2, 64 ± 7, and 60 ± 2 μA/cm² for 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively (Fig. 1A). Figure 1B shows current relative to the initial forskolin stimulation (5 min). Relative current in HEPES-only buffer was not statistically different: 73 ± 1, 81 ± 8, and 73 ± 2% for 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively.

In contrast to HCO₃⁻-free buffer, when experiments were performed in the presence of 25 mM HCO₃⁻ and 5% CO₂, 10 mM apical NH₄⁺ significantly inhibited cAMP-stimulated current within 5 min (Table 1). The overall magnitude of cAMP-stimulated current was significantly greater and showed less rundown in the presence of HCO₃⁻. Current magnitudes were 100 ± 4, 103 ± 4, and 101 ± 2 μA/cm² for the respective control conditions before apical ion substitution. Current magnitude 5 min after apical ion substitution was 101 ± 4, 97 ± 5, and 62 ± 3 μA/cm² for 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively (Fig. 1A). Current relative to the initial forskolin stimulation (5 min) is shown in Fig. 1B. Relative current in HCO₃⁻ buffer was 100 ± 1, 93 ± 6, and 61 ± 4% for 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively. There was no significant difference between 5 and 10 mM K⁺; however, current in the presence of 10 mM apical NH₄⁺ was significantly less than in the presence of 5 and 10 mM K⁺. This indicates that current inhibition was not simply due to an increase in extracellular K⁺-like cations at the apical membrane. Inhibition occurred in a dose-dependent manner, with reductions of 34, 44, and 56% by 5, 10, and 15 mM apical NH₄⁺, respectively, in cAMP-stimulated currents (data not shown). Significant inhibition (29 ± 0.01%) by 10 mM apical NH₄⁺ was also noted at 1 mM HCO₃⁻. Figure 1C shows NH₄⁺ inhibition of current obtained by Lw measurement; current relative to the initial forskolin-stimulated current is shown to allow for easy comparison with the data in Fig. 1B. Initial forskolin-stimulated current was 30 and 29 µA/cm² for the NH₄⁺- and K⁺-treated monolayers, respectively. At 5 min after apical ion substitution, current was 21 µA/cm² (71%) for the 10 mM NH₄⁺-treated monolayer and 27 µA/cm² (92%) for the 10 mM K⁺-treated monolayer. At 10 and 15 min, current for the NH₄⁺-treated monolayer was 19 µA/cm² (63%) and 18 µA/cm² (61%) and current for the K⁺-treated monolayer was 25 µA/cm² (87%) and 26 µA/cm² (87%).

Carbachol-stimulated Cl⁻ secretion is not affected by basolateral NH₄⁺ (31). Apical NH₄⁺ also does not significantly affect the peak carbachol response, even in the presence of HCO₃⁻ and CO₂ (Fig. 2). The relative mean Iw plots from three to four monolayers are shown synchronized in time so that the current peaks coincided. Although peak current is not affected, there is perhaps a trend for slight inhibition at longer durations. Relative peak current was 7.1 ± 0.1 for 10 mM apical K⁺ and 7.4 ± 0.4 for 10 mM apical NH₄⁺. Relative current at 20 min after carbachol was 1.35 ± 0.01 for 10 mM K⁺ and 1.15 ± 0.01 for 10 mM NH₄⁺.

Mole fraction effect of apical NH₄⁺ inhibition. We previously demonstrated that inhibition of Cl⁻ secretion by basolateral NH₄⁺ occurs in HCO₃⁻-free conditions and exhibits anomalous mole fraction behavior (48). In contrast, apical NH₄⁺ inhibition of current in HCO₃⁻ buffer does not show anomalous mole fraction behavior (Fig. 3). Under K⁺-NH₄⁺ mixed ionic conditions, the degree of current inhibition follows the absolute [NH₄⁺] in mixtures where K⁺ concentration + [NH₄⁺] = 10 mM. Figure 3 depicts current relative to the initial forskolin-stimulated current in 5 mM apical K⁺ vs. the mole fraction of K⁺ to NH₄⁺. Relative current was 0.97 ± 0.07 for 10 mM K⁺, 0.46 ± 0.03 for 10 mM NH₄⁺, and 0.54 ± 0.01 for 2 mM K⁺:8 mM NH₄⁺, the ratio that produced maximal inhibition with basolateral NH₄⁺ application (48).

