Calcium-sensing receptor stimulates $\mathrm{Cl}^-\text{-}$ and SCFA-dependent but inhibits cAMP-dependent $\mathrm{HCO}_3^-$ secretion in colon

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The extracellular calcium-sensing receptor (CaSR) is a well-conserved G protein-coupled cell surface receptor that is expressed in diverse tissues in mammalian (8) and marine (32) species. CaSR is a key regulator of tissue responses, not only for calcium homeostasis (7), but also for fluid balance (37, 38) and osmotic regulation (32). The primary physiological ligand for the CaSR is extracellularly ionized calcium ($\mathrm{Ca}^{2+}$), providing a mechanism for $\mathrm{Ca}^{2+}$ to function as a first messenger. The CaSR also functions as a general sensor of the extracellular milieu because of allosteric modification of $\mathrm{Ca}^{2+}$ affinity by polyamines, L-amino acids, oligo-peptides, pH, and ionic strength (46).

The CaSR is widely expressed in the gastrointestinal tract, including the epithelial cells that line the entire small and large intestine (9, 14, 16, 23) and regulate ion and fluid transport, thus raising the possibility that these receptors may have important roles in regulation of intestinal fluid and electrolyte movement in both health and disease. In enteric epithelial cells, CaSR has been identified on both the apical and basolateral membranes of human (16, 44) and rat colonocytes (9, 16). Receptors in both membrane domains of these polarized epithelia are functionally active and can be activated by $\mathrm{Ca}^{2+}$ (14, 16), amino acids and peptides (29, 49), polyamines (14, 16), and the specific pharmacological CaSR agonist R568 (25).

In colonic crypt cells, CaSR activation from either mucosal or serosal side inhibits net fluid secretion (14, 16, 25) and cyclic nucleotide accumulation (25) induced by synthetic/natural secretagogues. These secretagogues include forskolin (42) and guanylin (17), which generate cAMP and cGMP, respectively. CaSR activation also blocks the effects of bacterial enterotoxins (25) such as cholera toxin (21), a potent activator of membrane-bound adenylyl cyclase leading to elevated intracellular levels of cAMP, and heat stable Escherichia coli enterotoxin STa (19), which enhances cytosolic cGMP accumulation through the guanylyl cyclase C-type guanylin receptor. In the rat proximal and distal colon, activation of CaSR by R568 inhibits secretagogue-induced $\mathrm{Cl}^-$ secretion (12), either directly via inhibition of epithelial cell basolateral Na$^+$--K$^+$--2Cl$^-$ cotransporter (25) or indirectly via the enteric nervous system (12). In perfused colonic crypts, it also promotes apical Na$^+$--H$^+$ exchanger activity (25); thus it may stimulate Na$^+$ absorption in these cells. However, it remains to be elucidated whether CaSR regulates apical anion conductive pathway (e.g., cystic fibrosis transmembrane conductance regulator, CFTR).

Acute infectious diarrheal illnesses persist as an important worldwide public health problem. Approximately 600,000 children die each year, not necessarily because of an infection, but as a result of the associated dehydration and metabolic acidosis (4, 31). Reducing fluid and bicarbonate ($\mathrm{HCO}_3^-$) losses from acute diarrhea offers a major opportunity for improving child health globally.
Few studies have examined the role for the CaSR in regulating intestinal HCO₃⁻ secretion, which has been critically implicated in the pathophysiology of acute infectious diarrhea. For example, enhanced intestinal HCO₃⁻ secretion/loss associated with cholera and many other acute diarrheal illnesses can result in severe HCO₃⁻ deficit and metabolic acidosis (22, 34). The latter is another of the most common causes (in addition to dehydration and systemic volume depletion) of the morbidity and mortality associated with these clinical conditions. Whether CaSR activation inhibits secretagogue-induced HCO₃⁻ secretion as it does for the secretagogue-induced fluid and Cl⁻ secretion is not known.

Regulated HCO₃⁻ secretion is also required for mucosal defense against luminal acid (via neutralization) in the upper gastrointestinal (GI) tract and bacteria (via stimulation of mucus secretion and maintenance of intestinal barrier function) in the lower GI tract, the defect of which has been shown to be a risk factor for peptic ulcer diseases (2, 20, 28) and intestinal inflammation (24, 51, 52). The role of CaSR in basal and acid-induced HCO₃⁻ secretion is unknown although a recent study with gut-specific CaSR knockout mice suggests that this receptor is crucial for mucosal defense and immunity (15).

HCO₃⁻ secretion in the intestine can be the result of one or more HCO₃⁻ transporters. In rat distal colon, at least three distinct mechanisms of HCO₃⁻ secretion have been described (47, 48): 1) lumen Cl⁻-dependent HCO₃⁻ secretion associated with a brush-border Cl⁻/HCO₃⁻ exchange, 2) lumen short-chain fatty acid (SCFA)-dependent HCO₃⁻ secretion mediated by a SCFA/HCO₃⁻ exchange, and 3) cyclic nucleotide-induced HCO₃⁻ secretion as a result of activation of an anion-conductive pathway (e.g., CFTR). To date, there is no information whether CaSR regulates one or more of these HCO₃⁻ transport mechanisms.

