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Molecular Mechanisms of Calcium-sensing Receptor-mediated Calcium Signaling in the Modulation of Epithelial Ion Transport and Bicarbonate Secretion*

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Background: Calcium-sensing receptor (CaSR) plays a critical role in the regulation of epithelial ion transport.
Results: CaSR activators induce Ca^{2+} signaling and duodenal bicarbonate secretion (DBS).
Conclusion: CaSR triggers Ca^{2+}-dependent DBS, likely through receptor-operated channels, intermediate conductance Ca^{2+}-activated K^+ channels, and the cystic fibrosis transmembrane conductance regulator.
Significance: Dietary CaSR activators may modulate the physiological process of DBS that is critical for duodenal mucosal protection.

Epithelial ion transport is mainly under the control of intracellular cAMP and Ca^{2+} signaling. Although the molecular mechanisms of cAMP-induced epithelial ion secretion are well defined, those induced by Ca^{2+} signaling remain poorly understood. Because calcium-sensing receptor (CaSR) activation results in an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) but a decrease in cAMP levels, it is a suitable receptor for elucidating the mechanisms of $[Ca^{2+}]_{cyt}$-mediated epithelial ion transport and duodenal bicarbonate secretion (DBS). CaSR proteins have been detected in mouse duodenal mucosae and human intestinal epithelial cells. Spermine and Gd^{3+}, two CaSR activators, markedly stimulated DBS without altering duodenal short circuit currents in wild-type mice but did not affect DBS and duodenal short circuit currents in cystic fibrosis transmembrane conductance regulator (CFTR) knockout mice. Clotrimazole, a selective blocker of intermediate conductance Ca^{2+}-activated K^+ channels but not chromanol 293B, a selective blocker of cAMP-activated K^+ channels (KCNQ1), significantly inhibited CaSR activator-induced DBS, which was similar in wild-type and KCNQ1 knockout mice. HCO_3^- fluxes across epithelial cells were activated by a CFTR activator, but blocked by a CFTR inhibitor. CaSR activators induced HCO_3^- fluxes, which were inhibited by a receptor-operated channel (ROC) blocker. Moreover, CaSR activators dose-dependently raised cellular $[Ca^{2+}]_{cyt}$, which was abolished in Ca^{2+}-free solutions and inhibited markedly by selective CaSR antagonist calhex 231, and ROC blocker in both animal and human intestinal epithelial cells. Taken together, CaSR activation triggers Ca^{2+}-dependent DBS, likely through the ROC, intermediate conductance Ca^{2+}-activated K^+ channels, and CFTR channels. This study not only reveals that $[Ca^{2+}]_{cyt}$ signaling is critical to modulate DBS but also provides novel insights into the molecular mechanisms of CaSR-mediated Ca^{2+}-induced DBS.

Cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$)[4] plays an essential role in a variety of mammalian cells through the regulation of many biological functions, including neurotransmitter release, muscle contraction, gene regulation, cell proliferation, and apoptosis (1). Therefore, dysregulation of $[Ca^{2+}]_{cyt}$ homeostasis may result in pathological changes in many systems. Under physiological conditions, various mechanisms are controlling Ca^{2+} homeostasis in the human body, one of which is the calcium-sensing receptor (CaSR) (2). The CaSR is a plasma membrane protein initially cloned from bovine parathyroid cells. It is a member of the G protein-coupled receptor family and regulates the synthesis of parathyroid hormone in response to changes in serum Ca^{2+} concentrations (3–5).

CaSR activation elicits complex intracellular signaling events through the modulation of a wide range of intracellular mediators, including $G\alpha_{q/11}$ proteins and phospholipase C (PLC). These, in turn, stimulate both inositol trisphosphate production and PKC activation, which increases $[Ca^{2+}]_{cyt}$ (4, 5). Activation of the CaSR has been shown to increase $[Ca^{2+}]_{cyt}$ in different types of mammalian cells, especially in parathyroid cells, epithelial cells, osteocytes, cardiomyocytes, and smooth

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[4] The abbreviations used are: $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration; CaSR, calcium-sensing receptor; PLC, phospholipase C; $[Ca^{2+}]_o$, extracellular Ca^{2+}; DBS, duodenal bicarbonate secretion; ROC, receptor-operated channel; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel(s); CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc}, short circuit current; 2-APB, 2-aminoethoxydiphenyl borate; 5-HT, 5-hydroxytryptamine.

muscle cells (4, 5). In addition, CaSR activation can stimulate $G\alpha_i$ proteins and phosphodiesterase, leading to a decrease in cyclic AMP and cyclic GMP levels (4, 5).

It has been demonstrated that the CaSR is expressed along the entire gastrointestinal tract and plays a critical role in normal gut physiology (6). Recent studies have been mainly performed on its functions in modulating gastrin and gastric acid secretion and intestinal fluid and electrolyte transports (6–9). Extracellular Ca^{2+} ($[Ca^{2+}]_o$) stimulates gastric acid and bicarbonate secretion in the guinea pig (10, 11), suggesting that gastric surface epithelial cells are capable of sensing changes in Ca^{2+} to modulate gastric secretion, likely through CaSR activation. Although it is well documented that the CaSR inhibits intestinal transepithelial Cl^- secretion by blocking cyclic AMP signaling (7), little is known about the role of the CaSR in intestinal transepithelial HCO_3^- secretion, which is a critical factor in duodenal mucosal protection and mainly under the control of cyclic AMP and Ca^{2+} signaling. Although the physiological roles and molecular mechanisms of cyclic AMP-induced HCO_3^- secretion are relatively well defined, those induced by Ca^{2+} signaling remain poorly understood in most epithelia, especially in intestinal epithelia (12). Moreover, although it is known that Ca^{2+} signaling is critical for duodenal bicarbonate secretion (DBS), the molecular mechanisms controlling $[Ca^{2+}]_{cyt}$ homeostasis in duodenal epithelial cells are poorly understood.

In our previous studies, we proposed that Ca^{2+} and cyclic AMP signaling may play different roles in the regulation of intestinal transepithelial HCO_3^- and Cl^- secretion. We found that although cyclic AMP plays a major role in intestinal Cl^- secretion, Ca^{2+} signaling may be critical for transepithelial HCO_3^- secretion (13). However, activation of most well defined receptors expressed in intestinal epithelial cells usually increase both $[Ca^{2+}]_{cyt}$ and intracellular cyclic AMP levels, making it difficult to distinguish between $[Ca^{2+}]_{cyt}$- and cyclic AMP-regulated epithelial ion transports. Because CaSR activation results in an increase in $[Ca^{2+}]_{cyt}$ but a decrease in intracellular cyclic AMP levels (3–5), we hypothesized that the CaSR is a suitable receptor system for further delineating the role of $[Ca^{2+}]_{cyt}$- and cAMP-mediated intestinal epithelial ion transports in general and HCO_3^- secretion in particular.

Therefore, in this study, we sought to investigate CaSR modulation of $[Ca^{2+}]_{cyt}$-mediated DBS and the underlying mechanisms. We found that CaSR activation triggers Ca^{2+}-dependent duodenal transepithelial HCO_3^- secretion, likely through the receptor-operated channels (ROCs), the intermediate-conductance Ca^{2+}-activated K^+ channels (IK_{Ca}), and the cystic fibrosis transmembrane conductance regulator (CFTR) channels. This study not only reveals that $[Ca^{2+}]_{cyt}$ signaling is critical to modulate DBS but also provides novel insights into the underlying molecular mechanisms of CaSR-induced Ca^{2+}-dependent DBS.

EXPERIMENTAL PROCEDURES

Animal Study—The animal use protocol was approved by the University of California San Diego Committee on Investigations Involving Animal Subjects. All experiments were performed with adult Harlan C-57 black mice; homozygous CFTR knockout ($CFTR^{-/-}$) mice and their wild-type littermates ($CFTR^{+/+}$), which were established as described previously (13); and mice deficient in KCNQ1 ($kcnq1^{-/-}$) and their wild-type littermates ($kcnq1^{+/+}$), which were generated as described earlier (14).

