Expression of calcium-sensing receptor in rat colonic epithelium: evidence for modulation of fluid secretion

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The calcium-sensing receptor (CaSR) is a classic seven-transmembrane, G protein-coupled receptor (GPR) belonging to Family C of the superfamily of GPRs. The CaSR has been shown to recognize and respond to changes in extracellular calcium (Ca2+) concentrations and mediates many of the known effects of extracellular calcium on body cells. Both surface and crypt cells express CaSR transcripts and protein on both apical and basolateral surfaces. Raising Ca2+ elicits increases in intracellular calcium (Ca2+) in both surface and crypt cells with an EC50 of 2 mM. The Ca2+-induced increase in Ca2+ was associated with increases in inositol 1,4,5-trisphosphate and diacylglycerol, an inhibitor of phosphatidylinositol-phospholipase C, as well as by thapsigargin. Other CaSR agonists, Gd3+ and neomycin, mimicked these Ca2+-induced responses. Both luminal and bath Ca2+, Gd3+, and neomycin induced increases in Ca2+ in isolated perfused crypts. The stimulatory effect of forskolin on net fluid secretion in perfused crypts was abolished by increasing Ca2+ in either luminal or bath perfusates. Thus both apical and basolateral CaSR on crypt cells are functional and provide pathways modulating net intestinal fluid transport that may have important implications for the prevention and treatment of certain diarrheal diseases associated with elevated cAMP.

The extracellular calcium (Ca2+) sensing receptor, CaSR (4), is a classic seven-transmembrane-spanning, G protein-coupled receptor (GPR) belonging to Family C of the superfamily of GPRs. The CaSR has been cloned from several cells and tissues, including rat, human, and rabbit parathyroid and kidney as well as rat brain. The CaSR is not only activated by increases in Ca2+ but also by extracellular Mg2+ and other divalent cations like Gd3+ and neomycin. In addition, the EC50 for Ca2+-induced effects can be modulated by polyamines and aromatic amino acids (9, 27). Considerable evidence now suggests that this receptor mediates the influence of Ca2+ and the other CaSR agonists on the function of these tissues and cells (see recent review by Brown and MacLeod (6)). Various aspects of cellular function and processes modulated by CaSR have been reported, including increases in intracellular Ca2+ (Ca2+) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3), reductions in cellular cAMP, activation of mitogen-activated protein kinases, regulation of cell growth-differentiation, modulations of gene expression, and changes in salt and water homeostasis (6).

The CaSR is expressed in gastrointestinal tract, including colon (7, 14), suggesting multiple potential roles for this receptor in gastrointestinal biology. In the intestine, and colon in particular, previous studies have indicated that high Ca2+ dietary intake promotes colonic mucosal epithelial cell differentiation, decreases cell growth, and reduces risks for development of colorectal cancer [see recent summary by Bresalier (2) and also Refs. 17, 18, and 31]. There is also evidence that, as in the small intestine, the colon can absorb and secrete Ca2+ in response to changes in Ca2+ concentration as well as levels of 1,25-dihydroxyvitamin D3 (11, 12, 20, 23). These observations indicate that the colonic mucosal epithelium is equipped with a mechanism that is capable of recognizing and responding to changes in Ca2+ concentration and that the CaSR might function as this Ca2+-sensing mechanism.

To date, however, there has been no documentation of the functioning of this receptor in colon nor its involvement in the regulation of colonic function. In addition, despite the presence of CaSR transcripts and protein in the colon mucosal epithelium, there has been uncertainty with regard to the receptor distribution and localization within this tissue. We previously (7) suggested that the CaSR is present in the apical and basolateral membranes of rat colon crypt cells, although the CaSR distribution in the surface epithelium was unclear. Sheinin et al. (32) recently examined the CaSR immunolocalization in human large intestine using a monoclonal anti-CaSR antibody and found that the CaSR immunoreactivity was only present in certain enteroendocrine cells of the crypts.

In the present study, we further investigated the expression and localization of the CaSR in both rat and human, and human, and rabbit parathyroid and kidney as well as rat brain. The CaSR is not only activated by increases in Ca2+ but also by extracellular Mg2+ and other divalent cations like Gd3+ and neomycin. In addition, the EC50 for Ca2+-induced effects can be modulated by polyamines and aromatic amino acids (9, 27). Considerable evidence now suggests that this receptor mediates the influence of Ca2+ and the other CaSR agonists on the function of these tissues and cells (see recent review by Brown and MacLeod (6)). Various aspects of cellular function and processes modulated by CaSR have been reported, including increases in intracellular Ca2+ (Ca2+) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P3), reductions in cellular cAMP, activation of mitogen-activated protein kinases, regulation of cell growth-differentiation, modulations of gene expression, and changes in salt and water homeostasis (6).

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human colonic epithelial cells, the functioning of this receptor in rat colon, and its modulation of fluid movement in isolated perfused rat colonic crypts. The expression of CaSR in colon surface and crypt epithelial cells was verified by RT-PCR and Western blot analyses. In addition, immunofluorescence and immunohistochemistry studies demonstrated CaSR protein at both the apical and basolateral surfaces of rat colonic surface and crypt cells. Moreover, by measuring the CaSR agonist-induced changes in Ca$^{2+}$ and Ins(1,4,5)P$_3$ responses, we show that the CaSR is functionally active in both apical and basolateral domains of rat colonic epithelial cells. Finally, by study of the rate of fluid movement ($J_v$) in isolated perfused rat colonic crypts, we provide evidence that the colonic CaSR is a modulator of intestinal fluid secretion.

