Three distinct mechanisms of HCO$_3^-$ secretion in rat distal colon

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Three distinct mechanisms of HCO$_3^-$ secretion in rat distal colon have been studied thoroughly during the past three decades. Although most studies of colonic HCO$_3^-$ secretion have revealed evidence of lumen Cl$^-$ dependence, suggesting a role for apical membrane Cl$^-$/HCO$_3^-$ exchange, direct examination of HCO$_3^-$ secretion in isolated crypt from rat distal colon did not identify Cl$^-$/HCO$_3^-$ secretion but did reveal cAMP-induced, Cl$^-$-independent HCO$_3^-$ secretion. Studies were therefore initiated to determine the characteristics of HCO$_3^-$ secretion in isolated colonic mucosa to identify HCO$_3^-$ secretion in both surface and crypt cells. HCO$_3^-$ secretion was measured in rat distal colonic mucosa stripped of muscular and serosal layers by using a pH stat technique. Basal HCO$_3^-$ secretion (5.6 ± 0.03 μeq•h$^{-1}$•cm$^{-2}$) was abolished by removal of either lumen Cl$^-$ or bath HCO$_3^-$; this Cl$^-$/HCO$_3^-$ secretion was also inhibited by 100 μM DIDS (0.5 ± 0.03 μeq•h$^{-1}$•cm$^{-2}$) but not by 5-nitro-3(3-phenylpropyl-amino)benzoic acid (NPPB), a Cl$^-$ channel blocker. 8-Bromo-cAMP induced Cl$^-$-independent HCO$_3^-$ secretion (and also inhibited Cl$^-$-dependent HCO$_3^-$ secretion), which was inhibited by NPPB and by glibenclamide, a CFTR blocker, but not by DIDS. Isobutylate, a poorly metabolized short-chain fatty acid (SCFA), also induced a Cl$^-$-independent, DIDS-insensitive, saturable HCO$_3^-$ secretion that was not inhibited by NPPB. Three distinct HCO$_3^-$ secretory mechanisms were identified: 1) Cl$^-$/HCO$_3^-$ secretion associated with apical membrane Cl$^-$/HCO$_3^-$ exchange, 2) cAMP-induced secretion that was a result of an apical membrane anion channel, and 3) SCFA-dependent secretion associated with an apical membrane SCFA/HCO$_3^-$ exchange.

HCO$_3^-$ secretion has not been well characterized. Although most studies of colonic HCO$_3^-$ secretion have included an apical membrane Cl$^-$/HCO$_3^-$ exchange, direct examination of HCO$_3^-$ secretion in isolated crypt from rat distal colon did not identify Cl$^-$-independent HCO$_3^-$ secretion but did reveal cAMP-induced, Cl$^-$-independent HCO$_3^-$ secretion. Studies were therefore initiated to determine the characteristics of HCO$_3^-$ secretion in isolated colonic mucosa to identify HCO$_3^-$ secretion in both surface and crypt cells. HCO$_3^-$ secretion was measured in rat distal colonic mucosa stripped of muscular and serosal layers by using a pH stat technique. Basal HCO$_3^-$ secretion (5.6 ± 0.03 μeq•h$^{-1}$•cm$^{-2}$) was abolished by removal of either lumen Cl$^-$ or bath HCO$_3^-$; this Cl$^-$/HCO$_3^-$ secretion was also inhibited by 100 μM DIDS (0.5 ± 0.03 μeq•h$^{-1}$•cm$^{-2}$) but not by 5-nitro-3(3-phenylpropyl-amino)benzoic acid (NPPB), a Cl$^-$ channel blocker. 8-Bromo-cAMP induced Cl$^-$-independent HCO$_3^-$ secretion (and also inhibited Cl$^-$-dependent HCO$_3^-$ secretion), which was inhibited by NPPB and by glibenclamide, a CFTR blocker, but not by DIDS. Isobutylate, a poorly metabolized short-chain fatty acid (SCFA), also induced a Cl$^-$-independent, DIDS-insensitive, saturable HCO$_3^-$ secretion that was not inhibited by NPPB. Three distinct HCO$_3^-$ secretory mechanisms were identified: 1) Cl$^-$/HCO$_3^-$ secretion associated with apical membrane Cl$^-$/HCO$_3^-$ exchange, 2) cAMP-induced secretion that was a result of an apical membrane anion channel, and 3) SCFA-dependent secretion associated with an apical membrane SCFA/HCO$_3^-$ exchange.

HCO$_3^-$ secretion has often been identified (21). The driving force of fluid secretion in these models is active electrogenic Cl$^-$ secretion, which has been the subject of numerous in vitro investigations (1). In contrast, HCO$_3^-$ secretion that is induced by one or more secretagogues and second messengers has been studied less frequently and often is not observed in in vitro models. No adequate explanation has been proposed for the failure to observe HCO$_3^-$ secretion in vivo despite its presence in vivo.

Spatial separation of surface cell and crypt cell function has been a long-standing concept of colonic physiology. Absorptive processes are present in surface cells, and secretory processes are present in crypt cells (63). To study crypt cell function directly, we have developed methods for the isolation of J individual crypts that allow determination of fluid and electrolyte movement in cysts (25) and 2) apical membrane vesicles (AMV) from relatively pure crypt cell preparations that permit identification of specific transporters in crypt apical membranes (41). Using these experimental approaches, we have studied HCO$_3^-$ secretion in isolated microperfused crypts from the rat distal colon (25). In these studies, neither endogenous nor Cl$^-$-dependent HCO$_3^-$ secretion was identified, but vasoactive intestinal peptide (VIP), acetyl choline, and dibutyryl cAMP induced HCO$_3^-$ secretion that required HCO$_3^-$ in the serosal bath. This HCO$_3^-$ secretion was not altered by the removal of lumen Cl$^-$ but was inhibited by lumen 5-nitro-3-(3-phenylpropyl-amino)benzoic acid (NPPB), a nonspecific Cl$^-$ channel blocker (25). Because these studies were performed in isolated crypts, it is not known whether cAMP-induced HCO$_3^-$ secretion also occurs in surface cells and, if it does, whether the mechanism of HCO$_3^-$ secretion in surface cells is similar to or different from that in crypt cells.