The present study was initiated to examine the CaSR effects on HCO₃⁻ secretion ex vivo in isolated intact colonic mucosa of rats and mice using pH stat and short-circuit current (Iₑᵥ) measurements. Our experiments were designed in such a way that effects on basal, acid-induced, and secretagogue-induced HCO₃⁻ secretion were examined. Basal and acid-induced secretion was studied to provide information that may mimic physiological states, whereas secretagogue-induced secretion experiments were used to assess potential relevance of CaSR regulation in diarrhea. To provide insight into the mechanisms by which CaSR may regulate different HCO₃⁻ secretory processes, the effects of R568 on the Cl⁻/HCO₃⁻ and SCFA/HCO₃⁻ exchanges (which are electroneutral and thus can be measured by pH stat) and electrogenic HCO₃⁻ movement (thus can be measured by Iₑᵥ and pH stat) were examined, both in rats and in CaSR wild-type and mutant mice. We have previously demonstrated that CaSR agonists can inhibit fluid/Cl⁻ secretion (10, 12, 14, 16, 25) and mucosa inflammation (11, 15). These present studies show that CaSR agonists can also exert their effects on mucosal biology and physiology through differentially regulating HCO₃⁻ secretion.

**Materials and Methods**

**Animals and Tissue Preparations**

Experiments were performed using nonfasting male Sprague-Dawley rats and C57BL/6 mice. Rats weighing 100–400 g were obtained from Charles River Laboratories. Mice lacking CaSR expression in intestinal epithelial cells (CaSR⁻/⁻) and their CaSR⁻/+ littermates were bred and maintained in house at the University of Florida. CaSR⁻/⁻ mice were generated as previously described (36). Briefly, CaSR flox/flox mice were bred with transgenic mice expressing Cre recombinase under the control of the villin 1 promoter and genotyped before all experiments after an approximate 10–12 generations. Mice were used at 5–10 wk of age in accordance with the Animal Welfare Act and the Public Health Policy on Humane Care. Animals were fed and maintained on regular chow (Harlan) with free access to water before death. Animals were killed with standard CO₂ inhalation and killed by cervical dislocation. The colon was isolated, cut open along the mesenteric border into a flat sheet, and flushed with ice-cold Ringer solutions. Mucosa from the distal colon were carefully hand-stripped off of serosal, muscular, and submucosal layers as described (27), and a pair of adjacent mucosal segments was incised and mounted into Ussing chambers. In some experiments, stripped mucosa was incised longitudinally into two equal pieces to facilitate comparisons between control and treatment (see below). Either way, Iₑᵥ and transepithelial resistance differences between these two adjacent tissues were <15%. The use of animals as well as the protocol for isolating colon tissues were approved by the Institutional Animal Care and Use Committee (IACUC no. 201307567) at the University of Florida.

**Ussing Chamber Setup**

Stripped mucosal sheets were mounted into Ussing chambers (window area = 0.3–0.5 cm²; Physiologic Instruments). The mucosal side of tissue was bathed with an unbuffered HCO₃⁻-free Cl⁻ Ringer solution (see Table 1 for detailed composition) circulated by a gas lift with 100% O₂ while the serosal side was bathed with buffered Cl⁻ Ringer solution (pH 7.4) that contained 25 mM HCO₃⁻ and gassed with 5% CO₂/95% O₂. Each side contained 3–5 ml of solution, and the temperature of the solution was adjusted to and maintained at 37°C by heated water-jacketed reservoirs. Experiments were performed under short-circuit conditions (Voltage-Current Clamp, VCC MC8; Physiologic Instruments) to maintain the transepithelial potentials at 0 mV, except for a brief interruption at 20-s intervals for recording of open-circuit potential (Yᵥ, mV).

Two types of approaches were used in the measurement of colonic HCO₃⁻ secretion, namely measurement of lumen pH or pH stat titration of net alkalization by colonic mucosa. Except for limited experiments that require direct measurements of lumen pH (e.g., Experiment 1 and Experiment 3 below), luminal pH was maintained at 7.4 by the continuous infusion of 1 mM HCl or H₂SO₄ in the case of lumen Cl⁻–free conditions under the automatic control of a pH stat system (Bi-burette TIM 856 pH meter; Radiometer Analytical). The amount of the acid delivered per unit time per surface area was used to quantify HCO₃⁻ secretion by the mucosa. Measurements were made daily in 20–60 min increments or as noted.

**Table 1. The composition of Ringer solutions**

<table>
<thead>
<tr>
<th></th>
<th>Serosa</th>
<th>Lumen</th>
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<tbody>
<tr>
<td>Cl⁻</td>
<td>123</td>
<td>123</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>25</td>
<td>147</td>
</tr>
<tr>
<td>Isethionate</td>
<td>25</td>
<td>122</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>1</td>
<td>1</td>
</tr>
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<td></td>
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Compositions of constituents of all solutions are provided in mM; pH of all Cl⁻–containing solutions was adjusted to 7.4 with HCl, whereas that of Cl⁻–free solutions was adjusted with H₂SO₄. All solutions were gassed with 95% O₂/5% CO₂. HCO₃⁻–free solutions were gassed with 100% O₂. All solutions contained (in mM) 140.8 Na⁺, 5.9 Cl⁻, 5.2 K⁺, 0.5 Ca²⁺, and 0.5 Mg²⁺. Of note, the anion isethionate is not a short-chain fatty acid (SCFA) and was used to substitute Cl⁻ and/or HCO₃⁻.
recorded continuously, and mean values for consecutive 5- or 10-min periods were averaged. The rate of HCO₃⁻ secretion (J_HCO₃) is expressed as μeq h⁻¹ cm⁻². The Iₑ was measured in microamperes (μA) and converted into μeq h⁻¹ cm⁻². Tissue resistance (R, Ω/cm²) was calculated from Ohm’s law.