MATERIALS AND METHODS

Animals. Experiments were performed using adult male Sprague-Dawley rats (weighing 150–300 g) obtained from Charles River Laboratories and Taconic Farms. The use of rats as well as the protocol for isolating colon tissues and cells was approved by the Institutional Animal Care and Use Committee (IACUC # 2000-10253) at Yale.

Animals were fed and maintained on regular chow (PMI Nutrition International) with free access to water before the investigation. Rats were anesthetized and euthanized with isoflurane or pentobarbital and killed by cervical dislocation before colonectomy.

Isolation of surface and crypt cells from colon. The procedure for the isolation of surface and crypt cells from rat colon is based on the method previously described (21) with minor modifications. Colons were removed from rats and were cut open longitudinally to eliminate fecal pellets. After being dissected and washed, the tissues were ewered to expose the mucosal surface. To obtain surface cells, the everted colons were either scraped gently over the mucosa with a glass slide or incubated in Na-citrate buffer containing (in mM): 96 NaCl, 27 Na citrate, 0.8 KH$_2$PO$_4$, 5.6 Na$_2$HPO$_4$, and 1.5 D-glucose, pH 7.4. To obtain isolated crypts, the colonic segments were further incubated in Na-EDTA buffer containing (in mM): 96 NaCl, 1.5 KCl, 21 Na EDTA, 55 sorbitol, 22 sucrose, and 10 HEPES, pH 7.4. At the end of each incubation period, colonic segments were agitated for 30 s to release surface cells or individual crypts. Released cells or crypts were immediately mixed with 2 volumes of standard Ringer solution containing (in mM): 125 NaCl, 5.0 KCl, 1.0 CaCl$_2$, 1.2 MgSO$_4$, 2.0 NaH$_2$PO$_4$, 5.0 D-glucose, and 32 HEPES, pH 7.4. Cells were collected by centrifugation (2,000 rpm for 5 min in a Beckman Coulter Allegra 6R centrifuge), washed in initial Ringer solution, and resuspended in initial Ringer solution unless otherwise specified. Initial Ringer solution contains the same composition as standard Ringer except that Ca$^{2+}$ was reduced to 0.1 mM and Mg$^{2+}$ was either 0 mM or 0.5 mM. These solutions with reduced divalent cation concentrations were used to minimize stimulation of CaSR by Ca$^{2+}$ and Mg$^{2+}$ at basal state but still maintain cell viability and integrity. We validated the separation of surface and crypt cells by the differential expression of alkaline phosphatase activity as described elsewhere (21). In this study, the alkaline phosphatase activities (expressed as increase in absorbance (405 nm) per minute per milligram protein, mean ± SE) in the surface and crypt cell preparations were 0.40 ± 0.11 and 0.12 ± 0.02, respectively, which are consistent with a previous study (21).

Detection of CaSR transcripts in isolated surface and crypt cells by RT-PCR. Total RNA was isolated, separately, from surface and crypt cells as well as from rat kidneys (RNeasy Mini Kit, QIAGEN, Valencia, CA) and reverse transcribed into cDNA using oligo(dT)$_{12-18}$ primers (SuperScript premi

Detection of CaSR protein expression in isolated colonic surface and crypt cells by Western blot analysis. Cells were lysed for 15 min on ice in a buffer containing: 62.5 mM Tris, pH 6.8, 10% sucrose, 2% SDS, 5% 2-mercaptoethanol, 100 µM phenylmethylsulfonyl fluoride, and Mini Complete protease inhibitors (Roche). The lysates were briefly sonicated and were incubated at room temperature for 15 min before they were loaded onto a 6% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad) by electroblotting. The membranes were rinsed in Tris-buffered saline (TBS) and quenched with 5% nonfat milk in TBS containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Expression of CaSR was detected with an affinity-purified polyclonal antibody raised against a 22-amino acid region of the NH$_2$ terminus of the receptor (29). Incubation with primary antibody was made overnight in 5% nonfat milk containing TBS-T (1:100 dilution). After three 20-min washes at room temperature in TBS-T, membranes were blocked with milk, avidin, and biotin solutions and were incubated with anti-rabbit IgG secondary antibody conjugated to biotin (1:20,000 dilution) and with avidin/biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Each treatment was followed by three 20-min washes at room temperature in TBS-T. Immunoreactive signals were visualized by chemiluminescence (Amersham Pharmacia Biotech UK).

