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Research Article

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Ammonia Inhibits cAMP-regulated Intestinal Cl^- Transport

Asymmetric Effects of Apical and Basolateral Exposure and Implications for Epithelial Barrier Function

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Abstract

The colon, unlike most organs, is normally exposed to high concentrations of ammonia, a weak base which exerts profound and diverse biological effects on mammalian cells. The impact of ammonia on intestinal cell function is largely unknown despite its concentration of 4–70 mM in the colonic lumen. The human intestinal epithelial cell line T84 was used to model electrogenic Cl^- secretion, the transport event which hydrates mucosal surfaces and accounts for secretory diarrhea. Transepithelial transport and isotopic flux analysis indicated that physiologically-relevant concentrations of ammonia (as NH_4Cl) markedly inhibit cyclic nucleotide-regulated Cl^- secretion but not the response to the Ca^{2+} agonist carbachol. Inhibition by ammonia was 25-fold more potent with basolateral compared to apical exposure. Ion substitution indicated that the effect of NH_4Cl was not due to altered cation composition or membrane potential. The site of action of ammonia is distal to cAMP generation and is not due simply to cytoplasmic alkalization. The results support a novel role for ammonia as an inhibitory modulator of intestinal epithelial Cl^- secretion. Secretory responsiveness may be dampened in pathological conditions associated with increased mucosal permeability due to enhanced access of lumenal ammonia to the basolateral epithelial compartment. (*J. Clin. Invest.* 1995. 96:2142–2151.) Key words: secretions, intestinal • diarrhea • Na^+-K^+-$2Cl^-$ cotransporter • hydrogen ion concentration • cell membrane

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1. In this report, the terms "ammonia" and "total ammonia" are used interchangeably to indicate the sum of NH_4^+ and NH_3. When specific mechanisms of transport are discussed, the chemical formulas NH_4^+ and NH_3 will be used.

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Introduction

The weak base ammonia[1] exerts profound biological effects on mammalian cells (1). Plasma membranes are typically highly permeable to NH_3 but considerably less permeable to NH_4^+, and this characteristic accounts for the widespread experimental use of ammonium salts to manipulate intracellular pH (2). It is important to recognize, however, that the effects of ammonia on cell function may be more complex than simple alterations in cytoplasmic pH. Ammonia and other weak bases will accumulate within and raise the pH of acidic intracellular compartments, and thereby may induce cell vacuolization, impair lysosomal proteolysis, and perturb membrane recycling events (1, 3–6). Fortunately, the concentration of ammonia in extracellular fluids is low, and most cells are rarely, if ever, exposed to concentrations greater than 1 mM under physiological circumstances (1). The mammalian colon represents an interesting and important exception. Ammonia is formed in the gut lumen by bacterial action upon nitrogen-containing substances such as urea, and is a major component of colonic lumenal fluid, with concentrations in human and rat fecal dialysates reported to range between 4–70 mM depending upon dietary protein intake (7–9). Thus, the apical surface of colonocytes is normally bathed by a remarkably high concentration of ammonia. The basolateral aspect of the epithelium and the submucosa are also exposed to unusually high concentrations of ammonia, reflected by the presence of 1–2 mM ammonia in the mesenteric venous blood directly draining the colon (10). The impact of ambient ammonia on normal intestinal epithelial cell function is largely unknown.

Electrogenic Cl^- secretion by crypt epithelial cells is the fundamental means of intestinal surface hydration, and, as such, is thought to represent a primitive defense mechanism which "flushes" the mucosa in response to noxious stimuli (11). Defective regulation of Cl^- secretion accounts for the diarrheal manifestations of numerous infectious and inflammatory intestinal diseases (12, 13). In native intestine and in model intestinal epithelia such as the cryptlike human intestinal epithelial cell line T84, Cl^- secretion is regulated via cyclic nucleotide and Ca^{2+}-dependent signalling cascades (14, 15). In the present study, we examine the effect of ammonia on the Cl^- transport function of T84 cells. We find that ammonia selectively inhibits and inactivates cAMP- and cGMP- but not Ca^{2+}-regulated Cl^- secretion when applied to either the apical or basolateral epithelial surface. The inhibitory effect of ammonia is particularly profound with basolateral exposure. The data support a novel role for ammonia as an inhibitory modulator of intestinal epithelial Cl^- secretion. Pathological conditions associated with altered epithelial barrier function may result in dampened secretory responsiveness due to increased concentrations of lumenal ammonia gaining access to the basolateral compartment.

Methods

Cell culture and buffers. T84 cells obtained from American Type Culture Collection (ATCC, Rockville, MD) were grown to confluence on colla-

gen-coated permeable supports for 12- or 24-well culture dishes (Costar Corporation, Cambridge, MA) and maintained until steady-state transepithelial electrical resistance (TER)[2] was achieved. Flasks were passaged weekly, and monolayers for experiments were fed every 2–3 d. Over 500 monolayers (7–14 d after plating) composed of cells from passages 59 through 90 were used for the majority of these experiments. Additionally, some later experiments were performed utilizing lower passage cells kindly provided by Dr. Kim Barrett (University of California, San Diego). For experiments, monolayers were bathed in a Hepes–phosphate-buffered Ringer's solution (HPBR) containing 135 mM NaCl, 5 mM KCl, 3.33 mM NaH_2PO_4, 0.83 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM Hepes. Except where indicated, all ammonia-containing solutions consisted of HPBR modified by isotonic replacement of NaCl with NH_4Cl (0–135 mM). All solutions were warmed to 37°C and buffer pH was 7.4, except where indicated.

Transepithelial transport. Transepithelial potential difference (PD), TER, and short-circuit current (I_{sc}) were determined in monolayers grown on 0.33 cm^2 inserts using a dual voltage-current clamp (University of Iowa, Iowa City, IA) and apical and basolateral Ag/AgCl and calomel electrodes interfaced via "chopstick" KCl-agar bridges as reported previously (15, 16). In the T84 cell model, I_{sc} has been demonstrated repeatedly to be an accurate measure of electrogenic Cl^- secretion under a wide variety of experimental conditions (14–16). This proved to be true also for ammonia treatment of T84 cells, as determined by the isotopic flux studies described below. Transepithelial fluxes of ^{36}Cl and ^{22}Na were measured under short-circuit conditions by standard techniques using Snapwell Diffusion Chambers from Costar. Paired monolayers from the identical passage which did not vary in TER by > 15% were selected for flux measurements in apical-to-basolateral and basolateral-to-apical directions.

cAMP determinations. cAMP generation in response to forskolin was measured using ethanol extracts of cells from monolayers grown on permeable supports. A commercially available radioimmunoassay kit from Dupont NEN was used according to the manufacturer's instructions and as described (17).

Lactate dehydrogenase (LDH) release. After experimental manipulation, extracellular buffer was collected and LDH measured using a commercially available kit from Sigma Chemical Co. (St. Louis, MO) based on enzymatic oxidation of lactate and the simultaneous reduction of nicotinamide adenine dinucleotide, and an automated analyzer (COBAS; Roche Diagnostic System, Nutley, NJ). Total LDH was determined from 0.1% Triton X-100 extracts and LDH release expressed as percent of total LDH.

Ammonia determinations. Confluent monolayers were exposed to NH_4Cl buffer either apically or basolaterally under open circuit conditions, and after a 30–60 min interval, 100 μl samples were obtained from the *trans*-compartment. Ammonia was then determined using a commercially available kit from Sigma based on reductive amination of 2-oxoglutarate in the presence of glutamate dehydrogenase, measuring the decrease in absorbance at 340 nm of NADPH due to oxidation, according to the manufacturer's instructions.

Materials and statistical analysis. Radiochemicals were obtained from Dupont NEN (Boston, MA). All other chemicals were from Sigma Chemical Co. Data are reported as mean±SEM. Statistical analysis consisted of Student's *t*-test for paired or unpaired variates and two-way analysis of variance (ANOVA), where appropriate, with $P < 0.05$ considered significant.

Results

Ammonia does not affect basal TER or LDH release in unstimulated T84 monolayers. Confluent T84 monolayers bathed in HPBR displayed a basal TER of 1216±64 Ω-cm^2 and I_{sc} of 3.4±0.2 $\mu A/cm^2$ (n = 12 representative monolayers). In the absence of secretagogues, these parameters typically remain stable over at least a 2–3 h observation period (data not shown). Exposure of monolayers to ammonia did not affect basal TER (e.g., TER = 1033±79 and 1294±121 Ω-cm^2 after 30 min exposure of the apical surface to modified HPBR containing 135 mM NH_4Cl or the basolateral surface to HPBR containing 30 mM NH_4Cl, respectively, each n = 4). Because monolayer TER is a sensitive indicator of cell toxicity, such data indicate the absence of non-specific T84 cell injury due to ammonia. In further support of the absence of cell injury, LDH release was not increased by ammonia (1.5 vs. 2.2% for ammonia-treated *vs.* controls, n = 3 after a 60 min exposure to basolateral 10 mM NH_4Cl). With concentrations of apical NH_4Cl above 50 mM, the basal transepithelial PD became slightly more negative without a change in TER; this accounted for a small but significant increase in calculated I_{sc} (e.g., basal I_{sc} = 8.1±1.5 $\mu A/cm^2$ for monolayers bathed in standard HPBR on the basolateral side with modified HPBR containing 135 mM NH_4Cl apically, n = 4). The direction of the change in PD suggests that this deflection could be due to a small diffusion potential for NH_4^+.

