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

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Submitted 15 September 2014; accepted in final form 10 March 2015

Tang L, Peng M, Liu L, Chang W, Binder HJ, Cheng SX. Calcium-sensing receptor stimulates Cl\(^-\) - and SCFA-dependent but inhibits cAMP-dependent HCO\(_3\)^{-} secretion in colon. Am J Physiol Gastrointest Liver Physiol 308: G874–G883, 2015. First published March 19, 2015; doi:10.1152/ajpgi.00341.2014.—Colonic bicarbonate (HCO\(_3\)^{-}) secretion is a well-established physiological process that is closely linked to overall fluid and electrolyte movement in the mammalian colon. These present studies show that extracellular calcium-sensing receptor (CaSR), a fundamental mechanism for sensing and regulating ionic and nutrient compositions of extracellular milieu in the small and large intestine, regulates HCO\(_3\)^{-} secretion. Basal and induced HCO\(_3\)^{-} secretory responses to CaSR agonists were determined by pH stat techniques used in conjunction with short-circuit current measurements in mucosa from rat distal colon mounted in Ussing chambers. R568, a specific CaSR activator, stimulated lumen Cl\(^-\) - and short-chain fatty acid (SCFA)-dependent HCO\(_3\)^{-} secretion but inhibited cyclic nucleotide-activated HCO\(_3\)^{-} secretion. Consequently, at physiological conditions (either at basal or during lumen acid challenge) when electroneutral Cl\(^-\)/HCO\(_3\) and SCFA/HCO\(_3\)^{-} exchangers dominate, CaSR stimulates HCO\(_3\)^{-} secretion; in contrast, in experimental conditions that stimulate fluid and HCO\(_3\)^{-} secretion, e.g., when forskolin activates electrogenic cystic fibrosis transmembrane conductance regulator-mediated HCO\(_3\)^{-} conductance, CaSR activation inhibits HCO\(_3\)^{-} secretion. Corresponding changes in J\(_{HCO3}\) (mmol·h\(^{-1}\)·cm\(^{-2}\); absence vs. presence of R568) were 0.18 ± 0.03 vs. 0.31 ± 0.08 under basal nonstimulated conditions and 1.85 ± 0.23 vs. 0.45 ± 0.06 under forskolin-stimulated conditions. Similarly, 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; such effects were abolished in CaSR-null mice. These results suggest a new paradigm for regulation of intestinal ion transport in which HCO\(_3\)^{-} secretion may be fine-tuned by CaSR in accordance with nutrient availability and state of digestion and absorption. The ability of CaSR agonists to inhibit secretagogue-induced intestinal HCO\(_3\)^{-} secretion suggests that modulation of CaSR activity may provide a new therapeutic approach to correct HCO\(_3\) deficit and metabolic acidosis (4, 31). Reducing fluid and bicarbonate (HCO\(_3\)^{-}) losses from acute diarrhea offers a major opportunity for improving child health globally.

The extracellular calcium-sensing receptor (CaSR) (7) 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 extracellular ionized calcium (Ca\(^{2+}\)), providing a mechanism for 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 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 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 adenyl cyclase leading to elevated intracellular levels of cAMP, and heat stable Escherichia coli enterotoxin StA (19), which enhances cytosolic cGMP accumulation through the guanylate cyclase C-type guanylin receptor. In the rat proximal and distal colon, activation of CaSR by R568 inhibits secretagogue-induced 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).

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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 (HCO\(_3\)^{-}) losses from acute diarrhea offers a major opportunity for improving child health globally.

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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 (Isc) measurements. Our experiments were designed in such a way that effects on basal, acid-induced, and secretagogue-induced HCO₃⁻ secretions 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 Isc 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 Communicore Animal Facility. 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 colons were 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, Isc 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 (Vᵢₒ, 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 alkalinization 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 quantitate HCO₃⁻ secretion by the mucosa. Measurements were taken at 10.220.33.4 on May 30, 2017 http://ajpgi.physiology.org/ Downloaded from

<table>
<thead>
<tr>
<th>Table 1. The composition of Ringer solutions</th>
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<td></td>
</tr>
<tr>
<td>Cl⁻</td>
</tr>
<tr>
<td>HCO₃⁻</td>
</tr>
<tr>
<td>Isethionate</td>
</tr>
<tr>
<td>Isobutyrate</td>
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<tr>
<td>SO₄⁰⁻</td>
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Compositions 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. HCO₃⁻-containing solutions were gassed with 95% O₂-5% CO₂. HCO₃⁻-free solutions were gassed with 100% O₂. All solutions contained (in mM) 140.8 Na⁺, 5.0 glucose, 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 Iₒ 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⁺]₀, while the other piece was bathed with Cl⁻-Ringer solution that contained 0.5 mM [Ca²⁺]₀. Initially, tissues were bathed, both luminally and serosally, in HCO₃⁻-free solution. After 15–30 min, when stabilization was achieved and basal lumen 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, carbachol (CCH) was added to the serosal side before 15–30 min without significantly compromising tissue responses and integrity. After 15–30 min, when stabilization had been achieved, the CaSR agonist, R568, was added to the serosal or mucosal side of tissue, and changes in Iₒ and J_{HCO₃⁻} during the 15–30-min period ensuing after the addition of the agonist were determined.