Apical carbonic anhydrase is not involved. To determine if NH₄⁺ was affecting apical carbonic anhydrase, thereby producing the inhibitory effect in the presence of HCO₃⁻, we employed the carbonic anhydrase inhibitor methazolamide. Preincubation with 100 µM apical methazolamide did not signific-

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Fig. 2. Relative Iw in the presence of 25 mM HCO₃⁻ with 10 mM apical K⁺ (○) or 10 mM apical NH₄⁺ (●); n = 4 for each condition. Ca²⁺-stimulated Cl⁻ secretion was elicited 5 min after apical ion substitution by 100 µM carbachol at time 0. No significant effect on peak current was observed with 10 mM apical NH₄⁺.

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HCO₃⁻-DEPENDENT APICAL NH₄⁺ INHIBITION OF SECRETION

significantly affect cAMP-stimulated Cl⁻ current in the presence of 10 mM apical K⁺ or 10 mM apical NH₄⁺ (Fig. 4). Initial forskolin-stimulated currents (5 min at 5 mM K⁺) were 80 ± 3, 81 ± 8, 87 ± 2, and 87 ± 5 μA/cm² for each of the conditions. At 20 min of apical methazolamide and 5 min after apical substitution, currents were 63 ± 1, 63 ± 4, 44 ± 1, and 45 ± 2 μA/cm² for 10 mM K⁺, 10 mM K⁺ with methazolamide, 10 mM NH₄⁺, and 10 mM NH₄⁺ with methazolamide, respectively. Current relative to the initial forskolin-stimulated current in 5 mM K⁺ is depicted in Fig. 4. Relative current magnitude was 0.79 ± 0.02 and 0.79 ± 0.01 for 10 mM apical K⁺ in the absence and presence of methazolamide, respectively. At 10 mM NH₄⁺, relative current was 0.51 ± 0.01 and 0.54 ± 0.01 in the absence and presence of methazolamide, respectively.

Involvement of apical anion exchanger in apical NH₄⁺ inhibition of current. To determine the possible role of apical anion exchanger(s) in mediating the HCO₃⁻-dependent inhibitory effect of apical NH₄⁺, the disulfonic stilbene DIDS was used to inhibit anion exchangers. Figure 5 shows the effect of 500 μM apical DIDS on the apical NH₄⁺-induced inhibition of current. Two protocols were used: 1) DIDS was applied 10 min before apical ion substitution and current was measured after 5 min (Fig. 5A), and 2) DIDS was applied 5 min after apical ion substitution and current was measured 10 min later (Fig. 5B). Currents relative to the initial forskolin-stimulated (5-min) level are shown. Application of DIDS before apical NH₄⁺ prevented the inhibitory effect of DIDS. In the absence of DIDS, relative current was 1.05 ± 0.04, 0.88 ± 0.02, and 0.73 ± 0.03 for 5 mM apical K⁺, 10 mM apical K⁺, and 10 mM apical NH₄⁺, respectively. In the presence of DIDS, relative current was 1.11 ± 0.11, 0.99 ± 0.06, and 1.05 ± 0.08 for 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively. There was no statistical difference between the groups in the presence of DIDS.

In contrast to the preapplication of DIDS, apical application of DIDS 5 min after apical ion substitution was ineffective, blocking the inhibitory effect of apical NH₄⁺. Relative current was 1.06 ± 0.06, 0.83 ± 0.01, and 0.69 ± 0.04 in the absence of DIDS for apically applied 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively. With the postapplication of DIDS, relative current was 1.02 ± 0.03, 0.91 ± 0.02, and 0.71 ± 0.04 for apically applied 5 mM K⁺, 10 mM K⁺, and 10 mM NH₄⁺, respectively. The apical NH₄⁺ effect was not significantly different with the postapplication of DIDS. Together, these data suggest the involvement of an apical anion exchanger in mediating the HCO₃⁻-dependent inhibitory effect of apical NH₄⁺.