Ammonia inhibits and inactivates cAMP-dependent Cl^- secretion in T84 cells. Cyclic AMP mediated agonists such as the adenylate cyclase activator forskolin induce a rapid and sustained increase in I_{sc} in T84 cells which has been shown by others to represent activation of net Cl^- secretion (14, 18). The peak I_{sc} generated in response to a maximal stimulatory concentration of forskolin (10 μM) was found to be profoundly reduced in monolayers bathed in apical and basolateral buffer containing 10 mM NH_4Cl (bilateral exposure, Fig. 1 *A*). Unilateral exposure of monolayers to either apical or basolateral 10 mM NH_4Cl indicated that, while apical exposure exerted a small inhibitory effect on forskolin-stimulated I_{sc}, the effect of basolateral exposure alone was nearly equivalent to bilateral exposure (Fig. 1 *B*). In control monolayers, forskolin induces a rapid fall in TER in parallel with the increase in I_{sc} which is consistent with the activation of transcellular ion conductive pathways. NH_4Cl did not prevent this forskolin-elicited decrease in TER, but did attenuate it. For example, baseline TER was 1270±80 and 1420±120 Ω-cm^2 for monolayers bathed for 30 min in control and 10 mM basolateral NH_4Cl buffer, respectively (n.s.); by the time of peak I_{sc} after forskolin stimulation, TER had fallen to 540±30 and 820±80 Ω-cm^2 in control and ammonia-treated monolayers (n = 12, $P < 0.005$). The magnitude of this attenuation of the forskolin-induced change in TER was consistent with its effect on I_{sc}, although, because the forskolin-stimulated transepithelial PD is also affected by ammonia, the change in TER does not entirely account for the effect on I_{sc} and Cl^- secretion. Ammonia was found to inhibit peak I_{sc} throughout a dose-response curve to forskolin (Fig. 1 *C*). In monolayers pre-stimulated with forskolin, subsequent exposure to apical or basolateral NH_4Cl rapidly reduced the I_{sc} to a similar extent as monolayers pre-exposed to NH_4Cl followed by forskolin stimulation (Fig. 2). The inhibitory effect of ammonia on forskolin stimulated I_{sc} was also evident in monolayers bathed in HCO_3^-/CO_2 buffer rather than Hepes-buffered solutions (Fig. 3).

The effect of NH_4Cl could not be attributed to altered cation composition of the buffer, as the forskolin-stimulated I_{sc} was not inhibited using 30 mM *N*-methyl-D-glucamine (NMDG) Cl

2. *Abbreviations used in this paper:* CFTR, cystic fibrosis transmembrane conductance regulator; HPBR, Hepes-phosphate buffered Ringer's solution; I_{sc}, short-circuit current; MTAL, medullary thick ascending limb of Henle; PD, potential difference; TER, transepithelial resistance.

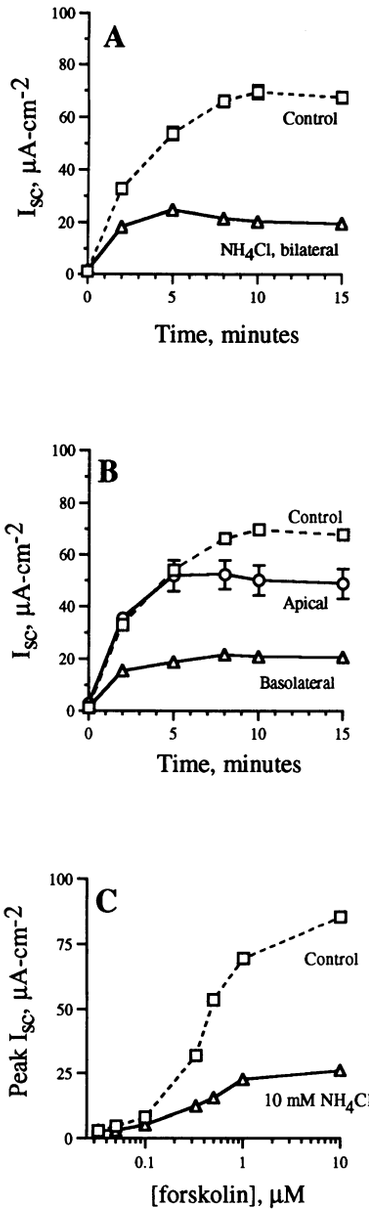


Figure 1. NH_4Cl inhibits cAMP-elicited short-circuit current (I_{sc}) in T84 cells. (*A*) Confluent monolayers grown on permeable supports were bathed in HPBR (control, *squares*) or modified HPBR containing 10 mM NH_4Cl (*triangles*) at pH 7.4 for 30 min. At time zero, monolayers were transferred to wells containing identical buffer plus forskolin (10 μM). The subsequent increase in I_{sc} reflects net electrogenic Cl^- secretion, and the peak I_{sc} achieved is markedly reduced in NH_4Cl^-treated monolayers ($n = 6-8$, $P < 0.0001$). (*B*) By a similar protocol, monolayers were exposed to HPBR (control, *squares*) or to modified HPBR containing 10 mM NH_4Cl in the apical (*circles*) or basolateral (*triangles*) compartment only (with standard HPBR in the contralateral compartment) for 30 min, followed by exposure to forskolin (10 μM). Each route of exposure inhibited the forskolin-elicited I_{sc} ($P < 0.0001$), but the inhibitory effect of basolateral exposure was more profound ($n = 6-8$). C) Dose-response relationship for activation of I_{sc} by forskolin. Monolayers were bathed in HPBR (control, *squares*) or basolateral 10 mM NH_4Cl (*triangles*) for 30 min and subsequently exposed to forskolin (0.03–10 μM). Peak forskolin-stimulated I_{sc} values obtained at 8–10 min after exposure to forskolin are shown (each point represents mean of duplicate monolayers).

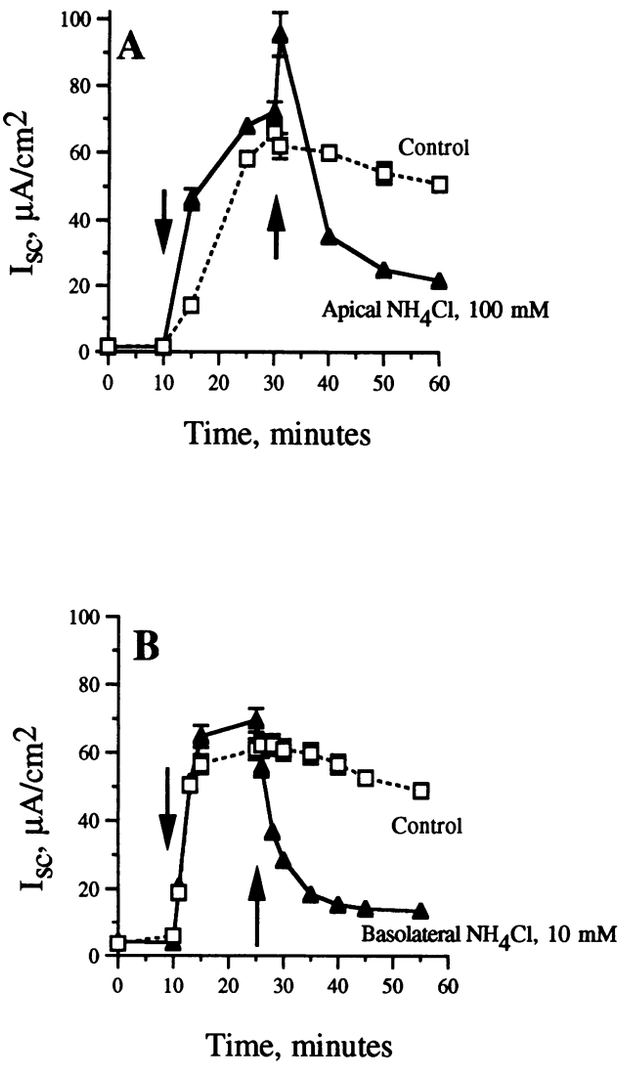


Figure 2. NH_4Cl inactivates forskolin-stimulated I_{sc} in T84 monolayers. Monolayers grown on permeable supports and bathed in HPBR were stimulated with 10 μM forskolin at the downward arrow. At the upward arrow, the apical (*A, triangles*) or basolateral (*B, triangles*) bath was replaced with modified HPBR containing 100 or 10 mM NH_4Cl, respectively. Compared with control monolayers (*squares*), the I_{sc} was markedly reduced by both apical and basolateral NH_4Cl (each $n = 6$). The transient increase in I_{sc} seen in response to apical exposure was consistently observed with 100 mM NH_4Cl but not with concentrations below 50 mM; the mechanism of this transient increase is not certain, but could be accounted for by a diffusion potential for NH_4^+.