**Experimental Design**

Seven experiments for HCO₃⁻ secretion were conducted. Lumen alkalinization response to [Ca²⁺]. Two equal pieces of mucosa from each colon that were obtained by longitudinal division along the antimesenteric border were mounted into two Ussing chambers. One piece was bathed with Cl⁻ Ringer solution that contained 1.2 mM [Ca²⁺]o, while the other piece was bathed with Cl⁻ Ringer solution that contained 0.5 mM [Ca²⁺]o. Initially, tissues were bathed, both luminally and serosally, in HCO₃⁻-free solution. After 15–30 min, when stabilization was achieved and basal luminal pH recordings performed, HCO₃⁻-free Ringer solution in the serosal side was replaced by 25 mM HCO₃⁻-containing Ringer solution, and changes in lumen pH were monitored and recorded for 15–30 min. In some tissues, carbobloc (CCH) was added to the serosal side before the experiment was concluded.

**Basal HCO₃⁻ secretion.** Two adjacent sheets of mucosa from each colon were mounted into two Ussing chambers and bathed luminally with HCO₃⁻-free Cl⁻ Ringer solution and serosally with HCO₃⁻-containing Cl⁻ Ringer solution. After 15–30 min, when Iₑ and J_HCO₃ had stabilized, the CaSR agonist, R568, was added to the serosal or mucosal side of tissue, and changes in Iₑ and J_HCO₃ during the 15–30-min period ensuing after the addition of the agonist were determined.

**Acid-induced HCO₃⁻ secretion.** Colonic mucosa from each animal was divided longitudinally into two equal pieces and mounted into two Ussing chambers. Acid (hydrochloride) was added to the lumen to lower pH by ~0.3–0.4 units, and changes in lumen pH were monitored. In response to luminal addition of acid, HCO₃⁻ is secreted, and pH in the lumen rises. When lumen pH rose above 7.4, another acid challenge was applied. After 15–30 min, when lumen pH responses had stabilized, one piece of mucosa was treated with R568 and the other with vehicle control, and changes in lumen pH responses during the 30–45-min period ensuing after the addition of the agonist were determined. Initial studies demonstrated that, under these experimental conditions, the tissue tolerated acid challenges for at least 60 min without significantly compromising tissue responses and integrity. Initial studies also established that acid-induced pH recovery reflects HCO₃⁻ secretion from tissue, as additions of acid to no-tissue controls or tissue controls without the presence of serosal HCO₃⁻ did not generate significant pH recovery. A lowering of 0.3–0.4 pH units was used because this is the range of luminal pH by which this part of the intestine normally varies (33). Initial rates of acid-induced pH recovery were used for comparison and were expressed as pH unit recovered min⁻¹ cm⁻².

**Secretagogue-induced HCO₃⁻ secretion.** To examine CaSR effect on stimulated HCO₃⁻ secretion, a model of forskolin-induced HCO₃⁻ secretion was employed to mimic choleretic effects. The secretagogue forskolin was used to increase tissue cAMP content rapidly, as the effect of choleretic toxin will not be seen for 1–2 h. Tissues were divided and treated as in the basal HCO₃⁻ secretion experiments except that, after 15–30 min, when Iₑ and J_HCO₃ had stabilized, forskolin was added to the serosal side of tissue for 15–30 min until Iₑ and J_HCO₃ had plateaued before R568 was then added to the serosal solution. Changes in Iₑ and J_HCO₃ during the 15–30-min period ensuing after the addition of the agonist were determined. Rodent distal colon displays Na⁺, K⁺, and Cl⁻ currents, in addition to HCO₃⁻ conductance. To reduce current interference from non-HCO₃⁻ conductance, before the recording of HCO₃⁻ secretory responses, a cocktail containing 10 μM amiloride and 5 mM barium was added to the mucosal side to selectively inhibit Na⁺ and K⁺ conductance, and 100 μM bumetanide was added to the serosal side to inhibit Cl⁻ secretory current (26, 41). Preliminary studies indicate that these transport inhibitors did not significantly affect J_HCO₃ response although Iₑ was inhibited by amiloride and stimulated by barium (see Table 2).

**Cl⁻/HCO₃⁻ exchange.** Mucosa from each colon was divided longitudinally into two equal pieces and mounted into two Ussing chambers. One piece of mucosa was bathed luminally with zero HCO₃⁻ Ringer solution that contained Cl⁻, while the other piece of mucosa was bathed luminally with zero HCO₃⁻ Ringer solution that did not contain Cl⁻. Both pieces of mucosa were bathed serosally with Ringer solution that contained Cl⁻ and HCO₃⁻. After 15–30 min, when J_HCO₃ had stabilized, R568 or vehicle control was added to the serosal or mucosal side of tissue, and changes in J_HCO₃ during the 15–30-min period ensuing after the addition of the agonist or vehicle were determined and averaged. When inhibitor was used, it was added at 30 min before the agonist. Cl⁻/HCO₃⁻ exchange activity was calculated as ΔJ_HCO₃ (the difference between J_HCO₃ in the presence minus absence of luminal Cl⁻) (18, 48).

