Am J Physiol Gastrointest Liver Physiol 292: G753–G766, 2007.
First published November 30, 2006; doi:10.1152/ajpgi.00225.2006.

The extracellular calcium-sensing receptor reciprocally regulates the secretion of BMP-2 and the BMP antagonist Noggin in colonic myofibroblasts

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Submitted 19 May 2006; accepted in final form 26 November 2006

Peiris D, Pacheco I, Spencer C, MacLeod RJ. The extracellular calcium-sensing receptor reciprocally regulates the secretion of BMP-2 and the BMP antagonist Noggin in colonic myofibroblasts. *Am J Physiol Gastrointest Liver Physiol* 292: G753–G766, 2007. First published November 30, 2006; doi:10.1152/ajpgi.00225.2006.—To understand whether postprandial extracellular Ca^{2+} (Ca_o^{2+}) changes were related to intestinal epithelial homeostasis, we performed array analysis on extracellular calcium-sensing receptor (CaSR)-expressing colonic myofibroblasts (18Co cells) and observed increases in bone morphogenetic protein (BMP)-2 transcripts. The present experiments demonstrated that regulated secretion of BMP-2 occurs in response to CaSR activation of these cells and revealed a new property of BMP-2 on the intestinal barrier. Activation by Ca_o^{2+}, spermine, $GdCl_3$, or neomycin sulfate of 18Co cells or primary isolates of myofibroblasts from the normal human colon stimulated both the synthesis (RT-PCR) and secretion (ELISA) of BMP-2. Transient transfection with short interfering RNA against CaSR completely inhibited BMP-2 secretion. Transient transfection with dominant negative CaSR (R185Q) increased the EC_{50} of Ca_o^{2+} (5.7 vs. 2.3 mM). Upregulation of BMP-2 transcript and secretion occurring within 3 h of CaSR activation was prevented by actinomycin D. CaSR-mediated BMP-2 synthesis and secretion required phosphatidylinositol 3-kinase activation (as assessed by phospho-Akt generation). Exogenous BMP-2 and conditioned medium from CaSR-stimulated 18Co cells accelerated restitution in wounded postconfluent Caco-2 cells. Exogenous BMP-2 and conditioned medium from CaSR-stimulated 18Co cells increased the transepithelial resistance of low- and high-resistance T-84 epithelial monolayers. CaSR stimulation of T-84 epithelia and colonic myofibroblasts downregulated the BMP family antagonist Noggin, as assessed by RT-PCR and Western blot analysis. Together, our data suggest that the CaSR mediates the effective concentration of BMP-2 in the intestine, which leads to enhanced repair and barrier development.

bone morphogenetic protein 2; barrier

THE AMOUNT of free ionized Ca^{2+} in reconstituted dried milk is ~7 mM (33). Perfusates containing 7–10 mM Ca^{2+} resulted in increases of extracellular Ca^{2+} (Ca_o^{2+}) from ~ 1 to 1.9 mM in the duodenal submucosal interstitium (33). These changes are of sufficient magnitude to be detected by the G protein-coupled, extracellular calcium-sensing receptor (CaSR) (5, 6, 19). The CaSR has been shown to be expressed on the epithelia of the stomach, small intestine, and colon (8–10). We speculated that if some cells in the lamina propria expressed the CaSR, then the postprandial changes in Ca_o^{2+} occurring regularly could stimulate these cells to signal to the luminal epithelia and perhaps contribute to epithelial homeostasis (25). One such cell type that might express the CaSR is the colonic subepithelial myofibroblast, which sits under the basement membrane and is physically poised to signal to the intestinal epithelia.

The CaSR was originally cloned from the parathyroid gland but was subsequently found to be widely expressed on cell types uninvolved in Ca^{2+} homeostasis (6). Evidence about the CaSR's physiological role in the gut is limited to the colon, where its activation by either Ca_o^{2+} or spermine inhibits forskolin-stimulated secretion (9, 10). High Ca^{2+} has been shown to inhibit β-catenin signaling on colonic cancer cell lines (7), and expression of the colonic CaSR is progressively lost during the transition from adenoma to carcinoma (7, 41). When activated in heterologous or endogenously expressing cells, the CaSR stimulates several MAPKs (MEK1, MEK2, ERK1, ERK2, p38, and JNK) using filamin A as a scaffold (21, 23, 26, 42, 43). Recently, it has been reported that CaSR activation "transactivates" the EGF receptor (EGFR) through a triple membrane-spanning signaling cascade (27).

Intestinal myofibroblasts are a resident cell type in the lamina propria (36, 37). Numerous studies have documented the constitutive secretion of a large number of growth factors from myofibroblasts with changes in growth factor secretion occuring during inflammatory bowel diseases (28, 29). The 18Co human colonic myofibroblast cell line has been extensively used to demonstrate signaling cascades from these cells in response to the proinflammatory cytokine IL-1β (31, 32) and secretes TGF-β isoforms (37).

Bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily. BMP-4 is a canonical Wnt target gene, and BMP-2 has recently been shown to be expressed in the epithelia of surface and crypt cells in the human and mouse colon (17). Noggin, the diffusible antagonist of BMP receptor (BMPR) interactions, has also been reported to be expressed in the human and mouse colon (17). Evidence for a functional role of BMP-2 in the intestine is scant as homozygous knockout mice for BMP-2 die at birth (44). A recent report (17) has suggested that exogenous BMP-2 stimulates the upregulation of some markers of differentiation and promotes apoptosis in colonic cell lines, but in vivo data showed equal rates of proliferation and apoptosis when mice were fed chimeric Noggin.

In the present study, we show that colonic myofibroblasts (18Co cells) express the CaSR and that activation of the CaSR results in the synthesis and secretion of BMP-2. We defined the

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signal transduction cascade activated by the CaSR to cause BMP-2 synthesis and secretion and showed, using a scrape-migration assay of postconfluent Caco-2 cells, that this BMP-2 is biologically active in accelerating restitution. Using T-84 colonic adenocarcinoma cells, which express the CaSR (14), grown on Transwells to model the intestinal barrier, we added exogenous BMP-2 and observed increases in transepithelial resistance (TER) that occurred with cells of both low and high resistances. We further determined that CaSR activation of myofibroblasts and the intestinal epithelia substantially down-regulates transcripts for BMP-4, a Wnt target, and eliminates the transcript and protein of Noggin, the BMP antagonist in both cell types. Therefore, the net effect of CaSR activation is to increase the effective concentration of BMP-2 in epithelia and subepithelia. The released BMP-2 accelerates the restitution and barrier development of the intestine. This represents a new function of BMP-2 in the adult intestine.

MATERIALS AND METHODS

Materials. Polyclonal antisera against phospho-Akt was obtained from Cell Signaling (Beverly, MA). Polyclonal antisera for CaSR, PA 1-1034, was purchased from ABR (Golden, CO). BMP-2 ELISA kits, anti-BMP2 antibody, and recombinant human (rh) BMP-2 were purchased from R&D Systems (Minneapolis, MN). Inhibitors such as GM-6001 (PKC inhibitor), AG-1478 (EGFR kinase inhibitor), AG-1295 (PDGF inhibitor), LY-2104002 [phosphatidylinositol 3-kinase (PI3K) inhibitor], SP-600125 (JNK kinase inhibitor), protein phosphatase 2 (PP2; Src family inhibitor), SB-203580 (p38 MAPK inhibitor), PD-1080510 (MEK1 inhibitor), KN-62 (Ca^{2+}/calmodulin-dependent protein kinase II inhibitor), and HCl bisindolylmeleimide I (PKC inhibitor) were purchased from Calbiochem-Novabiochem (San Diego, CA). The enhanced chemiluminiscense supersignal kit was from Pierce (Rockford, IL). Cell culture media, Minimun Essential Medium Alpha Medium, DMEM-F-12, DMEM, and RPMI 1640 medium, with or without Ca^{2+}, were obtained from GIBCO-BRL (Grand Island, NY). Protease inhibitors were from Boehringer Ingelheim.

Cell culture. 18Co, T-84, HT-29, SW480, and Caco-2 cell lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD). Stably transfected CaSR HEK cells were a gift from E. M. Brown (Harvard Medical School, Boston, MA). The 18Co line was cultured in Minimal Essential Medium Alpha supplemented with 10% FBS and 100 μU/ml penicillin and grown at 37°C in a humidified 5% CO_2 atmosphere. Cells were passaged weekly with 0.25% trypsin and used for experimentation within the first six passages. HT-29 and Caco-2 cells were cultured in DMEM supplemented with 10% or 20% FBS, respectively, and 100 μU/ml penicillin. T-84 cells were seeded to reach confluency in Transwells in DMEM-F-12 supplemented with 10% FBS and 100 μU/ml penicillin. Control human colonic myofibroblasts isolated by conventional protocols (28, 29) and transformed by telomerase were a generous gift from R. Mifflin (Galveston, Texas).

Generation of short interfering RNA against the CaSR. We found 13 potential short interfering (si)RNA target sites in the extracellular domain of the CaSR. Based on a GC content of >40 but <57%, we evaluated siRNA target sites by a BLAST analysis and then selected those that gave <14 hits. The present experiments used nucleotides 371–390 (5′-AACCTTGATGAGTTCTGCAAC-3′), to which were added complementary nucleotides to a T7 promoter primer. Sense and antisense oligonucleotide templates were generated, and transcribed siRNA was produced according to the manufacturer's protocol (Silencer siRNA Construction Kit, Ambion). Nucleotides 371–390 were scrambled, and a BLAST analysis was preformed to confirm the lack of specificity of this construct. T7 sites were added to this oligonucleotide template, and it was processed as described above. 18Co cells were transfected with 25–50 nM siRNA of either the scrambled construct or siRNA against the CaSR using Superfect according to the manufacturer's instructions (Superfect, Qiagen, Valencia, CA).

Transfection of 18Co cells with dominant negative CaSR. 18Co cells were transiently transfected with dominant negative CaSR (R185Q) using Superfect according to the manufacturer's instructions (Qiagen). Cells were then incubated for 18 h in serum-free, Ca^{2+}-free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM $CaCl_2$ and stimulated with high (5 mM) and low (0.5 mM) Ca^{2+}. At 18 h of stimulation, aliquots of media were centrifuged at 12,000 rpm at 4°C for 3 min, and supernatants were used for immediate BMP-2 ELISA determination.