Immunofluorescence and immunohistochemistry. The colons from male Sprague-Dawley rats (weighing 200–250 g) were perfused via the descending aorta with PBS, followed by 4% paraformaldehyde, and then by 12.5% sucrose in PBS solution. The same fixative was also used for a human colon sample taken via biopsy (graciously donated by Mary Kay Washington, Associate Professor of Pathology, Vanderbilt University Medical Center, in conjunction with the Vanderbilt-Ingram Cancer Center, Human Tissue Acquisition and Pathology Shared Resource Core). Tissues were kept overnight at 4°C in 30% sucrose in PBS. Tissues were then embedded in Tissue Tek optimum cutting temperature compound.
Cryosections were antigen retrieved by using Citra Microwave Solution (Biogenex, San Ramon, CA) followed by treatment with 1% SDS in PBS for 5 min to expose antigenic sites. After SDS treatment, slides were rinsed three times with PBS and incubated 30 min with 1% BSA/PBS followed by three 5-min washes in PBS. Slides were then incubated with 4% Seablock for 1 h at room temperature. A primary antibody was diluted 1% BSA/PBS and applied to sections for 1 h at room temperature followed by overnight incubation with primary affinity-purified anti-CaSR polyclonal antibody (1:100 dilution). For immunohistochemistry, slides were preincubated in peroxidase blocking reagent (DAKO, Carpentrya, CA) for 5 min and in protein blocking serum-free solution (DAKO) for 15 min before overnight incubation with the primary antibody. To assess non-specific staining, control experiments were performed by incubating the slides without primary antibody or with primary antigen absorbed antibody for 1 h at room temperature. Secondary antibody was diluted 1% BSA/PBS and applied to sections for 1 h at room temperature at the following dilutions: Alexa 594 anti-rabbit IgG (Molecular Probes, Eugene, OR) 1:5,000 diluted in 1% BSA/PBS, and peroxidase-conjugated goat anti-rabbit IgG (Vector Laboratories) 1:100 diluted in PBS, pH 7.4. The slides were then washed in two 5-min washes in high salt PBS + 2.8% NaCl then two 5-min washes in PBS. For immunohistochemistry, the color reaction was developed at room temperature for 5 min by using the DAKO AEC substrate system. The reaction was stopped by three rinses in water. Slides were then mounted using Vectashield (Vector Laboratories) and were examined with a Nikon Eclipse 800 Research microscope equipped with a charge-coupled device camera. Fluorescence and histochemical photomicrographs were stored in a computer and were processed using Adobe Photoshop 5.0 software.

Fluo 3 fluorescence imaging. Crypts were adhered to glass coverslips precoated with Cell-Tak (Collaborative Biomedical Products, Bedford, MA) and were loaded with fluo 3-AM (Molecular Probes). Following dye loading, the crypts were washed for 5 min and the coverslips were transferred to a perfusion chamber where the isolated crypt cells were imaged with a confocal laser scanning microscope (LSM 410, Carl Zeiss, Thornwood, NY) using a 40 × 1.4 oil immersion lens with infinity corrected optics. Dye molecules were excited with a multiline argon laser at a wavelength of 488 nm, and emission was detected in the wavelength range of 515–565 nm. Images of crypt cells (512 × 512 × 12 bit deep) were recorded before and after addition of stimulant, and each image was an average of eight sequential frames acquired at 2-s intervals. Neither solution changes per se nor addition of vehicle affected the fluorescent signals.

Measurement of Ca<sup>2+</sup> responses in cell suspensions. Changes in Ca<sup>2+</sup> in response to CaSR activation of isolated colon cells were monitored in a SPEX Fluoromax-3 spectrophotofluorometer (Jobin Yvon, Edison, NJ) using fluo 3 as an indicator as previously described (8). Cells were exposed to a solution of fluo 3-AM at 5 μM for 20 to 30 min at room temperature to allow uptake and ester hydrolysis. The cells were then washed at 37°C for −20 min to remove any extracellular dye that had not been taken up or had deesterified on the extracellular surface of the crypts. Fluorescence measurements (480 nm excitation; 520 nm emission; 5 nm band-pass) on cell suspensions were performed in a thermostatically regulated 2-ml cuvette maintained at 37°C with constant stirring in initial Ringer solution. In some experiments, Ca<sup>2+</sup> measurements were also performed on cells that were adhered to glass coverslips precoated with Cell-Tak. In this case, the coverslips were placed diagonally in the cuvette and changes in Ca<sup>2+</sup> signal were measured. This setup was employed when such experiments were required in which different drugs or buffers were applied to the same cells. Fluo 3 was selected because of its large change in intensity as a function of a change in Ca<sup>2+</sup> levels. However, because this is a single wavelength excitation/emission dye, the data are expressed in arbitrary units of fluorescence; variations in intensity due to variations in dye loading cannot be corrected. This may account for some of the variation found in basal fluorescence.

To estimate the kinetics of CaSR activation, we stimulated the receptor by additions of an agonist in increments to reach the desired concentrations as previously described in other studies (Refs. 1, 9, and 15; also see review in Ref. 6 for more references). The cumulative Ca<sup>2+</sup> response at a given concentration of the agonist was determined. If the peak increases in Ca<sup>2+</sup> are P1, P2, P3 . . . Pn at concentrations of the agonist in the bath solution corresponding to C1, C2, C3 . . . Cn, which were achieved by incremental additions of the agonist, the cumulative Ca<sup>2+</sup> response (Rn) at any given agonist concentration (Cn) is defined as the sum, P1 + P2 + P3 . . . + Pn. These cumulative Ca<sup>2+</sup> responses were plotted against the concentrations of the agonist added. EC<sub>50</sub> and Hill slope or Hill coefficient values were obtained by curve fitting the data using the equation

\[
R = 100/(1 + 10^{(\log EC_{50} - C \times \text{Hillslope})})
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where R is the % normalized maximal Ca<sup>2+</sup> response at a given Ca<sup>2+</sup> concentration C. The Hillslope describes the steepness of the curve. In a preliminary study, we have also estimated the kinetics of CaSR activation by additions of an agonist to the desired concentrations and compared with that of the stepwise protocol. Both protocols produced similar results.