SCFA/HCO₃⁻ exchange. Unless specifically described elsewhere, colon segments were prepared and treated as in the Cl⁻/HCO₃⁻ exchange studies except that they were bathed luminally with zero HCO₃⁻ Ringer solution that either contained or did not contain 25 mM isobutyrate. SCFA/HCO₃⁻ exchange activity was calculated as ΔJ_HCO₃ (the difference between J_HCO₃ in the presence minus absence of luminal isobutyrate) (18, 48).

**Cyclic nucleotide-dependent HCO₃⁻ secretion.** Colonic segments were prepared and treated as in the secretagogue-induced HCO₃⁻ secretion experiments except that they were bathed luminally with HCO₃⁻-free Ringer solution that contained no isobutyrate and no Cl⁻. Cyclic nucleotide-dependent HCO₃⁻ secretion was calculated as ΔJ_HCO₃ (the difference between J_HCO₃ after minus J_HCO₃ before the addition of forskolin) (18, 48). When inhibitor was used, it was added at 30 min before R568 or forskolin.

**Chemicals and Solutions**

Forskolin, 4,4′-disothiocyanato-2,2′-stilbenedisulfonic acid (DIDS), glibenclamide, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), amiloride, barium, and bumetanide were obtained from Sigma and Gly-H101 from Santa Cruz Biotechnology, whereas R-568 was from Tocris Bioscience. All stock solutions were prepared in DMSO. The detailed composition of Ringer solutions used in these studies is listed in Table 1. A combination of 5% CO₂/95% O₂ was used to oxygenate Ringer solutions that contained HCO₃⁻, whereas 100% O₂ was used to oxygenate those solutions that did not contain HCO₃⁻.

**Statistical Analysis**

Values are expressed as means ± SE. ΔJ_HCO₃, ΔIₑ, and ΔpH recovery rates refer to stimulated peak responses minus basal control levels. Data were analyzed by one-way ANOVA followed by Holm-Sidak’s post hoc test or, when appropriate, by the paired or unpaired two-tailed Student’s t-test using Microsoft Excel 2010 for Windows.

Table 2. Effect of transport inhibitors on basal HCO₃⁻ secretion in rat distal colon

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>D_J_HCO₃, μeq h⁻¹ cm⁻²</th>
<th>ΔIₑ, μA cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride, 10 μM</td>
<td>0.01 ± 0.01 (3)ns</td>
<td>−6.3 ± 10.3 (3)†</td>
</tr>
<tr>
<td>Bumetanide, 100 μM, lumen</td>
<td>0.02 ± 0.06 (3)ns</td>
<td>4.7 ± 0.7 (3)‡</td>
</tr>
<tr>
<td>DIDS, 100 μM, lumen</td>
<td>−0.03 ± 0.04 (4)*</td>
<td>4.6 ± 3.9 (4)ns</td>
</tr>
<tr>
<td>NPPB, 100 μM, lumen</td>
<td>−0.17 ± 0.06 (3)ns</td>
<td>−10.2 ± 0.8 (3)†</td>
</tr>
</tbody>
</table>

Data shown are means ± SE (n). *P < 0.05; †P < 0.01; ns P > 0.05 vs. control (before treatment). DIDS, 4,4′-disothiocyanato-2,2′-stilbenedisulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
REGULATION OF COLONIC HCO₃⁻ SECRETION BY CaSR

RESULTS

Basal HCO₃⁻ Secretion

Lowering extracellular Ca²⁺ concentration reduces and activation of CaSR by R568 enhances basal HCO₃⁻ secretion. The initial experiments performed examined the role of extracellular Ca²⁺ in modulation of HCO₃⁻ secretion under basal (nonstimulated) conditions. Mucosa was first bathed with HCO₃⁻-free, Cl⁻ Ringer solution before 25 mM HCO₃⁻ was added to the serosa. As shown in Fig. 1, there was no change in lumen pH; thus lumen alkalinization did not occur in the absence of a serosa-to-lumen-directed HCO₃⁻ gradient. Consistent with HCO₃⁻ secretion, subsequent addition of HCO₃⁻ ion to serosal side induced significant lumen alkalinization (Fig. 1A; means ± SE (n) absence vs. presence of HCO₃⁻ ion: −0.011 ± 0.002 (3) vs. 0.035 ± 0.002 (3) pH U·min⁻¹·cm⁻², P < 0.01). CCH, a known secretagogue for HCO₃⁻ secretion, was used as a positive control. CCH induced an initial transient increase followed by a sustained increase in lumen pH (Fig. 1A) characteristic of HCO₃⁻ secretion associated with cholinergic receptor activation (43).