Ussing Chamber Experiments in Vitro—The proximal duodenum removed from mice was immediately placed in ice-cold iso-osmolar mannitol with indomethacin (10 μM) solution. The duodenal tissue was stripped of seromuscular layers and then mounted in the Ussing chambers (window area, 0.1 cm^2). Experiments were performed under continuous short-circuited conditions (voltage current clamp, VCC 600, Physiologic Instruments, San Diego, CA), and luminal pH was maintained at 7.40 by the continuous infusion of 5 mM HCl under the automatic control of a pH-stat system (ETS 822, Radiometer America, Westlake, OH). Duodenal short circuit currents (I_{sc}) and HCO_3^- secretion were measured simultaneously as described previously (15). The rate of luminal bicarbonate secretion is expressed as micromolar per square centimeter per hour. The I_{sc} was measured in microamperes and converted into μEq per square centimeters per hour. After basal parameters were measured for a 30-min period, CaSR activators were added to both the mucosal and serosal sides of the Ussing chamber because the CaSR has been identified on both the apical and basolateral membranes of epithelial cells (7, 16). In some experiments, tissues were treated with inhibitors for 10 min after the baseline recording, followed by addition of CaSR activators. Electrophysiological parameters and bicarbonate secretion were recorded for a total of 90 min. During this experimental period, the vehicle did not significantly change I_{sc} and HCO_3^- secretion, as shown in our previous control experiments (15). The mucosal solution used in Ussing chamber experiments contained the following: 140 mM Na^+, 5.4 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 120 mM Cl^-, 25 mM gluconate, and 10 mM mannitol. The serosal solution contained the following: 140 mM Na^+, 5.4 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 120 mM Cl^-, 25 mM HCO_3^-, 2.4 mM HPO_4^{2-}, 2.4 mM $H_2PO_4^{2-}$, 10 mM glucose, and 0.01 mM indomethacin. The osmolalities for both solutions were ~300 mosmol/kg of H_2O.

Epithelial Cell Culture—As described previously (17, 18), SCBN, a duodenal epithelial cell line of canine origin (19), and Caco-2 and HEK-293 cells, human epithelial cell lines, were fed with fresh DMEM supplemented with 10% fetal bovine serum, L-glutamine, and streptomycin every 2–3 days. SW-480, a human colon cancer cell line, was fed with fresh L15 supplemented with 10% fetal bovine serum and streptomycin. After the cells had grown to confluence, they were replated onto 12-mm round coverslips (Warner Instruments Inc., Hamden, CT) and incubated for at least 24 h before use for $[Ca^{2+}]_{cyt}$ and pH_i measurements.

Measurement of $[Ca^{2+}]_{cyt}$ in Epithelial Cells by Digital Ca^{2+} Imaging—$[Ca^{2+}]_{cyt}$ levels in epithelial cells were measured by digital Ca^{2+} imaging as described previously (20). Cells grown on coverslips were loaded with 5 μM Fura-2/AM in physiological salt solution, described below, at room temperature (~22 °C) for 50 min and then washed for 30 min. Thereafter, the coverslips with epithelial cells were mounted in a perfusion chamber on a Nikon microscope stage (Nikon Corp., Tokyo,

Japan). The ratio of Fura-2/AM fluorescence with excitation at 340 or 380 nm ($F_{340/380}$) was followed over time and captured using an intensified charge-coupled device camera (ICCD200) and a MetaFluor imaging system (Universal Imaging Corp., Downingtown, PA). The physiological salt solution used in digital Ca^{2+} measurement contained the following: 140 mM Na^+, 5 mM K^+, 2 mM Ca^{2+}, 147 mM Cl^{2-}, 10 mM Hepes, and 10 mM glucose (pH 7.4). For the Ca^{2+}-free solution, Ca^{2+} was omitted, but 0.5 mM EGTA was added. The osmolality for all solutions was ~300 mosmol/kg of H_2O.

Measurement of HCO_3^- Fluxes in SCBN Cells—pH_i measurements in SCBN cells were applied as described previously (21). Briefly, cells grown on coverslips were incubated with 2 μM 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein-AM in physiological salt solution, described above, for 30 min at room temperature and then washed for 30 min. The ratio of 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein fluorescence with excitation at 495 or 440 nm ($F_{495/440}$) was captured using an intensified charge-coupled device camera and a MetaFluor imaging system. The NaCl/HCO_3^- solutions contained the following: 120 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 10 mM glucose equilibrated with 5% CO_2/95% O_2 (pH 7.4). In Na^+-free (Na^+-free/HCO_3^-) solutions, Na^+ was replaced with *N*-methyl-D-glucamine. In HCO_3^--free solutions, $NaHCO_3$ was replaced with NaCl (in NaCl/Hepes solution) or with *N*-methyl-D-glucamine (in Na^+-free/Hepes solution). In experiments in which cells were acidified, 30 mM NH_4C1 replaced an equal amount of *N*-methyl-D-glucamine. The ratio of the 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein fluorescence was calibrated in terms of pH_i by incubating the cells in a high K^+ solution (KCl replaced NaCl) and then permeabilizing the cells with 10 μM nigericin. Then the pH of the bathing solution was stepped between pH 6.3 and 7.4. The $F_{495/440}$ was linear over this pH range. Cells were first perfused with either the NaCl/HCO_3^- or NaCl/Hepes solution in the chamber for 15 min to allow the pH_i to stabilize. Then the cells were switched to Na^+-free/HCO_3^- or Na^+-free/Hepes for 5 min to remove Na^+ from the cells. The cells were then treated with the NH_4-containing solution for 5 min, and when the NH_4-containing solution was removed, cells were acidified to pH_i 6.0–6.5. Rates of pH_i recovery after treatment with drugs were calculated by linear regression analysis between pH 6.0 and 6.5.

Western Blot Analysis—The specific anti-CaSR antibody used in this study is an affinity-purified monoclonal antibody raised against a synthetic peptide corresponding to the extracellular domain (residues 214–235) of the human CaSR (Labome, Princeton, NJ). Its cross-reactivity with rodents, specificity, and applications have been described previously (7, 22). A Western blot analysis of mouse duodenal mucosae and intestinal epithelial cells was applied as described previously (15). PVDF membranes (Millipore Corp., Billerica, MA) with resolved proteins were incubated with the anti-CaSR antibody or GAPDH (1:5000, Ambion, Austin, TX). After washing with PBS plus 1% Tween (PBST), the rabbit anti-mouse secondary antibody was applied to the membranes, which were treated with a chemiluminescent solution (Fivephoton Biochemicals, San Diego, CA) and then exposed to x-ray film. Densitometric analysis of the blots was performed with the use of an AlphaImager digital imaging system (Alpha Innotech, San Leandro, CA).

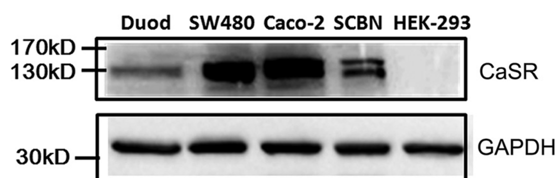

FIGURE 1. **Protein expression of the CaSR in mouse duodenal mucosa and human epithelial cells.** After mouse duodenal mucosal tissues (*Duod*), SCBN cells, SW-480 cells, and Caco-2 cells (human colonic epithelial cells), and HEK-293 cells (human epithelial cells used as a negative control) were lysed, Western blot analysis was performed to detect protein expression of the CaSR using a specific anti-CaSR monoclonal antibody. GAPDH was used as a loading control. These data are representative of three experiments with similar results.

Immunohistochemistry—Immunohistochemistry was carried out as described previously (7). Briefly, the slides with duodenal tissues from C-57 mice or with intestinal epithelial cells were incubated with an anti-CaSR monoclonal antibody (1:100 dilution, Labome). The primary antibodies were detected with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA) secondary antibodies. Immunoreactivity was detected using a horseradish peroxidase (3′-,3′-diaminobenzidine) kit (BioGenex, San Francisco, CA) followed by counterstaining with hematoxylin, dehydration, and mounting. Slides were then examined with a Nikon Eclipse 800 Research microscope. To demonstrate the CaSR specificity of the antibody labeling, a control experiment was performed in which the primary antibody was omitted. All incubations were performed at room temperature.

Chemicals and Solutions—Spermine, U-73122, genistein, $GdCl_3$, clotrimazole, and $CFTR_{inh}$-172 were purchased from Sigma. 2-Aminoethoxydiphenyl borate (2-APB) and chromanol 293B were purchased from Tocris Bioscience (Ellisville, MO). Fura-2/AM and 2′,7′-bis(2-carboxyethyl)-5-(and -6)carboxyfluorescein were from Invitrogen. Anti-CaSR monoclonal antibody (catalog no. MA1–934, a mouse mAb) was from Labome. The other chemicals were obtained from Fisher Scientific (Santa Clara, CA).

Statistical Analysis—Results are expressed as mean ± S.E. Differences between means were considered to be statistically significant at $p < 0.05$ using Student's *t* test or one-way analysis of variance followed by Newman-Keuls post hoc test, as appropriate.