Western blot analysis of the CaSR in 18Co cells. Immunoblot analysis for the CaSR was performed using confluent monolayers of 18Co cells that had been cultured in 10-cm plastic plates. Monolayers were rinsed once with ice-cold PBS containing 1 mM sodium vanadate and 25 mM NaF and scraped on ice into lysis buffer containing 10 mM Tris·HCl (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, 1% Triton X-100, 1 mM dithiothreitol, and a cocktail of protease inhibitors (10 μg/ml each of aprotinin, leupeptin, calpain, pepstatin, and soybean trypsin inhibitor as well as 100 μg/ml Pefabloc). NaF, sodium vanadate, and Pefabloc were freshly prepared on the same day of the experiments. Lysates were sonicated for 5 s and centrifuged at 10,000 *g* at 4°C for 10 min, and the resultant total cellular lysate in the supernatant was stored at −70°C for batch analysis. Protein concentrations were measured using the Bio-Rad protein assay. Aliquots of supernatant fractions containing 80 μg protein from 18Co cells and 5 μg protein of CaSR HEK cells were mixed with an equal volume of 2× SDS-Laemmli gel loading buffer under nonreducing conditions, incubated at 65°C for 30 min, and resolved electrophoretically on 6.5% acrylamide gels. Separated proteins were then transferred to Immobilon 0.2-μm membranes and incubated with blocking solution (PBS with 0.1% Tween 20 and 5% dry milk in 20 ml) for 1 h at room temperature. Blots were subsequently incubated overnight at 4°C with PA 1-1034 affinity-purified polyclonal antiserum at a 1:100 dilution. Blots were washed five times with PBS containing 0.1% Tween 20 at room temperature for 5 min each. Blots were further incubated with a 1:2,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG (Sigma) in blocking solution for 1 h at room temperature. Blots were then washed five times in washing solution, and bands were visualized by chemiluminescence according to the manufacturer's instructions (Supersignal, Pierce Chemical).

Phospho-Akt, total Akt, and Noggin Western blot analysis. For the determination of Akt or Noggin of 18Co cells, early passage cells were grown on six-well dishes. Cells were incubated for 18 h in serum-free, Ca^{2+}-free DMEM (as described above). This medium was removed and substituted with the same medium supplemented with 0.5 or 5 mM Ca^{2+}. At the end of the incubation period, cells were washed once with ice-cold PBS containing 1 mM sodium vanadate and 25 mM NaF, and 100 μl of ice-cold lysis buffer were added [20 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM glycerophosphate, and a cocktail of proteases inhibitors, as described above]. After being sonicated for 5 s, lysates were centrifuged at 10,000 *g* for 10 min at 4°C and processed as previously described (26, 27). Total Akt and phospho-Akt were detected by an overnight incubation with a 1:1,000 dilution of rabbit polyclonal antibodies against Akt or phospho-Akt in 1× PBS and 0.1% Triton X-100 with 5% BSA. Noggin was detected with a 1:500 dilution of primary antibody in PBS and Triton X-100 with 2.5% milk. Blots were washed for three 5-min periods at room temperature (1× PBS and 0.1% Triton X-100) and then incubated for 1 h with a secondary antibody conjugated to horseradish peroxidase (1:2,000) in blocking solution. Blots were then washed a second time (3 × 5 min). Bands were visualized by chemiluminiscence as previously described (25). Quantitation of the phosphorylation of Akt was done using an ImageQuant and a Personal Densitomenter (Molecular Dynamics).

ELISA determinations. Cells were incubated for 18 h in serum-free, Ca_o^{2+}-free DMEM containing 4 mM L-glutamine, 0.2% BSA, and 0.5 mM $CaCl_2$. For kinetic studies, this medium was removed and substituted with the same medium supplemented with $CaCl_2$ (5 mM) for 0, 3, 6, 12, 18, and 24 h. Based on preliminary results, the well-characterized CaSR agonists $GdCl_3$ (25 mM), neomycin sulfate (300 mM), and spermine (2 mM), inhibitors of various MAPKs, and PKC inhibitors were assessed at 18 h. At the end of the incubation period, aliquots of media were centrifuged at 12,000 rpm at 4°C for 3 min, and supernatants were used for immediate BMP-2 determinations. Similarly, HT-29, SW-480, and T-84 cells were incubated in serum-free and 0.5 mM Ca_o^{2+} DMEM over 18 h, and supernants were collected for immediate BMP-2 ELISA determination.

RT-PCR. Differential gene expression analysis was performed by semiquantitative PCR using a Mastercycler (Eppendorf, Hamburg, Germany). Total RNA of 18Co cells was isolated by TRIzol reagent by following manufacturer's instructions. The recovered RNA was quantitated by spectrophotometry, and aliquots of 1 μg total RNA from low (0.5 mM) or high (5 mM) Ca_o^{2+} were used for cDNA synthesis at 37°C for 1 h in a volume of 20 μl containing the following reagents: 5 mM dNTP mix, 10 μM oligo-dT primer (Invitrogen, Carlsbad, CA), 10 units RNase inhibitor (Roche), 4 units Omniscript reverse transcriptase (Qiagen), and 1× buffer RT (Qiagen). The PCR was prepared in a volume of 50 μl containing the following reagents: 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 1 μl template cDNA, and 1.0 units platinum *Taq* DNA polymerase (Invitrogen). The PCR cycle conditions were as follows: initial denaturation at 104°C for 60 s, followed by 35 amplification cycles denaturing at 104°C for 30 s, annealing at an amplicon-dependent temperature for 30 s, and elongation at 72°C for 1 min, followed by a final elongation at 72°C for 10 min. The primers for the CaSR were sense 5′-CGGGGTACCTTAAGCACCTACGGCATCTAA-3′ and antisense 5′-GCTCTAGAGTTAACGCGATCCCAAAGGGCTC-3′; the sequences used for BMP-2 were sense 5′-AGTAGCAATGAATTTTTAACC-3′ and antisense 5′-GTAGCAGAAACCCTTAGTTAG-3′; the sequences used for Noggin were sense 5′-GCAGCTGCTTCAGTAAGCG-3′ and antisense 5′-GCACTCGGAAATGGG-3′; and the sequences used for BMP-4 were sense 5′-TCCATGCTGTACG-3′ and antisense 5′-TGAGTGGATGGGAACGTGT-3′. Resulting bands were visualized on a 1.0% agarose gel stained with ethidium bromide and compared with a 100-bp DNA ladder (NEB) to confirm the predicted sizes. For the positive amplification control, we used the GAPDH gene (sense 5′-TTACTCGCACCCCTGGCCAAGG-3′ and antisense 5′-CTTACTCCTTGGAGGCCATG-3′).

TER. T-84 epithelial cells were seeded to form confluent monolayers to develop low electrical resistance on Transwell-permeable supports (area of 0.33 cm^2) in 24-well plates (Corning, Corning, NY). Transwell filters were precoated in our laboratory with rat collagen (Sigma). TER was measured using an epithelial voltmeter (WPI, Sarasota, FL) and STX2 electrodes. TER measurements were done at 24-, 48-, 72, and 120-h intervals in the presence of rhBMP-2. In additional experiments, we assessed the effect of apical vs. basolateral addition of rhBMP-2 for 72 h. Finally, to evaluate the biological activity of BMP-2 secreted from 18Co cells after activation, conditioned medium from 18Co cells was collected after 18 h of stimulation with 3 mM Ca_o^{2+} . This medium was added to T-84 cells, and TER was measured after 72 h of incubation in the presence or absence of antibody against BMP-2.

Scrape-wound assays. Caco-2 cells were allowed to grow to confluence on six-well Costar plates that had been scored across the well with a black marker. Cells were serum starved in low-Ca^{2+} medium (as described above) overnight before wounding. At *day 5* postconfluence, a sterile P20 tip was used to create a wound at right angles to the scored line. The width of the wound was measured under the microscope using a ×40 objective at the level of the scored mark. Ca^{2+}-supplemented medium, which was serum free (0.2% BSA), was added and replaced every 24 h. The width of each wound was measured every 6 h for 48 h. Restitution in the presence of CaSR activation occurred within 72 h in differentiated Caco-2 cells and within 48 h in subconfluent Caco-2 (~70% confluent) cells. Rates of restitution were calculated as the difference in the width at each time point compared with the starting width expressed per hour.

Statistics. Data are presented as means ± SE of at least 3 separate experiments. Data were analyzed by Student's *t*-test or ANOVA when appropriate. $P < 0.05$ was set as the statistically significant difference level.

RESULTS

CaSR expression in 18Co cells: RT-PCR and Western blot analysis. We first assessed whether CaSR expression was present on 18Co cells, a colonic myofibroblast cell line, by RT-PCR and Western blot analysis. Figure 1*A* shows the results of RT-PCR performed on deoxyribonuclease-treated total RNA (2 μg) obtained from the cells. Use of an intron-spanning primer pair amplified a cDNA product of the expected length (480 bp). Sequencing revealed 100% homology with the corresponding segment of human parathyroid CaSR. For Western blot analysis, we used total unfractionated lysates of myofibroblasts. Heterologously expressing CaSR HEK cells were used as a positive control. Under nonreducing conditions, strong immunoreactive bands were observed at >218 kDa, corresponding to the stably CaSR in transfected HEK cells, which is most likely representative of multimeric CaSR (Fig. 1*B*). These signals disappeared when the antibody was preincubated with the specific blocking peptide (data not shown). A

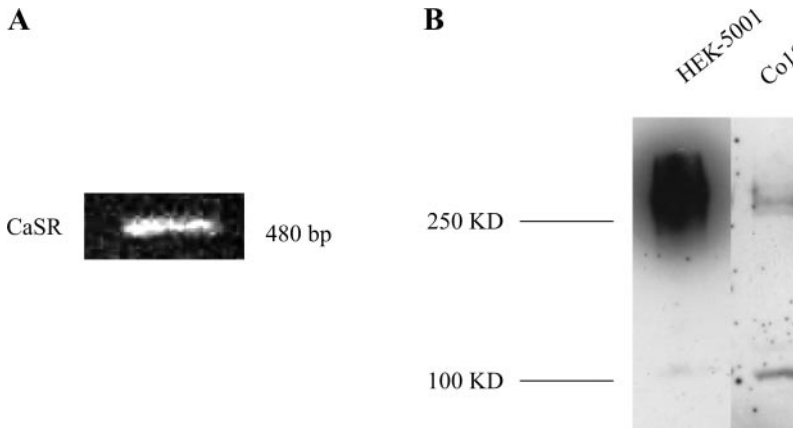


Fig. 1. Expression of extracellular calcium-sensing receptor (CaSR) in colonic myofibroblasts. *A*: RT-PCR showing the 480-bp product obtained with intron-spanning primers designed against the CaSR. *B*: Western blot analysis of 18Co and stably CaSR-transfected HEK cells (positive control) showed a multimeric CaSR-immunoreactive band at 250 kDa and a monomeric band at 120 kDa.

lower band of ~120 kDa was also observed, which would be consistent with monomeric CaSR; this band also was lost with preabsorption by the blocking antibody. Together, these data suggested to us that 18Co myofibroblasts express the CaSR.