Experiments were then performed to examine whether CaSR regulates basal HCO₃⁻ secretion. For this, the concentration of Ca²⁺ in the buffer was lowered from 1.2 mM to 0.5 mM, and HCO₃⁻ secretory response was compared. Extracellular Ca²⁺ is a physiological ligand of CaSR. Previous studies have shown that this maneuver reduces CaSR activity and that, at 0.5 mM Ca²⁺ concentration, CaSR is only minimally stimulated (12, 14, 16, 25). Reduction of [Ca²⁺]o from 1.2 mM to 0.5 mM significantly reduced lumen alkalinization rates [Fig. 1B; [Ca²⁺]o = 1.2 vs. 0.5 mM: 0.031 ± 0.002 (3) vs. 0.013 ± 0.002 (3) pH U·min⁻¹·cm⁻², P < 0.01]. As extracellular Ca²⁺ is also a known determinant of paracellular permeability of intestinal epithelium, additional experiments were performed to exclude the possibility that the reduced rate of lumen alkalinization noted at reduced [Ca²⁺]o was not simply caused by HCO₃⁻ "back leak" secondary to altered paracellular integrity; thus, transepithelial electrical resistances (TEER) were measured. No significant differences in TEER were noted between normal and reduced Ca²⁺-treated tissues (see details in Fig. 1 legend). Thus it is likely that extracellular Ca²⁺ (and CaSR) regulates transeellular and not paracellular HCO₃⁻ movement.

To further assess the role of CaSR as a regulator of HCO₃⁻ secretion, another set of experiments measured the rate of serosal to mucosal HCO₃⁻ flux (JHCO₃) and HCO₃⁻ secretory Iₑc and tested the effect of R568, a specific pharmacological CaSR agonist. A relatively low basal JHCO₃ was observed in the absence of R568. Addition of R568 to serosa (Fig. 1C) significantly stimulated JHCO₃. Similar but slightly less pronounced effects were also noted when R568 was added to lumen solutions (not shown). Such stimulatory changes were not observed in Iₑc [absence vs. presence of R568: 1.36 ± 0.40 (5) vs. 1.48 ± 0.44 (5) μeq·h⁻¹·cm⁻², P > 0.05]. These data suggested that CaSR stimulates electroneutral HCO₃⁻ secretion.

Acid-Induced HCO₃⁻ Secretion

Activation of CaSR by R568 enhances acid-induced HCO₃⁻ secretion. Acid-induced HCO₃⁻ secretion is a known mechanism that intestinal mucosa utilizes as a defense against acid-induced damage (2, 20, 28). Colonic mucosa is exposed to luminal acidic environment, generated as a result of bacteria...
induced a transient but significant stimulation of acid-induced HCO$_3^-$ secretion.

Secretagogue-Induced HCO$_3^-$ Secretion

Activation of CaSR by R568 inhibits secretagogue-induced HCO$_3^-$ secretion. HCO$_3^-$ secretion is markedly increased in cholera and other secretagogue-induced diarrheal diseases (22, 34). To assess whether CaSR stimulates HCO$_3^-$ secretion under these diseased conditions, the R568 effect was examined in a model of secretagogue-induced secretory diarrhea. In this study, forskolin was used to stimulate HCO$_3^-$ secretion, and HCO$_3^-$ secretory response was monitored by measuring HCO$_3^-$ secretory rate ($J_{\text{HCO}_3}$) (Fig. 3A) and by recording $I_{\text{sc}}$ (Fig. 3C). Forskolin stimulated both $J_{\text{HCO}_3}$ and $I_{\text{sc}}$; subsequent addition of R568 did not increase but rather decreased forskolin-induced HCO$_3^-$ secretion.

Cl$^-$-Dependent HCO$_3^-$ Secretion

Activation of CaSR stimulates Cl$^-$/HCO$_3^-$ exchange activity. Because the basal and acid-induced HCO$_3^-$ secretion were assayed in Cl$^-$-containing Ringer, it is likely that the CaSR effects reflect stimulation of a Cl$^-$-dependent HCO$_3^-$ secretory mechanism such as Cl$^-$/HCO$_3^-$ exchange (48). To address this possibility, Cl$^-$/HCO$_3^-$ exchange activity was measured and is shown in Fig. 4. Figure 4A demonstrates HCO$_3^-$ secretory responses to the presence vs. absence of lumen Cl$^-$. In agreement with previous studies (47, 48), a luminal Cl$^-$-dependent HCO$_3^-$ secretory mechanism (Cl$^-$/HCO$_3^-$ exchange) was observed. The latter was partially abolished by pretreatment with 100 μM DIDS added to lumen side (data not shown). Activation of CaSR by R568, added to serosal side, resulted in stimulation of the Cl$^-$/HCO$_3^-$ exchange activity (Fig. 4B). This Cl$^-$-dependent HCO$_3^-$ secretion was significantly higher in the presence than in the absence of R568 (Fig. 4B). A similar but less pronounced stimulatory effect of R568 was noted when this agonist was added to the mucosal solution (data not shown).

Fig. 2. Effect of CaSR agonist R568 on acid-induced HCO$_3^-$ secretion. A: representative of pH recovery responses in the presence vs. absence of R568 (10 μM, serosa). The addition of R568 is shown as indicated. B: summary of ΔpH recovery rates (stimulated peak values above basal levels before additions of R568 or vehicle). Basal levels of acid-induced pH recovery were 0.015 ± 0.001 pH U·min$^{-1}$·cm$^{-2}$. The tissue resistance (Ω·cm$^2$) and $I_{\text{sc}}$ (μEq·h$^{-1}$·cm$^{-2}$) at the end of 60-min experiments were 50 ± 12 and 1.74 ± 0.20 without challenge and 46 ± 10 and 1.97 ± 0.23 with acid challenges (P > 0.05). Data are means ± SE of 5 experiments. *P < 0.05 vs. control (with no R568).