RESULTS

Protein Expression of the CaSR in Mouse Duodenum Mucosal Tissues—To examine CaSR expression in mouse duodenum mucosa, both Western blot and immunohistochemistry analyses were performed. As shown in our Western blot analysis (Fig. 1), the antibody identified a significant band at ~120–130 kDa in lysates of mouse duodenum mucosal tissues, indicating protein expression of the CaSR (8, 23). Fig. 2*A* shows typical villous crypt structures of mouse duodenum mucosa with H&E staining. Fig. 2*B* shows representative images of CaSR immunohistochemistry in duodenum mucosa. Intense CaSR immunoreactivity (*brown*) was noted on both apical and basolateral

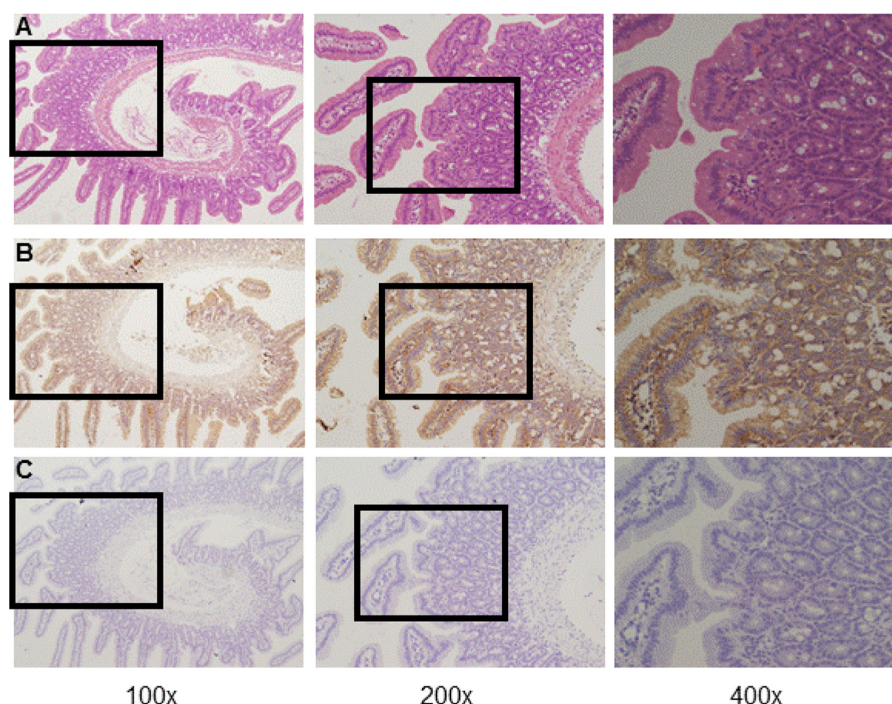

FIGURE 2. **Immunohistochemistry on sections obtained from mouse duodenal mucosal tissues.** *A*, H&E staining of mouse duodenal mucosa showing normal morphology and typical villous crypt structures. *B*, representative immunoreactivity of CaSR proteins (*brown*) in the villus and crypts of the duodenal mucosa at different magnifications. *C*, representative immunoreactivity without incubation of primary anti-CaSR antibody in the villus and crypts of duodenal mucosa as a negative control. Magnifications are ×100, ×200, and ×400 in the *left*, *center*, and *right panels*, respectively. These data are representative of at least three experiments with similar results.

membranes of the villous and crypt epithelial cells (Fig. 2*B*, *right panel*). However, no specific signal for the CaSR was observed when CaSR primary antibody was omitted (Fig. 2*C*). Therefore, cellular distribution and location of the CaSR in mouse duodenum mucosa was detected by immunohistochemistry.

Protein Expression of the CaSR in Intestinal Epithelial Cells—To examine CaSR expression in intestinal epithelial cells, both Western blot and immunohistochemistry analyses were performed on SCBN, SW-480, and Caco-2 cells, two human intestinal epithelial cell lines commonly used in the literature for physiological and pathological studies of intestinal ion transports. As shown by the Western blot analysis in Fig. 1, the antibody identified a strong band at ~120–130 kDa in both SW480 and Caco-2 cells. However, the antibody identified one band of ~120–130 kDa and another band of ~140–150 kDa in the SCBN cell line, which is similar to previous reports (8, 23), indicating CaSR protein expression in duodenal epithelial cells. However, our Western blot results show that the expression of CaSR protein is severalfold higher in human intestinal epithelial cells than in mouse duodenum mucosal tissues (Fig. 1), suggesting a higher CaSR expression in pure epithelial cells than in mucosal tissues that contain various cell types. To rule out the possible nonspecific staining of the CaSR in the tissues and cell lines, we also used parental HEK-293 cells as negative controls. Indeed, the antibody did not detect any CaSR expression in these cells (Fig. 1).

Fig. 3 shows representative images of CaSR immunocytochemistry in these epithelial cells. Intense CaSR immunoreactivity was noted in intestinal epithelial SCBN, SW-480, and Caco-2 cells (Fig. 3, *A*, *C*, and *E*) but not in epithelial HEK-293 cells (Fig. 3*G*). No specific signal for the CaSR was observed when the CaSR primary antibody was omitted (Fig. 3, *B*, *D*, *F*, and *H*). Therefore, by immunocytochemistry, the CaSR was verified in intestinal epithelial cells, which is consistent with its presence in the epithelial cells of rat duodenum mucosa (24).

Role of the CaSR in Regulating Duodenal HCO_3^- Secretion and I_{sc}—The CaSR has been functionally demonstrated along the entire gastrointestinal epithelium, where it plays an important role in the regulation of gastric acid and intestinal Cl^- secretion. Therefore, in our initial studies, Ussing chamber experiments were conducted to test whether the CaSR is involved in duodenal mucosal ion transports, especially DBS. Because it is now evident that CFTR channels are essential for transepithelial HCO_3^- and Cl^- secretion in most gastrointestinal epithelia (25, 26), both CFTR knockout and wild-type mice were used to test whether CaSR activation can modulate duodenal I_{sc} and HCO_3^- secretion. After basal I_{sc} and HCO_3^- secretion were recorded for 30 min, two commonly used CaSR activators, spermine (1 mM) and Gd^{3+} (0.5 mM), were added to both sides of the tissues because the CaSR is not restricted to one side of epithelial cells (7, 16). As shown in Fig. 4, *A* and *B*, in both CFTR knockout and wild-type mice, spermine and Gd^{3+} did not significantly affect duodenal basal I_{sc} ($p > 0.05$, $n = 6$). The net peak HCO_3^- secretion, calculated as the difference between the baseline and the peak value at 10 min, was used to describe the CaSR-activated HCO_3^- secretion. As shown in Fig. 4, *C* and *D*, both spermine and Gd^{3+} markedly stimulated DBS in wild-type mice ($p < 0.01$, $n = 6$), which was inhibited significantly by U73122 (10 μM), a selective PLC inhibitor ($p < 0.01$, $n = 6$). However spermine and Gd^{3+} did not stimulate DBS in CFTR knockout mice (not significant, $n = 6$). Therefore, these data