Short-chain fatty acids (SCFA) are the major anions in stool but are not present in the diet (8). SCFA are produced by fermentation of nonabsorbed carbohydrate by colonic bacteria. The primary SCFA are acetate, propionate, and butyrate, with butyrate being the primary nutrient for colonocytes (43). In addition to SCFA stimulation of fluid, Na$^+$, and Cl$^-$ absorption, SCFA also induce HCO$_3^-$ secretion (62). The model of butyrate stimulation of Na$^+$ and Cl$^-$ absorption is based on butyrate uptake across the apical membrane via a butyrate/HCO$_3^-$ exchange (or via nonionic diffusion) linked to apical membrane Na$^+$/$H^+$ and Cl$^-$/butyrate exchanges (4, 11, 32, 39). Detailed information regarding the mechanism of SCFA-induced HCO$_3^-$ secretion is limited.

The present study was initiated to examine HCO$_3^-$ secretion in isolated intact colonic mucosa with the use of pH stat methods to provide quantitation of HCO$_3^-$ secretion. The pH stat method with intact colonic mucosa identifies HCO$_3^-$ se-
cretion that may originate in surface or crypt cells. If the characteristics of \( \text{HCO}_3^- \) secretion were found to differ qualitatively from those previously described in isolated crypts, we would conclude that different mechanisms of \( \text{HCO}_3^- \) secretion exist in surface and crypt cells. In contrast, if the characteristics of \( \text{HCO}_3^- \) secretion in the present studies with the use of pH stat methods were identical to those observed in isolated perfused crypts, it would be uncertain whether \( \text{HCO}_3^- \) secretion is present in surface cells as well as in crypt cells. We report the identification, characterization, and interrelationship of three distinct modes of \( \text{HCO}_3^- \) secretion: \( \text{Cl}^- \) dependent, cAMP stimulated, and SCFA dependent.

**METHODS**

Nonfasting male Sprague-Dawley rats weighing 200–250 g were used in all experiments. Colonic mucosa was obtained from the distal colon after enucleation, and \( \text{HCO}_3^- \) transport studies were performed in mucosa that was stripped of muscular and submucosal layers as described by Fromm et al. (23). Mucosa was mounted between Ussing-type Lucite chambers as previously described (6). All experiments were approved by the Yale University Institutional Animal Care and Use Committee.

**pH stat recordings.** \( \text{HCO}_3^- \) secretion was quantitated with the use of Bi-burette TIM 856 (Radiometer Analytical, Villeurbanne, France), which titrates both above and below stat pH 7.4 with a hysteresis of 0.05 and thus titrates between pH 7.35 and 7.45 (i.e., the physiological limits of \( \text{pH} \) range for bodily fluids). With the use of this technique, the lumen solution \( \text{pH} \) was continuously maintained at a constant (or stat) \( \text{pH} \) by the addition of 0.025 N \( \text{H}_2\text{SO}_4 \). Pumps were programmed to operate in real time according to the \( \text{pH} \) changes of the lumen solution, with the pumps calibrated to deliver a minimum of 0.05 \( \mu\text{l} \) at a given time. Calibration of standard to stat \( \text{pH} \) was established by adding increasing concentrations of acid. A known quantity of \( \text{H}_2\text{SO}_4 \) added to a minimally buffered solution titrated against \( \text{HCO}_3^- \) provided a linear curve. The amount of \( \text{HCO}_3^- \) measured in the lumen solution was always within the linear range of this curve. The acid used for the titration was diluted in the same ionic solution as that used in that particular experiment to obtain a final concentration of 0.025 M. Colonic tissues were always exposed to a buffered solution on the bath side, while tissues on the lumen side were exposed to a minimally buffered solution (0.1 mM HEPES buffer, pH 7.4) (46). \( \text{HCO}_3^- \) secretion is equivalent to the amount of acid required to maintain \( \text{pH} \) 7.4. All experiments were performed under voltage-clamp conditions, and \( \text{HCO}_3^- \)-free solutions were gassed with 100% \( \text{O}_2 \) while \( \text{HCO}_3^- \)-containing solutions were gassed with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \). \( \text{HCO}_3^- \) secretion was expressed as microequivalents per hour per square centimeter.

Initial studies demonstrated that immediately after the tissue was mounted, \( \text{HCO}_3^- \) secretion was present in the absence of bath \( \text{HCO}_3^- \) but rapidly fell toward 0 within 20–30 min. If bath \( \text{HCO}_3^- \) was not added, \( \text{HCO}_3^- \) secretion remained close to 0. Addition of \( \text{HCO}_3^- \) to bath solution resulted in a rapid increase in \( \text{HCO}_3^- \) secretion that remained constant for \( \approx 60 \) min (Fig. 1). All experiments were performed during this 1-h steady-state period.

In experiments in which inhibitors were added to the mucosal solution, the specific drug was added during the initial steady-state period, \( \text{pH} \) was adjusted, and tissue was allowed to equilibrate for 30 min until a steady rate of \( \text{HCO}_3^- \) secretion was again observed. In experiments in which the inhibitor was added to the serosal side, the tissue was also equilibrated for 30 min to achieve a steady state of \( \text{HCO}_3^- \) secretion. In experiments in which isobutylate was used, 25 mM isobutylate solution was first neutralized with \( N \)-methyl-d-glucamine (NMDG) before use. The composition of the several solutions used in these experiments is presented in Table 1. In brief, in \( \text{Cl}^- \) -free experiments, isethionate was used as a substitute for \( \text{Cl}^- \); in \( \text{Na}^+ \) -free experiments, NMDG was used as a \( \text{Na}^+ \) substitute. Glucose (10 mM) was added to all solutions. Only one tissue from each animal was used for a specific experiment, and only one experimental condition was used with each tissue. All experiments were repeated at least four times.

**Ion flux studies.** Unidirectional \( \text{Na}^+ \) and \( \text{Cl}^- \) fluxes were measured in the lumen and bath \( \text{HCO}_3^- \) secretion: \( \text{Cl}^- \) dependent, cAMP stimulated, and SCFA dependent.