to replace 30 mM NaCl in the basolateral buffer (peak I_{sc} = 64.8±6.0 vs. 9.3±0.7 $\mu A/cm^2$ for 30 mM NMDG vs. NH_4^+, respectively, each $n = 3$). Although, in T84 cells, the forskolin-stimulated I_{sc} can be reduced by raising basolateral $[K^+]$ (a manipulation that depolarizes the basolateral membrane), the effect of NH_4Cl is not likely to be due to changes in membrane potential. For example, the inhibitory effect of replacing basolateral NaCl with KCl was far less than the inhibitory effect of equimolar NH_4Cl (e.g., peak forskolin-stimulated I_{sc} = 59.8±3.5 vs. 31.3±0.2 for monolayers bathed basolaterally in modified HPBR in which 10 mM NaCl was replaced by 10 mM KCl vs. 10 mM NH_4Cl, respectively, each $n = 3$). Moreover, as shown below, NH_4Cl did not inhibit the secretory response to the Ca^{2+} mediated agonist carbachol, which largely relies upon activation of basolateral K^+ conductance to drive electrogenic apical Cl^- exit in T84 cells (19, 20).

Several experiments were performed to confirm that the inhibition of the cAMP-elicited I_{sc} by NH_4Cl was wholly accounted for by decreased electrogenic Cl^- secretion. First, when monolayers were pre-treated with 30 μM bumetanide (to inhibit basolateral Na^+-K^+-$2Cl^-$ cotransport and thus net Cl^- secretion), there was minimal difference in the I_{sc} response to forskolin between control and NH_4Cl-treated monolayers (peak I_{sc} = 11.0±0.8 and 8.5±0.2 $\mu A/cm^2$ in the absence and presence of 10 mM basolateral NH_4Cl, respectively, each $n = 3$). Second, the forskolin-stimulated Isc was nearly abolished in Cl^- free (gluconate) buffer and not substantially affected by the presence

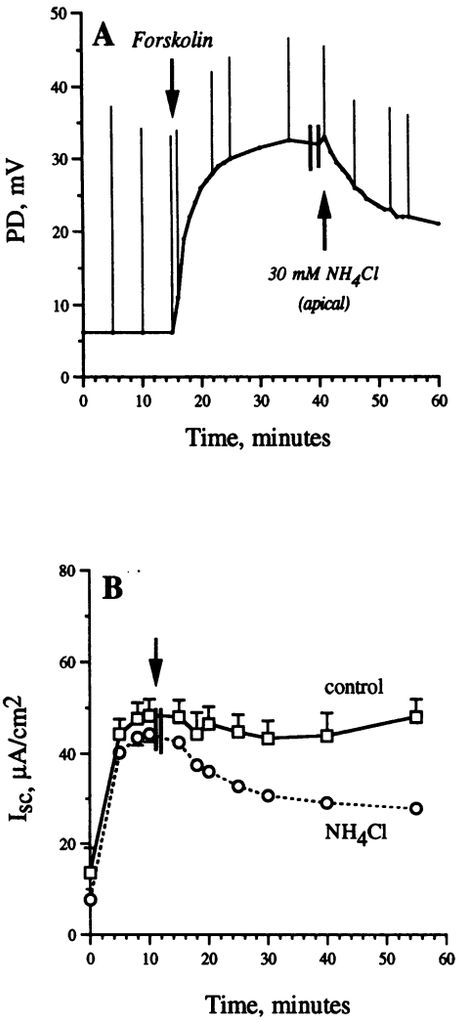


Figure 3. Inhibition of forskolin-stimulated I_{sc} by apical NH_4Cl in HCO_3^-/CO_2 buffer. Confluent T84 monolayers grown on permeable supports mounted in modified Ussing chambers bathed by HCO_3^- buffered Ringer's solution gassed with 95% O_2–5% CO_2 mixture. (*A*) Transepithelial potential difference (PD) in a representative experiment performed under open circuit conditions (expressed as absolute value of the apical-side negative PD). Vertical spikes denote deflection of PD to 25 μA external current pulse. Monolayer was treated with 10 μM forskolin at the downward arrow, evoking an increase in PD and a decrease in transepithelial resistance (TER) and thus an increase in I_{sc}. At the upward arrow, the apical bath was replaced with modified HPBR containing 30 mM NH_4Cl at pH 7.4, resulting in a rapid fall in PD and minimal change in TER, accounting for an approximately 40% decrease in I_{sc}. Note discontinuity of tracing at time of solution change as indicated by double bars. **B**) Short-circuit current (I_{sc}) and effect of apical NH_4Cl in HCO_3^-/CO_2 buffer. At time zero, monolayers were stimulated with 10 μM forskolin. At downward arrow the apical buffer was rapidly replaced with either 30 mM NH_4Cl (*open circles*) or control buffer (*open squares*). NH_4Cl is seen to inhibit I_{sc} by approximately 42% after 45 min. There is no effect of a simple solution change on I_{sc}. Data are mean±SEM for $n = 4$ paired monolayers each group ($F = 43.7$, $P < 0.05$ by ANOVA). Note discontinuity of tracing at time of solution change as indicated by double bars.

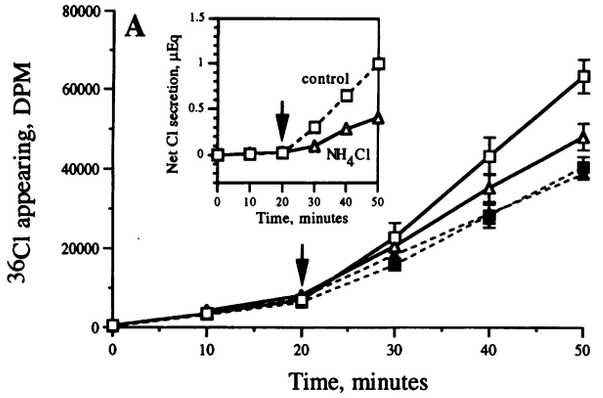
of ammonia (peak I_{sc} = 3.1±0.2 and 1.5±0.2 μA/cm² in the absence and presence of 10 mM basolateral $(NH_4)_2SO_4$, respectively, each $n = 3$). Third, treatment with apical amiloride (1 mM) did not alter the inhibitory effect of 10 mM basolateral NH_4Cl on forskolin-stimulated I_{sc} (peak I_{sc} = 19.1±2.6 and 14.3±1.3 μA/cm² in the absence and presence of amiloride, respectively, each $n = 3$). These data further exclude the possibility that rheogenic NH_4^+ secretion by T84 cells could account for the decreased forskolin-stimulated I_{sc}.

Furthermore, transepithelial fluxes of ^{36}Cl and ^{22}Na were performed to confirm that NH_4Cl inhibited net Cl^- secretion but did not affect net Na^+ transport (Fig. 4). In response to forskolin, fluxes of ^{36}Cl rapidly increased in both directions across the epithelium, with basolateral-to-apical flux exceeding apical-to-basolateral flux. Ammonia markedly attenuated the forskolin-elicited flux increase in the basolateral-to-apical (secretory) direction but had no effect on the increase in apical-to-basolateral flux. The decrease in net forskolin-stimulated flux of ^{36}Cl was proportional to the reduction in I_{sc} by NH_4Cl, and thus fully accounts for its effects. Forskolin did not increase net flux of ^{22}Na, and although the absorptive flux of Na^+ was slightly higher in the ammonia treated monolayers, this difference did not achieve statistical significance; an increase in absorptive Na^+ flux would in any event increase rather than decrease the Isc and thus could not account for the action of ammonia.

Collectively, these data indicate that, in T84 cells, NH_4Cl both inhibits activation of Cl^- secretion by forskolin in unstimulated monolayers and inactivates Cl^- secretion in forskolin-stimulated monolayers.

The effect of NH_4Cl did not appear to be due to simple cytoplasmic alkalization. The weak base imidazole has a much lower pK_a than ammonia such that at equal concentrations of total base, the concentration of the unprotonated form of imidazole exceeds that of NH_3 by approximately 50-fold. Nevertheless, 30 mM apical imidazole did not inhibit forskolin-stimulated I_{sc} (74.1±5.2 μA/cm², $n = 6$, compared to 70.1±3.2 μA/cm² for control monolayers, $n = 12$), and 30 mM basolateral imidazole exerted only a small inhibitory effect (60.3±0.7 μA/cm², $n = 6$) on peak I_{sc}. Furthermore, the weak acid butyrate (10 mM) did not affect peak forskolin-stimulated I_{sc} ($n = 6$ for basolateral, $n = 3$ for apical, data not shown), nor did it attenuate the inhibitory effect of basolateral NH_4Cl (peak I_{sc} = 19.5±2.4 vs. 19.6±2.8 μA/cm² for 10 mM basolateral NH_4Cl in the absence and presence of 10 mM butyrate, respectively, each $n = 6$, n.s.).