Fig. 3. Effect of CaSR agonist R568 on secretagogue-induced HCO$_3^-$ secretion and HCO$_3^-$ current. Shown are $J_{\text{HCO}_3}$ (A) and $I_{\text{sc}}$ (C) responses to forskolin (FSK, 500 nM, serosa) ± R568 (10 μM, serosa), assayed in lumen Cl$^-$-containing Ringer solution. The changes induced by FSK in the absence vs. presence of R568 are summarized in B ($\Delta J_{\text{HCO}_3}^{\text{FSK}}$) and D ($\Delta I_{\text{sc}}^{\text{FSK}}$). The basal tissue resistance and $I_{\text{sc}}$ under these conditions were as follows: 68 ± 4 Ω·cm$^2$ and 1.51 ± 0.22 μEq·h$^{-1}$·cm$^{-2}$. Data are means ± SE of 8 experiments. **P < 0.01 vs. control (with no R568); ###P < 0.01 vs. FSK.
SCFA-Dependent HCO$_3^-$ Secretion

**Activation of CaSR stimulates SCFA/HCO$_3^-$ exchange activity.** SCFA are present in the colon and induce HCO$_3^-$ secretion via the SCFA/HCO$_3^-$ exchange (47, 48). To assess whether CaSR stimulates HCO$_3^-$ secretion under these conditions, the next series of experiments examined isobutyrate-dependent HCO$_3^-$ secretion and its response to R568. Figure 5, A and B, shows representative tracings, and Fig. 5C presents a summary of HCO$_3^-$ secretory responses to the presence vs. absence of lumen SCFA (25 mM isobutyrate) with vs. without a serosa-to-mucosa-directed HCO$_3^-$ gradient. SCFA-dependent HCO$_3^-$ secretion was present and required serosal HCO$_3^-$, consistent with HCO$_3^-$ secretion mediated by SCFA/HCO$_3^-$ exchange. Similar to the R568 effect on the Cl$^-$ dependence of lumen HCO$_3^-$ exchange, isobutyrate-dependent HCO$_3^-$ secretion was significantly stimulated by activation of CaSR by R568 (Fig. 5D).

**cAMP-Dependent HCO$_3^-$ Secretion**

**Activation of CaSR by R568 inhibits cAMP-dependent HCO$_3^-$ secretion.** In contrast to R568 stimulation of basal HCO$_3^-$ secretion, HCO$_3^-$ secretion was inhibited by R568 under forskolin-stimulated condition (see Fig. 3). It is uncertain how R568 produces this inhibition. Because R568 inhibited neither Cl$^-$-dependent nor SCFA-dependent HCO$_3^-$ secretion (see Figs. 4–5), it is unlikely that the effect of the CaSR agonist is via inhibition of either of these anion exchanges. Rather, a Cl$^-$/SCFA-independent HCO$_3^-$ transport mechanism might be responsible. One such mechanism is a cyclic nucleotide-dependent electrogenic channel (e.g., CFTR)-mediated HCO$_3^-$ secretion (48). Thus, to address this latter possibility, stimulation of HCO$_3^-$ secretion by forskolin and its inhibition following addition of R568 were reassessed in lumen Cl$^-$/SCFA-free solutions. As shown in Fig. 6A, a low rate of HCO$_3^-$ transport was noted under basal condition before the addition of forskolin. Addition of forskolin significantly stimulated HCO$_3^-$ secretion. The subsequent addition of R568 almost completely reversed forskolin-induced HCO$_3^-$ secretion. Similar changes were observed in experiments that determined changes in $I_{sc}$ (Fig. 6C). Forskolin stimulated $I_{sc}$, subsequent addition of R568 inhibited HCO$_3^-$ secretion. Removal of the serosa to mucosa HCO$_3^-$ gradient significantly diminished both basal and forskolin-stimulated $I_{sc}$ (data not shown); in the absence of serosal HCO$_3^-$, forskolin and R568 failed to stimulate or to inhibit $I_{sc}$, respectively (data not shown). Pretreatment with luminal glibenclamide (100 μM) or GlyH-101 (10 μM), CFTR channel blockers, and NPPB (100 μM), an anion channel inhibitor, added either before the addition of forskolin or R568, abolished the forskolin stimulatory and R568 inhibitory effects on $I_{sc}$ (data not shown).
not shown). As a consequence, these results suggest that R568 inhibits cAMP-dependent, glibenclamide/GlyH-101/NPPB-sensitive electrogenic HCO$_3^-$/H$_2$CO$_3^-$ secretion.

Effect of CaSR Knockout

R568 fails to stimulate Cl$^-$- and SCFA-dependent and inhibit cAMP-dependent HCO$_3^-$ secretion in colon of CaSR-null mouse. R568 is a specific pharmacological agonist that has been widely used to stimulate CaSR. To verify that the effects of R568 occurred via the CaSR, additional studies on the effect of R568 were performed in intestinal epithelium-specific CaSR knockout mice (see Fig. 7). In this study, intestinal epithelium-specific CaSR knockout mice were used together with their wild-type littermates. Activation of CaSR by R568 stimulated Cl$^-$- and SCFA-dependent HCO$_3^-$ secretion and inhibited cAMP-dependent HCO$_3^-$ secretion in colon mucosa of wild-type mice (Fig. 7, A–C); such effects were abolished in CaSR-null mice (Fig. 7, D–F). These results indicate that the R568 effect on HCO$_3^-$ secretion is mediated by the CaSR.
effects occur via activating the CaSR in the intestinal epithelium.