**RESULTS**

\( \text{Cl}^- \) -dependent \( \text{HCO}_3^- \) secretion. The initial experiment was performed to determine whether there was endogenous secretion of \( \text{HCO}_3^- \), i.e., in the presence of a nominally \( \text{HCO}_3^- \)-free solution in the serosal or bath solution of rat distal colon. Under conditions in which the colonic mucosa was bathed on both the lumen and bath sides with a nominally \( \text{HCO}_3^- \)-free Ringer solution, only a minimal rate of \( \text{HCO}_3^- \) secretion was observed (0.3 ± 0.03 \( \mu\text{eq} \cdot \text{h}^{-1} \cdot \text{cm}^{-2} \)) in the presence of lumen \( \text{Cl}^- \).
The addition of HCO$_3^-$ to the bath solution resulted in a substantial rate of HCO$_3^-$ secretion (5.7 ± 0.04 μeq h$^{-1}$ cm$^{-2}$), indicating that basal HCO$_3^-$ secretion required bath HCO$_3^-$ (Fig. 2). Because the methodology of these pH stat studies requires an unbuffered or minimally buffered lumen solution (i.e., one that is nominally HCO$_3^-$-free), experiments could not be performed to assess whether the presence of HCO$_3^-$ in the lumen solution modified HCO$_3^-$ secretion. To establish that the observed rate of HCO$_3^-$ secretion was not masking the actual rate of basal secretion as a result of its partial neutralization by simultaneous proton secretion, e.g., K$^+$/H$^+$ exchange, experiments were performed in which potential proton secretion into the lumen solution was inhibited. Table 2 demonstrates that in the presence of 1 mM orthovanadate, an inhibitor of P-type ATPases, HCO$_3^-$ secretion was significantly increased by ~7%, suggesting that HCO$_3^-$ secretion was only minimally reduced by parallel K$^+$/H$^+$ exchanged H$^+$ secretion (i.e., H$^+$/K$^+$-ATPase). HCO$_3^-$ secretion was also measured in experiments in which apical membrane Na$^+$/H$^+$ exchange was inhibited by the removal of lumen Na$^+$, by the addition of 1 mM amiloride to the lumen solution, and by both the removal of lumen Na$^+$ and the addition of amiloride. Inhibition of Na$^+$/H$^+$ exchange in each of these three experimental conditions resulted in a minimal though significant decrease in the rate of HCO$_3^-$ secretion (Table 2). As a result, the observed HCO$_3^-$ secretion represented net HCO$_3^-$ secretion.

To determine whether basal HCO$_3^-$ secretion was Cl$^-$ dependent, we measured HCO$_3^-$ secretion in the absence of lumen Cl$^-$. As shown in Fig. 3, the absence of Cl$^-$ from the lumen solution resulted in almost complete elimination of basal HCO$_3^-$ secretion (0.6 ± 0.02 μeq h$^{-1}$ cm$^{-2}$), indicating that basal HCO$_3^-$ secretion was lumen Cl$^-$ dependent. Thus basal HCO$_3^-$ secretion is completely dependent on lumen Cl$^-$ and as a result is referred to as Cl$^-$-dependent HCO$_3^-$ secretion.

To establish whether Cl$^-$-dependent HCO$_3^-$ secretion involves an apical membrane Cl$^-$/HCO$_3^-$ exchange, we performed experiments with an anion exchange inhibitor, DIDS (100 μM). DIDS, which at 100 μM concentration is an inhibitor of Cl$^-$/anion exchanges (40, 57), completely inhibited HCO$_3^-$ secretion (0.5 ± 0.04 μeq h$^{-1}$ cm$^{-2}$), which is consistent with a Cl$^-$/HCO$_3^-$ exchange being closely associated with HCO$_3^-$ movement across the apical membrane (Fig. 3). Experiments were also performed to assess the effect of acetazolamide, a carbonic anhydrase (CA) inhibitor, on Cl$^-$-dependent HCO$_3^-$ secretion. Acetazolamide (100 μM) substantially reduced Cl$^-$-dependent HCO$_3^-$ secretion (Fig. 3).

### Table 1. Composition of several solutions

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Bath Ringer</th>
<th>HCO$_3^-$ free Ringer</th>
<th>Lumen Cl$^-$/HCO$_3^-$ containing</th>
<th>Cl$^-$ free</th>
<th>SCFA containing</th>
<th>SCFA containing Cl$^-$ free</th>
<th>Na$^+$ free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>140</td>
<td>119.8</td>
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<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>HPO$_4^{2-}$</td>
<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
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<td>2.4</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td>H$_2$PO$_4$</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>25</td>
<td>25</td>
<td>140</td>
<td>115</td>
<td>25</td>
<td>140</td>
<td>140</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Cyclamide</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
<td>5.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Isethionate</td>
<td>25</td>
<td>25</td>
<td>140</td>
<td>115</td>
<td>25</td>
<td>140</td>
<td>140</td>
</tr>
</tbody>
</table>

Compositions of constituents of all solutions are given in mM; pH of all Cl$^-$/HCO$_3^-$ containing solutions was adjusted to 7.4 with HCl, while that of Cl$^-$-free solutions was adjusted with H$_2$SO$_4$. HCO$_3^-$ containing solutions were gassed with 95% O$_2$-5% CO$_2$; HCO$_3^-$-free solutions were gassed with 100% O$_2$. Both lumen and bath solutions also contained (in mM) 10 glucose, 5.2 K$^+$, 1.2 Ca$^{2+}$, and 1.2 Mg$^{2+}$. All lumen solutions were HCO$_3^-$ free and contained 0.1 mM HEPES (pH 7.4). SCFA, short-chain fatty acid; NMDG, N-methyl-D-glucamine.