The inhibitory effect of ammonia on Cl^- secretion occurs at physiologically relevant concentrations and displays marked asymmetry in basolateral/apical dose dependence. In native intestine, the lumenal concentration of ammonia far exceeds that in mesenteric venous blood. Thus, the apical membrane of native intestinal epithelial cells is normally exposed to considerably higher concentrations of ammonia than the basolateral membrane. Because the initial experiments in T84 cells described above suggested that basolateral NH_4Cl exerted a much greater inhibitory effect upon cAMP-regulated Cl^- secretion than apical exposure, we examined dose-response relationships for the inhibitory effect of apical and basolateral NH_4Cl. As shown in Fig. 5, the effect of NH_4Cl on forskolin-stimulated I_{sc} in T84 cells displayed prominent asymmetry. Basolateral exposure ($IC_{50} \sim 3$ mM) was approximately 25-fold more potent than apical ($IC_{50} \sim 80$ mM). Thus, substantial inhibition of cAMP-dependent Cl^- secretion is seen to occur at physiologically-relevant concentrations of apical or basolateral ammonia. Inhibition of secretion by ammonia occurred irrespective of whether forskolin was administered on the same or opposite side as ammonia (data not shown), thus excluding the possibility that ammonia interacts with the agonist itself.



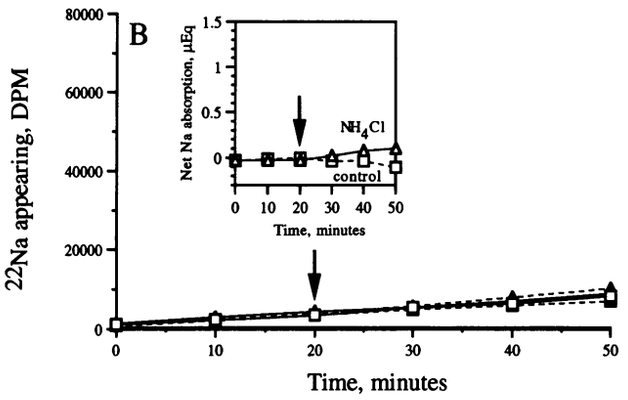


Figure 4. Effect of NH_4Cl on transepithelial fluxes of ^{36}Cl and ^{22}Na. Monolayers grown on permeable supports mounted in modified Ussing chambers and gassed with 100% O_2. Fluxes were performed under short-circuited conditions in control monolayers (*squares*) and monolayers treated with 10 mM basolateral NH_4Cl for 30 min (*triangles*). Radioisotope was added to either the apical or basolateral bath 20 min before flux determinations. At 10-min intervals, 500 μl was withdrawn from the *trans*-compartment for liquid scintillation counting and replaced by 500 μl fresh buffer. In the large panels, fluxes are shown in both the basolateral-to apical (*open symbols*) or apical-to-basolateral (*filled symbols*) directions. Forskolin (10 μM) is added at the downward arrows. The insets indicate net transepithelial flux. (*A*) ^{36}Cl fluxes increase in both directions across the epithelium in response to forskolin. Although NH_4Cl does not affect flux in the apical-to-basolateral direction, the flux in the basolateral-to-apical direction is substantially inhibited ($F = 32.4$, $P < 0.005$). Net secretory flux, shown as μEq of Cl^- in the inset, increases markedly with forskolin, and this increase in net secretion is reduced in monolayers treated with NH_4Cl. The reduction in net Cl^- secretion fully accounted for the reduction in I_{sc} in NH_4Cl^-treated monolayers. For each flux direction, data are $n = 8$ for controls and $n = 4$ for NH_4Cl^-treated monolayers. (*B*) ^{22}Na fluxes are not increased in response to forskolin, and there is no significant effect of NH_4Cl on flux in either direction across the monolayers. In the inset, net absorptive flux in μEq is shown. The absorptive Na^+ flux is trivially higher in NH_4Cl^-treated monolayers after forskolin stimulation; net Na^+ flux in this direction would increase rather than inhibit the I_{sc} and thus does not contribute to the observed ammonia inhibition of forskolin-stimulated I_{sc}. For each flux direction, data are $n = 4$ for control and $n = 3$ for NH_4Cl^-treated monolayers.

Confluent T84 monolayers were found to serve as substantial barriers to the transepithelial movement of ammonia, as evidenced by measurement of ammonia in either the apical or basolateral buffer after exposure to high concentrations of

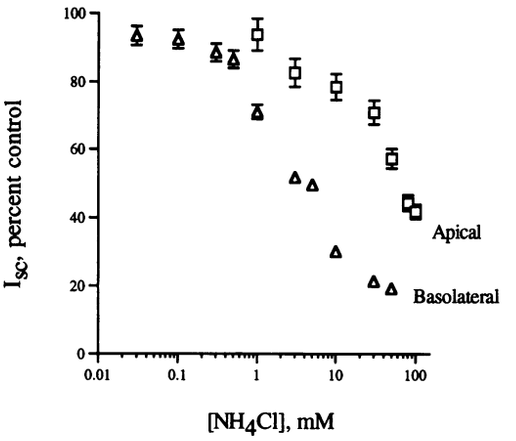


Figure 5. NH_4Cl inhibits forskolin-stimulated I_{sc} in asymmetric fashion. Dose-response data shown for monolayers exposed to 0.03–100 mM NH_4Cl apically (*squares*) or basolaterally (*triangles*) for 30 min followed by treatment with 10 μM forskolin. Peak increase in I_{sc} is shown as percent control. Basolateral exposure is seen to be approximately 25-fold more potent than apical exposure in inhibiting I_{sc}. Data are mean±SEM for $n = 4$ (apical) and $n = 4$–16 (basolateral) for each point.

ammonia in the *trans*-compartment under open circuit conditions. For example, after 30–60 min exposure to 100 mM apical NH_4Cl, the concentration in the basolateral compartment was only 0.74±0.06 mM ($n = 6$), and after a 30–60 min exposure to 30 mM basolateral NH_4Cl, the concentration in the apical compartment averaged 0.13 mM ($n = 2$).

Time dependence of inhibition of Cl^- secretion by ammonia and reversal by washout. The inhibitory effect of ammonia on forskolin-stimulated I_{sc} is rapid in onset. Peak I_{sc} was 50% reduced after 5 min pre-incubation with 10 mM NH_4Cl and 80% reduced after 30 min (Fig. 6). Incubation of monolayers in modified media containing 10 mM NH_4Cl for 24 h reduced the peak I_{sc} by nearly 90%. The dose-response relationship for ammonia inhibition of secretion was similar between 30 min and 24 hr exposure time (Fig. 6, *inset*). Near-normal I_{sc} responses to forskolin were rapidly restored within minutes of washout of NH_4Cl (Fig. 7), demonstrating both reversibility of the ammonia effect and the absence of nonspecific toxicity due to ammonia.

Ammonia inhibits cGMP-dependent secretion but does not inhibit Ca^{2+}-dependent Cl^- secretion or cAMP-Ca^{2+} synergy. In T84 cells and in native intestine, Cl^- secretion can be evoked by a variety of agonists acting via Ca^{2+}-dependent signaling cascades in addition to those acting via cyclic nucleotide dependent pathways. We therefore examined the effects of NH_4Cl on the I_{sc} evoked by the Ca^{2+}-mediated agents, carbachol. In contrast to its inhibitory effect on cAMP-dependent secretion, ammonia slightly enhanced the peak I_{sc} response to carbachol (Fig. 8 *A*). In T84 cells, cAMP and Ca^{2+}-mediated agonists are known to interact in synergistic fashion, together eliciting a peak I_{sc} that is substantially higher than the sum of their responses alone (21). As shown in Fig. 8 *B*, the synergistic I_{sc} response to carbachol was similar in ammonia-treated or control monolayers after prior stimulation with forskolin. The secretory response to the cGMP-dependent heat-stable enterotoxin of *E. coli* was inhibited by basolateral NH_4Cl in similar fashion as seen with forskolin (Fig. 8 *C*). These data indicate that the effects of ammonia on regulated Cl^- secretion are agonist-