Ca_o^{2+} and other CaSR agonists stimulate BMP-2 synthesis and secretion. To determine whether activating the CaSR had an effect on BMP-2 secretion from endogenously CaSR-expressing human colonic myofibroblasts, we incubated these cells in serum-free medium supplemented with different amounts of Ca_o^{2+} for 18 h and then measured secreted BMP-2 in the medium using ELISA (Fig. 2*A*, *left*). In low-Ca_o^{2+} medium (0.5 mM Ca^{2+}), there was no detectable BMP-2 in the medium. Increasing Ca_o^{2+} by a modest amount to 1 mM stimulated BMP-2 secretion, which was 47% of the maximal

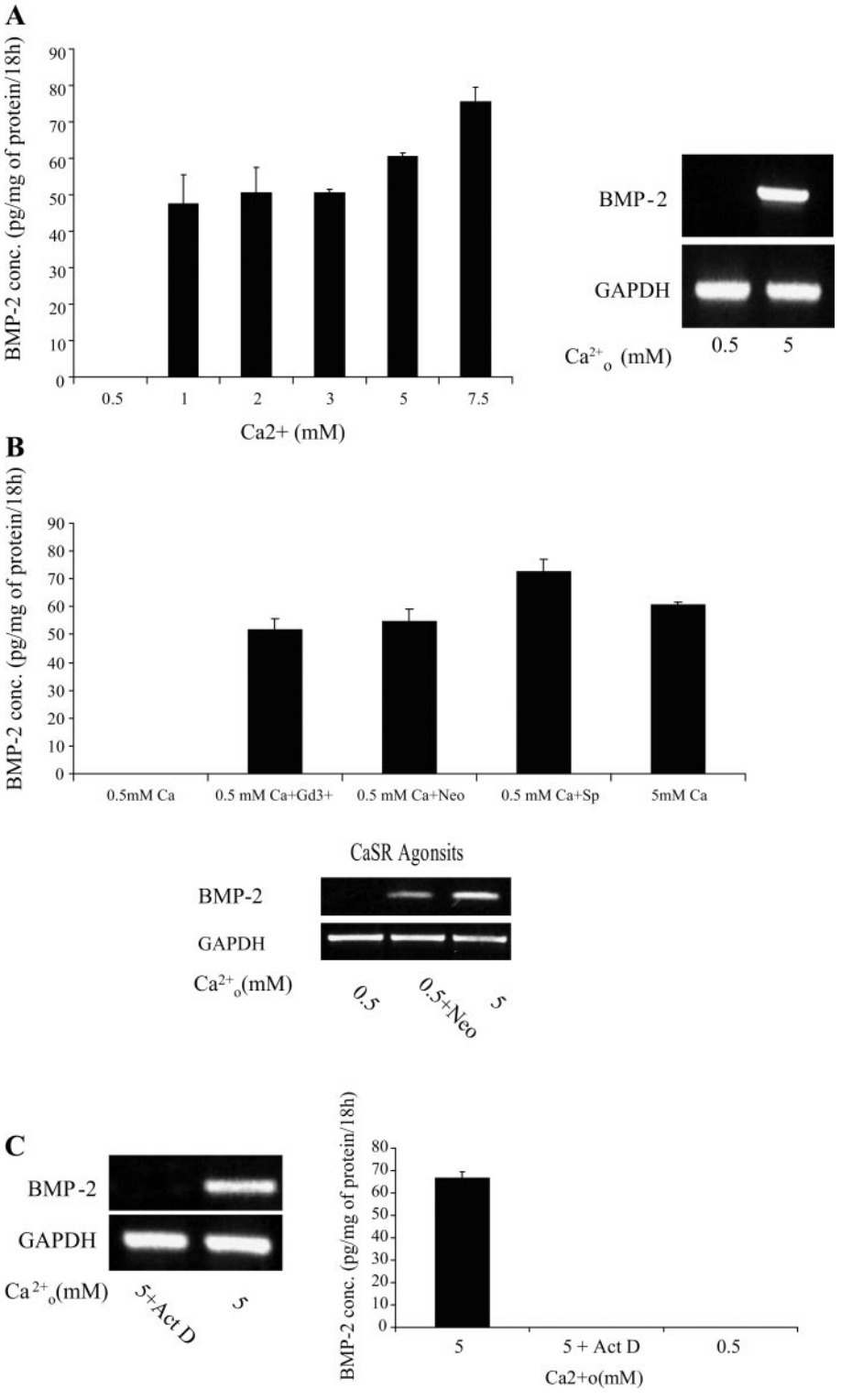


Fig. 2. Effect of different extracellular Ca^{2+} (Ca_o^{2+}) concentrations ($[Ca^{2+}]_o$) and polycationic agonists on the synthesis and secretion of bone morphogenetic protein (BMP)-2 from colonic myofibroblasts. *A*: dose-response of Ca_o^{2+} on BMP-2 secretion. Cells were treated for 18 h, and conditioned media were assessed for BMP-2 by ELISA. In low Ca_o^{2+} (0.5 mM), no measurable BMP-2 was observed (*left*). The amount of BMP-2 in 7.5 mM Ca_o^{2+} was greater than that in 3 mM Ca_o^{2+} ($P < 0.05$, $n = 5$). Ca_o^{2+} (5 mM) induced the expression of BMP-2 from the 18Co cell line as assessed by RT-PCR (*right*). *B*: effect of different agonists on the synthesis and secretion of BMP-2. *Top*, Gd^{3+} (25 μM), neomycin (Neo; 300 μM), and spermine (Sp; 2 mM) induced BMP-2 secretion from 18Co cells to comparable levels as with 5 mM Ca_o^{2+}. *Bottom*, RT-PCR showed that both 5 mM Ca_o^{2+} and neomycin induced the expression of BMP-2. *C*: effect of inhibition of RNA synthesis with actinomycin D (Act D) on BMP-2 synthesis and secretion. Cells were treated with 5 μg/ml actinomycin D together with 5 mM Ca_o^{2+}. Actinomycin D completely downregulated the Ca^{2+}-induced BMP-2 synthesis as assessed by RT-PCR (*left*) as well as secretion (*right*).

stimulation measured in response to 7.5 mM Ca_o^{2+} (47 ± 8 vs. 75 ± 5 pg·mg protein^{-1}·18 h^{-1}, $P < 0.05$, $n = 5$). While there were no differences in the amounts of BMP-2 secreted in the range of 1–3 mM Ca_o^{2+} (47 ± 8 vs. 48 ± 5 vs. 50 ± 1 pg·mg protein^{-1}·18 h^{-1}), there was more BMP-2 secreted in response to 7.5 mM Ca_o^{2+} (75 ± 4 pg·mg protein^{-1}·18 h^{-1}, $P < 0.05$, $n = 5$) compared with 3–5 mM Ca_o^{2+}. We next determined whether high Ca_o^{2+}-stimulated BMP-2 release occurred at the level of transcription (Fig. 2*A*, *right*). BMP-2 transcripts were upregulated by high Ca_o^{2+}, whereas no transcripts were observed at 0.5 mM Ca_o^{2+}.

We also determined the effect of other well-characterized polyvalent cation agonists of the CaSR on BMP-2 secretion from 18Co cells (Fig. 2*B*). In low-Ca_o^{2+} medium, the addition of either $GdCl_3$ (25 μM), neomycin sulfate (300 μM), or spermine (2 mM) all stimulated robust secretion of BMP-2 from these colonic myofibroblasts. Amounts of BMP-2 secreted were not different compared with stimulation elicited by 5 mM Ca_o^{2+}, which was used as a positive control during each experiment.

We then assessed whether the high-Ca^{2+} or CaSR agonist neomycin reflected the stimulation of transcription and secretion from 18Co cells. We used RT-PCR with specific BMP-2 primers to compare the presence of BMP-2 transcripts in 18Co cells treated with either low (0.5 mM) or high (5.0 mM) Ca_o^{2+} for 18 h, as described above (Fig. 2*B*, *left*). Neomycin increased BMP-2 transcripts and protein, as did high Ca_o^{2+}. Incubation with actinomycin D (5 μg/ml) for 6 h prevented both the appearance of BMP-2 transcripts and protein (Fig. 2*C*).

Effect of siRNA and dominant negative CaSR on BMP-2 secretion. To understand whether the CaSR mediated the stimulation of BMP-2 secretion, we transiently transfected 18Co cells with siRNA designed against the CaSR. In separate experiments, we compared the effect of dominant negative CaSR (R185Q). BMP-2 concentrations after different concentrations of Ca^{2+} challenge were determined 48 h after cell transfection. The interfering RNA duplex (25 nM) completely inhibited Ca_o^{2+}-stimulated BMP-2 secretion, whereas scrambled siRNA had no effect on BMP-2 secretion (Fig. 3*A*). Transient transfection with R185Q right shifted the dose-response curve of wild-type cells (Fig. 3*B*). The EC_{50} for cells transfected with the wild type was 2.3 mM, whereas the EC_{50} for cells transfected with R185Q was 5.7 mM.

We then asked if the rate of synthesis and secretion of BMP-2 from these cells was first order using RT-PCR and ELISA. As shown in Fig. 4, the upregulation of the BMP-2 cDNA product was rapid; within 3 h of Ca^{2+} treatment, the cDNA product was observed (Fig. 4*A*). Interestingly, this was also manifested at the protein level as 1,600 ng/mg protein of BMP-2 was exported into the medium after 3 h of incubation with high Ca^{2+} (Fig. 4*B*). The stimulation of BMP-2 transcripts and the secretion of BMP-2 protein continued at the same rate for 18 h and began to lessen by ~40% by 24 h.

Together, these data suggested to us that activating the CaSR on 18Co colonic myofibroblasts stimulated the synthesis and secretion of BMP-2.