### Table 2. Effect of apical membrane Na$^+$/H$^+$ exchange and H$^+$/K$^+$-ATPase inhibition on Cl$^-$-dependent HCO$_3^-$ secretion

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Tissues</th>
<th>HCO$_3^-$ Secretion, μeq h$^{-1}$ cm$^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>5.7±0.04</td>
</tr>
<tr>
<td>Na$^+$ free</td>
<td>6</td>
<td>5.4±0.05*</td>
</tr>
<tr>
<td>Amiloride (1 mM)</td>
<td>6</td>
<td>5.4±0.05*</td>
</tr>
<tr>
<td>Na$^+$ free + amiloride (1 mM)</td>
<td>6</td>
<td>5.4±0.05*</td>
</tr>
<tr>
<td>Orthovanadate (1 mM)</td>
<td>5</td>
<td>6.1±0.06*</td>
</tr>
</tbody>
</table>

Values are means ± SE. All substitutions and additions were made to the lumen solution. Na$^+$ was replaced by N-methyl-D-glucamine. *P < 0.02 compared with control; †P < 0.001 compared with control.
NPPB (100 μM) did not alter Cl⁻-dependent HCO₃⁻ secretion (Fig. 3). Because NPPB inhibits Cl⁻ (as well as other anion) channel function, but not Cl⁻/anion exchanges, the failure of NPPB to alter Cl⁻-dependent HCO₃⁻ secretion is consistent with the close association of an apical membrane Cl⁻/HCO₃⁻ exchange process with Cl⁻-dependent HCO₃⁻ secretion that is not a result of the coupling of a Cl⁻/HCO₃⁻ exchange with an apical membrane Cl⁻ channel.

The observation that the removal of lumen Na⁺ or the addition of amiloride (Table 2) did not increase the rate of Cl⁻-dependent HCO₃⁻ secretion suggests that in the conditions required to perform these pH stat studies (i.e., the presence of a bath-to-lumen HCO₃⁻ gradient), apical membrane Na⁺/H⁺ exchange is either absent or not coupled to Cl⁻/HCO₃⁻ exchange. To establish Na⁺/H⁺ exchange function in the presence of a bath-to-lumen HCO₃⁻ gradient, we performed Na⁺ and Cl⁻ isotopic flux studies in the presence of HCO₃⁻-containing Ringer solution in the bath side and HCO₃⁻-free, minimally buffered solution in the lumen side. HCO₃⁻-containing solution was bubbled with CO₂, and HCO₃⁻-free solution was bubbled with O₂. Table 3 shows data demonstrating the absence of net Na⁺ absorption (−1.0 ± 0.4 μeq·h⁻¹·cm⁻²) and net Cl⁻ absorption (5.1 ± 0.2 μeq·h⁻¹·cm⁻²). Of interest is that the rate of Cl⁻-dependent HCO₃⁻ secretion is approximately equal to that of net Cl⁻ absorption and is consistent with the presence of a Cl⁻/HCO₃⁻ exchange.

cAMP-stimulated HCO₃⁻ secretion. Increases in mucosal cAMP have multiple effects on colonic ion transport, including stimulation of HCO₃⁻ secretion in experiments with isolated crypts (25) and downregulation of apical membrane Cl⁻/HCO₃⁻ exchange (3). As a result, it was possible that cAMP might stimulate HCO₃⁻ secretion and/or inhibit Cl⁻-dependent HCO₃⁻ secretion in the present studies with isolated intact colonic mucosa that included both surface and crypt cells. Therefore, the effect of 8-bromo-cAMP on HCO₃⁻ secretion was characterized. As shown in Fig. 5, in the absence of lumen Cl⁻, HCO₃⁻ secretion was minimal, but the addition of 0.5 mM 8-bromo-cAMP resulted in a marked increase in HCO₃⁻ secretion. To determine whether cAMP-stimulated HCO₃⁻ secretion required bath HCO₃⁻, as was demonstrated for Cl⁻-dependent HCO₃⁻ secretion (Fig. 2), the rate of HCO₃⁻ secretion in the presence of 8-bromo-cAMP was established in the absence of bath HCO₃⁻. Figure 5 demonstrates that cAMP-stimulated HCO₃⁻ secretion was markedly inhibited by the removal of bath HCO₃⁻.

To assess whether cAMP-stimulated HCO₃⁻ secretion was associated with an apical membrane anion channel, we also examined the effect of NPPB, a nonspecific Cl⁻ channel blocker. The effect of NPPB on Cl⁻-dependent HCO₃⁻ secretion was also determined.
blocker. Figure 6 demonstrates that HCO$_3^-$ secretion in the presence of 8-bromo-cAMP was completely inhibited by 100 µM NPPB (0.5 ± 0.03 µeq·h$^{-1}$·cm$^{-2}$), indicating that cAMP mediates HCO$_3^-$ secretion via apical membrane anion channels. Because NPPB at 100 µM concentration is a nonspecific Cl$^-$ channel blocker (24, 57), studies were also performed with 300 µM glibenclamide, a concentration at which glibenclamide is a relatively specific inhibitor of CFTR, and with 1 mM DIDS, a concentration at which DIDS inhibits the outwardly directed Cl$^-$ conductance. Figure 6 demonstrates that cAMP-stimulated HCO$_3^-$ secretion was completely inhibited by 300 µM glibenclamide but was not altered by 1 mM DIDS (4.9 ± 0.08 µeq·h$^{-1}$·cm$^{-2}$), suggesting a role for CFTR in cAMP-induced HCO$_3^-$ secretion. To exclude an effect of glibenclamide on apical membrane K$^+$ channels, the addition of 5 mM barium to the luminal solution was evaluated, but it did not affect HCO$_3^-$ secretion (data not shown).