Because DIDS is generally an irreversible inhibitor, we also employed the DIDS derivative DNDS. Preapplication of 100 μM DNDS partially prevented the NH₄⁺-induced inhibition of current (Fig. 5C). Application of ≤500 μM DNDS after apical NH₄⁺ was without effect, similar to the data obtained using DIDS (not shown). Preapplication of the structurally unrelated anion-exchange inhibitor niflumic acid at 25 μM completely blocked apical NH₄⁺-induced current inhibition (Fig. 5C). For this set of experiments, current relative to the initial forskolin-stimulated current (under control conditions or in the presence of DNDS or niflumic acid) was 0.71 ± 0.06 for NH₄⁺, 0.90 ± 0.06 for 100 μM DNDS followed by NH₄⁺, and 1.1 ± 0.04 for 25 μM niflumic acid followed by NH₄⁺. DNDS did not affect current, similar to the data shown for DIDS; however, niflumic acid reduced forskolin-stimulated current (from 38 ± 3.9 to 13 ± 1.6 μA/cm²), even in the absence of HCO₃⁻. Thus this niflumic acid effect is likely due to its known inhibitory effect on cyclooxygenase (3) and not to an inhibitory action on anion exchange. The ability of niflumic acid to prevent the inhibitory effect of NH₄⁺ in the presence of HCO₃⁻ is, however, most likely related to its inhibitory effect on anion exchange.

To further elucidate the potential effects of an apical anion exchanger in mediating the NH₄⁺ inhibition of current, we chose to compare the degree of inhibition in apical Cl⁻ and nominally Cl⁻-free conditions. Figure 6 shows current relative to the initial forskolin-stimulated current for 10 mM apical K⁺ and 10 mM apical NH₄⁺ with or without apical Cl⁻. Relative current values were 0.90 ± 0.1 for 10 mM K⁺ and 0.61 ± 0.03 for 10 mM NH₄⁺ in Cl⁻-containing apical solution. When gluconate was used as an anion replacement for Cl⁻ in the apical medium, relative current values were 0.55 ± 0.07 for 10 mM K⁺ and -0.33 ± 0.11 for 10 mM NH₄⁺. The relative...
magnitude of current reduction was greatly enhanced (~3-fold) by Cl\textsuperscript{-}-free apical medium: a difference of 0.94 vs. 0.35 for Cl\textsuperscript{-}-containing apical medium (Fig. 6). Under nominally Cl\textsuperscript{-}-free conditions, it is likely that an active anion exchanger would favor running backward. Thus the enhanced magnitude of current inhibition by apical NH\textsubscript{4}\textsuperscript{+} in apically Cl\textsuperscript{-}-free condition is consistent with the involvement of an apical anion exchanger.

Detection of anion exchanger in T84 cell RNA. RT-PCR was used to determine the identity of the putative apical anion (Cl\textsuperscript{-}/base) exchanger in T84 cells. Transcripts for SLC26A3 (DRA) and SLC26A6 (PAT-1) are abundantly expressed in T84 cells (Fig. 7A). Human colon RNA was used as control. In the absence of reverse transcriptase, no band was detected with either primer pair, indicating the specificity of the RT-PCR. Bands were of the predicted size for the respective mRNA. To confirm the RT-PCR results, Northern blots for DRA and PAT1 were also performed. mRNA for DRA and PAT1 are detected in T84 cells (Fig. 7B). DRA and PAT1 are also detected at the protein level in T84 cell lysate (Fig. 7C).

Fig. 5. Apical preapplication of known inhibitors of anion exchange prevents apical NH\textsubscript{4}\textsuperscript{+} inhibition of cAMP-stimulated current. A: DIDS pretreatment. Relative current with apical K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} was measured in the absence or presence of 500 \( \mu \)M DIDS. Apical DIDS was applied 10 min before apical ion substitution, and current was measured 5 min after apical ion substitution. B: DIDS posttreatment. Relative current with apical K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} was measured in the absence or presence of 500 \( \mu \)M DIDS. Apical DIDS was applied 5 min after apical ion substitution, and current was measured 10 min after DIDS application. C: as with DIDS, apical preapplication of 100 \( \mu \)M 4,4\textsuperscript{-}dinitrostilbene-2,2\textsuperscript{-}disulfonic acid (DNDS) partially prevents apical NH\textsubscript{4}\textsuperscript{+}-induced inhibition of current. Apical preapplication of the structurally unrelated inhibitor niflumic acid at 25 \( \mu \)M completely prevents NH\textsubscript{4}\textsuperscript{+}-induced inhibition as well. In both cases, inhibitors were applied 10 min before application of apical NH\textsubscript{4}\textsuperscript{+}. \( n = 4 \). *Significantly different from respective 10 mM K\textsuperscript{+} condition.