We determined whether other $G_{q/11}$ agonists stimulated BMP-2 secretion from 18Co cells. ADP (1 μM), endothelin (1 nM), and angiotensin (1 μM) were added to 18Co cells, and the medium was screened by ELISA after an 18-h challenge. No BMP-2 was stimulated after any of these challenges; in contrast, cells treated with 3 mM Ca^{2+} robustly stimulated BMP-2 (66.9 ± 2 pg·mg protein^{-1}·18 h^{-1}, $P < 0.05$, $n = 3$). RT-PCR was performed on comparably treated cells and confirmed increased BMP-2 in response to CaSR activation, whereas no BMP-2 was generated after either ADP or endothelin treatment (data not shown).

Pharmacological basis of the CaSR stimulation of BMP-2 secretion. In further experiments, we determined the effect of different kinase and MAPK inhibitors on CaSR-stimulated BMP-2 secretion. These experiments are shown in Fig. 5. Cells were serum starved for 18 h and then challenged with 5 mM Ca^{2+} for 18 h, and the conditioned medium was used for ELISA. As before, no BMP-2 was detectable in low Ca^{2+} (0.5 mM) medium, whereas in 5 mM Ca_o^{2+} medium, a robust amount of BMP-2 was secreted (65 ± 3 pg·ml^{-1}·18 h^{-1}, $P < 0.05$, $n = 5$; Fig. 5*A*). An inhibitor of PI3K, LY-810103 (10 μM), obliterated the high-Ca^{2+} stimulation of BMP-2 secretion (3 ± 2 pg·mg protein^{-1}·18 h^{-1}, $P < 0.05$, $n = 5$). No

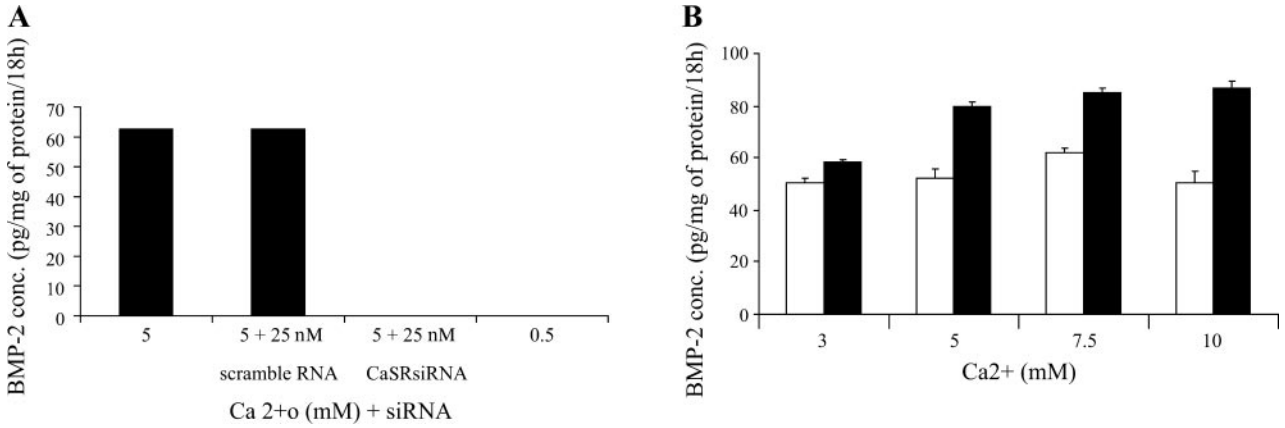


Fig. 3. Effect of short interfering (si)RNA against the CaSR or dominant negative CaSR (R185Q) on Ca^{2+}-stimulated secretion of BMP-2. *A*: effect of siRNA against the CaSR. Ca^{2+}-mediated BMP-2 secretion was blocked in cells transfected with 25 nM siRNA against the CaSR, whereas BMP-2 secretion was not affected in cells transfected with scrambled siRNA. *B*: effect of R185Q on BMP-2 secretion. Open bars, 18Co transfected with R185Q; solid bars, control cells with wild-type CaSR. Ca_o^{2+}-mediated BMP-2 secretion was reduced in cells with dominant negative CaSR, and the EC_{50} was shifted to the right compared with control cells.

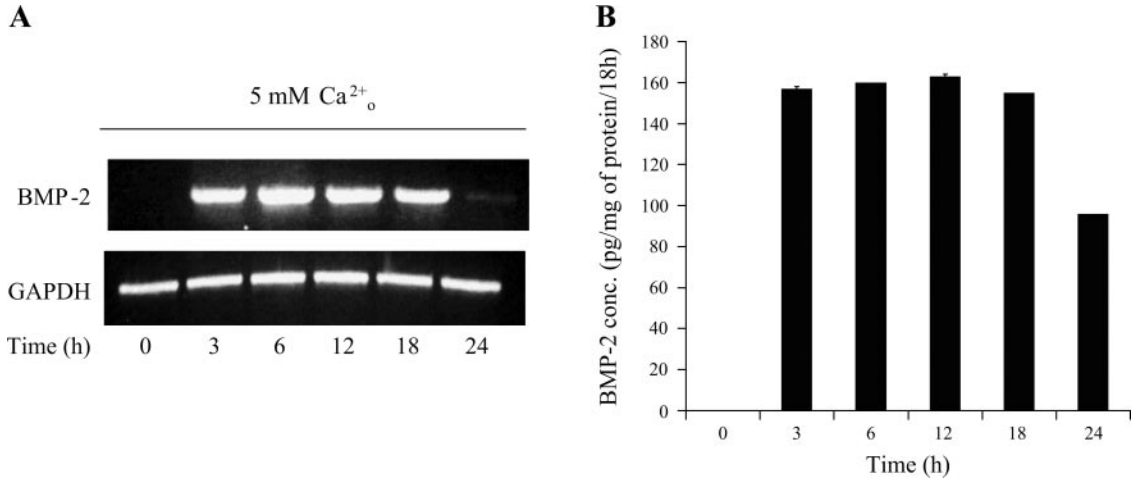


Fig. 4. Synthesis and secretion of BMP-2 at different time points over 24 h. *A*: synthesis of BMP-2 over 24 h. Upregulation of the BMP-2 transcript was observed at 3 h and was maximal at 6 h. *B*: secretion of BMP-2 over 24 h. Consistent with the RT-PCR results, BMP-2 protein was also increased at 3 h, remained constant for 18 h, and then declined.

effect on BMP-2 secretion in response to CaSR activation was observed in the presence of a calmodulin kinase inhibitor (50 μM KN-62), a JNK inhibitor (10 μM SP-60456), or a broad-spectrum PKC inhibitor (1 μM GF-190203X1). Selective tryphostin inhibitors of either EGFR kinase (500 nM AG-1478) or PDGF kinase (500 nM AG-1725) also had no effect on high-Ca^{2+} stimulation of BMP-2 release from 18Co cells. Selective inhibition of MEK1 and MEK2 using PD-1080108 (10 μM) also had no effect on the amount of BMP-2 secreted in response to high Ca^{2+} (58 ± 10 pg·mg protein^{-1}·18 h^{-1}, not

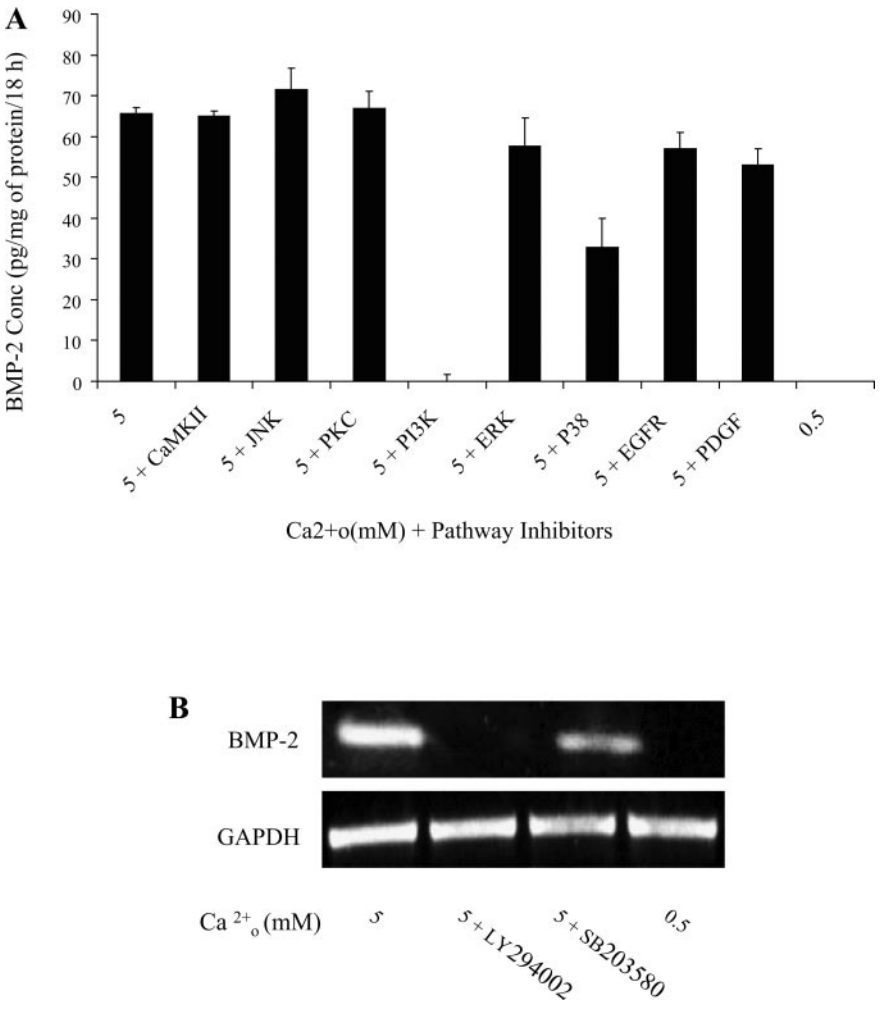


Fig. 5. Effect of MAPK and PKC inhibitors on basal and Ca^{2+}-stimulated BMP-2 secretion from 18Co cells. *A*: cells were incubated with 5 mM Ca_o^{2+} with or without the PKC inhibitor GF-190203X1 (1 μM), MEK inhibitor PD-98095 (10 μM), p38 inhibitor SB-203580 (10 μM), JNK inhibitor SP-600125 (10 μM), phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (20 μM), EGF receptor (EGFR) kinase inhibitor AG-1478 (1 μM), PDGF inhibitor AG-1295 (1 μM), or Ca^{2+}/calmodulin-dependant kinase II (CaMII) inhibitor KN-62 (10 μM) for 18 h. BMP-2 was measured in the conditioned medium. *B*: RT-PCR of BMP-2 showing comparable inhibitory effects of p38 and PI3K inhibitors at the transcriptional level.