Acid-induced HCO₃⁻ secretion. Colon 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 acidification, 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 to test the effect of transport inhibitors on basal and stimulated HCO₃⁻ secretion. 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.

Chemicals and Solutions

Forskolin, 4,4'-disothiocyano-2,2'-stilbenedisulfonic acid (DIDS), glibenclamide, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 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 those solutions that did not contain HCO₃⁻. When inhibitor was used, it was added at 30 min before R568 or forskolin.

Statistical Analysis

Values are expressed as means ± SE. ΔJ_{HCO₃⁻}, ΔIₒ, 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

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<tr>
<th>Transport Inhibitor</th>
<th>ΔJ_{HCO₃⁻}, μEq·h⁻¹·cm⁻²</th>
<th>ΔIₒ, μA/cm²</th>
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<tbody>
<tr>
<td>Amiloride, 10 µM, lumen</td>
<td>0.01 ± 0.01 (3)ns</td>
<td>-6.3 ± 1.0 (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>
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<tr>
<td>DIDS, 100 µM, lumen</td>
<td>-0.08 ± 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)*</td>
<td>-10.2 ± 0.8 (3)†</td>
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Data shown are means ± SE (n). *P < 0.05; †P < 0.01; ns P > 0.05 vs. control (before treatment). DIDS, 4,4'-disothiocyano-2,2'-stilbenedisulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid.
or GraphPad Prism version 6 for Windows (GraphPad Software). \( P < 0.05 \) was considered significant.

**RESULTS**

**Baseline HCO\(_3\) Secretion**

Lowering extracellular Ca\(^{2+}\) concentration reduces and activation of CaSR by R568 enhances baseline HCO\(_3\) secretion. The initial experiments performed examined the role of extracellular Ca\(^{2+}\) in modulation of HCO\(_3\) secretion under baseline (nonstimulated) conditions. Mucosa was first bathed with HCO\(_3\)-free, Cl\(^-\) Ringer solution before 25 mM HCO\(_3\) 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 presence of a serosa-to-lumen-directed HCO\(_3\) gradient. Consistent with HCO\(_3\) secretion, subsequent addition of HCO\(_3\) ion to serosal side induced significant lumen alkalinization [Fig. 1A; means \( \pm \) SE (n) absence vs. presence of HCO\(_3\) ion: \(-0.011 \pm 0.002\) (3) vs. \(0.035 \pm 0.002\) (3) pH U·min\(^{-1}\)·cm\(^{-2}\), \( P < 0.01\)]. CCH, a known secretagogue for HCO\(_3\) 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 HCO\(_3\) secretion associated with cholinergic receptor activation (43).

Experiments were then performed to examine whether CaSR regulates basal HCO\(_3\) secretion. For this, the concentration of Ca\(^{2+}\) in the buffer was lowered from 1.2 mM to 0.5 mM, and HCO\(_3\) secretory response was compared. Extracellular Ca\(^{2+}\) is a physiological ligand of CaSR. Previous studies have shown that this maneuver reduces CaSR activity and that, at 0.5 mM Ca\(^{2+}\) concentration, CaSR is only minimally stimulated (12, 14, 16, 25). Reduction of [Ca\(^{2+}\)]\(_o\) from 1.2 mM to 0.5 mM significantly reduced lumen alkalinization rates [Fig. 1B; [Ca\(^{2+}\)]\(_o\) = 1.2 vs. 0.5 mM: 0.031 \( \pm \) 0.002 (3) vs. 0.013 \( \pm \) 0.002 (3) pH U·min\(^{-1}\)·cm\(^{-2}\), \( P < 0.01\)]. As extracellular Ca\(^{2+}\) 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 [Ca\(^{2+}\)]\(_o\) was not simply caused by HCO\(_3\) "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 Ca\(^{2+}\)-treated tissues (see details in Fig. 1 legend). Thus it is likely that extracellular Ca\(^{2+}\) (and CaSR) regulates transcellular and not paracellular HCO\(_3\) movement.