Fig. 6. Apical NH\textsubscript{4}\textsuperscript{+} inhibitory effect in HCO\textsubscript{3}\textsuperscript{-} buffer is augmented under apical Cl\textsuperscript{-}-free conditions. Relative currents are shown with apical K\textsuperscript{+} or NH\textsubscript{4}\textsuperscript{+} with Cl\textsuperscript{-} or gluconate as the anion (\( n = 4 \)). Apical NH\textsubscript{4}\textsuperscript{+} inhibition of cAMP-stimulated Cl\textsuperscript{-} current is enhanced ~3-fold in apical Cl\textsuperscript{-}-free conditions (brackets), which is consistent with involvement of an apical anion exchanger in mediating the HCO\textsubscript{3}\textsuperscript{-}-dependent apical NH\textsubscript{4}\textsuperscript{+} effect. Apical ion shift occurred just after the 5-min time point and 5 min after forskolin addition. \( \bullet \), 10 mM K\textsuperscript{+}; \( \downarrow \), 10 mM NH\textsubscript{4}\textsuperscript{+}; \( \blacktriangle \), Cl\textsuperscript{-}-free 10 mM K\textsuperscript{+}; \( \blacktriangledown \), Cl\textsuperscript{-}-free 10 mM NH\textsubscript{4}\textsuperscript{+}.
Apical anion-exchange activity was determined in T84 monolayers by assessment of DIDS-sensitive apical $^{36}$Cl$^{-}$ uptake. Uptake was determined in the presence and absence of apical NH$_4^+$, DIDS-sensitive uptake was 7.7 ± 1.0 and 66 ± 3 cpm·min$^{-1}$·cm$^{-2}$ in the absence of apical NH$_4^+$ and in the presence of 10 mM apical NH$_4^+$, respectively (Fig. 8). Apical NH$_4^+$ significantly increased DIDS-sensitive apical $^{36}$Cl$^{-}$ uptake. NH$_4^+$ stimulation of exchange activity could be the result of a direct effect on exchanger protein and/or an indirect effect via increased pH$_i$.

**Association of CFTR with DRA and PAT1.** CFTR R-domain phosphorylation has been shown to increase the association of the SLC26 STAS domain with the R-domain in an overexpression system with “flag-tagged” protein expression (24). This study focuses on the effect of apical NH$_4^+$ on cAMP-stimulated secretion; thus we sought to determine if CFTR was in close proximity and associated with DRA or PAT1 when these cells were in a secretory state. Figure 9 depicts the association of CFTR with DRA as well as with PAT1 in T84 cells. DRA (Fig. 9A) and PAT1 (Fig. 9B) are immunoprecipitated with CFTR by anti-CFTR. Previous reports demonstrated association of CFTR with SLC26 family members only using expression systems in which CFTR and the anion exchangers are overexpressed. Detection of DRA and PAT1 coimmunoprecipitation with CFTR in native cells has proven rather difficult because of low signal intensity. Despite this difficulty, coimmunoprecipitation is observed in Fig. 9, A and B, despite the expected low band intensity. Control immunoprecipitation without anti-CFTR antibody produced no detectable signal for DRA or PAT1 (not shown).

The NH$_4^+$ interaction site is located in the COOH-terminal region of DRA (8), which happens to be the general location of the STAS domain. The positive effect of SLC26 family members on CFTR activity could thus be diminished if NH$_4^+$ reduced the STAS-R-domain interaction. Exposure of secreting T84 cell monolayers to 10 mM apical NH$_4^+$ may indeed decrease the association of CFTR with DRA and PAT1. Although the present method of assessment is not optimum, i.e., normalizing to the respective amount of CFTR that was immunoprecipitated (Fig. 9, A and B), coimmunoprecipitation of DRA is estimated to be reduced ~15% and coimmunoprecipitation of PAT1 ~24% with apical NH$_4^+$.