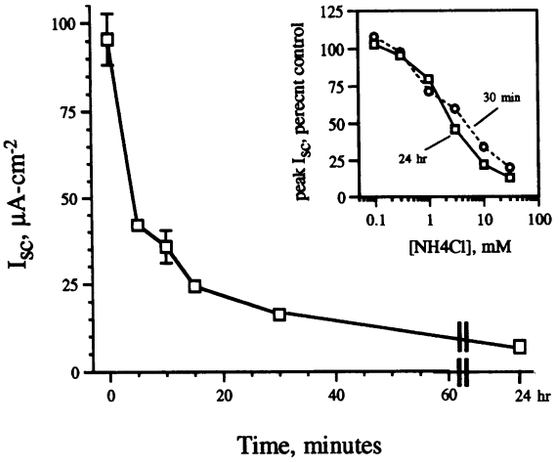


Figure 6. Effect of time of exposure to basolateral NH_4Cl on subsequent response to 10 μM forskolin. Monolayers were exposed to HPBR containing 10 mM forskolin for 0, 5, 10, 15, or 30 min and subsequently treated with forskolin. Peak I_{sc} is shown for $n = 3$ (mean±SEM). For 24 h exposure, monolayers were treated with standard culture media plus 10 mM NH_4Cl basolaterally for 24 h and then treated with 10 μM forskolin in HPBR containing 10 mM NH_4Cl ($n = 6$). Note discontinuity of *x*-axis. (*Inset*) Dose-response relationship for inhibition of peak forskolin stimulated I_{sc} for monolayers exposed to ammonia for 30 min (*circles*) or 24 h (*squares*). There is a suggestion of displacement of the curve to the left after 24 h, but this is not statistically significant. Data are mean for $n = 5$ and $n = 6$ for 30 min and 24 h, respectively. SEM was < 3% such that error bars smaller than symbol size.

specific, including agonists mediated via cyclic nucleotide dependent pathways but not Ca^{2+}-mediated agonists.

Ammonia inhibits cAMP-dependent Cl^- secretion at a site distal to cAMP. To exclude effects of ammonia on the regulated activity of adenylate cyclase, we examined the Isc evoked by

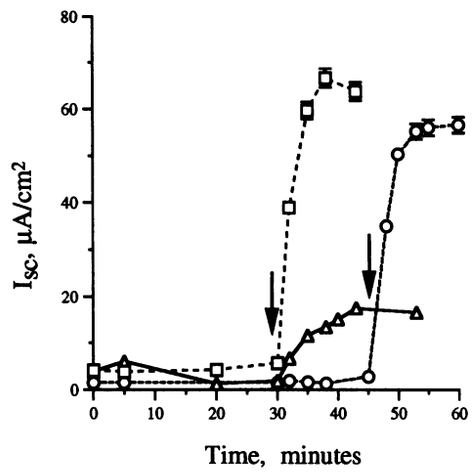


Figure 7. Washout of NH_4Cl restores I_{sc} response to forskolin. Monolayers were bathed in HPBR (*squares*) or 10 mM NH_4Cl for 30 min (*triangles*, $n = 4$ and *circles*, $n = 3$). At the first arrow, control monolayers and a subset of NH_4Cl^-treated monolayers (*triangles*) were treated with 10 μM forskolin. Substantial inhibition of the secretory response is evident. The remaining NH_4Cl^-treated monolayers (*circles*) were extensively washed with HPBR without NH_4Cl and 15 min later were treated with forskolin. The I_{sc} response to forskolin is largely restored following washout of NH_4Cl (86% of control monolayer, n.s.).

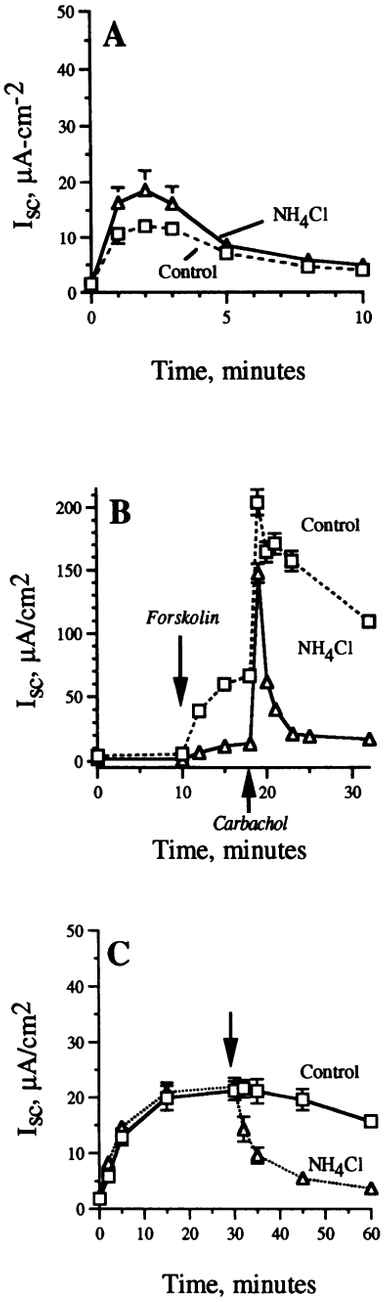


Figure 8. Inhibitory effect of NH_4Cl is agonist-selective. (*A*) Response to the Ca^{2+} mediated agonist carbachol (0.1 mM) added at time zero to monolayers bathed in HPBR (control, *squares*) or basolateral 10 mM NH_4Cl for 30 min (*triangles*). I_{sc} response is enhanced in the NH_4Cl treated monolayers (by ANOVA, $F = 4.94$, $P < 0.05$). Data are $n = 11$ for NH_4Cl and n = 16 for controls. (*B*) Preservation of synergistic response to carbachol in monolayers pre-stimulated with forskolin. Control monolayers (*squares*) and monolayers bathed with 10 mM basolateral 10 mM NH_4Cl for 30 min (*triangles*) were treated with forskolin as indicated by the downward arrow. The I_{sc} response to this cAMP agonist was inhibited, as before. At the time indicated by the upward arrow, carbachol (0.1 mM) was added. The carbachol-induced increase in secretion (ΔI_{sc}) is equivalent between control and NH_4Cl^-treated monolayers and is far greater than the ΔI_{sc} to carbachol in the absence of forskolin (*A*). Data are $n = 4$, each group. (*C*) NH_4Cl inactivates the I_{sc} response to the cGMP-mediated agonist heat stable enterotoxin (ST_a, 1 μM). Monolayers were pretreated with ST_a, applied apically, for 30 min. At the time indicated by the arrow, the basolateral buffer was changed to modified HPBR containing 10 mM NH_4Cl. The I_{sc} is rapidly inhibited, compared to control monolayers (*squares*). Data are $n = 6$, each group. In data not shown, the I_{sc} response to ST_a was also inhibited in monolayers pre-exposed to NH_4Cl.

the cell-permeant cAMP analogue 8-bromo-cAMP (0.1 mM). Because the Isc response to 8-Br-cAMP was also inhibited (by 61.6±0.8% for 30 mM basolateral NH_4Cl, $n = 6$), this suggested that ammonia was acting to inhibit secretion at a site distal to cAMP generation in the cyclic-nucleotide dependent signalling cascade. To further exclude effects at the level of adenylate cyclase, we examined cAMP generation in response to a half-maximal and supramaximal dose of forskolin in control and ammonia-treated monolayers (Fig. 9). Basal cAMP levels were 0.5±0.1 μM pmol per monolayer ($n = 3$). A half-maximal