Parallel studies performed in the presence of lumen Cl$^-$ provided an opportunity to determine whether cAMP also modifies Cl$^-$-dependent HCO$_3^-$ secretion. cAMP-stimulated HCO$_3^-$ secretion was not altered by the removal of lumen Cl$^-$ (4.9 ± 0.09 vs. 5.1 ± 0.01 µeq·h$^{-1}$·cm$^{-2}$), indicating that cAMP-stimulated HCO$_3^-$ secretion was Cl$^-$ independent and suggesting that cAMP inhibited Cl$^-$-dependent HCO$_3^-$ secretion. Additional experiments were performed with anion transport inhibitors to confirm that cAMP modified Cl$^-$-dependent HCO$_3^-$ secretion. Figure 7 demonstrates that NPPB inhibited HCO$_3^-$ secretion to a rate identical to that observed in the absence of lumen Cl$^-$ (0.5 ± 0.03 vs. 0.4 ± 0.04 µeq·h$^{-1}$·cm$^{-2}$). In contrast, 100 µM DIDS did not inhibit cAMP-stimulated HCO$_3^-$ secretion. Although acetazolamide markedly inhibited Cl$^-$-dependent HCO$_3^-$ secretion (Fig. 3), cAMP-induced HCO$_3^-$ secretion in the presence of lumen Cl$^-$ was only minimally reduced by acetazolamide (Fig. 7). These results provide compelling evidence that Cl$^-$-dependent HCO$_3^-$ secretion is absent in the presence of cAMP. Thus cAMP both induced Cl$^-$-independent HCO$_3^-$ secretion via an anion channel and inhibited Cl$^-$-dependent HCO$_3^-$ secretion.

SCFA-dependent HCO$_3^-$ secretion. In vivo studies of HCO$_3^-$ secretion previously demonstrated that SCFAs increase HCO$_3^-$ secretion but did not establish the mechanism of its effects (62). To assess the effect of the transport of a SCFA rather than its metabolism, we performed these SCFA studies with isobutyrate, a poorly metabolized SCFA. In another series of studies, 25 mM isobutyrate was added to a luminal Cl$^-$-free solution, and both the rate and the characteristics of HCO$_3^-$ secretion were determined. The addition of 25 mM isobutyrate significantly enhanced HCO$_3^-$ secretion to 5.7 ± 0.05 µeq·h$^{-1}$·cm$^{-2}$ (Table 4), a rate that is identical to that of Cl$^-$-dependent HCO$_3^-$ secretion (Fig. 3). Isobutyrate-dependent HCO$_3^-$ secre-
isobutyrate and lumen Cl

...we performed kinetic experiments. Figure 8 demonstrates that

and acetazolamide inhibited Cl

in the presence of both lumen isobutyrate and lumen Cl

increasing concentrations of isobutyrate in the absence of

...HCO$_3^-$ secretion was substantially reduced by the removal of bath HCO$_3^-$ (0.5 ± 0.03 vs. 5.6 ± 0.03 μeq·h$^{-1}$·cm$^{-2}$), and this rate was similar to the rate of Cl$^-$-dependent HCO$_3^-$ secretion in the absence of bath HCO$_3^-$.

...lumen Cl$^-$ represented the same or different HCO$_3^-$ secretory processes. HCO$_3^-$ secretion in the presence of both lumen isobutyrate and lumen Cl$^-$ was no greater than the rate of HCO$_3^-$ secretion observed in the presence of either lumen isobutyrate or lumen Cl$^-$ alone. To establish whether isobutyrate inhibited Cl$^-$-dependent HCO$_3^-$ secretion or whether isobutyrate-dependent HCO$_3^-$ secretion was not manifest in the presence of Cl$^-$, we compared the effect of anion transport inhibitors on HCO$_3^-$ secretion in the presence of both lumen isobutyrate and lumen Cl$^-$ with that observed in the presence of lumen Cl$^-$ alone. Although DIDS and acetazolamide inhibited Cl$^-$-dependent HCO$_3^-$ secretion (Fig. 3 and Table 4), HCO$_3^-$ secretion in the presence of both isobutyrate and lumen Cl$^-$ was not substantially altered by either DIDS or acetazolamide. To exclude the possibility that isobutyrate induced HCO$_3^-$ secretion that was mediated by anion channels, we also examined the effect of NPPB. NPPB (100 μM) did not affect HCO$_3^-$ secretion in the presence of isobutyrate and lumen Cl$^-$.

These results establish that in the presence of both isobutyrate and Cl$^-$, HCO$_3^-$ secretion is insensitive to DIDS, acetazolamide, and NPPB, and thus isobutyrate both stimulated Cl$^-$-independent HCO$_3^-$ secretion and inhibited Cl$^-$-dependent HCO$_3^-$ secretion.

To characterize further SCFA-dependent HCO$_3^-$ secretion, we performed kinetic experiments. Figure 8 demonstrates that increasing concentrations of isobutyrate in the absence of lumen Cl$^-$ resulted in an enhanced rate of HCO$_3^-$ secretion with evidence of saturation kinetics with a kinetic constant $K_m$ for isobutyrate of 2.9 ± 0.14 mM and a $V_{max}$ of 5.9 ± 0.08 μeq·h$^{-1}$·cm$^{-2}$. These results permit the conclusion that isobutyrate-dependent HCO$_3^-$ secretion is mediated by an apical membrane NPPB-insensitive, DIDS-insensitive, Cl$^-$-independent, carrier-mediated transport mechanism. Thus isobutyrate-

dependent HCO$_3^-$ secretion is likely secondary to apical membrane SCFA/HCO$_3^-$ exchange.

Because the data presented in Table 4 indicate that Cl$^-$-dependent HCO$_3^-$ secretion was inhibited by isobutyrate, a final series of experiments were designed to assess the effect of isobutyrate on cAMP-stimulated HCO$_3^-$ secretion. Figure 6 shows that in the absence of isobutyrate, cAMP-induced HCO$_3^-$ secretion was NPPB sensitive. When isobutyrate was present together with cAMP, HCO$_3^-$ secretion was not altered by NPPB (5.7 ± 0.05 vs. 5.7 ± 0.07 μeq·h$^{-1}$·cm$^{-2}$). Because these experiments were performed in the absence of lumen Cl$^-$, HCO$_3^-$ secretion was Cl$^-$ independent and NPPB insensitive. These characteristics are those of SCFA-dependent HCO$_3^-$ secretion and not those of cAMP-stimulated HCO$_3^-$ secretion, suggesting that isobutyrate also inhibited cAMP-stimulated HCO$_3^-$ secretion.