DISCUSSION

These present results provide the basis for a new model for regulation of HCO$_3^-$ secretion in the mammalian colon in that activation of CaSR by R568 stimulated basal and acid-induced HCO$_3^-$ secretion, but, in contrast, R568 inhibited cyclic nucleotide-mediated HCO$_3^-$ secretion. Our studies also indicate that the enhancement of HCO$_3^-$ secretion is mediated via stimulation of electroneutral Cl$^-$/HCO$_3^-$ and SCFA/HCO$_3^-$ exchanges that are localized on the apical membrane of colonic surface epithelial cells; in contrast, the CaSR inhibitory action is a consequence of CaSR inhibition of a cAMP-dependent, lumen glibenclamide/GlyH-101/NPPB-sensitive electrogenic HCO$_3^-$ epithelial cells; in contrast, the CaSR inhibitory action is a consequence of CaSR inhibition of a cAMP-dependent, lumen glibenclamide/GlyH-101/NPPB-sensitive electrogenic HCO$_3^-$ exchange coupled to Na$^+$/H$^+$ exchange, which is also localized in surface cells. The ability of CaSR agonists to stimulate both anion exchanges (see Figs. 4, 5, and 7) as well as Na$^+$/H$^+$ exchange (25) suggests that, in addition to stimulation of HCO$_3^-$ secretion, CaSR may function as a mechanism to enhance electrolyte and fluid absorption. Interestingly, activation of CaSR also stimulates colonic acid-induced HCO$_3^-$ secretion (see Fig. 2). One could speculate that this latter function may also neutralize H$^+$ from bacterial fermentation and/or Na$^+$/H$^+$ exchange, further increasing solute absorption.

Fig. 8. Cellular model of CaSR regulation of HCO$_3^-$ secretion by CaSR is depicted in Fig. 8.

According to the present model of colonic ion function (50), absorptive processes are primarily localized to surface cells, whereas secretory processes are primarily present in crypt cells. Because Cl$^-$/dependent HCO$_3^-$ exchange and SCFA-dependent HCO$_3^-$ exchange are present only in surface cells and are absent in crypts (see Refs. 47 and 48 and also in review by Binder, et al. (3)), Cl$^-$/dependent HCO$_3^-$ secretion and SCFA-dependent HCO$_3^-$ secretion are most likely both surface cell functions. In contrast, CFTR-mediated HCO$_3^-$ secretion is generally considered to represent a crypt cell function. Thus, in addition to mediating colonic HCO$_3^-$ secretion, the primary function of these two anion exchanges in these absorptive surface cells is to absorb solutes/electrolytes. SCFA absorption is mediated by SCFA/HCO$_3^-$ exchange, and NaCl absorption is the result of Cl$^-$/HCO$_3^-$ exchange coupled to Na$^+$/H$^+$ exchange, which is also localized in surface cells. The ability of CaSR agonists to stimulate both anion exchanges (see Figs. 4, 5, and 7) as well as Na$^+$/H$^+$ exchange (25) suggests that, in addition to stimulation of HCO$_3^-$ secretion, CaSR may function as a mechanism to enhance electrolyte and fluid absorption. Interestingly, activation of CaSR also stimulates colonic acid-induced HCO$_3^-$ secretion (see Fig. 2). One could speculate that this latter function may also neutralize H$^+$ from bacterial fermentation and/or Na$^+$/H$^+$ exchange, further increasing solute absorption.

Fig. 8. Cellular model of CaSR regulation of HCO$_3^-$ secretion in colonocytes of rat distal colon. Top: R568 acting via CaSR causes enhancement of HCO$_3^-$ secretion in surface epithelial cells by stimulation of luminal Cl$^-$/dependent HCO$_3^-$ secretion via apical Cl$^-$/HCO$_3^-$ exchange and stimulation of luminal SCFA-dependent HCO$_3^-$ secretion mediated by apical SCFA/HCO$_3^-$ exchange. Bottom: CaSR reduces HCO$_3^-$ secretion in the crypt epithelial cells through inhibition of a cAMP-dependent HCO$_3^-$ secretory process that may involve a 5-nitro-2-(3-phenylpropylamino)benzolic acid/glibenclamide-sensitive apical anion channel such as cystic fibrosis transmembrane conductance regulator (CFTR) and/or a basolateral HCO$_3^-$ entry mechanism(s). +, stimulation; −, inhibition.

Consistent with a recent in vivo study in rat perfused duodenum (1), these present studies demonstrated that CaSR activation stimulated basal HCO$_3^-$ secretion in ex vivo colonic mucosa. These findings may have important physiological significance, as HCO$_3^-$ secretion is an integral part of mucosal defense mechanisms. HCO$_3^-$ secretion is required for mucin secretion by goblet cells to establish a layer of mucus overlying the epithelium (35), an initial defense barrier that limits pathogen invasion. Defects in HCO$_3^-$ secretion have been shown to impair the formation of the mucus layer and compromise the integrity of the intestinal barrier, leading to bacteria translocation and development of intestinal inflammation (24, 51, 52). Thus the ability for CaSR to stimulate HCO$_3^-$ secretion under basal conditions suggests that, through modulating mucus secretion and barrier function, this well-conserved nutrient-sensing receptor may play a role in intestinal immune function. Indeed, mice deficient in CaSR with deficient regulated HCO$_3^-$ secretion in the colon have altered barrier integrity, enhanced bacteria translocation, and increased inflammation (15, 30), whereas enteral nutrients, including the CaSR-activating nutrients/minerals, calcium, spermine, and tryptophan, have been shown to improve intestinal permeability and immunity (39, 40) and inflammation (5, 6, 11, 13).