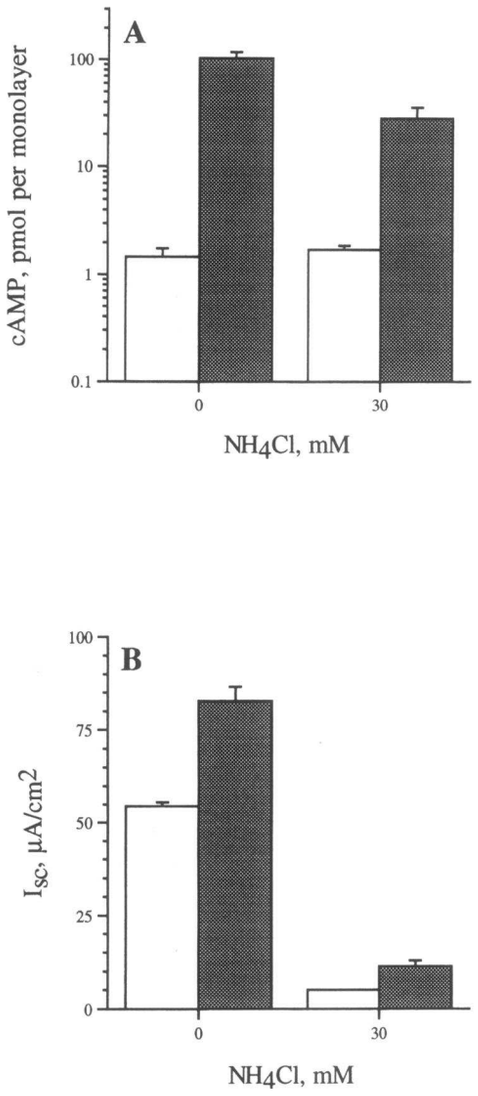


Figure 9. The inhibitory effect of NH_4Cl is not due to impaired cAMP generation. (*A*) Cellular levels of cAMP generated in response to an approximately half-maximal I_{sc} stimulatory dose of forskolin (0.5 μM, *unshaded bars*) and a maximal I_{sc} stimulatory dose of forskolin (10 μM, *shaded bars*) in monolayers bathed in control HPBR or 30 mM basolateral NH_4Cl for 30 min (each $n = 6-10$). Data are mean±SEM. There is no effect of NH_4Cl on cAMP generation in response to 0.5 μM forskolin. With 10 μM forskolin, peak cAMP generation is reduced ($P < 0.005$) in NH_4Cl^-treated monolayers, but levels are 50-fold elevated over basal values, far in excess of that required for a peak I_{sc} response (see text). (*B*) Peak I_{sc} for monolayers bathed in control HPBR or 30 mM basolateral NH_4Cl for 30 min and then exposed to either 0.5 μM (*unshaded bars*) or 10 μM (*shaded bars*) forskolin. Data are mean±SEM for $n = 4-6$ each group. For 0.5 μM forskolin, the peak I_{sc} is dramatically reduced in NH_4Cl^-treated monolayers ($P < 0.001$) despite similar cAMP content. In monolayers exposed to 30 mM NH_4Cl and 10 mM forskolin (in which cAMP levels are 50-fold elevated over basal levels), the peak I_{sc} is far lower than that achieved with the fourfold elevation of cAMP in control monolayers treated 0.5 μM forskolin ($P < 0.001$).

stimulatory dose of forskolin (0.5 μM), elicited a threefold increase in cAMP levels with no difference between control monolayers and monolayers pre-exposed to 30 mM basolateral NH_4Cl. However, the I_{sc} induced by 0.5 μM forskolin was markedly attenuated in ammonia-treated monolayers despite the fact that cAMP levels were identical to control monolayers. A supramaximal dose of forskolin (10 μM) elicited a 200-fold increase in cAMP in control monolayers and a 50-fold increase above basal levels in ammonia-treated monolayers, a difference that was statistically significant. However, a 50-fold increase in cAMP (to ~ 30 pmol per monolayer) is far in excess of that required to achieve peak cAMP-dependent secretory responses in T84 cells (17) and maximum physiological responses in other cell systems (22). As shown in Fig. 9, only a threefold increase in cAMP levels in control monolayers elicited an I_{sc} of ~ 55 $\mu A/cm^2$ in control monolayers (0.5 μM forskolin), whereas a 50-fold increase in cAMP levels in response to 10 μM forskolin could only elicit an I_{sc} of less than 12 $\mu A/cm^2$ in monolayers treated with 30 mM basolateral NH_4Cl. Thus, while ammonia may impair peak cAMP generation in response to a supramaximal dose of forskolin, this effect cannot account for the observed inhibiton of cAMP-regulated I_{sc}.

Discussion

Little is known about the potential interaction between NH_3/NH_4^+ and the regulated transport of other ions. In principle, ammonia could affect such processes directly (i.e., by competing for transport sites or altering membrane potential) or indirectly (by altering cytoplasmic or endosomal pH, proteolysis, or membrane cycling) (23). It may be particularly important to define such interactions in cells and organs which are exposed to high concentrations of ammonia under normal or pathological conditions. The colon is a rich reservoir of ammonia, and the present study demonstrates that physiologically relevant concentrations of ammonia substantially and selectively inhibit cyclic nucleotide-regulated Cl^- secretion in the T84 human intestinal epithelial cell line. In preliminary experiments using isolated rat colonic mucosal sheets, lumenal ammonia has been found to reduce the basal transepithelial PD and inhibit the forskolin-stimulated I_{sc}, also possibly due to inhibition of Cl^- secretion (M. Prasad and W. Silen, unpublished observations). Other Cl^- secretory gastrointestinal epithelia may be exposed to substantial concentrations of ammonia under certain circumstances. For example, the lumenal concentration of ammonia in the stomach of individuals infected with *Helicobacter pylori* may reach as high as 30 mM and may correlate with the extent of gastric injury (24, 25). Yanaka et al. previously observed that NH_4Cl reduces the basal gastric mucosal PD, an effect which was also attributed to reduced Cl^- transport, although the effects of ammonia on agonist-regulated Cl^- secretion was not specifically addressed in that study (25).

Epithelial cells separate biological compartments, a fundamental characteristic termed barrier function. Barrier function relies on the ability of the junctional complex and the apical plasma membrane to selectively restrict the passive permeation of molecules on the basis of size and charge (26). Apical membranes of certain barrier epithelia have been found to display extremely low permeability to NH_3, far lower than that of simple unilamellar lecithin vesicles (27–29). These include the apical membrane of epithelial cells from the medullary thick ascending limb of Henle (MTAL) and from stomach. In the case of MTAL, this unusual property appears to facilitate renal tubular transport of NH_4^+ (and thus urinary acid excretion) by preventing dissipation of transepithelial ammonia gradients (27). For gastric parietal and chief cells, the relative impermeance of their apical membranes to NH_3 (as well as NH_4^+ and

CO_2) may be relevant to the ability of the stomach to withstand the harsh lumenal environment created by its acid-peptic secretions as well as the ammonia formed by urea-splitting *H. pylori* (29). The low permeability properties of these barrier epithelia may be due to an unusual lipid composition of the exofacial leaflet of the apical membrane bilayer (28). A recent preliminary report suggests low ammonia permeability in the apical membrane of isolated perfused rabbit colonic crypts (30). Permeability of human colonocyte plasma membranes is not yet defined.

Apical and basolateral membranes exhibit marked asymmetry in the surface expression of ion channels, pumps, and transporters (31, 32). Thus, any effects of ammonia on intestinal epithelial cell function might depend upon which cell surface is exposed, the relative permeability of apical and basolateral membranes to NH_3, and whether or not transport pathways for NH_4^+ are present. Here, we observed that basolateral ammonia was at least 20-fold more potent in inhibiting Cl^- secretion than apical ammonia. Thus, in comparison to the basolateral membrane, the apical membrane of these cells serves as a more effective barrier in limiting the inhibitory action of ammonia on the T84 secretory apparatus. However, high concentrations of apical ammonia were found to exert small inhibitory effects on cAMP-evoked secretion. Thus, in contrast to MTAL and gastric parietal/chief cells (and possibly rabbit colonic crypts), T84 cell apical membranes are not entirely ammonia impermeant.

The normal colon absorbs substantial quantities of ammonia, approximately 250 mmol daily, and is the major source of the ammonia found in peripheral blood (10, 33, 34). Thus, regardless of the precise permeability properties of crypt cells alone (native or T84 model crypt cells), the epithelium as a whole clearly does not behave as an absolute permeability barrier to ammonia. There may be heterogeneity in ammonia permeability among the various cells types that constitute native epithelia. Colonic surface epithelial cells are thought to represent the site of active colonic Na^+ absorption. Cation transport pathways in the apical membrane of these cells are potential routes of permeation for lumenal NH_4^+. The exact concentration of ammonia in the basolateral compartment is unknown, but is likely to be higher than the 1–2 mM normally present in colonic mesenteric venous blood. The mechanism of ammonia absorption by the colon is not fully understood. Distal ileum is thought to possess transport pathways for the active absorption of NH_4^+ (35). Cohen et al., however, observed that in rat distal colon, NH_3 is ~ 400 times more permeant than NH_4^+, and suggested that passive absorption of NH_3 may be quantitatively more important than transport of NH_4^+ at physiological fecal pH (33). Moreover, they observed that ammonia absorption is enhanced in the presence of HCO_3^- secretion, possibly due to titration of lumenal NH_4^+ and HCO_3^- into NH_3 and CO_2. Our data indicate that inhibition of secretion by ammonia occurs in both Hepes and HCO_3^-/CO_2 buffer; future experiments will address whether HCO_3^-/CO_2 potentiates inhibition by apical ammonia.