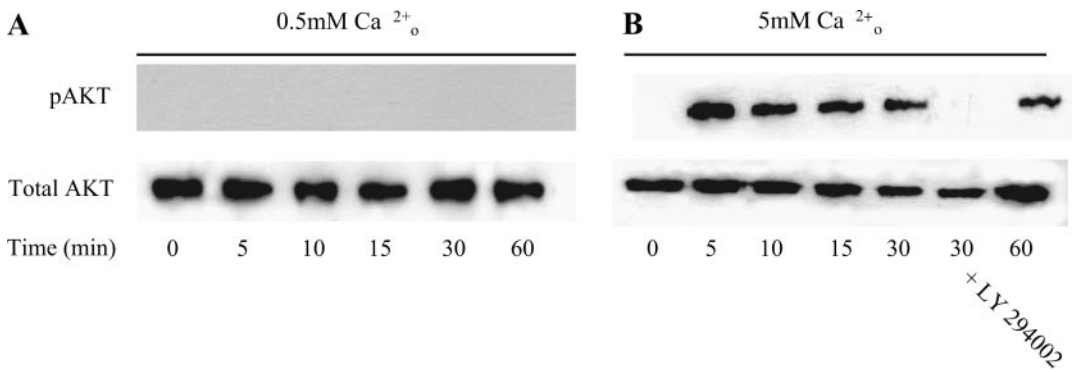


Fig. 6. Time course of the phosphorylation of the downstream target of the PI3K pathway Akt by high $[Ca^{2+}]_o$. *A*: low $[Ca^{2+}]_o$ did not induce the phosphorylation of Akt (pAkt). Total Akt levels showed equal loading. *B*: Akt was maximally phosphorylated at 5 min in response to high $[Ca^{2+}]_o$ and declined over the next 45 min.

significant, $n = 5$). A p38 MAPK inhibitor, SB-208230 (10 μM), reduced the amount of BMP-2 secreted in response to high Ca^{2+} (11 ± 8 pg·mg protein^{-1}·18 h^{-1}, $P < 0.05$, $n = 5$). Together, these data suggested to us that the CaSR activation of BMP-2 secretion required the stimulation of both PI3K and p38 MAPK. Furthermore, these data suggested that neither EGFR nor PDGF transactivation by the CaSR played a role in the high-Ca^{2+} stimulation of BMP-2 secretion from colonic myofibrobasts. We then assessed the effect of PI3K and p38 MAPK inhibitors on 5 mM Ca_o^{2+}-stimulated secretion of BMP-2 from Co18 cells. As shown in Fig. 5*B*, PI3K inhibition in the presence of high Ca^{2+} obliterated BMP-2 transcripts, whereas p38 MAPK inhibition attenuated BMP-2 transcripts.

Ca_o^{2+} stimulation of Akt in colonic myofibroblasts. Because inhibition of PI3K substantially inhibited the CaSR stimulation of BMP-2 secretion from Co18 cells, we went on to assess the impact of high Ca^{2+} addition on changes in phospho-Akt (also known as PKB). Akt is immediately downstream of PI3K, so it can serve as a index of PI3K induction. As shown in Fig. 6*A*, the addition of 0.5 mM Ca^{2+} did not stimulate the appearance of immunoreactive bands against an antibody for phospho-Akt. The effect of 5 mM Ca^{2+} added to colonic myofibroblasts is shown in Fig. 6*B*. The immunoreactivity of phospho-Akt was increased rapidly from 5 to 10 min after the addition of Ca^{2+} but declined over the next 45 min. The inclusion of LY-2104002 (10 μM) with cells lysed 30 min after Ca^{2+} (5 mM) addition prevented the appearance of phospho-Akt, consistent with the inhibition of the PI3K cascade. These findings suggested to us that CaSR activation was stimulating PI3K, leading to the phosphorylation of the kinase Akt. They further suggested to us that the PI3K inhibitor LY-2104002 prevented this activation.

Effect of CaSR activation on Noggin expression (RT-PCR and Western blot analysis) in colonic myofibroblasts. We then asked if activation of these cells influenced the expression of Noggin, the diffusible antagonist of BMPR(s), or the expression of other BMP family members. As shown in Fig. 7*A*, expression of the transcript for Noggin was absent in myofibroblasts incubated with 5 mM Ca^{2+} to activate the CaSR. Western blot analysis confirmed that Noggin was present constitutively and lost after high-Ca^{2+} treatment (Fig. 7*B*). Expression of BMP-4 was also reduced in cells exposed to high Ca_o^{2+} (Fig. 7*C*).

Primary human myofibroblasts express CaSR and BMP-2. To confirm that the present findings were not an artifact of using an established cell line, human colonic myofibroblasts were isolated by a standard protocol (29) and then treated with telomerase. These cells were then screened by RT-PCR for the repertoire of colonic myofibroblast markers (36). As shown in Fig. 8*A*, control myofibroblasts expressed transcripts for smooth muscle actin, smooth muscle myosin, and vimentin but lacked expression of the intermediate filament marker desmin. These human primary isolates also expressed the CaSR, as assessed by RT-PCR of the intron-spanning primer set used for 18Co cells (Fig. 8*B*). When treated with low (0.5 mM) or high (5 mM) Ca_o^{2+} for 18 h, these cells showed little BMP-2 transcript in low Ca^{2+}, which increased with high Ca_o^{2+} (Fig. 8*C*). Together, these results suggested that primary human colonic myofibroblasts, when challenged with high Ca^{2+}, upregulate transcripts for BMP-2.

Effect of BMP-2 on epithelial restitution and barrier function. To assess a role for CaSR-mediated BMP-2 secretion from myofibroblasts, we first employed an in vitro wound healing/restitution assay using differentiated Caco-2 cells at *day 5* postconfluence. We modified this assay from standard "scratch wound" protocols and used postconfluent Caco-2 monolayers to model the restitution of an epithelial barrier. We determined the rate of cell migration after wounding in response to low (0.5 mM) and high (3 mM) Ca_o^{2+} with conditioned medium from 18Co cells treated with 3 mM Ca^{2+} for 18 h, as in the above experiments. We then assessed the effect of adding exogenous BMP-2 (0.5 ng/ml) to the wounded monolayers in 0.5 mM Ca_o^{2+}. Exogenous rhBMP-2 substantially increased the rate of migration (143 ± 24 vs. 36 ± 17 μm/h, $n = 12$, $P < 0.05$). Conditioned medium from 18Co cells challenged with high Ca^{2+} added to wounded Caco-2 cells also accelerated the rate of restitution (187 ± 19 vs. 36 ± 17 μm/h, $n = 6$, $P < 0.05$). This stimulation was no different than that found when Caco-2 cells were challenged with 3 mM Ca^{2+} (230 ± 79 μm/h). The conditioned medium stimulation of restitution was attenuated by a 1:50 dilution of anti-BMP-2 antibody (94 ± 13 vs. 187 ± 19 μm/h, $n = 6$, $P < 0.05$). Together, these results suggested to us that BMP-2 released from activated myofibroblasts was biologically active in accelerating restitution in a model of modest wounding.

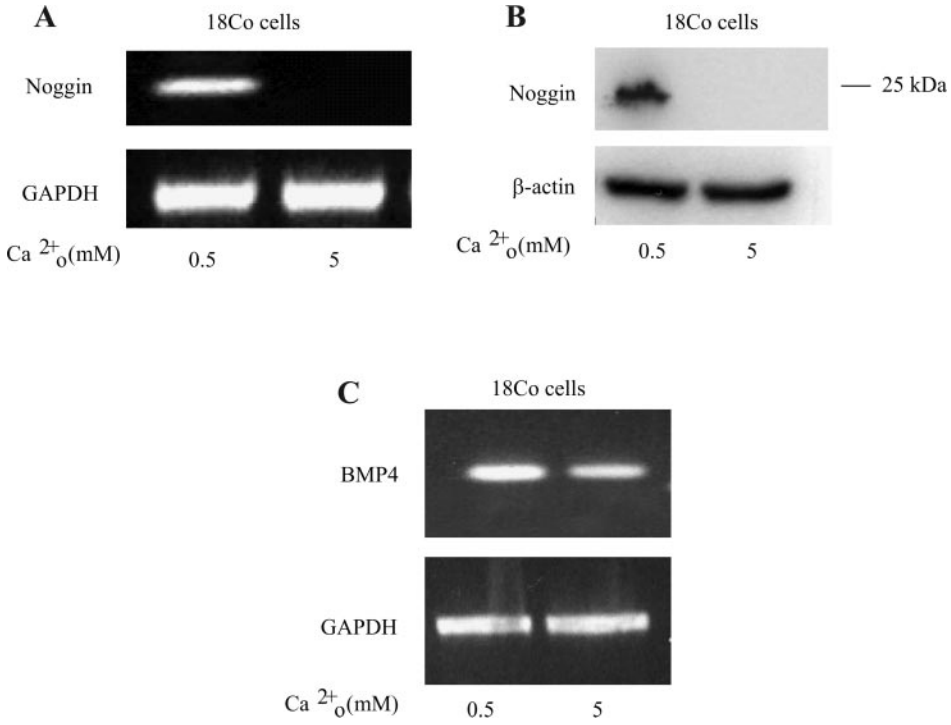


Fig. 7. Effect of $[Ca^{2+}]_o$ on the synthesis and secretion of Noggin and secretion of BMP-4. *A*: effect of $[Ca^{2+}]_o$ on the synthesis of Noggin in 18Co cells. *B*: effect of $[Ca^{2+}]_o$ activation on Noggin protein from 18Co cells. Cells were stimulated with either 0.5 or 5 mM $[Ca^{2+}]_o$, and lysates were prepared. *C*: effect of $[Ca^{2+}]_o$ on BMP-4 transcripts in 18Co cells.