To further assess the role of CaSR as a regulator of HCO\(_3\) secretion, another set of experiments measured the rate of serosal to mucosal HCO\(_3\) flux (\(J_{HCO3}\)) and HCO\(_3\) secretory I\(_{sc}\) and tested the effect of R568, a specific pharmacological CaSR agonist. A relatively low basal \(J_{HCO3}\) was observed in the absence of R568. Addition of R568 to serosa (Fig. 1C) significantly stimulated \(J_{HCO3}\). 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 I\(_{sc}\) absence vs. presence of R568: 1.36 \( \pm \) 0.40 (5) vs. 1.48 \( \pm \) 0.44 (5) \( \mu \)eq·h\(^{-1}\)·cm\(^{-2}\), \( P > 0.05\)]. These data suggested that CaSR stimulates electroneutral HCO\(_3\) secretion.

**Acid-Induced HCO\(_3\) Secretion**

Activation of CaSR by R568 enhances acid-induced HCO\(_3\) secretion. Acid-induced HCO\(_3\) 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 HCO₃⁻ secretion.

Secretagogue-Induced HCO₃⁻ Secretion

Activation of CaSR by R568 inhibits secretagogue-induced HCO₃⁻ secretion. HCO₃⁻ secretion is markedly increased in cholera and other secretagogue-induced diarrheal diseases (22, 34). To assess whether CaSR stimulates HCO₃⁻ 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 HCO₃⁻ secretion, and HCO₃⁻ secretory response was monitored by measuring HCO₃⁻ secretory rate (JHCO₃) (Fig. 3A) and by recording Iₛₑ (Fig. 3C). Forskolin stimulated both JHCO₃ and Iₛₑ; subsequent addition of R568 did not increase but rather decreased forskolin-induced HCO₃⁻ secretion.

Cl⁻-Dependent HCO₃⁻ Secretion

Activation of CaSR stimulates Cl⁻/HCO₃⁻ exchange activity. Because the basal and acid-induced HCO₃⁻ secretion were assayed in Cl⁻-containing Ringer, it is likely that the CaSR effects reflect stimulation of a Cl⁻-dependent HCO₃⁻ secretory mechanism such as Cl⁻/HCO₃⁻ exchange (48). To address this possibility, Cl⁻/HCO₃⁻ exchange activity was measured and is shown in Fig. 4. Figure 4A demonstrates HCO₃⁻ secretory responses to the presence vs. absence of lumen Cl⁻. In agreement with previous studies (47, 48), a luminal Cl⁻-dependent HCO₃⁻ secretory mechanism (Cl⁻/HCO₃⁻ 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⁻/HCO₃⁻ exchange activity (Fig. 4B). This Cl⁻-dependent HCO₃⁻ 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).

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Fig. 2. Effect of CaSR agonist R568 on acid-induced HCO₃⁻ secretion. A: representatives 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/cm²) and Iₛₑ (μeq h⁻¹·cm⁻²) at the end of 60-min experiments were 50 ± 1.2 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 HCO₃⁻ secretion and HCO₃⁻ current. Shown are JHCO₃ (A) and Iₛₑ (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 (ΔJHCO₃) and D (ΔIₛₑ). The basal tissue resistance and Iₛₑ under these conditions were as follows: 68 ± 4 Ω/cm² 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.
whether CaSR stimulates HCO$_3^-$ secretion. Similar to the R568 effect on the Cl$^-$/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 electrolyte 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 not 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).
Fig. 6. Activation of CaSR inhibits cAMP-dependent HCO\textsubscript{3} secretion and HCO\textsubscript{3} current. Shown are J\(_{\text{HCO}_3}\) (A) and I\(_{\text{sc}}\) (C) responses to FSK (500 nM, serosa) ± R568 (10 μM, serosa), assayed in lumen Cl\textsuperscript{-}-free Ringer solution. The changes induced by FSK in the absence vs. presence of R568 are summarized in B (ΔJ\(_{\text{HCO}_3}\)/FSK) and D (ΔI\(_{\text{sc}}\)/FSK). The basal tissue resistance and I\(_{\text{sc}}\) under these conditions were 103 ± 3 Ωcm\(^2\) and 0.87 ± 0.05 μeq h\(^{-1}\) cm\(^{-2}\). Data are means ± SE of 4–7 experiments. **P < 0.01 vs. control (1st bar); ##P < 0.01 vs. FSK alone (2nd bar).

not shown). As a consequence, these results suggest that R568 inhibits cAMP-dependent, glibenclamide/GlyH-101/NPPB-sensitive electrogenic HCO\textsubscript{3} secretion.

Effect of CaSR Knockout

R568 fails to stimulate Cl\textsuperscript{-}- and SCFA-dependent and inhibit cAMP-dependent HCO\textsubscript{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\textsuperscript{-}- and SCFA-dependent HCO\textsubscript{3} secretion and inhibited cAMP-dependent HCO\textsubscript{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
effects occur via activating the CaSR in the intestinal epithelium.