Basolateral membranes of T84 cells and native colonocytes possess multiple permeability pathways for cations such as Na^+ and K^+, pathways which are also likely to transport NH_4^+. One such pathway, the basolateral Na^+-K^+-$2Cl^-$ cotransporter, is the transport element largely responsible for the cellular uptake and accumulation of Cl^- above its chemical equilibrium (36). In renal epithelial cells, NH_4^+ is transported efficiently via the Na^+-K^+-$2Cl^-$ cotransporter, probably by competing for the K^+ transport site (37). Seagrave and co-workers observed in dispersed acinar cells from rat submandibular gland that NH_4Cl induced an increase in intracellular Cl^- content and that this increase was largely bumetanide-inhibitable (38). Paulais and Turner reported that acute exposure to NH_4Cl in rat submandibular acini caused a transient rather than a sustained increase in pH_i (39). The rapid recovery in pH_i after the initial alkalization phase was shown to be bumetanide-sensitive. These data are consistent with ammonia entry both by non-ionic diffusion of NH_3 (resulting in initial cytoplasmic alkalization) as well as by bumetanide-sensitive Na^+-K^+(NH_4^+)-$2Cl^-$ cotransport (resulting in subsequent acidification). In preliminary experiments, we found that the pH_i of T84 cells responds to basolateral ammonia exposure in similar fashion as dispersed salivary gland cells, with initial cytoplasmic alkalization followed by bumetanide-sensitive acidification (40). Thus, the enhanced inhibitory potency of basolateral NH_4Cl on Cl^- secretion may be due to enhanced basolateral membrane permeability to total ammonia, with NH_4^+ entry via the basolateral Na^+-K^+-$2Cl^-$ cotransporter occurring in addition to non-ionic diffusion of NH_3. NH_4^+ may also permeate the basolateral membrane through the Na^+-K^+ ATPase or via conductive K^+ channels. We considered the possibility that basolateral ammonia could inhibit Cl^- secretory responses by reducing the driving force for apical Cl^- exit, because high concentrations of NH_4Cl may depolarize the basolateral cell membrane. This seems unlikely. First, 50% inhibition of secretion was evident at even low concentrations of NH_4Cl ($\leq$ 3 mM) and this effect was not altered by lowering basolateral [K^+]. Secondly, cation substitution studies indicated that inhibition of secretion by NH_4^+ was much greater than observed by raising basolateral [K^+] to a similar or higher concentration. Moreover, the Cl^- secretory response to Ca^{2+} agonists, which relies largely on activation of basolateral K^+ channels to hyperpolarize the basolateral membrane and increase the driving force for Cl^- exit, was not affected by ammonia.

Several lines of evidence suggest that the inhibitory effect of NH_4Cl on cAMP-regulated Cl^- secretion cannot be explained by simple cytoplasmic alkalization. Equimolar concentrations of the unprotonated form of weak bases have the same effect on cytosolic pH because of the high membrane permeability of unprotonated bases in general (41). However, we found that apical or basolateral imidazole did not inhibit Cl^- secretion as effectively as ammonia. Moreover, the weak acid butyrate, which acidifies the cytosol, did not itself affect Cl^- secretion, nor did it attenuate the inhibitory effect of NH_4Cl. Finally, preliminary observations indicate that the inhibition of secretion by basolateral NH_4Cl occurs at cytoplasmic pH values above baseline (during the initial alkalization phase) as well as at pH values that are at or below baseline (during the subsequent acidification phase) (40). Studies to address in greater detail the relationship of cytoplasmic pH changes to inhibition of secretion by ammonia and to explore the effect of weak bases other than imidazole are currently in progress.

The secretory response to the cGMP-mediated agonist ST_a was also inhibited by ammonia but the response to the Ca^{2+}-mediated agonist carbachol was preserved, and possibly augmented. Thus, the inhibitory effects of ammonia on regulated Cl^- transport in T84 cells were apparently selective for cyclic nucleotide but not Ca^{2+}-mediated agonists. Ammonia interferes with cAMP-dependent signalling in some cell systems. In the

slime mold Dictyostelium discoidium, for example, ammonia inhibits cellular differentiation by inhibiting activation of adenylate cyclase induced by the binding of extracellular cAMP to its cell surface receptor, an effect which may be related to the ability of ammonia to raise the pH of acidic intracellular compartments (41). Ammonia also inhibits receptor-mediated cAMP production in primary astrocyte cultures (42). Whether ammonia directly affects adenylate cyclase itself is not entirely clear, as both inhibitory and stimulatory effects have been described, depending upon assay conditions (43). We found that ammonia inhibits Cl^- secretion evoked by both forskolin and 8-bromo cAMP. Moreover, in ammonia-treated cells, forskolin increased cAMP levels several-fold above that required to generate maximal or near-maximal Cl^- secretory responses (17). Thus, it is likely that the site of ammonia's inhibitory action on regulated Cl^- secretion in T84 cells occurs at a regulatory site distal to membrane-receptor interactions and to adenylate cyclase in the cAMP signalling cascade. The synergistic interaction of cAMP and Ca^{2+}-dependent agonists was also preserved despite substantial inhibition in the secretory response to cAMP agonists alone. That is, the response to carbachol was markedly amplified to the same extent by prior exposure to forskolin irrespective of the presence or absence of ammonia. This finding implies that the site at which ammonia perturbs cAMP-regulated Cl^- secretion occurs downstream from the point at which cAMP synergistically enhances Ca^{2+}-dependent secretory responses.

The selectivity of ammonia action for cyclic nucleotide mediated agonists is strikingly similar to the effect of cytoskeletal stabilization on cAMP regulated Cl^- secretion (44). We previously reported that the F-actin stabilizer phalloidin inhibits cAMP-dependent Cl^- secretion but not Ca^{2+}-dependent responses or cAMP-Ca^{2+} synergy in T84 cells (44–47). Such data suggested that intestinal epithelial Cl^- secretion is profoundly influenced, if not directly regulated, by remodeling of actin microfilaments. Ammonia has been reported to reduce F-actin and alter degranulation and receptor recycling in leukocytes (48), and thus it is conceivable that its inhibitory effect on cAMP-regulated Cl^- secretion in T84 cells is due to perturbation of regulated actin polymerization cascades. It is possible that Na^+-K^+-$2Cl^-$ cotransport may be regulated in part by rapid recruitment of additional cotransporters or associated regulatory elements to the cell surface from cytoplasmic stores (49). Because weak bases can perturb plasma membrane cycling events (1), ammonia may interfere with the regulated insertion of new cotransporter units into the basolateral membrane. Alternatively, ammonia may enhance endocytotic retrieval of plasmalemmal cotransporters. It has recently been proposed that regulation of the cAMP-dependent apical membrane Cl^- channel (the cystic fibrosis transmembrane conductance regulator gene product, CFTR) may also involve rapid alterations in cell surface expression (50), and thus CFTR also represents a potential target of ammonia action in T84 cells.

Because physiologically relevant concentrations of ammonia were found to inhibit electrogenic Cl^- transport both in T84 cells (the present study) and in rat colon (unpublished observations), endogenous ammonia produced by the normal bacterial flora of the colon could play a novel and heretofore unsuspected role as an inhibitory regulator of intestinal salt and water secretion, perhaps serving to dampen epithelial secretory responsiveness to lumenal bacteria and their potentially diarrheogenic toxins and metabolites. Conversely, conditions associated with decreased lumenal ammonia could produce, in a sense, a colonic secretory diathesis and could account in part for antibiotic-associated diarrhea in Clostridium difficile toxin-negative patients or contribute to the severe diarrhea associated with orally administered antibiotics and laxatives used to diminish lumenal production of ammonia in cases of hepatic coma. Given the normally-high lumenal concentrations of ammonia, conditions associated with decreased intestinal barrier function and enhanced epithelial permeability (e.g., inflammation, infection, ischemia) could increase local concentrations of ammonia in the submucosal (basolateral) compartment. Our data indicate that the effects of basolateral ammonia are far more profound than apical ammonia, probably due to selective differences in membrane permeability. Thus, in pathological states, ambient ammonia could interfere with appropriate activation of epithelial secretion and exacerbate tissue injury by impairing this important mucosal defense. Because millimolar concentrations of ammonia also appear to impair synaptic transmission and neurotransmitter release (51) and affect inflammatory cell function (48), the potential impact of ammonia on integrative neurohumoral and immune regulation of epithelial transport and barrier function may be substantial and thus merits further investigation.

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References

1. Dean, R., W. Jessup, and C. Roberts. 1984. Effects of exogenous amines on mammalian cells, with particular reference to membrane flow. *Biochem. J.* 217:27–40.

2. Boron, W. 1983. Transport of H+ and of ionic weak acids and bases. *J. Membr. Biol.* 72:1–16.

3. Ohkuma, S., J. Chudzik, and B. Poole. 1986. The effects of basic substances and acidic ionophores on the digestion of exogenous and endogenous proteins in mouse peritoneal macrophages. *J. Cell Biol.* 102:959–966.

4. Ohkuma, S., and B. Poole. 1981. Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J. Cell Biol.* 90:656–664.

5. Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* 90:665–669.

6. deDuve, C., T. deBarsy, B. Poole, A. Trouet, P. Tulkens, and F. vanHoof. 1974. Lysosomotropic agents. *Biochem. Pharm.* 23:2495–2531.

7. Lin, H., and W. Visek. 1991. Colon mucosal cell damage by ammonia in rats. *J. Nutr.* 121:887–893.

8. Lin, H., and W. Visek. 1991. Large intestinal pH and ammonia in rats: dietary fat and protein interactions. *J. Nutr.* 121:832–843.

9. Lupton, J., and L. Marchant. 1989. Independent effects of fiber and protein on colonic luminal ammonia concentration. *J. Nutr.* 119:235–241.