Coimmunostaining of T84 monolayers stimulated with forskolin demonstrates that DRA (Fig. 9C) and PAT1 (Fig. 9D) each are located in general proximity to CFTR at the apical membrane when the cells are actively secreting Cl$^-$.  

**DISCUSSION**

Previous studies in rat and human colon as well as in cultured epithelial cell lines have demonstrated that NH$_4^+$ can inhibit Na$^+$ absorption (6, 7) as well as Cl$^-$ secretion (6, 16, 31, 32, 37, 48). The T84 secretory colonic cell line was used to show that basolateral NH$_4^+$ can affect cAMP- and cGMP-dependent Cl$^-$ secretion but not carbachol-induced Ca$^{2+}$- dependent secretion (31). Inhibition of cAMP-activated $I_{sc}$ by luminal NH$_4^+$ was observed in rat and human colon in the presence of HCO$_3^-$ (32); however, little effect of 10 mM apical NH$_4^+$ was observed in T84 cells in HCO$_3^-$-free conditions (37). Higher levels (50 –100 mM apical NH$_4^+$) did cause current inhibition in HCO$_3^-$-free conditions (37); however, this effect is likely due to diffusion to the basolateral side, because the inhibitory effect cannot be readily reversed unless the basolateral solution is exchanged (unpublished observations). We have studied the apical effect of NH$_4^+$ on T84 cells in the presence of HCO$_3^-$-containing buffer.
The data presented here demonstrate that apical NH$_4^+$ inhibits cAMP-stimulated secretion in an HCO$_3^-$-dependent manner through a mechanism that is consistent with an NH$_4^+$ effect on an apical anion exchanger. The following data support this conclusion: 1) Apical NH$_4^+$ at 10 mM does not affect current in HCO$_3^-$-free conditions. 2) The inhibitory effect of apical NH$_4^+$ is blocked by preapplication of apical DIDS, DNDS, and niflumic acid. 3) The magnitude of apical NH$_4^+$ inhibition is increased under apical Cl$^-$-free conditions. 4) The “known” apical anion exchangers DRA and PAT1 are present in these cells and appear to associate with CFTR.

NH$_3$ is a weak base (pK$_a$ = 9.2) and, with some notable exceptions (21, 42), can diffuse across the plasma membrane. However, at physiological pH, ~98% of NH$_3$ exists in the protonated form, i.e., NH$_4^+$. The NH$_2$/NH$_4^+$ prepulse method has been used extensively as a means of altering pH$_i$. In T84 cells, net NH$_3$ or net NH$_4^+$ entry can occur across the basolateral membrane; however, only net NH$_4^+$ entry is observed across the apical membrane (16, 48). Thus apical NH$_3$/NH$_4^+$ application in T84 cells leads to an alkalinization of pH$_i$. However, the observed change in pH$_i$ in the previous studies did not correlate with the changes in Cl$^-$ secretion (16). Moreover, Hrnjez et al. (16) reported that basolateral NH$_4^+$ did not alter apical Cl$^-$ conductance (CFTR) but did affect basolateral K$^+$ conductance. We later showed that basolateral NH$_4^+$ affects multiple targets on the basolateral membrane (48).

HCO$_3^-$-dependent apical NH$_4^+$ effect. Interestingly, in T84 cells, apical NH$_4^+$ inhibition of cAMP-stimulated Cl$^-$ secretion is HCO$_3^-$ dependent (Fig. 1). The inhibition is not due to an increase in “K$^+$-like” ions at the apical membrane, because the 10 mM NH$_4^+$-induced inhibition is significantly greater than the corresponding 10 mM K$^+$ control. As in previous studies with basolateral NH$_4^+$ application, the peak secretory response to carbachol stimulation was unaffected by apical NH$_4^+$, even in the presence of HCO$_3^-$ (Fig. 2).