To understand whether CaSR-mediated BMP-2 played a role in intact intestinal epithelia, we used T-84 cells, a colonic epithelial cell line that differentiates in a polarized fashion to model intact epithelia. We added rhBMP-2 to both the apical and basolateral sides of T-84 cells that had been seeded on collagen-coated Transwells and measured TER daily. As shown in Fig. 9*A*, untreated T-84 cells manifested a stepwise increase in TER with time. The addition of rhBMP-2 (0.5 ng/ml) to these cells increased TER at 24 h (335 ± 27 vs. 140 ± 30 Ω/cm^2, $P < 0.05$, $n = 8$), which was substantially higher at 48 h compared with untreated control cells (830 ± 107 vs. 330 ± 110 Ω/cm^2, $P < 0.05$, $n = 8$). Increasing the concentration of BMP-2 added (1, 10, and 30 ng/ml) caused greater increases in TER at 48 h, which were maximal at 0.5 ng/ml and decreased at 30 ng/ml. In parallel experiments, BMP-2 was added to T-84 cells to assess proliferation by MTT absorbance. After a 72-h exposure to 0.5 ng/ml BMP-2, the MTT absorbance of cells was no different than T-84 cells that

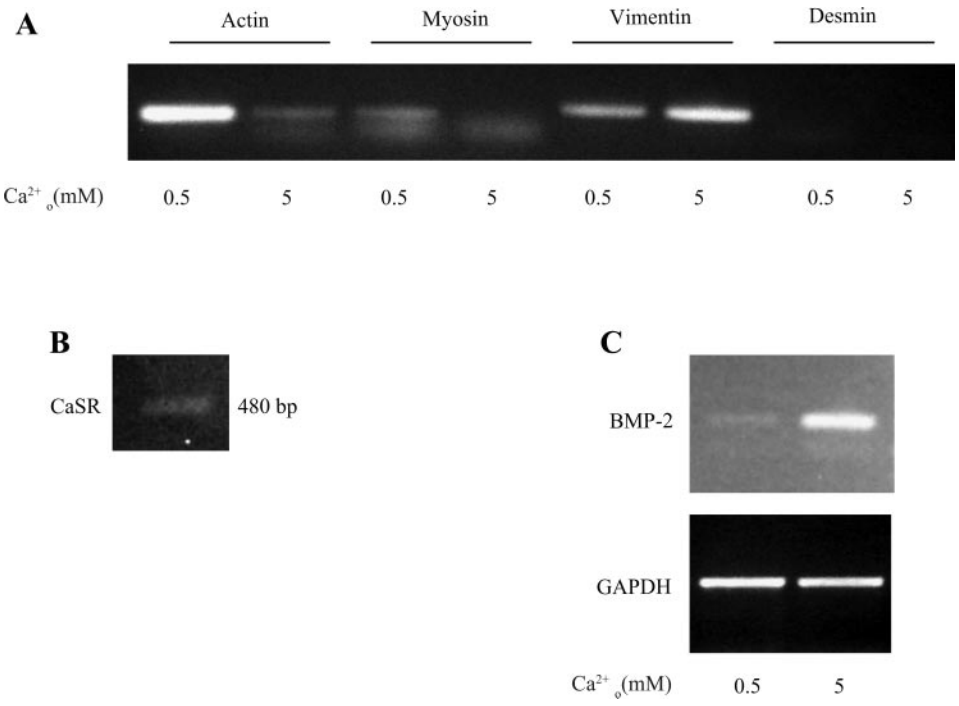


Fig. 8. Primary human colonic myofibroblasts express CaSR and BMP-2 after Ca^{2+} challenge. *A*: RT-PCR of smooth muscle actin, smooth muscle myosin, and vimentin. Colonic myofibroblasts do not show desmin transcripts. *B*: RT-PCR of the 480-bp CaSR product. *C*: RT-PCR of BMP-2 from colonic myofibroblasts treated with low (0.5 mM) or high (5 mM) Ca^{2+} for 18 h.

had received DMEM-F-12 (0.532 ± 0.018 vs. 0.580 ± 0.020, $n = 3$). Enhanced TER in the presence of BMP-2 was unlikely due to stimulated proliferation of T-84 cells.

Because others have shown that the BMPR1 and BMPR2 are present in colonic epithelia, we determined the effect of adding rhBMP-2 (0.5 ng/ml) to apical, basolateral, or both sides of T-84 cells by measuring TER (Fig. 9*B*). BMP-2 added apically at *time 0* had a TER at 24 h that was fourfold higher than that of untreated cells (401 ± 23 vs. 88 ± 8 Ω/cm^2, $P < 0.5$, $n = 3$). This increase was preserved for the next 48 h. The TER of cells to which BMP-2 was added to the apical side was threefold higher than that of untreated control cells (1,332 ± 76 vs. 400 ± 45 Ω/cm^2 at 72 h, $P < 0.05$, $n = 3$). The basolateral addition of BMP-2 to these cells also increased TER compared with untreated control cells. Within 24 h of this treatment, TER was greater than that in untreated cells (188 ± 6 vs. 88 ± 10 Ω/cm^2 at 24 h, $P < 0.05$). After 48 and 72 h, TER was still greater than that in untreated cells (313 ± 54 vs. 202 ± 27 Ω/cm^2 at 48 h, $P < 0.05$, $n = 3$). These data suggested to us that the BMP-2 stimulation of TER could occur when either apical or basolateral sides were stimulated.

We then examined the effect of exogenous BMP-2 addition to high-resistance T-84 cells (Fig. 9*C*). Untreated cells had a resistance of 1,500 ± 100 Ω/cm^2. As before, BMP-2 (0.5 ng/ml) was added to either the apical or basolateral compartments. Within 24 h of this addition, TER of the cells treated with basolateral BMP-2 was greater than that of cells treated apically (2,250 ± 25 vs. 1,760 ± 100 Ω/cm^2, $P < 0.05$, $n = 6$). This suggested to us that since higher-resistance cells reflected a better representation of adult intestinal epithelia, it was likely that the BMP-2 signaling cascade responsible for the increase in TER could be activated from the basolateral membrane rather than from the apical side.

Since colonic myofibroblasts are juxtaposed at the basement membrane of the lamina propria, BMP-2 released from such cells would be expected to interact with the basolateral membrane of the intestinal epithelia. Accordingly, we added "conditioned medium" from 18Co cells treated with 3 mM Ca^{2+} to T-84 cells seeded on Transwells and compared the effect on TER over 48 h of nonconditioned, serum-free medium containing 3 mM Ca^{2+} with conditioned medium with various dilutions of neutralizing antibody against BMP-2 (Fig. 9*D*). Within 72 h of the addition of the conditioned medium, TER was higher compared with conditioned medium with a 1:50 dilution of anti-BMP-2 antibody (720 ± 14 vs. 548 ± 21 Ω/cm^2, $P < 0.05$, $n = 3$). These results suggested that BMP-2 secreted from myofibroblasts after CaSR activation was biologically active in stimulating increases in TER of the T-84 model membrane.

Together, these data suggested to us that CaSR activation of the myofibroblast stimulates the synthesis and secretion of BMP-2, which then interacts with its receptors, presumably on the basolateral membrane of the intestinal epithelium, to stimulate an increase in TER.

Effect of CaSR activation on BMP-2, Noggin, and BMP-4 expression in intestinal epithelial cell lines. In the remaining experiments, we asked if activating the CaSR influenced the production of BMP-2, Noggin, and BMP-4 from the intestinal epithelia. In CaSR-expressing intestinal epithelial cell lines (HT-29, SW-480, and T-84) after serum starvation, low (0.5 mM) or high (3 or 5 mM) Ca_o^{2+} was applied to cells, conditioned media were harvested, and ELISA was preformed. As shown in Fig. 10*A*, HT-29 and SW-480 cells secreted a constitutive amount of BMP-2. Increasing Ca_o^{2+} (3 and 5 mM) from 0.5 mM Ca_o^{2+} increased the amount of BMP-2 secreted (HT-29: 168 ± 11 vs. 105 ± 20 pg/ml; SW-480: 104 ± 12 vs. 63 ± 12 pg/ml). Constitutive secretion of BMP-2 from T-84 cells is shown in Fig. 10*B*. TER of these cells was 1,500–1,800 Ω/cm^2. More BMP-2 was present in the apical compartment (91.1 ± 2 pg·ml^{-1}·18 h^{-1}) compared with the basolateral compartment (56.1 ± 2 pg·ml^{-1}·18 h^{-1}) of these cells.

Because the T-84 cells grown on Transwells were used in earlier experiments, we restricted our remaining experiments to these cells. T-84 cells with a TER of 1,000 Ω/cm^2 were serum deprived for 18 h and then challenged for a further 18 h with either 0.5 or 3 mM Ca_o^{2+} (added to both the apical and basolateral sides), and RNA was prepared. The results of the RT-PCR for BMP-2 and BMP-4 are shown in Fig. 10*C*. Consistent with the above ELISA results, constitutive conditions (0.5 mM Ca_o^{2+}) revealed a cDNA product for BMP-2 that was more intense after 3 mM Ca_o^{2+} was added to the cells. High Ca_o^{2+} reduced the intensity of the BMP-4 transcript compared with low Ca_o^{2+} After the Ca_o^{2+} challenge, both the cDNA and protein for Noggin were absent (Fig. 10*D*). This suggested that CaSR stimulation diminished both the transcription and secretion of Noggin from epithelial cells.

DISCUSSION

The present experiments demonstrated that an established and characterized colonic myofibroblast cell line, 18Co, synthesizes and secretes BMP-2 in response to activation of the CaSR. Interfering RNA against the CaSR prevented the Ca^{2+}-stimulated increases in BMP-2 from these cells. Primary cultures of human colonic myofibrobasts expressed the CaSR and high Ca^{2+} stimulated BMP-2 production. The CaSR-mediated secretion of BMP-2 was prevented by PI3K inhibition. Conditioned medium from CaSR-stimulated myofibroblasts added to the basolateral compartment increased the TER of T-84 cells grown on Transwells; this effect was blocked by an antibody against BMP-2. Activating the CaSR on myofibroblasts downregulated the BMP antagonist Noggin. Western blot analysis demonstrated that high Ca_o^{2+} extinguished Noggin protein from these cells. Stimulating the CaSR on T-84 epithelial cells also downregulated the transcripts for Noggin and BMP-4. Exogenous BMP-2 added to low-resistance T-84 cells increased their resistance and did not stimulate proliferation, whereas the addition of BMP-2 to the basolateral compartment of high-resistance cells further increased resistance. Thus, activating the CaSR on subepithelial and epithelial cells resulted in a coordinate response to increase the net effective BMP-2 concentration. These increases in BMP-2 concentration promoted increased TER of the model epithelial cells.