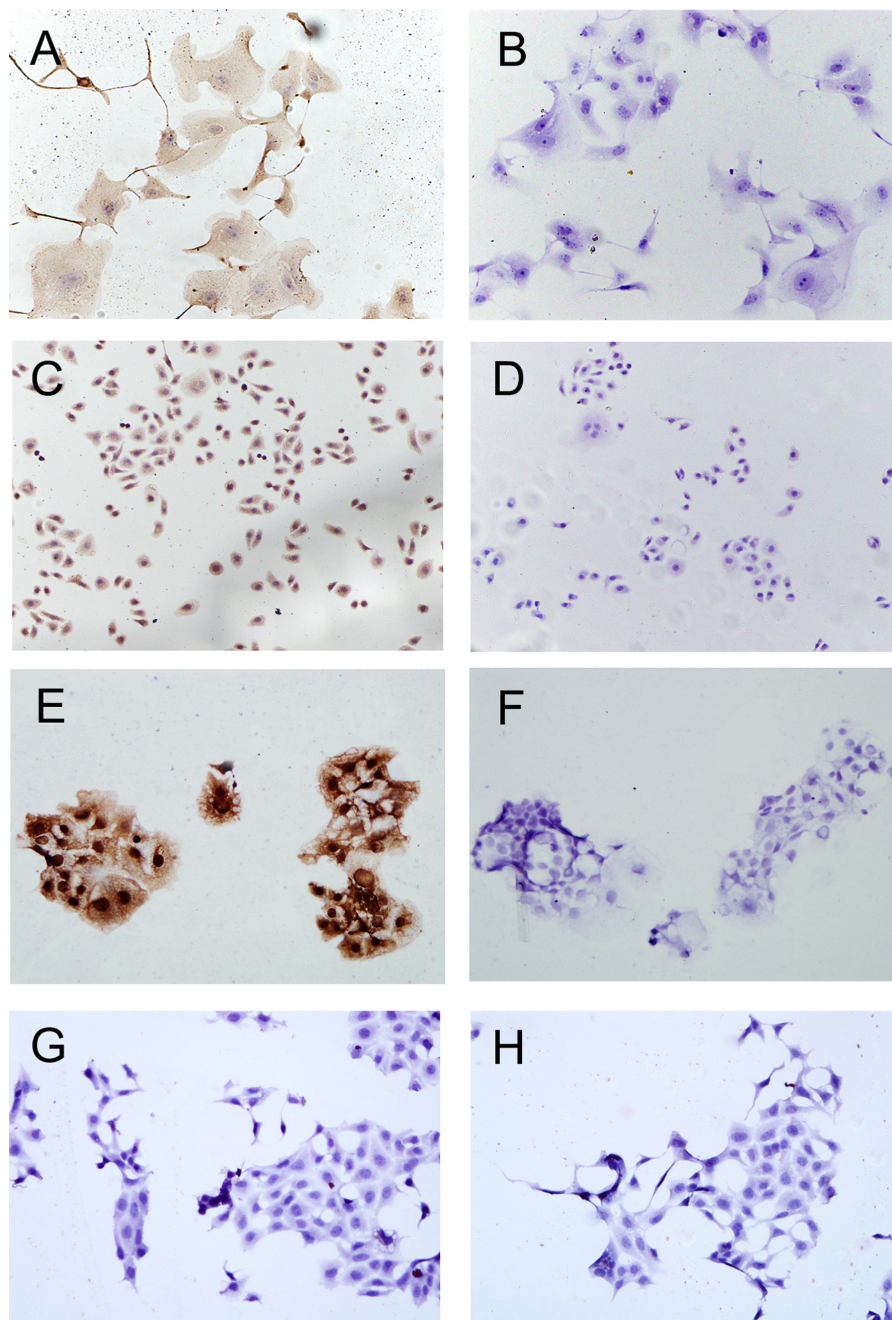

FIGURE 3. **Immunohistochemistry of CaSR proteins on intestinal epithelial cells.** *A* and *B*, representative immunoreactivity of CaSR proteins with (*A*) or without (*B*) primary anti-CaSR antibody in SCBN cells. *C* and *D*, representative immunoreactivity of CaSR proteins with (*C*) or without (*D*) primary anti-CaSR antibody in human colonic epithelial SW-480 cells. *E* and *F*, representative immunoreactivity of CaSR proteins with (*E*) or without (*F*) primary anti-CaSR antibody in human colonic epithelial Caco-2 cells. *G*, representative immunoreactivity with primary anti-CaSR antibody in human epithelial HEK-293 cells as a negative control. Magnification is ×100 for all images. These data are representative of at least three experiments with similar results.

suggest that CaSR activation selectively stimulates DBS through the PLC pathway and CFTR channels.

Involvement of ROC and IK_{Ca} in CaSR-mediated HCO_3^- Secretion—It is well known that cation channels are expressed in intestinal epithelia and that Ca^{2+} signaling is critical to modulate epithelial ion transport, likely through the activation of intermediate Ca^{2+}-activated K^+ channels (IK_{Ca} or KCNN4) (15). We further examined whether ROC and IK_{Ca} are involved in CaSR-mediated DBS. As shown in Fig. 5*A*, chromanol 293B (10 μM), a selective blocker of cAMP-activated K^+ channels (KCNQ1) (27–29), did not significantly affect spermine-induced net peak DBS (not significant, $n = 6$). However, clotrimazole (30 μM), a selective blocker of IK_{Ca} (30, 31), markedly inhibited spermine-induced net peak DBS ($p < 0.01$, $n = 6$). 2-APB (100 μM), a commonly used blocker of ROC (32), also markedly inhibited spermine-induced net peak DBS ($p < 0.01$, $n = 6$) (Fig. 5*A*). Moreover, when spermine-induced net peak DBS were compared between KCNQ1 knockout and wild-type mice, no significant differences were found between these two types of mice (NS, $n = 6$) (Fig. 5*B*). Again, spermine (1 mM) did not significantly affect the basal duodenal I_{sc} of both KCNQ1 knockout and wild-type mice (NS, $n = 6$) (Fig. 5*C*). Therefore, our

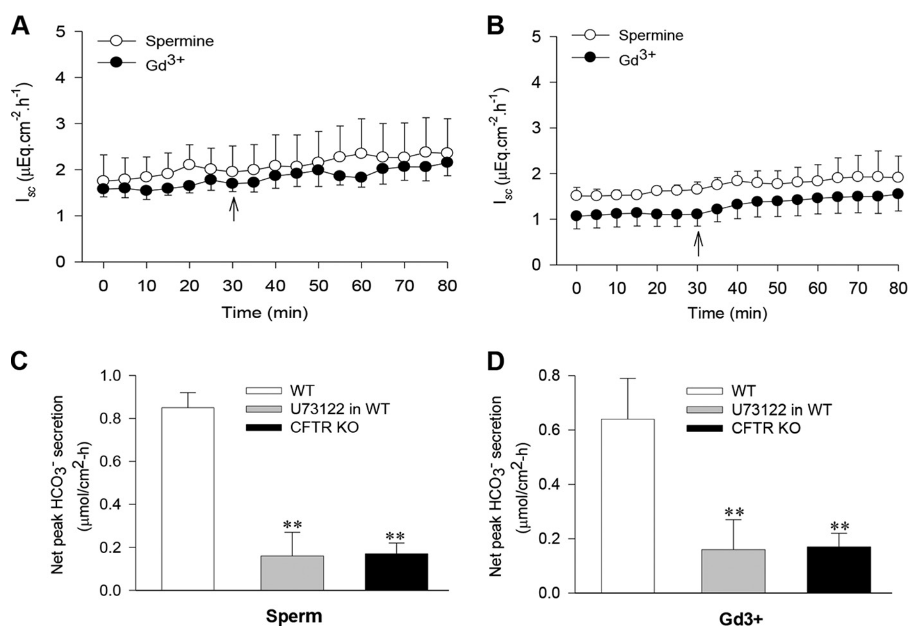

FIGURE 4. **Effects of different CaSR activators on duodenal I_{sc} and DBS in CFTR KO and WT mice.** *A*, time course of duodenal I_{sc} in wild type mice after addition of spermine (1 mM) or Gd^{3+} (0.5 mM) to Ussing chambers as indicated by the *arrow*. *B*, time course of duodenal I_{sc} in CFTR knockout mice after addition of spermine or Gd^3 to Ussing chambers as indicated by the *arrow*. *C*, spermine-stimulated net peak DBS in the presence or the absence of U73122 (10 μM) in wild-type mice and in CFTR knockout mice. *D*, Gd^{3+}-stimulated net peak DBS in the presence or the absence of U73122 in wild-type mice and in CFTR knockout mice. Values are expressed as mean ± S.E. for five to six experiments. **, $p < 0.01$ *versus* each activator-stimulated net peak DBS in WT mice.