The HCO$_3^-$-dependent apical NH$_4^+$ inhibition of current is distinct from basolateral NH$_4^+$ current inhibition, thus indicating another mechanism of action. Basolateral NH$_4^+$ inhibition of Cl$^-$ secretion exhibits anomalous mole fraction behavior (48), thus indicating competition between K$^+$ and NH$_4^+$. However, the HCO$_3^-$-dependent apical inhibition follows the NH$_4^+$ concentration and does not display anomalous behavior (Fig. 3). Also, significant inhibition occurs with as little as 2 mM NH$_4^+$ (72% of that at 10 mM NH$_4^+$:10 mM K$^+$) compared with >90% with mole fractions $<$0.6 with basolateral NH$_4^+$ (Fig. 3) (48). Mayol et al. (32) reported an IC$_{50}$ of ~5 mM for apical NH$_4^+$ inhibition of cAMP-stimulated current in rat distal colon. This is in close agreement with the data presented here corresponding to 5 mM K$^+$:5 mM NH$_4^+$ in Fig. 3, which is 59% of the value for 5 mM K$^+$. In rat distal colon, apical NH$_4^+$ inhibition of Cl$^-$ secretion appeared to be related to an apical membrane K$^+$ conductance (32). However, the extent to which K$^+$ secretion may have contributed to the measured current was unclear. In contrast, Cermak et al. (6) found little effect of luminal NH$_4^+$ on Cl$^-$ flux in rat colon. It is unlikely that an apical K$^+$ conductance is a contributing factor in this study,
because T84 cells do not demonstrate K⁺ secretion. Nevertheless, given the normal [NH4⁺] in the colonic lumen, little cAMP-dependent Cl⁻ secretion should occur, if crypt lumen [NH4⁺] is similar to large lumen [NH4⁺]. To our knowledge, crypt lumen [NH4⁺] has not been determined.

Carbonic anhydrase. Carbonic anhydrase has been shown to facilitate NH₃ transport across the sarcolemma in white muscle (15). In addition, inhibition of carbonic anhydrase can reduce the change in voltage response to HCO₃⁻ in corneal endothelium (19). To test if carbonic anhydrase activity was related to the HCO₃⁻-dependent NH₄⁺ inhibition of Cl⁻ secretion, we used methazolamide to inhibit apical carbonic anhydrase. Methazolamide pretreatment did not prevent apical NH₄⁺ inhibition of current (Fig. 4). Thus it is unlikely that apical carbonic anhydrase is involved in the inhibitory response.

Apical anion exchangers. A number of anion exchangers have been shown to be sensitive to NH₄⁺ as well as changes in pHᵢ (2, 8, 17, 49). To address the possible involvement of an apical anion exchanger in mediating the HCO₃⁻-dependent apical NH₄⁺ current inhibition, we used the disulfonic stilbene DIDS as an inhibitor. Preapplication of DIDS entirely prevented current inhibition (Fig. 5A). Interestingly, when DIDS was applied after apical NH₄⁺, the inhibitory effect was not reversed (Fig. 5B). Preapplication of 100 μM DNDS partially prevented current inhibition by NH₄⁺ (Fig. 5C). Similarly, NH₄⁺ inhibition of current was entirely prevented by preapplication of 25 μM niflumic acid (another commonly used blocker of anion exchange). The role of anion exchange was further implicated by the experiment in Fig. 6, where the magnitude of current inhibition increased nearly threefold under apical Cl⁻-free conditions, which are known to reverse the direction of apical Cl⁻/base exchange.

Humphreys et al. (17) showed that NH₄⁺ activates the anion exchanger AE2/SLC4A2 but does not affect the AE1/SLC4A1 isoform. AE2 is expressed in the colon; however, it appears predominantly on the basolateral side (1). At least three members of the SLC26 family of anion exchangers are localized to the apical membrane in polarized epithelia: DRA/SLC26A3 (5, 13), PAT1/SLC26A6 (22, 44), and pendrin/SLC26A4 (35, 40). Of these, DRA and PAT1 are expressed in the intestine, with DRA showing abundant expression in the colon and less expression in the small intestine (28). PAT1 displays a pattern of expression that is opposite that of DRA (44). Our results demonstrate the expression of both isoforms in T84 cells (Fig. 7). Ramirez et al. (38) did not find evidence of an apical Cl⁻/HCO₃⁻ exchanger in T84 cells; however, in their study, pHᵢ was “considerably” below normal values: a pHᵢ at which DRA would likely be inhibited (8). DRA is known to be inhibited by intracellular acidification and activated by NH₄⁺ (8), making DRA a candidate for involvement in the apical NH₄⁺ inhibition of current we have observed. The NH₄⁺ sensitivity of PAT1 has not been explored but is likely inhibited by NH₄⁺ on the basis of sequence homology to the newly cloned SLC26A9, which is inhibited by NH₄⁺ (49). The DIDS sensitivity of DRA is controversial. Chernova et al. (8) reported that human DRA expressed in Xenopus oocytes is relatively insensitive to DIDS (26% inhibition at 500 μM DIDS). Because 500 μM DIDS and 100 μM DNDS prevented the apical NH₄⁺-induced inhibition, our data might suggest the involvement of PAT1 over DRA in mediating the NH₄⁺-induced effect on current. However, the sensitivity of DRA to DIDS may depend on the environment in which it is expressed.