Intestinal subepithelial myofibroblasts are located at the interface between the epithelia and lamina propria lying along the basement membrane (36, 37). They are physically poised to signal to the epithelia. Previous extensive studies using a human colonic myofibroblast line, 18Co, have determined the signaling cascades responsible for IL-1-mediated induction of cyclooxygenase (COX)-2 (12, 32) and COX-2 transcript stabilization (31) as well as the role these cells play in innate immune responses of the intestine (37). We therefore used

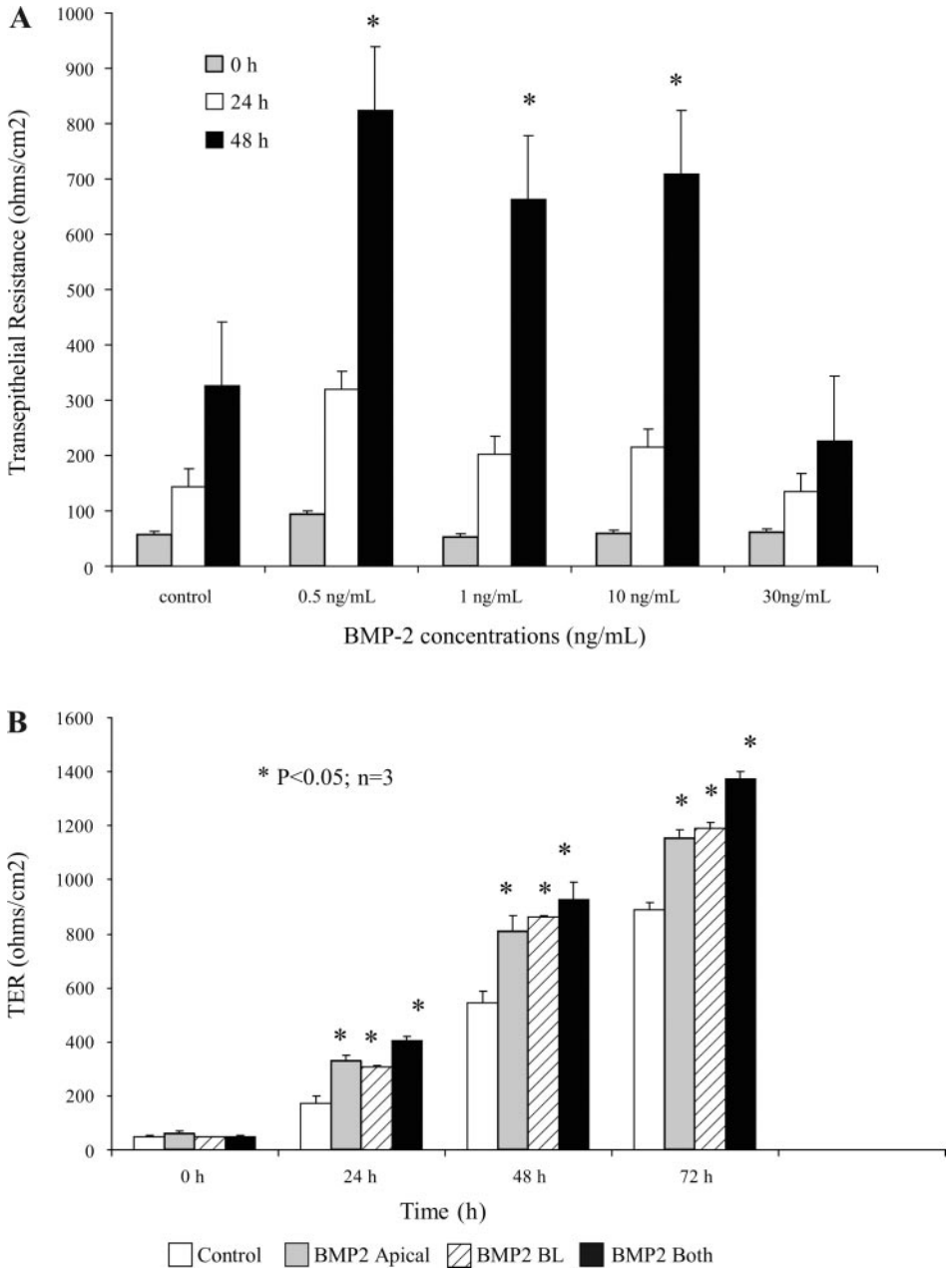


Fig. 9. Effect of the addition of exogenous BMP-2 on epithelial barrier function. Transepithelial resistance (TER) was measured daily. *A*: different concentrations of recombinant human (rh)BMP-2 were added to the apical and basolateral (BL) sides. Media were changed daily. *B*: effect of the addition of rhBMP-2 (0.5 ng/ml) to the apical, BL, or both sides. *C*: rhBMP-2 (0.5 ng/ml) added to high-resistance T-84 cells. TER of cells treated with BL BMP-2 was greater than that of cells treated apically ($P < 0.05$, $n = 6$). *D*: conditioned medium obtained from CaSR-stimulated 18Co cells (3 mM Ca_o^{2+}) was added to T-84 cells with or without anti-BMP-2 antibody (1:50 dilution). TER was measured compared with serum-free medium (3 mM Ca_o^{2+}). At 72 h, TER of cells treated with conditioned medium was higher ($P < 0.05$, $n = 3$) than that of cells treated with 3 mM Ca_o^{2+}, and this effect was reversed with the addition of BMP-2-neutralizing antibody.

primary isolates of human colonic myofibroblasts that expressed smooth muscle actin, smooth muscle myosin, and vimentin, but were negative for desmin (36). These cells also expressed CaSR transcripts. Earlier studies (8, 14) of CaSR expression in the mammalian intestine reported that CaSR transcripts were abundant in the submucosa of the rat ileum. Our present experiments demonstrating the expression of the CaSR on a colonic myofibroblast cell line as well as telomerase-treated cultures of human colonic myofibroblasts demonstrate that colonic myofibroblasts, by expressing the CaSR, are equipped to respond to postprandial changes in Ca_o^{2+} that occur in the lamina propria.

Postprandial stimulation of the CaSR on colonic myofibroblasts may contribute to intestinal homeostasis. Perfusates containing 10 mM Ca^{2+} have been shown to cause increases of Ca_o^{2+} from ~1 to 1.9 mM in the rat duodenal submucosal intestitium (33). While such putative stimulation would be transient, our present experiments demonstrate that CaSR activation resulted in the stimulation of synthesis and secretion of BMP-2 within 3 h. Evidence that the stimulation of the CaSR on this colonic myofibroblast cell line resulted in BMP-2 synthesis and secretion came from three lines of evidence. First, siRNA against the CaSR prevented the high Ca^{2+}-stimulated increases in BMP-2 protein. Recently, siRNA against CaSR has been used in human aortic endothelia (45). Second, other well-characterized polyvalent CaSR agonists such as $GdCl_3$, neomycin sulfate, and spermine (6) all stimulated BMP-2 synthesis and secretion. Finally, transient transfection with R185Q, a well-characterized dominant negative CaSR (2, 40), substantially reduced the amount of BMP-2 secreted over a range of Ca_o^{2+} concentrations. Because there was no basal secretion of BMP-2, calculation of the EC_{50} for

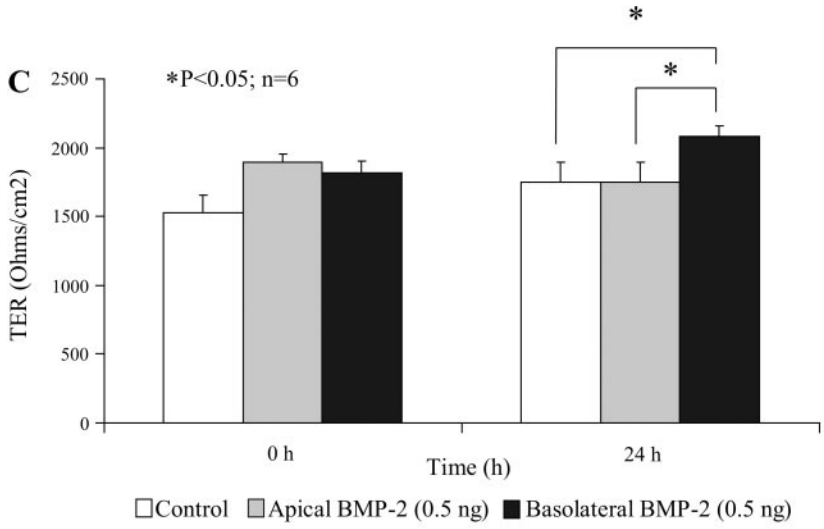
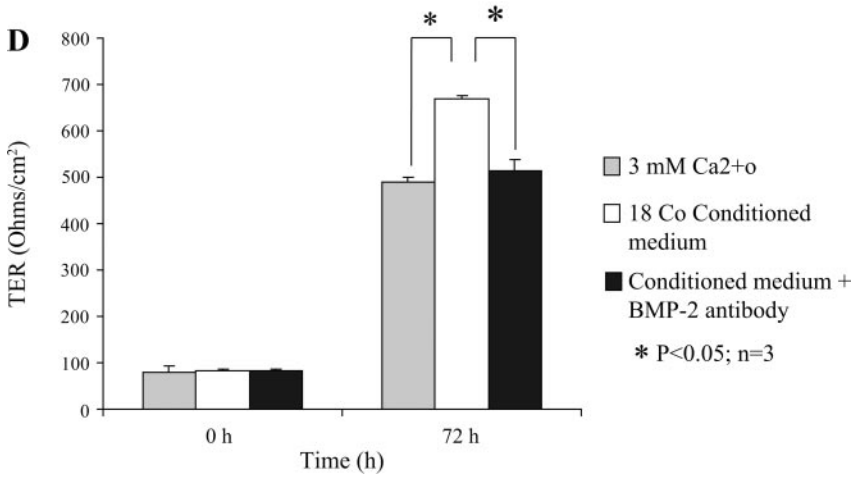


Fig. 9.—Continued



Ca_o^{2+} was higher (2.3 mM) than that in other endogenously expressing cells (40, 42, 43). Yet, in the cells transfected with R185Q, the EC_{50} was shifted to the right (5.7 mM). A right shift is strongly indicative of an event mediated by the CaSR and has been characterized using both parathyroid hormone-related peptide secretion and mobilization of intracellular Ca^{2+} as functional indexes of either transiently transfected or endogenously expressing cell lines (2, 40). Together, these effects strongly suggest that the high Ca_o^{2+}-stimulated BMP-2 secretion from colonic myofibroblasts was mediated by the CaSR.