10. Silen, W., H. Harper, D. Mawdsley, and W. Weirich. 1955. Effect of antibacterial agents on ammonia production within the intestine. *Proc. Soc. Exp. Biol. Med.* 88:138–140.

11. Halm, D. R., and R. A. Frizzell. 1990. Intestinal chloride secretion. *In* Textbook of Secretory Diarrhea. E. Lebenthal and M. E. Duffey, editors. Raven Press, New York. 47–58.

12. Field, M., M. C. Rao, and E. B. Chang. 1989. Intestinal electrolyte transport and diarrheal disease (first of two parts). *N. Engl. J. Med.* 321:800–806.

13. Field, M., M. C. Rao, and E. B. Chang. 1989. Intestinal electrolyte transport and diarrheal disease (second of two parts). *N. Engl. J. Med.* 321:879–883.

14. Dharmsathaphorn, K., J. A. McRoberts, K. G. Mandel, L. D. Tisdale, and H. Masui. 1984. A human colonic tumor cell line that maintains vectorial electrolyte transport. *Am. J. Physiol.* 246:G204–G208.

15. Dharmsathaphorn, K., and J. L. Madara. 1990. Established cell lines as model systems for electrolyte transport studies. *Methods Enzymol.* 192:354–389.

16. Madara, J. L., S. Colgan, A. Nusrat, C. Delp, and C. Parkos. 1992. A simple approach to measurement of electrical parameters of cultured epithelial

monolayers: use in assessing neutrophil–epithelial interactions. *J. Tiss. Cult. Meth.* 14:209–216.

17. Strohmeier, G. R., S. Reppert, W. Lencer, and J. Madara. 1995. The A_{2b} adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. *J. Biol. Chem.* 270:2387–2394.

18. Dharmsathaphorn, K., K. G. Mandel, H. Masui, and J. A. McRoberts. 1985. Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line: direct participation of a basolaterally located Na^+,K^+,Cl^- cotransport system. *J. Clin. Invest.* 75:462–471.

19. Dharmsathaphorn, K., and S. J. Pandol. 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. *J. Clin. Invest.* 77:348–354.

20. Venglarik, C. J., R. J. Bridges, and R. A. Frizzell. 1990. A simple assay for agonist-regulated Cl and K conductances in salt-secreting epithelial cells. *Am. J. Physiol.* 259:C358–C364.

21. Cartwright, C. A., J. A. McRoberts, K. G. Mandel, and K. Dharmsathaphorn. 1985. Synergistic action of cyclic AMP and calcium mediated chloride secretion in a colonic epithelial cell line. *J. Clin. Invest.* 76:1837–1842.

22. Francis, S., and J. Corbon. 1994. Structure and function of cyclic nucleotide-dependent protein kinases. *Annu. Rev. Physiol.* 56:237–272.

23. Good, D. 1994. Ammonium transport by the thick ascending limb of Henle's loop. *Annu. Rev. Physiol.* 56:623– 647.

24. Treibling, A. T., M. Korsten, J. Dlugosz, F. Paronetto, and C. Lieber. 1991. Severity of Helicobacter-induced gastric injury correlates with gastric juice ammonia. *Dig. Dis. Sci.* 36:1089–1096.

25. Yanaka, A., H. Muto, S. Ito, and W. Silen. 1993. Effects of ammonium ion and ammonia on function and morphology of in vitro frog gastric mucosa. *Am. J. Physiol.* 265:G277–G288.

26. Schneeberger, E., and R. Lynch. 1992. Structure, function, and regulation of cellular tight junctions. *Am. J. Physiol.* 262:L647–L661.

27. Kikeri, D., A. Sun, M. Zeidel, and S. Hebert. 1989. Cell membranes impermeable to NH_3. *Nature (Lond.).* 339:478–480.

28. Priver, N., E. Rabon, and M. Zeidel. 1993. Apical membrane of gastric parietal cell: water, proton, and nonelectrolyte permeabilities. *Biochemistry.* 32:2459–2468.

29. Waisbren, S., J. Geibel, I. Modlin, and W. Boron. 1994. Unusual permeability properties of gastric gland cells. *Nature (Lond.).* 368:332–335.

30. Singh, S. K., J. Geibel, H. Binder, and W. Boron. 1995. An apical permeability barrier to NH_3 in isolated, perfused colonic crypts. *Gastroenterol.* 108:A325.

31. Molitoris, B., and W. Nelson. 1990. Alterations in the establishment and maintenance of epithelial cell polarity as a basis for disease processes. *J. Clin. Invest.* 85:3–9.

32. Schwartz, G. J., J. Barasch, and Q. Al-Awqati. 1985. Plasticity of functional epithelial polarity. *Nature (Lond.).* 318:368–371.

33. Cohen, R., R. Stephenson, and G. Feldman. 1988. Bicarbonate secretion modulates ammonium absorption in rat distal colon in vivo. *Am. J. Physiol.* 254:F657–F667.

34. Wolpert, E., S. Phillips, and W. Summerskil. 1971. Transport of urea and ammonia production in the human colon. *Lancet.* 1387–1390.

35. Mossberg, S., and G. Ross. 1967. Ammonia movement in the small intestine: preferential transport by the ileum. *J. Clin. Invest.* 46:490–498.

36. Haas, M. 1994. The Na-K-Cl cotransporters. *Am. J. Physiol.* 267:C869–C885.

37. Tsuruoka, S., M. Takeda, K. Yoshitomi, and M. Imai. 1993. Cellular heterogeneity of ammonium ion transport across the basolateral membrane of the hamster medullary thick ascending limb of Henle's loop. *J. Clin. Invest.* 92:1881–1888.

38. Seagrave, J., S. Barker, M. Curry, and J. Martinez. 1992. Effects of NH_4Cl and dimethylamine on Cl^- fluxes in resting and stimulated rat submandibular acinar cells. *Am. J. Physiol.* 263:G558–G565.

39. Paulais, M., and R. Turner. 1992. Beta-adrenergic upregulation of the Na^+-K^+-$2Cl^-$ cotransporter in rat parotid acinar cells. *J. Clin. Invest.* 89:1142–1147.

40. Matthews, J., M. Prasad, A. Resnick, C. Awtrey, J. Smith, and B. Hrnjez. 1995. Ammonia inhibits cAMP-elicited intestinal epithelial Cl secretion: role of non-ionic diffusion and Na-K-2Cl cotransport. *Gastroenterol.* 108:A306.

41. Davies, L., M. Satre, J. Martin, and J. Gross. 1993. The target of ammonia action in Dictyostelium. *Cell.* 75:321–327.

42. Liskowsky, D., L. Norenberg, and M. Norenberg. 1986. Effect of ammonia on cyclic AMP production in primary astrocyte cultures. *Brain Research.* 386:386–388.

43. Khachatrian, L., A. Howlett, and C. Klein. 1987. Ammonium sulfate modifies adenylate cyclase and the chemotactic receptor of Dictyostelium discoideum. *J. Biol. Chem.* 262:8071–8076.

44. Shapiro, M., J. Matthews, G. Hecht, C. Delp, and J. L. Madara. 1991. Stabilization of F-actin prevents cAMP-elicited Cl^- secretion in T84 cells. *J. Clin. Invest.* 87:1903–1909.

45. Matthews, J. B., C. S. Awtrey, and J. L. Madara. 1992. Microfilament-dependent activation of $Na^+/K^+/2Cl^-$ cotransport by cAMP in intestinal epithelial monolayers. *J. Clin. Invest.* 90:1608–1613.

46. Matthews, J. B., C. S. Awtrey, R. Thompson, T. Hung, K. J. Tally, and J. L. Madara. 1993. Na/K/2Cl cotransport and Cl secretion evoked by heat-stable enterotoxin is microfilament-dependent in T84 cells. *Am. J. Physiol.* 265:G370–G378.

47. Matthews, J. B., C. S. Awtrey, K. J. Tally, and J. A. Smith. 1994. Dynamic role of microfilaments in intestinal Cl secretion. *Am. J. Surg.* 167:21–26.

48. Brunkhorst, B., and R. Niederman. 1991. Ammonium decreases human polymorphonuclear leukocyte cytoskeletal actin. *Inf. Immun.* 59:1378–1386.

49. Matthews, J. B., J. Smith, K. Tally, C. Awtrey, H. Nguyen, J. Rich, and J. Madara. 1994. Na-K-2Cl cotransport in intestinal epithelial cells: influence of Cl efflux and F-actin on regulation of cotransporter activity and bumetanide binding. *J. Biol. Chem.* 269:15703–15709.

50. Prince, L., R. Workman, and R. Marchase. 1994. Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc. Natl. Acad. Sci. USA.* 91:5192–5196.

51. Raabe, W. 1993. Effects of hyperammonemia on neuronal function: NH_4+, IPSP, and Cl^- extrusion. *Adv. Exp. Med. Biol.* 341:71–82.