Although pHᵢ would be increased with apical NH₄⁺ (via apical NH₃ entry in T84 cells), it is unlikely that the change in pHᵢ is directly causing current inhibition via CFTR activity. Willumsen and Boucher (45) reported that Iₖc in nasal epithelium is increased by intracellular alkalization (luminal NH₄⁺) and reduced by intracellular acidification (serosal NH₄⁺). Prasad et al. (37) also noted a transient increase in Iₖc with apical NH₄⁺ in T84 cells, although this was at a high concentration (100 mM). In addition, NH₄⁺-induced changes in pHᵢ occur in the absence of HCO₃⁻. One possible explanation for apical NH₄⁺-induced current inhibition would be that the microenvironment near the intracellular face of the membrane is becoming acidic on activation of anion-exchange activity because of HCO₃⁻ extrusion. Indeed, HT29-C1 cells show acidic pHᵢ at the apical membrane cytoplasmic surface (30). However, this hypothesis is inconsistent with the observed increase in inhibition under apical Cl⁻-free conditions, where the driving force favors HCO₃⁻ entry, rather than exit.

Although some controversy exists, DRA and PAT1 have been reported to be electrogenic (34), with DRA having a stoichiometry of at least 2Cl⁻:HCO₃⁻ and PAT1 being the opposite with 2HCO₃⁻:Cl⁻. Thus, in a model where DRA activity is predominant, NH₄⁺ stimulation of DRA would produce an increase in positive current. This could explain the decrease in negative current we observe with apical NH₄⁺. However, under apical Cl⁻-free conditions, where DRA would be expected to run in the opposite direction (e.g., Cl⁻ out, HCO₃⁻ in), negative current would increase, rather than decrease. In the case of PAT1, which would generate a negative current, inhibition of PAT1 could produce the observed results. In this case, under apical Cl⁻-free conditions, a positive current might be generated only if there were sufficient PAT1 activity, despite the presence of NH₂⁺. It is obvious that the relative interaction of NH₄⁺ with DRA and PAT1 and the extent of an NH₄⁺ effect under conditions where exchange activity is “forced” to run in reverse are fundamental to a full understanding of the observed effect in terms of the electrogenic nature of the two exchangers. This, however, is likely a moot point, inasmuch as two recent studies have convincingly demonstrated that DRA (25) and PAT1 (9) indeed carry out electroneutral Cl⁻/HCO₃⁻ exchange. Given electroneutral exchange activity, the current changes we observe are most likely due to changes in Cl⁻ channel activity, because this is the major conductive cAMP-stimulated pathway in T84 cells.