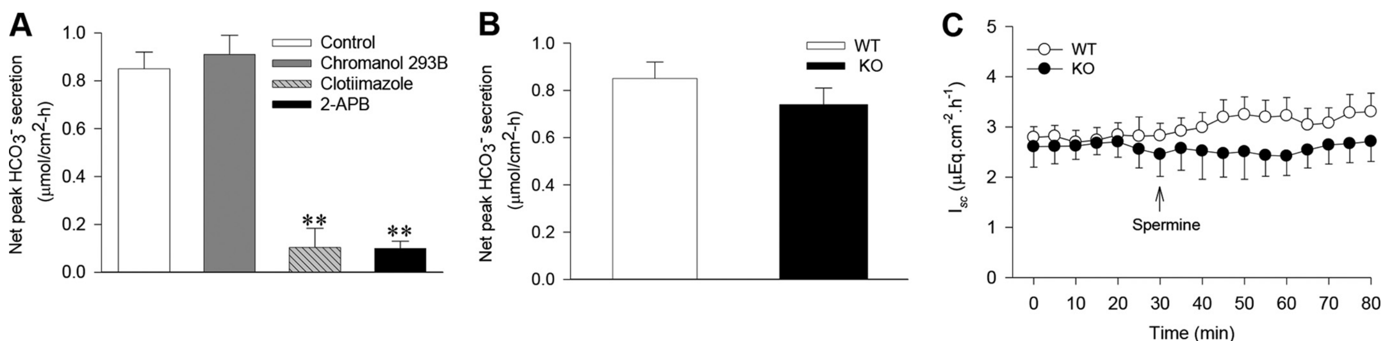

FIGURE 5. **Effects of spermine and ion channel blockers on duodenal I_{sc} and DBS in KCNQ1 KO and WT mice.** *A*, spermine-stimulated net peak DBS in the presence or the absence of chromanol 293B (10 μM), clotrimazole (30 μM), or 2-APB (100 μm) in wild-type mice. *B*, comparison of spermine-stimulated net peak DBS in KCNQ1 knockout and wild-type mice. *C*, comparison of the time course of duodenal I_{sc} in KCNQ1 knockout and wild-type mice after addition of spermine to Ussing chambers as indicated by the *arrow*. Spermine was used at 1 mM in *A–C*. Values are expressed as mean ± S.E. for six experiments. **, $p < 0.01$ *versus* the control (spermine-stimulated net peak DBS in wild-type mice).

data indicate that Ca^{2+} signaling, ROC, and IK_{Ca}, but not cAMP signaling and KCNQ1, are involved in CaSR-mediated DBS.

CaSR Activation-induced HCO_3^- Fluxes across SCBN Cells—Because expression and function of CFTR channels has been well established in SCBN cells (18), they are commonly used for studies of small intestinal epithelial ion transports (18, 33, 34). We first tested the role of CFTR in HCO_3^- fluxes in SCBN cells. To this end, cells were treated with NH_4Cl in Na^+-free/HCO_3^- solution that caused the pH_i first to increase (because of the entry of the weak base NH_3) and then to decrease when the NH_4 was washed from the bath. The cells remained acidic in the Na^+-free/HCO_3^- solution, in which the pH_i was kept relatively stable but recovered when cells were returned to NaCl/HCO_3^- solution (Fig. 6*A*), likely because of the operation of Na^+/H^+ exchange and other Na^+- and HCO_3^--dependent pH_i regulators. To test for the ability of HCO_3^- to permeate though CFTR, genistein (50 μM), a commonly used CFTR activator (35), was added to cells that were acidified in Na^+-free/HCO_3^- solution. We observed that pH_i quickly recovered, and further recovery occurred after adding back NaCl/HCO_3^- solution (Fig. 6*B*). To examine whether genistein indeed activates HCO_3^- fluxes through the CFTR, cells were pretreated with $CFTR_{inh}$-173 (10 μM), a commonly used CFTR blocker (35), which reversed genistein-induced pH_i recovery in Na^+-free/HCO_3^- solution (Fig. 6*D*). To test whether the genistein-activated, Na^+-independent recovery of pH_i was HCO_3^--dependent, these experiments were also repeated in Na^+-free and HCO_3^--free Hepes-buffered solutions in which the acidified cells responded to genistein with only a slight effect on pH_i, but a sustained pH_i recovery occurred when Na^+ was present in the Hepes solution (data not shown). Together, these findings are consistent with genistein-regulated HCO_3^- fluxes through the CFTR in the presence of extracellular HCO_3^-.

To test for the role of the CaSR in modulating HCO_3^- fluxes through the CFTR, similar experiments were performed with spermine (1 mM), which was added to cells acidified in Na^+-

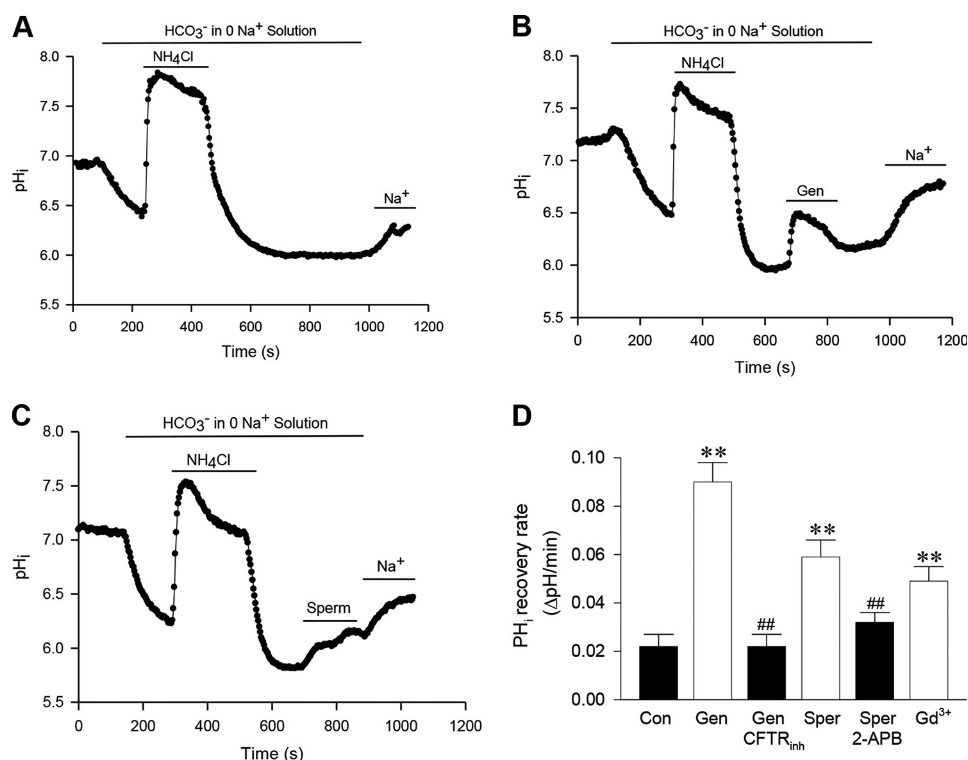

FIGURE 6. **Effects of different CaSR modulators and ion channel blockers on HCO_3^- fluxes in SCBN cells.** *A*, control time course of pH_i changes induced by NH_4Cl in Na^+-free/HCO_3^- solution. Treatment of cells with 30 mM NH_4Cl in the solution caused the pH_i first to increase and then to decrease when the NH_4Cl was washed out. The cells remained acidic, and the pH_i was relatively stable in Na^+-free/HCO_3^- solution, but the pH_i began to recover when the cells were returned to NaCl/HCO_3^- solution (Na^+). *B*, genistein-induced HCO_3^- fluxes through CFTR channels. The time course of pH_i changes in SCBN cells was similar to the control in *A*. However, after the NH_4Cl was washed out, genistein (*Gen*, 50 μM) was added to the cells acidified in Na^+-free/HCO_3^- solution, and the pH_i began to recover, but further recovery occurred after adding back NaCl/HCO solution (Na^+). *C*, spermine-induced HCO_3^- fluxes through CFTR channels. The time course of pH_i changes was similar to *B*, but spermine (*Sper*, 1 mM) was added to the cells acidified in Na^+-free/HCO_3^- solution. *D*, summary data showing the effects of different CaSR modulators and ion channel blockers on HCO_3^- fluxes in SCBN cells. $CFTR_{inh}$-173 (10 μM), 2-APB (100 μM), and Gd^{3+} (0.5 mM) were applied to the experiments. Values are expressed as mean ± S.E. of 40–50 cells for each group. **, $p < 0.01$ *versus* control (*Con*) in *A*; ##, $p < 0.01$ *versus* their corresponding activators in *B* and *C*.

free/HCO_3^- solution. As shown in Fig. 6*C*, spermine, like genistein, induced pH_{ii} recovery, which was reversed by 2-APB (100 μM). Similarly, Gd^{3+} (0.5 mM) induced pH_i recovery in Na^+-free/HCO_3^- solution (Fig. 6*D*). However, spermine and Gd^{3+} did not induce a significant pH_i recovery in Na^+-free, Hepes-buffered solutions (data not shown). These data from single cell studies are in agreement with those from a duodenal tissue study, indicating that ROC and CFTR channels are involved in CaSR-mediated transepithelial HCO_3^- secretion.

CaSR Activation Induces Ca^{2+} Signaling in Epithelial Cells—It is well documented that CaSR activation inhibits the intracellular cyclic AMP pathway in intestinal epithelial cells. However, little is known about Ca^{2+} signaling downstream of CaSR activation in these cells. In addition, although it is known that Ca^{2+} signaling is a critical regulator for DBS, so far Ca^{2+} signaling in duodenal epithelial cells is poorly understood. We therefore monitored $[Ca^{2+}]_{cyt}$ changes in epithelial cells stimulated with different CaSR activators.