DISCUSSION

HCO$_3^-$ secretion is an important component of normal colonic fluid and electrolyte movement and is a major fraction of the fluid secreted in several diarrheal diseases (21). Despite the essential role of HCO$_3^-$ secretion both in health and in diarrhea, the mechanism of HCO$_3^-$ movement in general and HCO$_3^-$ secretion in particular in both the small and large intestines has been studied relatively infrequently, especially compared with...
the extensive experimental studies of Cl− secretion. Several factors have probably contributed to this relative understudy of colonic HCO3− transport. 1) There are technical difficulties involved in the determination of HCO3−, e.g., the inability to use isotopes to measure HCO3− flux at pH 7.4. 2) HCO3− secretion has often been studied in in vitro studies by determining so-called residual flux (Jr), i.e., the portion of the short-circuit current that is not accounted for by measured Na+, Cl−, and K+ fluxes (18). The use of Jr to determine HCO3− movement is indirect and is subject to significant experimental variability. 3) HCO3− secretion, when estimated from Jr, can be highly variable in vitro experiments and is often substantially less than that observed in vivo studies. An adequate explanation for the lower rates of HCO3− secretion observed in in vitro studies compared with those observed in vivo studies has not been identified.

Several investigators have used an alternate experimental approach, pH stat, to determine HCO3− movement in vitro in several intestinal epithelia (13, 19, 20, 26, 33, 45, 46, 48, 52). In this method, low concentrations of acid are added to maintain the mucosal solution at a fixed pH (i.e., 7.4). HCO3− secretion is assumed to be equivalent to the amount of acid added to maintain the designated stat pH. This general approach has been used to determine HCO3− movement in gastric, duodenal, ileal, and colonic mucosa (13, 19, 20, 26, 33, 45, 46, 48, 52, 56). We adapted this methodology to study HCO3− secretion in the rat distal colon to complement prior studies of HCO3− secretion in isolated colonic crypt glands (25). HCO3− secretion determined with this approach could theoretically be underestimated if there were also simultaneous proton secretion. In the present experiments, proton secretion resulted in <10% underestimation of HCO3− secretion, as inhibition of H+·K+·ATPase resulted in only a minimal but significant increase in HCO3− secretion (Table 2).

The model of NaCl absorption in the rat distal colon that has been developed during the past 20 years is the presence in the apical membrane of parallel ion exchangers, Na+/H+ exchange and Cl−/HCO3− exchange, coupled with intracellular pH (pHi) (7). The relationship between Cl−/HCO3− exchange and Na+/H+ exchange in the rat distal colon is complex. Ion fluxes performed under voltage-clamp conditions in the absence of ion gradients have generally revealed approximately equal rates of net Na+ and net Cl− absorption (3, 4, 7), and inhibition of Na+/H+ exchange by 1 mM amiloride decreases net Cl− absorption (3, 35). Coupling of these two ion exchangers has been demonstrated in studies with brush-border vesicles prepared from rabbit ileum as well as in luminal perfusion experiments in human ileum and rat colon (30, 37, 60). Nonetheless, both exchangers are functionally independent of Cl− and Na+, because ion uptake studies with the use of AMV prepared from rat distal colon have demonstrated Na+/H+ exchange and Cl−/HCO3− exchange activities that are independent of Cl− and Na+, respectively (40, 42). Furthermore, in the proximal small intestine, HCO3− absorption is a result of an apical membrane Na+/H+ exchange that is present in the absence of an anion exchanger (61), while in the colon, when the rate of Cl−/HCO3− exchange is greater than that of Na+/H+ exchange, both Cl−-dependent HCO3− secretion and net NaCl absorption have been observed (17).

The present studies demonstrate Cl−-dependent HCO3− secretion with the use of pH stat methodology that requires that either no buffer or a very low buffering capacity be present in the lumen solution. Table 2 demonstrates that the removal of lumen Na+ and/or the addition of 1 mM amiloride to the lumen solution did not increase HCO3− secretion. The failure to observe an enhancement in HCO3− secretion after inhibition of apical membrane Na+/H+ exchange may be a result of the fact that in the presence of bath-to-lumen HCO3− gradient, an apical membrane Na+/H+ exchange is absent. To address this possibility, we measured Na+ and Cl− isotopic flux under the identical experimental conditions that were used in the HCO3− secretion studies, i.e., the presence of a bath-to-lumen HCO3− gradient (Table 3). These studies demonstrated an absence of net Na+ absorption and a rate of net Cl− absorption that was approximately equal to that of HCO3− secretion determined in the pH stat studies (5.1 ± 0.2 vs. 5.7 ± 0.04 μeq·h−1·cm−2). The rate of net Cl− absorption in the present experiments is approximately equal to that observed in several prior studies of Cl− movement across the rat distal colon (3, 4, 7, 58). These experiments provide compelling evidence that the presence of a HCO3− gradient results in inhibition of net Na+ absorption and explains the failure to observe an increase in Cl−-dependent HCO3− secretion after experimental maneuvers to inhibit apical membrane Na+/H+ exchange (Table 3). One possible explanation for this observation is that HCO3− movement from bath to cell (in the absence of adequate luminal buffering) raises pHi, thus reducing the driving force for Na+·H+ exchange. Future studies of pHi in surface cells of rat distal colon are required to explore these relationships.

Investigation of colonic HCO3− secretion has been undertaken in several species, including humans, during the past 50 years (14, 15, 17, 19, 20, 25, 38, 52). Most of these in vivo studies have used a luminal perfusion methodology and have largely made similar observations: HCO3− secretion appears to be an active transport process as luminal concentrations of HCO3− increase above plasma concentrations, and the electrical potential difference is lumen negative (14, 15, 17, 38). In general, HCO3− secretion has been established as lumen Cl−-dependent and is attributed to an apical membrane Cl−/HCO3− exchange. Evidence in support of an apical membrane Cl−/HCO3− exchange as the mechanism of colonic HCO3− secretion has also been provided by observations in congenital chloridorrhea, a diarrheal disorder associated with high stool Cl− concentrations (27, 36). Luminal perfusion studies in these patients established the absence of Cl−-dependent HCO3− exchange in the ileum and the colon (but not in the jejunum) (2, 59), and mutations in the DRA (downregulated in adenoma) gene have been identified as the cause of congenital chloridorrhea (34, 36). Furthermore, DRA has been identified as an anion transporter (47). Studies in normal colon have also established the presence of HCO3−-dependent Cl− absorption (4). Although Cl−/HCO3− exchange is coupled to Na+/H+ exchange, resulting in electroneutral NaCl absorption, overall net HCO3− secretion is observed when the rate of Cl−/HCO3− exchange is greater than that of Na+/H+ exchange (17).