Perhaps the most appealing aspect of these data derives from the described, but poorly understood, functional interaction between CFTR and apical anion exchangers. Inasmuch as HCO₃⁻ can be secreted via CFTR or anion exchange, the precise nature of the interaction is difficult to assess (12). DRA and PAT1 are associated physically (29) and functionally with CFTR (13, 24, 26, 28, 29). As stated above, the degree of association of each anion exchanger with CFTR when DRA and PAT1 are present would impact Cl⁻ secretion. Lee et al. (26) reported that forskolin increased the activity of anion exchange independent of Cl⁻ channel activity in T84 cells. Interestingly, they also found that the upregulation of anion-exchange activity by forskolin in pancreatic ducts required the presence of wild-type CFTR, inasmuch as no upregulation was seen in pancreatic ducts from ΔF508/ΔF508...
CFTR mice. Our earlier work indicated that \( \text{NH}_4^+ \) did not have a significant impact on apical \( \text{Cl}^- \) conductance (CFTR) in permeabilized T84 monolayers (37); however, this work was done in the absence of \( \text{HCO}_3^- \). CFTR has a reported \( \text{HCO}_3^- \)-to-\( \text{Cl}^- \) current ratio of 0.14–0.24 (18, 27). Interestingly, the conductance ratio appears to be variable depending on the intracellular ion composition and mode of activation of CFTR (39, 43, 46). Thus one possibility given the data in Fig. 1A is that \( \text{NH}_4^+ \) acting through anion exchange is affecting the \( \text{HCO}_3^- \)-to-\( \text{Cl}^- \) conductance ratio of CFTR, because apical \( \text{NH}_4^+ \) reduces current magnitude to the level observed in the absence of \( \text{HCO}_3^- \). The STAS domain of the anion exchangers has been shown to interact with the regulatory domain of CFTR, with R-domain phosphorylation enhancing this interaction (24). Colocalization of CFTR within general proximity to PAT1 and, to a lesser extent, to DRA was observed at the apical membrane in cAMP-stimulated T84 cells. Although not the focus of the present study, it will be interesting in future studies to determine the role of cAMP stimulation in the trafficking of DRA and PAT1 as well as potential cotraficking with CFTR. It is intriguing that the STAS domain and the \( \text{NH}_4^+ \) interaction site reside in the COOH-terminal tail of DRA (8). It remains to be determined what effect \( \text{NH}_4^+ \) might have on the STAS-R-domain interaction. Because of the low signal intensity associated with detection of SLC26 coimmunoprecipitation with CFTR, previous studies used heterologous expression systems in which CFTR and the anion exchangers are overexpressed. Although qualitatively our data appear to suggest that \( \text{NH}_4^+ \) may reduce the degree of association between CFTR and the exchangers (Fig. 9), we are rather hesitant to state this strongly because of the low signal intensity. Unfortunately, available DRA and PAT1 antibodies do not provide immunoprecipitation efficiency sufficient for the reciprocal coimmunoprecipitation experiment. Quantitative assessment will no doubt require overexpression of the exchanger and CFTR in experiments similar to those described by Ko et al. (24), where select flag-tagged “bits and pieces” of SLC26 family members and CFTR were used to characterize anion-exchanger STAS domain association with the CFTR R-domain.

The interaction of \( \text{NH}_4^+ \) with an anion exchanger tightly coupled to CFTR raises intriguing questions, particularly in tissues such as the colon, which are faced with relatively high and variable \( \text{NH}_4^+ \) levels. DRA and PAT1 indeed may play an important role in mediating the apical effect of \( \text{NH}_4^+ \). The exact extent and mechanism whereby \( \text{NH}_4^+ \) may influence association of DRA and/or PAT1 to CFTR will require significant additional studies.

In summary, apical \( \text{NH}_4^+ \) inhibits cAMP-stimulated \( \text{Cl}^- \) secretion in an \( \text{HCO}_3^- \)-dependent manner. 1) The nature of apical \( \text{NH}_4^+ \) inhibition is distinct from that of basolateral \( \text{NH}_4^+ \) inhibition, which is not \( \text{HCO}_3^- \) dependent. 2) The apical \( \text{NH}_4^+ \) effect follows \( \text{[NH}_4^+] \) and does not exhibit anomalous mole fraction behavior, as does the basolateral effect of \( \text{NH}_4^+ \). 3) Apical carbonic anhydrase does not appear to be involved in the \( \text{NH}_4^+ \) effect. 4) The involvement of an apical anion exchanger(s) in the apical \( \text{NH}_4^+ \) inhibitory effect is implicated, because inhibition is prevented by preapplication of DIDS, DNDS, or niflumic acid and augmented in apical \( \text{Cl}^- \)-free solution. We conclude that the \( \text{HCO}_3^- \) dependence of apical \( \text{NH}_4^+ \) inhibition of cAMP-stimulated secretion in T84 cells is due to the action of \( \text{NH}_4^+ \) on an apical anion exchanger, likely SLC26A3 (DRA) and/or SLC26A6 (PAT1).

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