Following a short exposure to Ca^{2+}-free solutions for 3 min, cells were superfused with different concentrations of $[Ca^{2+}]_o$ (1.0–4.0 mM), which are close to the EC_{50} (~2.0 mM) for CaSR activation (23). Although $[Ca^{2+}]_o$ at 1.0 mM did not affect basal $[Ca^{2+}]_{cyt}$, significant increases were seen when $[Ca^{2+}]_o$ increased to 4.0 mM (Fig. 7, *A* and *B*). Although $[Ca^{2+}]_o$ is an endogenous CaSR activator, it may enter healthy cells through the store-operated Ca^{2+} entry pathway (32, 36) or may even directly leak into unhealthy cells. We also used the CaSR activator spermine and found that it dose-dependently increased $[Ca^{2+}]_{cyt}$ in SCBN cells (Fig. 7, *C* and *D*). Moreover, both $[Ca^{2+}]_o$- and spermine-induced $[Ca^{2+}]_{cyt}$ signaling was inhibited markedly by calhex 231 (3 μM), a selective CaSR antagonist (Fig. 7, *E* and *F*). These results provide direct evidence for the CaSR-mediated increase in $[Ca^{2+}]_{cyt}$ in duodenal epithelial cells.

The CaSR is a member of the G protein-coupled receptor family, and its activation mobilizes different Ca^{2+} sources in different cell types (4, 5). Therefore, we sought to elucidate the mechanisms of $[Ca^{2+}]_{cyt}$ mobilization by CaSR activation in SCBN cells. To test whether ROCs are involved in CaSR activation, cells were superfused with spermine (3 mM) in the presence or the absence of 2 mM $[Ca^{2+}]_o$. As shown in Fig. 8*A*, spermine induced a significant increase in $[Ca^{2+}]_{cyt}$ in $[Ca^{2+}]_0$-containing solutions, but not in $[Ca^{2+}]_0$-free solution. We further examined CaSR activation-mediated $[Ca^{2+}]_0$ entry mechanisms in SCBN cells. Spermine (3–10 mM) significantly elevated $[Ca^{2+}]_{cyt}$ in $[Ca^{2+}]_0$-containing solutions (Fig. 7, *C* and *D*). However, treatment with 2-APB (100 μM) (Fig. 8, *C* and *D*) or SKF96365 (10 μM) (Fig. 7, *E* and *F*), two commonly used ROC inhibitors (37), significantly inhibited spermine-induced

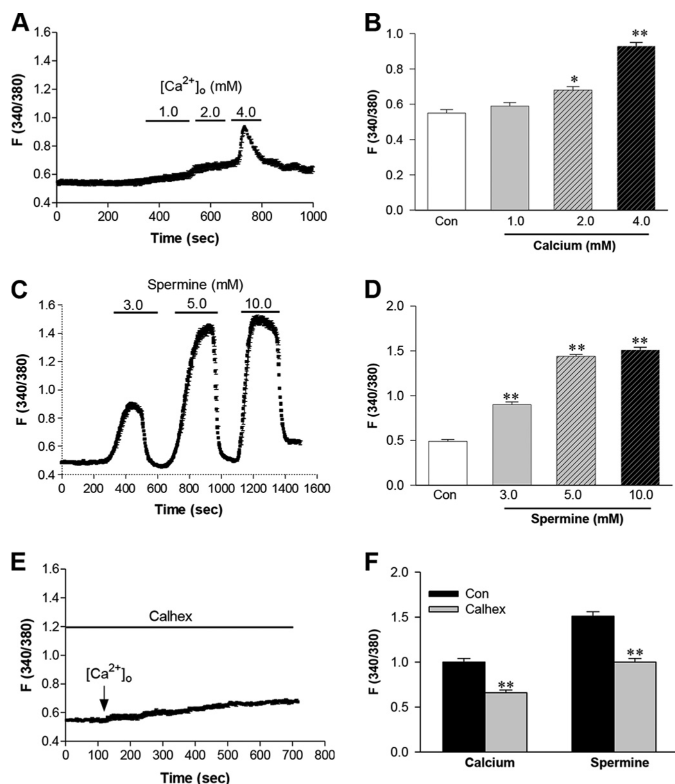

FIGURE 7. **Dose-dependent stimulation of $[Ca^{2+}]_{cyt}$ by the CaSR agonist and inhibition by the CaSR antagonist in SCBN cells.** After SCBN cells were loaded with Fura-2/AM, $[Ca^{2+}]_{cyt}$ in the cells was measured by a digital Ca^{2+} imaging system. *A*, time course of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of $[Ca^{2+}]_o$. *B*, summary data showing a dose-dependent peak $[Ca^{2+}]_{cyt}$ response to $[Ca^{2+}]_o$ stimulation. *C*, time course of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of spermine in 2 mM $[Ca^{2+}]_o$-containing normal physiological solutions. *D*, summary data showing dose-dependent peak $[Ca^{2+}]_{cyt}$ responses to spermine stimulation. *E*, time course of 4 mM $[Ca^{2+}]_o$-induced $[Ca^{2+}]_{cyt}$ changes in the presence of calhex 231 (3 μM) in normal physiological solutions. *F*, summary data showing the inhibitory effect of calhex 231 on 4 mM $[Ca^{2+}]_o$- or 5 mM spermine-induced $[Ca^{2+}]_{cyt}$ changes. Values are expressed as mean ± S.E. of 40–50 cells. *, $p < 0.05$; **, $p < 0.01$ *versus* the baselines before stimulation (*Con*) in *B* and *D* or *versus* the controls in the absence of calhex 231 in *F*.

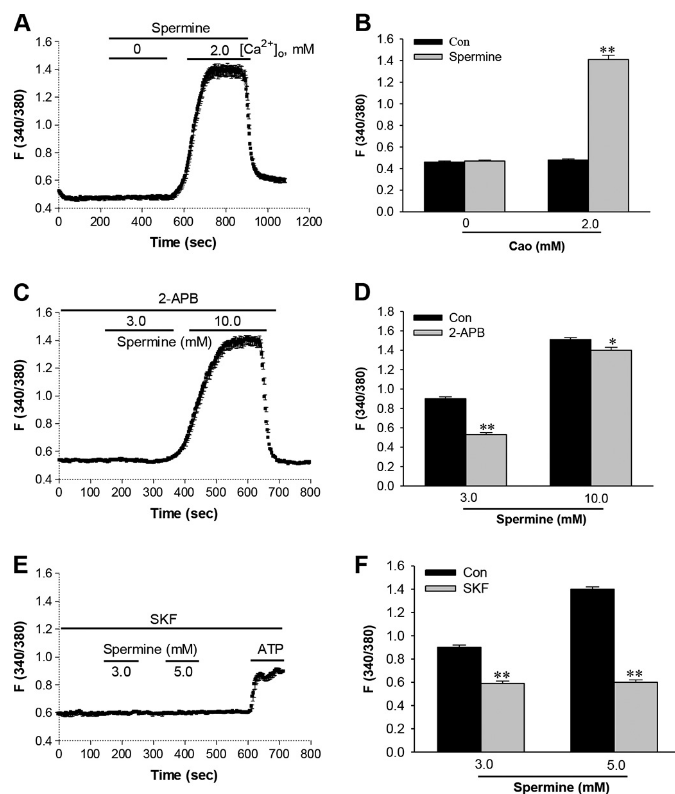

FIGURE 8. **Spermine-mediated $[Ca^{2+}]_o$ entry through ROCs in SCBN cells.** *A*, time course of $[Ca^{2+}]_{cyt}$ changes induced by spermine (5 mM) in $[Ca^{2+}]_o$-free or 2 mM $[Ca^{2+}]_o$-containing normal physiological solutions. *B*, summary data showing peak $[Ca^{2+}]_{cyt}$ responses to spermine in $[Ca^{2+}]_o$ or 2 mM $[Ca^{2+}]_o$-containing normal physiological solutions. *C*, time course of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of spermine (3 and 10 mM) in the presence of 2-APB (100 μM). *D*, summary data showing the effect of 2-APB on spermine-induced increase in $[Ca^{2+}]_{cyt}$. *E*, time course of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of spermine (3 and 5 mM) in the presence of SKF96365 (*SKF*, 10 μM). It is noteworthy that the cells still responded to 10 μM ATP although the spermine-induced increase in $[Ca^{2+}]_{cyt}$ was abolished by SKF96365. *F*, summary data showing the effect of SKF96365 on the spermine-induced increase in $[Ca^{2+}]_{cyt}$. Values are expressed as mean ± S.E. of 40–50 cells for each group. *, $p < 0.05$; **, $p < 0.01$ *versus* the baselines before stimulation of spermine (*Con*) in *B* or *versus* the controls in the absence of inhibitors in *D* and *F*.