Measurement of Ca<sup>2+</sup> responses in luminal and basolaterally perfused colonic crypts. Measurements of Ca<sup>2+</sup> were also conducted in perfused isolated colonic crypts that were either enzymatically dissected as described above or dissected by using a hand dissection technique that was previously described (33). Following isolation, the crypts were mounted on a series of glass pipettes and perfused in vitro as previously described (16). After establishing perfusion, the bath temperature was raised to 37°C and maintained for the remainder of the experiment. Ca<sup>2+</sup> was measured by using fluo 3-AM with the identical protocols to those described above. The measuring system consisted of a high-speed video imaging system that allows continuous recording of Ca<sup>2+</sup> (Universal Imaging). The collected data and images were stored on the hard disc of the computer. For measurements, at least five separate areas were monitored simultaneously online per crypt. For studies involving luminal Ca<sup>2+</sup> responses, we changed the perfusion solution along the apical surface of the crypt while maintaining a constant perfusion rate as monitored by luminal diameter, particle contact time, length of the crypt, and collection time in the distal end of the crypt. Using this technique, we had complete control of the combination of bath and luminal perfusate and could directly change the ionic strength on one or both surfaces simultaneously.

Measurement of J<sub>v</sub> in isolated perfused colonic crypts. Determination of fluid movement in the isolated perfused crypt followed a method that we have previously developed (33). Briefly, following isolation of the cryp by either a hand dissection or by the same EDTA dissection technique outlined above, the crypt was transferred to the stage of an
inverted microscope and mounted on a series of concentric glass micropipettes. Following cannulation of both ends of the crypt, perfusion was established on both the luminal and basolateral membranes. For the luminal perfusion, rates were maintained at ~4–8 nl/min, whereas the basolateral perfusion was at 3 ml/min (which equates to 3 bath exchanges/s). The length, diameter, and contact time for particles were recorded, and the time to fill a calibrated collection pipette was used to determine the luminal perfusion. The luminal perfusates contained 10 μCi/ml methoxy-[3H]inulin (New England Nuclear, Boston, MA). Jv was calculated as previously described (33) as the rate at which the effluent accumulated in the collection pipette and the concentrations of methoxy-[3H]inulin in the perfusate and effluent. Positive Jv values (nl·mm−1·min−1) indicate net fluid movement from lumen to bath (absorption), and negative values indicate fluid movement from bath to lumen (secretion). The bath was routinely assayed for methoxy-[3H]inulin, and experiments were discarded if the bath concentration exceeded background.

Determinations of Ins(1,4,5)P3 levels. Measurements of Ins(1,4,5)P3 were performed by using an Ins(1,4,5)P3 binding background. Measurements were discarded if the bath concentration exceeded the background.

RESULTS

Although previous studies have identified CaSR transcripts and protein in the colon (7, 32), there remains uncertainty as to the specific colonic epithelial cells expressing the CaSR as well as the sidedness (apical or basolateral) of expression in these cells. Thus we further investigated the expression and localization of the CaSR in rat colonic surface and crypt cells.

Figure 1A shows that PCR products of the expected size, i.e., 383 bp, were amplified by CaSR-specific primers from transcripts isolated from both surface and crypt cells. Nucleotide sequencing of the PCR products from colon crypts revealed >99% identity with the corresponding sequence of the full-length CaSR cDNA from rat kidney. Thus both surface and crypt cells express CaSR transcripts.

An affinity-purified polyclonal antibody that is specific for the CaSR (29) identified a specific band of ~135 kDa in lysates of both surface and crypt cells (Fig. 1B), verifying CaSR protein expression in both of these colonic epithelial regions. The Western blot was performed under reducing conditions, and the band at ~135 kDa represents the monomer form of CaSR. However, when blots were performed under nonreducing conditions (i.e., absence of 2-mercaptoethanol), bands at both 135 and 200–235 kDa were observed, representing, respectively, the monomer and dimer forms of CaSR (data not shown). The CaSR signals detected in surface and crypt cells of colon were similar to that from the kidney (Fig. 1B), which is known to express high levels of CaSR (4, 30). To assess which specific cells, and which membranes in these cells, express CaSR protein, we examined the receptor immunolocalization (29). CaSR antigenic sites were made accessible in frozen sections by “antigen retrieval” treatment before immunodetection. Figure 2, A–D show the immunofluorescence and immunohistochemistry of CaSR in the rat distal colon epithelial cells. Both apical and basolateral aspects of surface and crypt cells exhibited intense CaSR staining (Fig. 2, B–D). A similar pattern of CaSR immunostaining was observed in proximal colon epithelial cells (not shown). To assess if CaSR is expressed in human colonic crypts, we stained a colon biopsy specimen with the CaSR-specific antibody. Figure 2, E and F show representative differential interference contrast and immunoflu-
Fig. 2. Localization of CaSR in surface and crypt epithelial cells of colons. Phase (A) and immunofluorescence (B) images (magnification ×400) of rat distal colon stained with anti-CaSR antibody are shown. Note that the fluorescence (rhodamine) is evident in both apical (white arrows) and basolateral (green arrows) aspects of surface and crypt cells. C and D show immunohistochemistry of rat distal colon stained with anti-CaSR antibody and the immunoperoxidase technique. Intense CaSR staining of apical (black arrows) and basolateral (red or green arrows) surfaces was observed in both crypt (C) and surface (D) cells (magnification ×400). E and F show differential interference contrast (E) and CaSR-specific rhodamine fluorescence (F) images (magnification ×600) of a human colonic crypt from a biopsy. Note that CaSR fluorescence patterns in rat and human crypts are similar.
orescence images of the same human colonic crypt and demonstrate that the CaSR staining pattern in human colonic crypt is essentially identical to that in rat.