Several specific models of HCO3− secretion have been identified in different epithelia (13, 17, 28, 29, 49, 53, 55). The source of HCO3− can be either endogenous synthesis from CO2 and H2O by enzymatic action of CA or bath HCO3− with transport across the basolateral membrane. In human and bovine airway cells, HCO3− secretion is linked to endogenous CO2 production with HCO3− movement across the apical mem-
brane and proton movement across the basolateral membrane via Na/H exchange (49). HCO₃⁻ secretion that is linked to endogenous CO₂ production has been reduced by inhibition of CA (38). In addition to Cl⁻/HCO₃⁻ exchange, additional mechanisms proposed for the movement of HCO₃⁻ across the intestinal apical membrane include 1) CFTR with a finite HCO₃⁻ conductance, 2) a non-CFTR HCO₃⁻ channel, and 3) CFTR coupled to Cl⁻/HCO₃⁻ exchange (13, 26, 29, 53, 54).

Recent studies have suggested a close association between CA-II and apical membrane Cl⁻/anion exchangers (i.e., AE1, DRA) (50, 51) and may explain a portion of acetazolamide inhibition of HCO₃⁻ secretion. Although acetazolamide does not have an effect on Cl⁻/HCO₃⁻ exchange in membrane vesicle systems (32), recent studies have indicated that acetazolamide inhibits Cl⁻/HCO₃⁻ exchange to a greater degree than that which can be attributed to CA inhibition, suggesting that CA and apical membrane Cl⁻/HCO₃⁻ exchange are linked (50, 51). Although Cl⁻-dependent HCO₃⁻ secretion was inhibited by acetazolamide (Fig. 3), acetazolamide did not inhibit or only minimally inhibited cAMP-induced and SCFA-dependent HCO₃⁻ secretion, respectively (Fig. 7 and Table 4), which indicates that apical membrane anion channel and SCFA/HCO₃⁻ exchange are not coupled to CA-II. Although the molecular identity of SCFA/HCO₃⁻ exchange is not known, these results permit the speculation that neither AE1 nor DRA encodes the SCFA/HCO₃⁻ exchange protein.

The paradigm of colonic ion function is presently a separation of crypt cell and surface cell function (63). Absorptive processes are largely confined to surface cells, while secretory processes are localized in crypt cells. HCO₃⁻ secretion has been studied successfully in isolated colonic crypts, and those studies failed to identify basal or Cl⁻-dependent HCO₃⁻ secretion (25). This observation is consistent with the localization of Cl⁻/HCO₃⁻ exchange to apical membranes of surface cells but not to those of crypt cells (40). Furthermore, the finding of Cl⁻-independent HCO₃⁻ secretion in the crypt contrasts with in vitro experiments with intact mucosa in which HCO₃⁻ secretion was identified as Cl⁻ dependent. These observations indicate that Cl⁻-dependent HCO₃⁻ secretion is a surface cell function, while Cl⁻-independent secretion is most likely a crypt cell function.

cAMP-stimulated HCO₃⁻ secretion both in isolated crypts (25) and in the present studies with intact mucosa that included both surface and crypt cells. HCO₃⁻ secretion as determined in these pH stat studies could represent HCO₃⁻ that was secreted from crypt and/or surface cells. Because cAMP-stimulated Cl⁻ secretion is most likely predominantly restricted to crypt cells (1), cAMP-stimulated HCO₃⁻ secretion might also be an exclusive crypt cell function. However, it is not known whether HCO₃⁻ secretion is also generated from surface cells, because intact mucosa containing both surface and crypt cells was used in the present studies. Such a conclusion requires the study of surface cells independently of crypt cells.

Besides the induction of HCO₃⁻ secretion in colonic crypts and possibly in colonic surface cells, cAMP has several other effects on colonic ion transport (1, 3, 22). cAMP stimulates both active Cl⁻ secretion and active K⁺ secretion (1, 22). Electroneutral NaCl absorption is also inhibited by increased intramucosal concentrations of cAMP (3); the reduction of electroneutral NaCl absorption is most likely a result of its inhibition of Na⁺/H⁺ exchange and/or Cl⁻/HCO₃⁻ exchange.

In the present studies, cAMP not only stimulated HCO₃⁻ secretion but also was associated with downregulation of Cl⁻-dependent HCO₃⁻ secretion, which most likely was a result of cAMP inhibition of apical membrane Cl⁻/HCO₃⁻ exchange (Fig. 7). Although in intact mucosa cAMP appears to completely inhibit apical DIDS-sensitive, Cl⁻-dependent HCO₃⁻ secretion, cAMP has not been reported to inhibit the Cl⁻/HCO₃⁻ exchange activity of either DIDS-sensitive AE1 or relatively DIDS-insensitive DRA when they are expressed in the in vitro recombinant expression system (12). The possibility that cAMP-stimulated HCO₃⁻ secretion represented the coupling of an apical membrane Cl⁻/HCO₃⁻ exchange to CFTR was also excluded by demonstrating that the removal of luminal Cl⁻ did not affect cAMP-induced HCO₃⁻ secretion in either the absence (3.4 ± 0.05 vs. 3.6 ± 0.09 μeq·h⁻¹·cm⁻²) or presence (4.8 ± 0.02 vs. 5.2 ± μeq·h⁻¹·cm⁻²) of bath Cl⁻. This indicates that cAMP-stimulated HCO₃⁻ secretion was a result of an apical membrane anion channel and was not due to the coupling of a Cl⁻/HCO₃⁻ exchange to an apical membrane anion channel (Figs. 6 and 7).