$[Ca^{2+}]_0$ entry, indicating that the CaSR-mediated Ca^{2+} entry pathway in SCBN cells involves the ROC.

The functional activity of the CaSR was also characterized in human intestinal epithelial cells. As shown in Fig. 9, $[Ca^{2+}]_o$ dose-dependently increased $[Ca^{2+}]_{cyt}$ in SW-480 and Caco-2 intestinal epithelial cells with CaSR expression (Fig. 9, *A*, *B*, and *D*) but not in HEK-293 cells without CaSR expression (Fig. 9, *C* and *D*), confirming functional expression of the CaSR in human intestinal epithelial cells. Moreover, in SW-480 cells, spermine did not alter $[Ca^{2+}]_{cyt}$ in $[Ca^{2+}]_0$-free solutions but induced a marked $[Ca^{2+}]_{cyt}$ rise in $[Ca^{2+}]_0$-containing solutions (Fig. 9, *E* and *F*), which was inhibited significantly by 2-APB (100 μM) (Fig. 9, *G* and *H*), further indicating an important role of the ROC in CaSR-mediated Ca^{2+} entry in human intestinal epithelial cells.

DISCUSSION

In this study, we demonstrated a novel role for the CaSR in controlling $[Ca^{2+}]_{cyt}$ signaling in duodenal epithelial cells to regulate Ca^{2+}-dependent DBS and advance our understanding of the molecular mechanisms underlying CaSR-mediated $[Ca^{2+}]_{cyt}$ rise in these cells and Ca^{2+}-dependent transepithelial HCO_3^- secretion.

The CaSR is a member of the pheromone class of G-protein-coupled receptors that is expressed in a variety of tissues throughout the body and has been identified to mediate a wide array of physiological effects (3–5). In the parathyroid gland, it is responsible for regulating body calcium homeostasis by modulating the levels of parathyroid hormone and calcium in the circulation (2, 38). Following the cloning of the CaSR from bovine parathyroid cells in 1993 (2), studies were conducted to determine the expression of the receptor. The CaSR has been shown to be expressed along the entire gastrointestinal tract, where it has many physiological roles, such as modulation of gastrin and gastric acid secretion, intestinal fluid, and ion transports by sensing the concentrations of electrolytes, amino acids, and polyamines (2, 9, 39). Although the CaSR has been cloned for two decades, only one previous study implicated its role in pancreatic HCO_3^- secretion (40), and another study suggested its possible involvement in L-glutamate-mediated DBS (41). So far, CaSR-me-

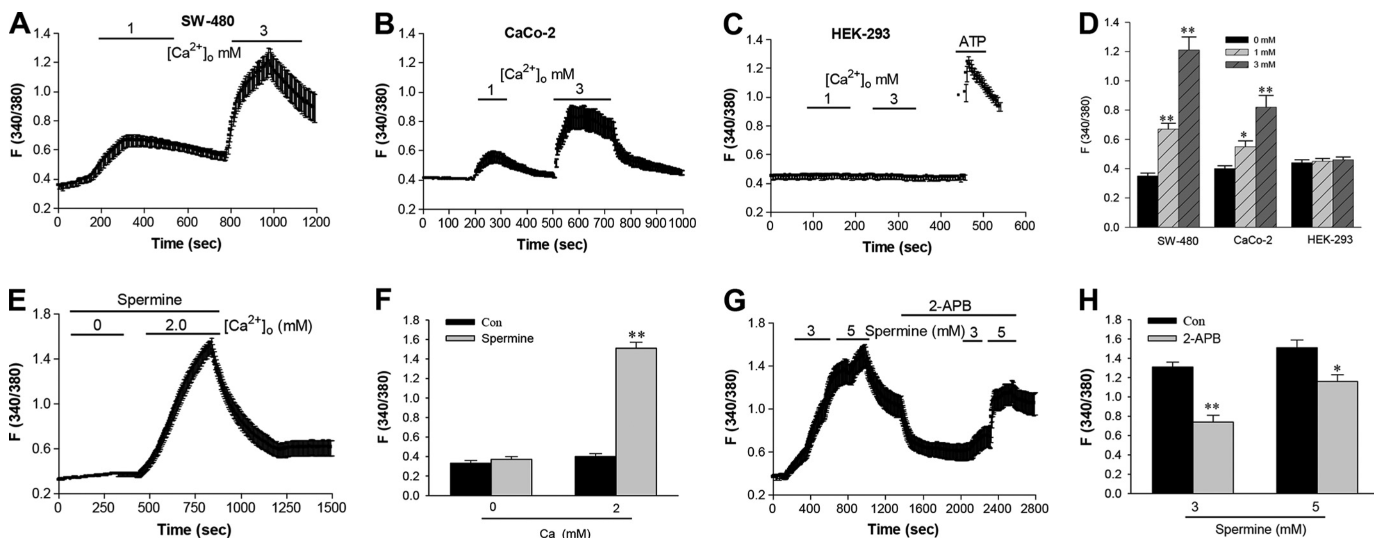

FIGURE 9. **Functional expression of the CaSR in human epithelial cells.** *A–C*, time courses of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of $[Ca^{2+}]_o$ in SW-480, Caco-2, and HEK-293 cells. ATP (10 μM) was used as a positive control. *D*, summary data showing dose-dependent peak $[Ca^{2+}]_{cyt}$ responses to $[Ca^{2+}]_o$ stimulation (0, 1, and 3 mM) in human epithelial cells. *E*, time course of $[Ca^{2+}]_{cyt}$ changes induced by spermine (5 mM) in $[Ca^{2+}]_o$-free or 2 mM $[Ca^{2+}]_o$-containing normal physiological solutions. *F*, summary data showing peak $[Ca^{2+}]_{cyt}$ responses to spermine in $[Ca^{2+}]_o$ or 2 mM $[Ca^{2+}]_o$-containing solutions. *G*, time course of $[Ca^{2+}]_{cyt}$ changes induced by different concentrations of spermine (3 and 5 mM) in the presence or the absence of 2-APB (100 μM) in $[Ca^{2+}]_o$-containing solutions. *H*, summary data showing the effect of 2-APB on the spermine-induced increase in $[Ca^{2+}]_{cyt}$. Values are expressed as mean ± S.E. of 30–40 cells. *, $p < 0.05$; **, $p < 0.01$ *versus* the baselines before stimulation (*Con*) in *A–C* and *E* or *versus* the controls in the absence of 2-APB in *H*.

diated intestinal transepithelial HCO_3^- secretion and the underlying molecular mechanisms are largely unknown.

The DBS is critical to defend the vulnerable duodenal epithelium against various aggressive factors (42, 43). The importance of DBS in protecting duodenal mucosa has been confirmed in patients with duodenal ulcer whose acid-stimulated DBS is only 41% of that in healthy subjects (44). The DBS is impaired in the duodenal tissues from patients with cystic fibrosis, suggesting a pivotal role of the CFTR in mediating the DBS (45). Because the CaSR has been demonstrated to regulate gastric secretion and intestinal Cl^- secretion, it is reasonable to infer that it may also modulate intestinal HCO_3^- secretion. We applied both CaSR agonists and antagonists in two models of duodenal mucosal tissues and intestinal epithelial cells and confirmed that CaSR activation indeed stimulates duodenal transepithelial HCO_3^- secretion, which is consistent with a previous observation that perfusion of Ca^{2+} and spermine increased DBS in anesthetized rats (41). However, that study did not further test whether Ca^{2+} and spermine stimulate the DBS through CaSR activation in the duodenum. Therefore, our study provides novel insights into the CaSR-mediated DBS.