After verifying that CaSR transcripts and protein were present in both surface and crypt cells, we next determined if this receptor is functionally active. We assessed Ca\(^{2+}\) responses to established CaSR agonists Ca\(^{2+}\), Gd\(^{3+}\), and neomycin. Increases in Ca\(^{2+}\) by these agonists have been commonly observed in many different cells and tissues (6). Figure 3A shows confocal images of the same crypts from rat distal colon loaded with fluo 3 and superfused with a Ringer solution containing 0.1 mM or 1.1 mM Ca\(^{2+}\). Increasing Ca\(^{2+}\) from 0.1 to 1.1 mM evoked an increase in Ca\(^{2+}\) in virtually all of the cells in the crypts. We also quantified these changes in Ca\(^{2+}\) in both surface and crypt cells before and after addition of different CaSR agonists. As shown in Fig. 3, B and C, application of Ca\(^{2+}\), extracellular Gd\(^{3+}\), or neomycin elicited a marked transient increase in Ca\(^{2+}\) concentration in both surface (Fig. 3B) and crypt cells (Fig. 3C). These Ca\(^{2+}\) responses are comparable to those found in HEK-293 cells stably transfected with CaSR (1, 27).

We next examined Ca\(^{2+}\) responses to activation of apical or basolateral CaSR. Single intact crypts were preloaded with fluo 3 and perfused in vitro with Ca\(^{2+}\), Gd\(^{3+}\), or neomycin either luminally or basolaterally as described previously (33). The changes in Ca\(^{2+}\) before and after receptor activation were recorded in a videomicroscope and quantified. Figure 4, A and C show representative recordings of Ca\(^{2+}\) responses to basolateral and apical CaSR activation by elevating Ca\(^{2+}\). Both luminal and basolateral increases in Ca\(^{2+}\) from 0.1 mM to 2.1 mM produced a transient peak Ca\(^{2+}\) response, followed by a sustained Ca\(^{2+}\) increase. A similar response profile was also observed for Gd\(^{3+}\) and neomycin (data not shown). The changes in Ca\(^{2+}\) transients by Ca\(^{2+}\), Gd\(^{3+}\), and neomycin were quantitated and are shown in Fig. 4, B and D. These results demonstrate that each of the three CaSR agonists raised fluo 3 fluorescence equivalently when added either to the luminal or bath perfusate.

CaSR-mediated Ca\(^{2+}\) transients were prevented (Fig. 5A) by prior treatment of cells with U-73122, a specific inhibitor of phosphatidylinositol-phospholipase C (PI-PLC). U-73122 by itself had no effect on basal Ca\(^{2+}\). Thus the Ca\(^{2+}\) transients induced by the CaSR agonists were not the result of altered cell Ca\(^{2+}\) entry but were due to receptor-mediated activation of PI-PLC. Consistent with direct involvement of PI-PLC stimulation by CaSR, there was a rapid increase in Ins(1,4,5)P\(_3\) accumulation following stimulating the

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**Fig. 3.** Intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) responses to agonist activation of CaSR in epithelial cells of rat colons. Changes in Ca\(^{2+}\) were measured by fluo 3 fluorescence. A: confocal images of fluo 3 fluorescence in rat colonic crypts superfused with a Ringer solution containing 0.1 or 1.1 mM extracellular Ca\(^{2+}\) (Ca\(^{2+}\)). B and C: representative changes in Ca\(^{2+}\) recorded by spectrofluorometry in isolated surface (B) and crypt (C) epithelial cells in response to addition of 2 mM Ca\(^{2+}\), 200 \(\mu\)M Gd\(^{3+}\), or 200 \(\mu\)M neomycin (Neo). Agonist was added (arrow) immediately before Ca\(^{2+}\) transient and remained for the duration of recording. The initial Ca\(^{2+}\) in these studies contained 0.1 mM Ca\(^{2+}\).
cells by elevating Ca\textsuperscript{2+} (Fig. 5B). Similar Ins(1,4,5)P\textsubscript{3} responses were also seen with neomycin and Gd\textsuperscript{3+} (Fig. 5C). In addition, we examined Ca\textsuperscript{2+} responses after depletion of the Ins(1,4,5)P\textsubscript{3}-sensitive Ca\textsuperscript{2+} pool with thapsigargin. Thapsigargin abolished the Ca\textsuperscript{2+}-induced increase in Ca\textsuperscript{2+} (Fig. 5, D and E), indicating that the rise in Ca\textsuperscript{2+} is produced intracellularly from the release of thapsigargin-sensitive Ca\textsuperscript{2+} stores.