Importantly, both HCO$_3^-$ and Cl$^-$ secretion are markedly induced in cyclic nucleotide-mediated secretory diarrheas (e.g., cholera) (22, 34). Although these secretory responses may be helpful in enhancement of the defensive mucus layer so as to limit pathogen invasion and also to flush out toxins, overproduction and secretion of these anions by the intestine under these pathological conditions is harmful and may result in dehydration, alkali deficit, and metabolic acidosis (22, 34). Systemic volume depletion and metabolic acidosis are the two major causes of death associated with acute diarrheal illnesses, especially in infants and young children. The ability of CaSR agonists both to inhibit cyclic nucleotide-stimulated Cl$^-$ (10, 12, 14, 16, 25) and HCO$_3^-$ secretion (see Figs. 6 and 7) and to promote Cl$^-$ and SCFA absorption (see Figs. 4, 5, and 7) as well as Na$^+$ absorption (25) suggests that this class of drugs may provide a unique therapeutic approach to prevent and treat these potentially lethal diarrheal illnesses. Because CaSR agonists are naturally occurring nutrients, CaSR-based anti diarrheal therapies would be of particular utility among actively growing infants and children (13).

In the present study, the net increases in $I_{sc}$ induced by forskolin ($\Delta I_{sc}^{FSK}$) were greater than those in net $J_{HCO3}$ ($\Delta J_{HCO3}^{FSK}$) [compare the gray-colored columns in Figs. 3D and 6D (mean values: 3.6 and 1.5 μeq·h$^{-1}$·cm$^{-2}$) vs. Figs. 3B and 6B (mean values: 1.7 and 0.2 μeq·h$^{-1}$·cm$^{-2}$)]. These differences cannot be explained by a non-steady-state flux.
period, as all measurements were made after 15–30 min when both $I_{EC}$ and $J_{HCO3}^+$ had stabilized and were in steady state. The most likely explanation is that a component of $\Delta I_{EC}^{FSK}$ represents forskolin-induced $Cl^-$ secretion even though serosal bumetanide was present in both experiments. Serosal bumetanide was employed to prevent (or at least to reduce) such a contribution from forskolin-induced $Cl^-$ secretion. It is known that, in rat distal colon, bumetanide does not completely suppress $Cl^-$ secretion induced by forskolin (41). Only $\sim$70% of such $Cl^-$ secretion was inhibited by bumetanide; the remainder of the $Cl^-$ secretion was mediated by a $Cl^-/HCO3^-$ exchange located in the basolateral membrane of colonicocytes (41). Consistent with this, we found that the non-$HCO3^-$ portion of $\Delta I_{EC}^{FSK}$ was greater when a serosa to mucosa transepithelial $Cl^-$ gradient was present than when a $Cl^-$ gradient was absent (compare 88% in Fig. 6 vs. 53% in Fig. 3).

Although most experiments were performed with a reduced concentration of $Ca^{2+ \text{o}}$ to minimize background activation of the receptor before R568 addition, under normal $Ca^{2+ \text{o}}$ condition, the effects of R568 were qualitatively similar, albeit with a slightly less pronounced effect (also see Fig. 6 of Ref. 12). As a result, the data from the present study suggest that the calcimimetic R568 may have physiological relevance and clinical utility. Indeed, this same class of drug has been employed successfully to inhibit parathyroid hormone secretion in hyperparathyroid patients, where $Ca^{2+ \text{o}}$ in the serum can be either $<1.0 \text{ mM}$ (secondary hyperparathyroidism) or $>1.5 \text{ mM}$ (primary hyperparathyroidism) [see a review by Tfelt-Hansen and Brown (46)].

In summary, the present in vitro studies confirmed the presence of at least three distinct mechanisms for $HCO3^-$ secretion in rodent distal colon, i.e., luminal $Cl^-$–dependent $HCO3^-$ secretion, SCFA-dependent $HCO3^-$ secretion, and cAMP-activated $HCO3^-$ secretion. Furthermore, CaSR agonists differently regulate $HCO3^-$ secretion, depending on the physiological state of the intestine and the specific transporter in question. During physiological conditions when electroneutral $Cl^-/HCO3^-$ and SCFA/HCO3$^-$ exchanges dominate, CaSR enhances $HCO3^-$ secretion; however, in experimental conditions that result in stimulation of fluid and $HCO3^-$ secretion that also occurs in cholera in which enteric $CFTR$-mediated $HCO3^-$ conductance is dominant, CaSR also inhibits $HCO3^-$ secretion. We suggest that both of these two regulatory processes induced by CaSR are potentially beneficial. Whereas the stimulatory effect may help expand the mucus layer, the inhibition of channel-mediated $HCO3^-$ secretion may be of particular clinical significance, as it may reduce and minimize $HCO3^-$ losses in diarrhea.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

30. Macleod RJ. Extracellular calcium-sensing receptor/PTH knockout mice colons have increased Wnt/β-catenin signaling, reduced non-canonical Wnt signaling, and increased susceptibility to azoxymethane-induced aberrant crypt foci. Lab Invest 93: 520–527, 2013.