Following our observation that CaSR activation induces DBS, we aimed to elucidate the underlying mechanisms, as established previously for pancreatic HCO_3^- secretion (40). We demonstrate that CaSR activation raises $[Ca^{2+}]_{cyt}$ in SCBN, SW-480, and Caco-2 cells, likely by evoking $[Ca^{2+}]_0$ entry through the ROC. The SCBN cell model was used in this study because this cell line is the only well characterized nontransformed duodenal epithelial cell line (17, 18); because it expresses functional CFTR channels and has been used widely in the study of Ca^{2+}-dependent Cl^- secretion (18, 33); because it secretes HCO_3^-, as demonstrated by this study and others (18); and because it expresses CaSR protein. Our data indicate that CaSR activation can raise $[Ca^{2+}]_{cyt}$, which then opens the IK_{Ca} and stimulates HCO_3^- fluxes through the CFTR in duodenal epithelial cells. Because the physiological roles and molecular mechanisms of $[Ca^{2+}]_{cyt}$-induced HCO_3^- secretion remain poorly understood in most epithelia (12), this study focuses on CaSR-mediated Ca^{2+} signaling in intestinal epithelial cells. CaSR activity is demonstrated in canine duodenal epithelial SCBN cells and human intestinal epithelial SW-480 and Caco-2 cells with CaSR expression but not in human epithelial HEK-293 cells without CaSR expression. These data strongly support an important role of the CaSR in regulating the $[Ca^{2+}]_{cyt}$-dependent function in both human and animal duodenal epithelial cells.

Although the CFTR has been thought to be principally activated by cyclic AMP, Ca^{2+} signaling can activate the CFTR or potentiate cyclic AMP-mediated CFTR activation (12). The $[Ca^{2+}]_{cyt}$ elevation can stimulate mitochondrial ATP production, which is necessary for the process of epithelial HCO_3^- secretion (46). During the activation of CFTR, PKA uses ATP to phosphorylate and activate the R domain of CFTR (47). Therefore, the rise in $[Ca^{2+}]_{cyt}$ can activate apical CFTR channels. It is also known that a rise in $[Ca^{2+}]_{cyt}$ modulates the activities of Cl^-/HCO_3^- exchangers, Na^+/H^+ exchangers, and Na^+-HCO_3^- cotransport in epithelial cells (20, 48–51). The Ca^{2+}-activated chloride channel has been suggested to contribute to HCO_3^- secretion in some epithelia (12). We reported previously that $[Ca^{2+}]_{cyt}$ activates basolateral IK_{Ca} in murine duodenal epithelium to provide a driving force for HCO_3^- secretion (15). This study combining selective pharmacological inhibitors and knockout mice is in good agreement with other reports on the CaSR-Ca^{2+}-IK_{Ca} pathway in the vascular system (52), further supporting our pervious notion that IK_{Ca} plays an essential role in Ca^{2+}-mediated DBS (15). All of these actions of $[Ca^{2+}]_{cyt}$ in epithelial cells may contribute to the molecular mechanisms underlying Ca^{2+}-mediated transepithelial HCO_3^- secretion.

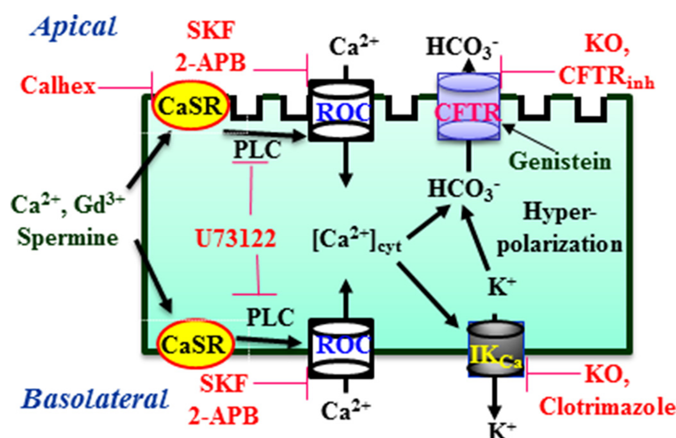

FIGURE 10. **Proposed model for CaSR-mediated Ca^{2+}-dependent intestinal epithelial HCO_3^- secretion through activation of ROC-IK_{Ca}-CFTR channels.** Dietary/nutrient CaSR modulators stimulate the plasma membrane CaSR of intestinal epithelial cells. CaSR activation elicits signaling events through modulation of intracellular mediators such as PLC, which induces $[Ca^{2+}]_o$ entry through the ROCs. An increase in $[Ca^{2+}]_{cyt}$ in epithelial cells activates the IK_{Ca} to lead to cell hyperpolarization, providing a driving force for transepithelial HCO_3^- secretion through the CFTR channels. *Black arrows*, simulation; *red lines*, inhibition; *thick lines*, physiological processes; *thin lines*, pharmacological activators or inhibitors or genetic knockout used for this study.

However, our data demonstrate that CaSR-$[Ca^{2+}]_{cyt}$-IK_{Ca}-CFTR is a major pathway involved in CaSR-mediated DBS observed in this study (Fig. 10).

It is generally assumed that the regulatory mechanisms involved in intestinal epithelial HCO_3^- and Cl^- secretion are similar, but this notion has never been fully studied and confirmed. Epithelial HCO_3^- and Cl^- secretion is mainly under the control of cyclic AMP and Ca^{2+} signaling, which may interact and cross-talk to regulate epithelial ion transports (25, 42, 53, 54). Previous studies demonstrated that most well known secretagogues, such as forskolin, ACh, 5-HT, and PGE_2, usually stimulate intestinal HCO_3^- and Cl^- secretion in parallel (20, 42, 55, 56). It is not known, however, whether epithelial HCO_3^- and Cl^- secretion has to occur in parallel and whether they are regulated by the same or different signaling/mechanisms. Notably, estrogen inhibits forskolin- and carbachol-induced rat colonic Cl^- secretion (57). However, we revealed that estrogen stimulates DBS in humans and mice likely through Ca^{2+} signaling without altering basal duodenal I_{sc}, an index primarily of epithelial Cl^- secretion (13, 58, 59). Therefore, estrogen may play different roles in regulating intestinal HCO_3^- and Cl^- secretion. These findings suggest that epithelial HCO_3^- and Cl^- secretion may not be always triggered in parallel and they may not be regulated by the same signaling/mechanism. We therefore propose that different regulatory mechanisms may exist for intestinal HCO_3^- and Cl^- secretion. Ca^{2+} signaling may play a key role in HCO_3^- secretion, but cyclic AMP may play a major role in Cl^- secretion. Indeed, in this study, CaSR activation, resulting in an increase in $[Ca^{2+}]_{cyt}$ but a decrease in intracellular cyclic AMP (4–6, 9), leads to a specific DBS without simultaneously altering duodenal I_{sc}. Moreover, IK_{Ca} rather than cyclic AMP-activated K^+ channels (KCNQ1) are found to be involved in CaSR-mediated DBS, indicating that a sole Ca^{2+} signaling in the absence of cyclic AMP can trigger the DBS. Therefore, this study not only confirms the pivotal role of $[Ca^{2+}]_{cyt}$ as primary signaling in transepithelial HCO_3^- secretion but also further supports our notion that intestinal HCO_3^- and Cl^- secretion can be triggered independently by different signaling/mechanisms (13).

What is the physiological relevance of this study? Food nutrients, such as dietary calcium, spermine, and L-amino acids, are CaSR activators that regulate gastric acid secretion, intestinal fluid, and ion transports. Here we confirmed a novel physiological role of these nutrients, namely DBS stimulation, and elucidated the underlying mechanisms. Because the DBS is critical for duodenal mucosal protection, these dietary CaSR modulators may also be involved in this physiological process through the $[Ca^{2+}]_{cyt}$-IK_{Ca}-CFTR cascade (Fig. 10). Because CaSR-mediated Ca^{2+} signaling can also stimulate Ca^{2+}-activated chloride channel-dependent epithelial secretion that is independent of the CFTR, these CaSR modulators might be used to restore fluid secretion defects in cystic fibrosis disease (60). Moreover, understanding whether different cell signaling triggers distinct intestinal epithelial ion secretion is important for the development of better drugs that can specifically target either intestinal the HCO_3^- or Cl^- secretion pathway. The medications that specifically trigger intestinal HCO_3^- secretion to protect gastrointestinal tract would not increase Cl^- secretion, which might induce diarrhea. Also, the medications that specifically inhibit intestinal Cl^- secretion to treat diarrhea would not reduce HCO_3^- secretion, which might induce gastrointestinal injury.

CONCLUSION

On the basis of this study, we conclude that dietary calcium and spermine could activate the CaSR in duodenal epithelial cells to specifically trigger Ca^{2+}-dependent DBS that protects mucosa, CaSR activation-induced Ca^{2+} entry through ROC is critical to trigger DBS, and Ca^{2+} signaling regulates DBS, likely through activation of IK_{Ca} and CFTR channels. This study not only reveals that $[Ca^{2+}]_{cyt}$ signaling is critical for CaSR-induced DBS but also provides novel insights into the molecular mechanisms of $[Ca^{2+}]_{cyt}$ signaling-mediated transepithelial HCO_3^- secretion.

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