The dose-response relationships for Ca\textsuperscript{2+}-elicited increases in Ca\textsuperscript{2+} are shown in Fig. 6. Figure 6A shows a representative recording of Ca\textsuperscript{2+} responses to 1.0-mM increments of Ca\textsuperscript{2+} ranging from 0.1 mM to 5.1 mM. Ca\textsuperscript{2+} > 2.1 mM produced sustained increases in Ca\textsuperscript{2+} in addition to transient peak Ca\textsuperscript{2+} responses. The changes in Ca\textsuperscript{2+} transients were quantitated and are shown in Fig. 6B. Increases in Ca\textsuperscript{2+} led to increases in Ca\textsuperscript{2+}, with a near-maximal response at Ca\textsuperscript{2+} concentrations of 4.0–5.0 mM and a half maximal response (i.e., EC\textsubscript{50}) at ~ 2.0 mM.

To further assess the functional relevance of the CaSR expression in the colon epithelial cells, particularly its potential role in modulating intestinal fluid movement in certain diarrheal states, the effect of either luminal or basolateral CaSR activation on J\textsubscript{v} was determined in isolated rat distal colonic crypts in both basal and forskolin-stimulated states. Figure 7 summarizes the changes in J\textsubscript{v} in perfused colonic crypts in the absence and presence of 100 \mu M forskolin and before and after raising Ca\textsuperscript{2+} from 0.5 to either 2.0 (Fig. 7, A and B) or 5.0 (Fig. 7, C and D) mM in the bath (Fig. 7, A and C) or lumen (Fig. 7, B and D) perfusate. In the absence of forskolin, the mean J\textsubscript{v} values were between 0.372 and 0.382 nl-mm\textsuperscript{-1}-min\textsuperscript{-1}, indicating net fluid absorption. Exposure to forskolin induced net fluid secretion; J\textsubscript{v} averaged between −0.195 and −0.202 nl-mm\textsuperscript{-1}-min\textsuperscript{-1}. Raising Ca\textsuperscript{2+} from 0.5 to 2 or 5 mM in either the bath (Fig. 7, A and C) or lumen (Fig. 7, B and D) perfusate reversed net fluid secretion induced by forskolin, resulting in net fluid absorption; the mean J\textsubscript{v} for Ca\textsuperscript{2+} from 0.5 to 2 mM and from 0.5 to 5 mM were 0.315 ± 0.016 and 0.328 ± 0.018 nl-mm\textsuperscript{-1}-min\textsuperscript{-1} for basolateral application and 0.302 ± 0.017 and 0.300 ± 0.024 nl-mm\textsuperscript{-1}-min\textsuperscript{-1} for luminal application.

**DISCUSSION**

Our data demonstrate that CaSR is both expressed and functionally active in the colon epithelium and that this colonic CaSR is a modulator of intestinal fluid transport. We and others (7, 32) have shown the presence of CaSR transcripts and protein in colon. In the present study, we extend these observations by demonstrating that CaSR transcripts and protein are expressed in isolated surface and crypt epithelial cells from rat distal colon. Immunohistochemistry and immunofluorescence showed the presence of CaSR on both apical and basolateral surfaces of these epithelial
This receptor protein was also found in a similar pattern in crypts from a human colon biopsy specimen, providing support for potential roles of the CaSR in human colon. The increases in \(\text{Ca}^{2+}\) and \(\text{Ins}(1,4,5)P_3\) to apical or basolateral application of the CaSR agonists \(\text{Ca}^{2+}\), \(\text{Gd}^{3+}\), and neomycin demonstrate that the CaSR in colon is functional on polarized domains of these epithelial cells. Our studies also demonstrated that the \(\text{Ca}^{2+}\) transients induced by \(\text{Ca}^{2+}\), \(\text{Gd}^{3+}\), and neomycin were mediated by activation of PLC, generation of \(\text{Ins}(1,4,5)P_3\), and \(\text{Ca}^{2+}\) release from thapsigargin-sensitive \(\text{Ca}^{2+}\) stores. The functional relevance of this receptor in colon physiology and pathophysiology is suggested by our \(J_{\text{f}}\) studies performed in isolated perfused crypts in which activation of luminal or basolateral CaSR by 2 or 5 mM \(\text{Ca}^{2+}\) diminished forskolin-stimulated fluid secretion.

The CaSR localization in rat colonic epithelium shown here is consistent with the distribution pattern in crypts previously reported in rat by Chattopadhyay et al. (7) but additionally demonstrates distinct apical and basolateral receptor expression in surface cells. In contrast, Sheinin et al. (32) recently examined the CaSR immunolocalization in the large intestine of humans and showed receptor immunoreactivity only in epithelial cells at the base of crypts. Since the CaSR protein was found in the present study in a similar pattern in colonic crypts from both rat and human, differences in CaSR localization between our study and that of Sheinin et al. are unlikely to be due to species differences. The most likely explanation is differences in experimental conditions, including use of polyclonal vs. monoclonal antibody, cryosections vs. paraffin sections, and with vs. without “antigen retrieval” pretreatment. In previous studies (29, 30), antigen retrieval was critical to identifying CaSR expression in rat kidney using our well-studied polyclonal antibody against the CaSR. Further support for our CaSR ex-