**DISCUSSION**

These present results provide the basis for a new model for regulation of HCO₃⁻ secretion in the mammalian colon in that activation of CaSR by R568 stimulated basal and acid-induced HCO₃⁻ secretion, but, in contrast, R568 inhibited cyclic nucleotide-mediated HCO₃⁻ secretion. Our studies also indicate that the enhancement of HCO₃⁻ secretion is mediated via stimulation of electroneutral Cl⁻/HCO₃⁻ and SCFA/HCO₃⁻ 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 HCO₃⁻ secretory process primarily located in the crypt cells. A model for this differential regulation of colonic HCO₃⁻ 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⁻/HCO₃⁻ exchange and SCFA-dependent HCO₃⁻ 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⁻/HCO₃⁻ secretion and SCFA-dependent HCO₃⁻ secretion are most likely both surface cell functions. In contrast, CFTR-mediated HCO₃⁻ secretion is generally considered to represent a crypt cell function. Thus, in addition to mediating colonic HCO₃⁻ 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/HCO₃⁻ exchange, and NaCl absorption is the result of Cl⁻/HCO₃⁻ 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 HCO₃⁻ secretion, CaSR may function as a mechanism to enhance electrolyte and fluid absorption. Interestingly, activation of CaSR also stimulates colonic acid-induced HCO₃⁻ 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.

Consistent with a recent in vivo study in rat perfused duodenum (1), these present studies demonstrated that CaSR activation stimulated basal HCO₃⁻ secretion in ex vivo colonic mucosa. These findings may have important physiological significance, as HCO₃⁻ secretion is an integral part of mucosal defense mechanisms. HCO₃⁻ secretion is required for mucin defense mechanisms. HCO₃⁻ secretion is required for mucin secretion (see Figs. 6 and 7) and to stimulate mucous defense mechanisms. HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 HCO₃⁻ 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 antidiarrheal therapies would be of particular utility among actively growing infants and children (13).

In the present study, the net increases in Iₑ induced by forskolin (ΔIₑFSK) were greater than those in net J₅₂ (ΔJ₅₂FSK) [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.

![Cellular model of CaSR regulation of HCO₃⁻ secretion in colonocytes of rat distal colon. Top: R568 acting via CaSR causes enhancement of HCO₃⁻ secretion in surface epithelial cells by stimulation of luminal Cl⁻/HCO₃⁻ secretion via apical Cl⁻/HCO₃⁻ exchange and stimulation of luminal SCFA-dependent HCO₃⁻ secretion mediated by apical SCFA/HCO₃⁻ exchange. Bottom: CaSR reduces HCO₃⁻ secretion in the crypt epithelial cells through inhibition of a cAMP-dependent HCO₃⁻ secretory process that may involve a 5-nitro-2-(3-phenylpropylamino)benzoic acid/glibenclamide-sensitive apical anion channel such as cystic fibrosis transmembrane conductance regulator (CFTR) and/or a basolateral HCO₃⁻ entry mechanism(s). +, stimulation; -, inhibition.](http://ajpgi.physiology.org/doi/abs/10.1152/ajpgi.00341.2014?journalCode=ajpgi)
period, as all measurements were made after 15–30 min when both $I_{sc}$ and $J_{HCO_3}$ had stabilized and were in steady state. The most likely explanation is that a component of $\Delta I_{sc}^{FSK}$ 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 ~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 colonocytes (41). Consistent with this, we found that the non-HCO3− portion of $\Delta I_{sc}^{FSK}$ 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 Ca2+ to minimize background activation of the receptor before R568 addition, under normal Ca2+ 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 Ca2+ condition in the serum can be either <1.0 mM (secondary hyperparathyroidism) or >1.5 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., lumen 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 electrogenic 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.

ACKNOWLEDGMENTS

We extend our gratitude to Sadasivan Vidyasagar, Liangjie Yin, and Xiangrong Sun for technical support on pH stat measurements, Megan McIntyre, Tao Huang, Peng Yi, and Sunan Donepudi for technical assistance on short-circuit current recordings, and Svea Cheng for artistic assistance in figure design.

GRANTS

This work was supported by Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health K08HD079674 (S. Cheng), Children’s Digestive Health and Nutrition Foundation award 00102979 (S. Cheng), and Children’s Miracle Network (S. Cheng). S. Cheng was a recipient of the North American Society of Pediatric Gastroenterology, Hepatology, and Nutrition Foundation’s “Fellow to Faculty Transition Award in Inflammatory Bowel Disease” 2012-2013.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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