The mechanism of the uptake of SCFA across the apical membrane of colonocytes has been controversial, with evidence of both an apical membrane butyrate/HCO₃⁻ exchange and nonionic diffusion (11, 32). It is not unlikely that both mechanisms are operative, with their relative contribution to overall SCFA uptake varying as a function of species and one or more experimental conditions. Several in vivo studies have observed HCO₃⁻ secretion in association with butyrate absorption support the possibility that butyrate uptake across the apical membrane occurs via a SCFA/HCO₃⁻ exchange. Table 4 demonstrates that isobutyrate stimulates Cl⁻-independent HCO₃⁻ secretion that is insensitive to both DIDS and NPPB. The presence of saturation kinetics (Fig. 8) provides compelling support for a carrier-mediated exchange process.

Fig. 9. Cellular model of HCO₃⁻ secretion in colonocytes of rat distal colon. Three distinct modes of HCO₃⁻ secretion were identified in rat distal colon: 1) Cl⁻-dependent HCO₃⁻ secretion is mediated by apical membrane Cl⁻/HCO₃⁻ exchange; the specific apical membrane protein responsible for Cl⁻/HCO₃⁻ exchange is uncertain, is indicated as AE1 (anion exchange), and could represent either AE1 or DRA (downregulated in adenoma). 2) SCFA-dependent HCO₃⁻ secretion is mediated by apical membrane SCFA/HCO₃⁻ exchange whose molecular identity has not been established. 3) cAMP-stimulated HCO₃⁻ secretion is mediated by apical membrane CFTR. cAMP also inhibited Cl⁻-dependent HCO₃⁻ secretion, while SCFA downregulated both Cl⁻-dependent HCO₃⁻ secretion and cAMP-induced HCO₃⁻ secretion.
that is responsible for butyrate absorption and HCO$_3^-$ secretion. The failure of DIDS to inhibit butyrate-dependent HCO$_3^-$ secretion parallels prior observations of apical membrane vesicles in which butyrate/HCO$_3^-$ exchange was also DIDS insensitive (32).

SCFA-dependent HCO$_3^-$ secretion potentially could be secondary either to an apical membrane SCFA/HCO$_3^-$ exchange or to SCFA-mediated proton uptake across the apical membrane. The available evidence supports a critical role for an anion exchange in isobutyrate-dependent HCO$_3^-$ secretion. First, prior studies of [${}^{14}$C]butyrate uptake by AMV prepared from rat distal colon established the presence of a DIDS-insensitive butyrate/HCO$_3^-$ exchange (32). Second, in these studies with AMV, an outward-directed pH gradient in the absence of HCO$_3^-$/CO$_2$ resulted in only minimal butyrate uptake (32), an observation inconsistent with the presence of a H$^+$/SCFA cotransport mechanism. Third, nonionic diffusion of SCFA across the apical membrane could also result in intracellular acidification, but such a process would not result in the saturation kinetics shown in Fig. 8. In addition, there was only minimal butyrate uptake across AMV via nonionic diffusion in the AMV studies (32). Fourth, in the pH stat studies of colonic HCO$_3^-$ secretion, removal of bath HCO$_3^-$/CO$_2$ completely inhibited SCFA-dependent HCO$_3^-$ secretion (Fig. 3), which provides additional evidence against the possibility that SCFA-H$^+$ cotransport across the apical membrane is responsible for isobutyrate-dependent HCO$_3^-$ secretion in the present studies.

Similarly to cAMP, butyrate modifies several different parameters of colonic ion transport and manifests some specific interactions with cAMP (4, 5, 10, 16, 31). First, butyrate enhances electroneutral NaCl absorption (4). Second, butyrate downregulates both cAMP- and cGMP-induced active Cl$^-$ secretion but does not modify intracellular Ca$^{2+}$- or cAMP-dependent NaCl absorption (5). Third, although stimulation of butyrate-dependent NaCl absorption is closely linked to Na$^+$/H$^+$ exchange, cAMP does not inhibit butyrate-stimulated NaCl absorption (5). There is evidence that SCFA-dependent electroneutral NaCl absorption is not altered by cAMP as a result of cAMP activation of the NHE2 isoform, which mediates butyrate-dependent, but not HCO$_3^-$, absorption (31).

The present studies identified additional effects of butyrate on colonic ion transport as butyrate blocked both cAMP-induced HCO$_3^-$ secretion and Cl$^-$-dependent HCO$_3^-$ secretion (Table 4). Evidence that isobutyrate inhibited cAMP-induced HCO$_3^-$ secretion was provided by the demonstration that HCO$_3^-$ secretion was NPPB sensitive in the absence of isobutyrate (0.4 ± 0.004 μeq·h$^{-1}$·cm$^{-2}$) but was NPPB insensitive in its presence (5.7 ± 0.07 μeq·h$^{-1}$·cm$^{-2}$). In addition, isobutyrate also downregulates Cl$^-$-dependent HCO$_3^-$ secretion, based on the observation that HCO$_3^-$ secretion in the absence of isobutyrate is Cl$^-$ dependent and DIDS sensitive (Fig. 7). After the addition of isobutyrate, HCO$_3^-$ secretion is both Cl$^-$ independent and DIDS insensitive. It thus appears that SCFA downregulates HCO$_3^-$ secretion that is regulated either by cAMP-induced anion channels or by Cl$^-$/anion exchanges. The mechanisms by which SCFA downregulates Cl$^-$-dependent and cAMP-induced HCO$_3^-$ secretion have not been established. However, SCFA uptake across the apical membrane, via either SCFA/HCO$_3^-$ exchange or SCFA-mediated proton influx, would undoubtedly lower pH, thus resulting in a decrease in Cl$^-$-dependent and/or cAMP-induced HCO$_3^-$ secretion. Delineation of these events requires additional studies, including studies of pH regulation by SCFA.

In conclusion, the present studies demonstrate the presence of three distinct HCO$_3^-$ transport processes: Cl$^-$-dependent, SCFA-dependent, and cAMP-stimulated HCO$_3^-$ secretion (Fig. 9). There is a substantial interrelationship between these three HCO$_3^-$ transport mechanisms: cAMP downregulates Cl$^-$-dependent HCO$_3^-$ secretion, while the SCFA isobutyrate inhibits both cAMP-induced and Cl$^-$-dependent HCO$_3^-$ secretion.