![Fig. 5. Evidence for functional coupling of colonic CaSR to phosphatidylinositol (PI)-phospholipase C (PLC)-inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]-Ca\textsuperscript{2+} signaling pathway. Unless otherwise specified, measurements were made using rat colonic epithelial cells containing both surface and crypt cells. A: Ca\textsuperscript{2+} responses to agonist activation of CaSR in the absence or presence of the PI-PLC inhibitor U-73122. Measurements of Ca\textsuperscript{2+} responses were made in cells attached to glass coverslips. The additions of drugs and their concentrations are indicated by arrows. Neo, neomycin. B: time-dependent changes in intracellular \(\text{Ins}(1,4,5)P_3\) accumulation in response to \(\text{Ca}^{2+}\) elevation from 0.1 to 2.1 mM. Data shown are average of 3 separate experiments. C: similar \(\text{Ins}(1,4,5)P_3\) responses to \(\text{Ca}^{2+}\), \(\text{Gd}^{3+}\), and neomycin (n = 4; *P < 0.01 vs. Ringer control). D: a representative tracing of the time-dependent changes in \(\text{Ca}^{2+}\) with basolateral application of 1 \(\mu\text{M}\) thapsigargin followed by 2 mM \(\text{Ca}^{2+}\) and then the \(\text{Ca}^{2+}\) ionophore ionomycin. Note that thapsigargin resulted in a small increase in \(\text{Ca}^{2+}\) consistent with \(\text{Ca}^{2+}\) release from stores. After thapsigargin, 2.1 mM \(\text{Ca}^{2+}\) failed to elicit an increase in \(\text{Ca}^{2+}\), but addition of ionomycin resulted in the expected large increase in \(\text{Ca}^{2+}\). E: summary of the \(\text{Ca}^{2+}\) responses to CaSR agonists added to either luminal or basolateral solutions in the presence or absence of pretreatment with 1 \(\mu\text{M}\) thapsigargin (Thap). *P < 0.01 with vs. without thapsigargin.
Note that activation of CaSR by Ca\textsuperscript{2+} negative value indicates secretion.

in rat colonic epithelial cells. Ca\textsuperscript{2+}/H\textsubscript{2}O\textsubscript{3} \textsubscript{100}/H\textsubscript{11001} kinetic parameters, data were curve monitored as in Fig. 3B. A: representative tracing of the patterns of the Ca\textsuperscript{2+} responses. The additions of Ca\textsuperscript{2+} are marked with arrows; at each arrow, the concentration of Ca\textsuperscript{2+} was increased in 1-mM increment. The initial concentration of Ca\textsuperscript{2+} was 0.1 mM. B: transient Ca\textsuperscript{2+} responses in A were plotted against the concentrations of Ca\textsuperscript{2+} added. All of the transient responses were normalized to the maximal cumulative Ca\textsuperscript{2+} response at 7.6 mM Ca\textsuperscript{2+}. To derive kinetic parameters, data were curve fit using the equation R = 100/[1 + 10\textsuperscript{AFU}/EC\textsubscript{50} \textsubscript{Cai} \times Hillslope)], where R is the % normalized maximal Ca\textsuperscript{2+} response at a given Ca\textsuperscript{2+} concentration C and Hillslope is the steepness of the curve. The best fit to this expression was at EC\textsubscript{50} = 1.98 mM and Hillslope = 2.37. A near-maximal Ca\textsuperscript{2+} response was at Ca\textsuperscript{2+} concentrations of ~5 mM. The calculated values agree well with those derived graphically (r\textsuperscript{2} = 0.97).

Fig. 6. Dose-response relationship for Ca\textsuperscript{2+}-elicited Ca\textsuperscript{2+} responses in rat colonic epithelial cells. Ca\textsuperscript{2+}-evoked increases in Cai were monitored as in Fig. 3B. A: representative tracing of the patterns of the Ca\textsuperscript{2+} responses. The additions of Ca\textsuperscript{2+} are marked with arrows; at each arrow, the concentration of Ca\textsuperscript{2+} was increased in 1-mM increment. The initial concentration of Ca\textsuperscript{2+} was 0.1 mM. B: transient Ca\textsuperscript{2+} responses in A were plotted against the concentrations of Ca\textsuperscript{2+} added. All of the transient responses were normalized to the maximal cumulative Ca\textsuperscript{2+} response at 7.6 mM Ca\textsuperscript{2+}. To derive kinetic parameters, data were curve fit using the equation R = 100/[1 + 10\textsuperscript{AFU}/EC\textsubscript{50} \textsubscript{Cai} \times Hillslope)], where R is the % normalized maximal Ca\textsuperscript{2+} response at a given Ca\textsuperscript{2+} concentration C and Hillslope is the steepness of the curve. The best fit to this expression was at EC\textsubscript{50} = 1.98 mM and Hillslope = 2.37. A near-maximal Ca\textsuperscript{2+} response was at Ca\textsuperscript{2+} concentrations of ~5 mM. The calculated values agree well with those derived graphically (r\textsuperscript{2} = 0.97).

expression pattern in both surface and crypt cells in rat colon is given both by the receptor-mediated responses in Ca\textsuperscript{2+} and Ins(1,4,5)P\textsubscript{3} to classic CaSR agonists and by the responses to luminal and bath application of agonists in isolated perfused colonic crypts. It should be noted, however, that the present study has not defined which of the CaSRs in the surface cells was activated. An experimental setup analogous to that used for crypt cells is required.

The EC\textsubscript{50} value for Ca\textsuperscript{2+} of 2.0 mM is in the same range as the EC\textsubscript{50} value for Ca\textsuperscript{2+} (1.6 mM) reported in a previous study (11) that measured unidirectional transmural Ca\textsuperscript{2+} flux (J\textsubscript{m-w}) in isolated rat colon mucosa. It is likely that the influence of extracellular levels of Ca\textsuperscript{2+} on these two different aspects of colon function is mediated by the same mechanism for Ca\textsuperscript{2+}, i.e., the CaSR. The EC\textsubscript{50} value for Ca\textsuperscript{2+} observed in isolated colon epithelial cells is also comparable with that observed in isolated parathyroid cells (25, 28), plasma ionized Ca\textsuperscript{2+}-induced increases in urinary Ca\textsuperscript{2+} excretion by human kidney (13), and Ca\textsuperscript{2+}-mediated decreases in cAMP accumulation in isolated rat cortical thick ascending limb cells (10). The Ca\textsuperscript{2+} EC\textsubscript{50} value for the colonic CaSR is, however, somewhat lower than those observed for CaSR expressed in Xenopus oocytes, HEK-293, cells or cultured keratinocytes [see recent review by Brown and MacLeod (6) and also Refs. 1 and 4]. Although these differences may reflect tissue- and/or species-specific alterations in Ca\textsuperscript{2+} affinity, they may also be attributable in part to experimental conditions used; for example, ionic strength and pH of incubation buffers and the presence of amino acids in the medium for cell cultures are known to affect Ca\textsuperscript{2+} sensing (9, 26).

The major function of the colon is to absorb fluid. In kidney, modulation of salt and fluid absorption by CaSR has been demonstrated in various tubular segments where the receptor is localized (5, 29). To assess if the colonic CaSR is a modulator of intestinal fluid...
movement, in the present study we measured changes in $J_c$ in isolated perfused colonic crypts using the in vitro microperfusion technique (33). We found that activation of either the apical or basolateral CaSR reversed net fluid secretion elicited by forskolin stimulation to net absorption (see Fig. 7). This reversal of forskolin-stimulated net fluid secretion may be due to either a reduction in secretory flux or a stimulation of absorptive flux. The mechanism for modulation of forskolin-stimulated fluid secretion may be due to CaSR-mediated reduction in intracellular cAMP accumulation as observed in cortical thick ascending limb (10) and in several other cell types [see review (6)]. In thick ascending limb, $\text{Ca}^{2+}$ at concentrations $<2 \text{ mM}$ increases cAMP destruction but higher $\text{Ca}^{2+}$ concentrations diminish cAMP production by adenylyl cyclase. This novel pathway for modulating intestinal fluid secretion through the colonic CaSR may lead to new therapies for prevention or treatment of certain clinical diarrheal diseases (i.e., cholera and other cAMP-associated diarrheal diseases). Clearly, further studies are needed to understand which transporter activity is altered and what exact mechanisms are involved in this process.

Even though the present study has identified the colonic CaSR as a potential modulator of regulated intestinal fluid transport, the high expression of CaSR in both luminal and basolateral surfaces of colon epithelium may also indicate physiological roles and functions in addition to regulating fluid absorption/secretion. The CaSR is abundantly expressed in the parathyroid gland, intestines, and absorption/secretion. The CaSR is abundantly expressed and functions in addition to regulating $\text{Ca}^{2+}$ homeostasis. There is evidence that colon, like duodenum, is also capable of absorbing $\text{Ca}^{2+}$ (11, 12, 20), whose magnitude depends on the concentrations of $\text{Ca}^{2+}$ and 1,25-dihydroxyvitamin D$_3$ (11, 12). The presence of CaSR at the basolateral border of the colon epithelial cells is consistent with roles for the receptor in sensing $\text{Ca}^{2+}$ concentration in blood or extracellular fluid and in potentially providing a negative feedback response to this divalent mineral being absorbed via the active transcellular pathway. In the kidney, vitamin D treatment increased the expression of CaSR mRNA (3). Vitamin D can also increase protein kinase C activity (24, 35). The latter has been shown to modulate the CaSR function in vitro (1, 28). Therefore, intestinal CaSR may also be implicated in regulation of intestinal $\text{Ca}^{2+}$ absorption by 1,25-dihydroxyvitamin D$_3$.

The CaSR in colon may also be regulating epithelial cell proliferation and differentiation. The epithelium of the colon as well as the small intestine is in a state of constant renewal. Cells proliferate and become differentiated as they migrate out of the base of the crypt to the surface. Thus cells at the base of the crypt are highly proliferative but less differentiated, and cells at the surface of the colon epithelium, on the other hand, are highly differentiated with little or no proliferation. In this regard, the CaSR in keratinocytes and certain other cells has been shown to modulate proliferation/differentiation and to alter the activities of mitogen-activated protein kinases and tyrosine kinases associated with cell proliferation (19, 22, 34, 36). Thus it is possible that the CaSR may be mediating the high dietary $\text{Ca}^{2+}$ responses of colonic mucosal epithelium in promoting cell differentiation, decreasing cell growth, and reducing the risk for development of colorectal cancer (2, 17, 18, 31).

Of particular interest in the colon is the intense CaSR immunoreactivity localized on the apical aspects of cells (see Fig. 2, B–D). The apical localization is consistent with potential roles for this receptor in sensing and responding to changes in luminal $\text{Ca}^{2+}$ and other receptor agonists such as amino acids and polyamines. Demonstration of functional CaSR in the colon shows that this tissue represents yet another potential site for regulating $\text{Ca}^{2+}$ and fluid homeostasis.

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