BMPs are members of the TGF-β family of proteins. TGF-β is known to play a role in colon cancer (3, 35, 38). BMP-4 expression occurs exclusively in the intravillus mesenchyme in mammals (18). Inhibition of BMP signaling by transgenic expression of Noggin resulted in ectopic crypt units perpendicular to the crypt-villus axis, which phenocopied juvenile polyposis (18). Targeted disruption of BMP-2, BMP-4, BMPR1a, and BMPR11 are all embryonically lethal (44). BMP-2 has recently been reported to be present in the adult human colon (17). Immunochemistry demonstrated increasing expression of BMP-2 from the crypt to epithelial surface cells with the most intense staining on mature colonocytes of the surface epithelium. BMPR1a, BMPR1b, BMPR11, and Noggin, the diffusible inhibitor of BMP signaling, have also been shown to be present in the same colonic epithelial cells that express BMP-2. The addition of exogenous BMP-2 to colonic tumor cell lines uniformly inhibited proliferation. Together, these findings suggested that a putative role of BMP-2 in the adult intestine is to inhibit proliferation or increase apoptosis (17). Our present results demonstrating that BMP-2 was not secreted under basal conditions from colonic myofibroblasts, whereas constitutive secretion of BMP-2 was present from HT-29, SW480, and T-84 cells, are in accord with these in vivo observations of BMP-2 expression.

Little is understood about the molecular determinants of BMP-2 synthesis and secretion from intestinal or subepithelial cells. While low Ca_o^{2+} (0.1–0.4 mM) has been reported to increase BMP-2 and BMP-4 transcripts in mandibular-derived human bone cells (34), these concentrations are insufficient to activate the CaSR. BMP-2 transcripts increased in human mesenchymal stem cells upon treatment with PGE_2. However, the effects of this agent on BMP-2 secretion were not determined. (35). In the present experiments, we observed a pharmacological distinctiveness of the CaSR-mediated synthesis and secretion of BMP-2. Only inhibition of PI3K (complete) and p38 MAPK (partial) had effects on BMP-2 secretion. In

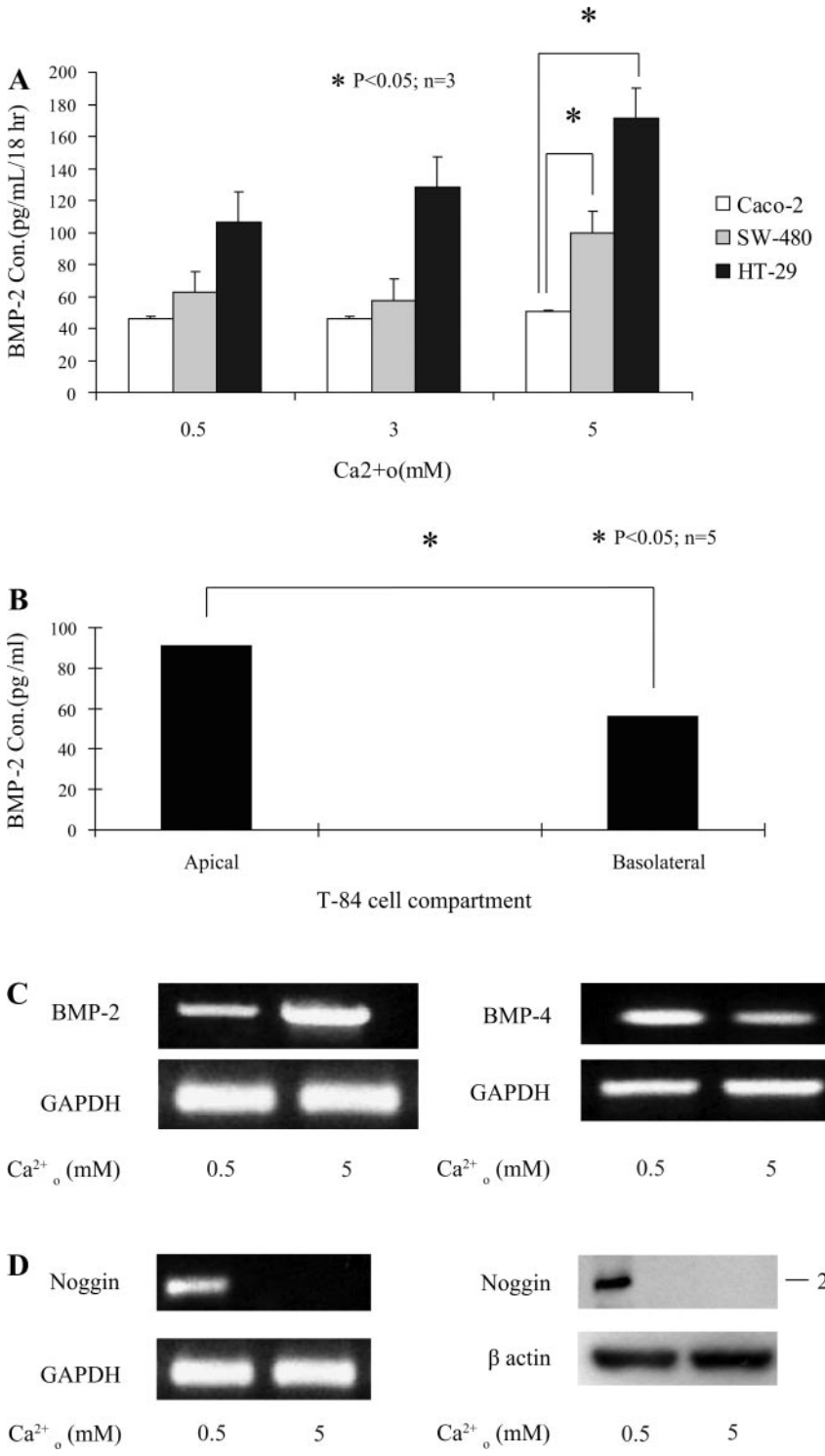


Fig. 10. Secretion and synthesis of BMP-4 and Noggin from epithelial cell lines. *A*: effect of different $[Ca^{2+}]_o$ on the secretion of BMP-2 from Caco-2, HT-29, and SW-480 cells. $*P < 0.05$; $n = 3$. *B*: secretion of BMP-2 from T-84 cells. More BMP-2 was present in the apical compartment compared with the BL compartment ($*P < 0.05$). *C*: effect of the CaSR on transcripts of BMP-2 and BMP-4 in T-84 cells. *D*: expression of Noggin from T-84 cells. High Ca_o^{2+} completely downregulated both transcripts (*left*) and protein (*right*) from T-84 cells.

other endogenously expessing cells, CaSR activation stimulates PI3K-Akt (30, 43), Ras, mitogen-activated kinase kinase 4 and 7 (5), and p38 MAPK (26). Our present results demonstrated that the CaSR-mediated synthesis and secretion of BMP-2 requires the stimulation of PI3K but was independent of calmodulin kinase, JNK, PKC, MEK1, and MEK2. Indeed, tryphostin inhibitors of the EGFR had no effect on the CaSR stimulation of BMP-2 synthesis and secretion, suggesting to us that the CaSR-stimulated transactivation of the EGFR (27) was not involved in BMP-2 secretion. The p38 MAPK inhibitor SB-203580 attenuated the CaSR-mediated BMP-2 secretion. While this inhibitor is selective for the α- and β-isoforms of p38 (26, 42), the isoforms of p38 stimulated by the CaSR in colonic myofibroblasts are not known. Since inhibition of the EGFR had no effect on CaSR-stimulated BMP-2 secretion, it is unlikely in myofibroblasts that PI3K activation or p38 MAPK

stimulation occurred proximate or distal to EGFR transactivation by the CaSR. This suggested to us that PI3K-Akt and p38 activation by the CaSR might occur in parallel.

The expression of Noggin, the diffusible BMP antagonist (35, 38), was extinguished by CaSR activation in both myofibroblasts and intestinal epithelial cells. The loss of Noggin transcript was mirrored by the extinction of Noggin protein. This strongly suggested to us that the CaSR mediated the loss of Noggin expression in both cell types. The CaSR-mediated signal transduction responsible for this effect was distinctive compared with BMP-2, since inhibition of PI3K had no effect on Noggin transcript, whereas inhibition of MEK1, MEK2, JNK, or p38 MAPK during CaSR activation all appeared to increase the PCR product for Noggin (data not shown). However, the net effect, CaSR stimulating BMP-2 synthesis and secretion while inhibiting Noggin in both cell types, was to increase the effective concentration of BMP-2. An earlier study (17) used intraperitoneal injections of recombinant mouse Noggin/Fc to show a reduction in both proliferation and apoptosis in the colon and small intestine of treated mice. Our present data therefore suggest that BMP-2, like TGF-β, promotes increased barrier function of the intestine. Our present data also suggest to us that BMP-2 homeostasis is exquisitely regulated, given the reciprocal effects that stimulating the CaSR generates on Noggin and BMP-2 in both subepithelial and epithelial cells.

Noggin, the BMP antagonist, became undetectable after CaSR stimulation of 18Co myofibroblasts and T-84 epithelial cells. Previously, BMP-2 has been shown to cause a time- and dose-dependent increase in Noggin transcripts and protein in osteoblast enriched-cells from rat calvariae (15). Such stimulation was thought to limit BMP action in osteoblasts. In general, Noggin expression is rapidly induced following the activation of BMPRs and therefore acts as a feedback antagonist (15, 34, 39). Our present results suggest that CaSR-induced BMP-2 is not acting in an autocrine fashion to decrease Noggin since Western blot analysis revealed that 15 min after high-Ca^{2+} application Noggin was absent. We do not know whether a Ca_o^{2+}-mediated protease (24) or whether CaSR-mediated MAPK stimulation is responsible for Noggin reduction. However, at longer times (>15 min), the Noggin transcript was also substantially reduced in both cell types. Both FGF-18 and bFGF diminish Noggin expression in chondrocytes (39). In contrast with the established FGF-BMP axis during osteogenesis and chondrogenesis, our present data suggest a reciprocal relationship between BMP-2 and Noggin following CaSR activation in both subepithelial myofibroblasts and intestinal epithelia. The net effect of this reciprocity is to increase the effective concentration of BMP-2.

CaSR stimulation of 18Co myofibroblasts and T-84 intestinal epithelia reduced transcripts for BMP-4, an established target of T cell factor/β-catenin. In vivo analysis of mice overexpressing Noggin has led to the suggestion that tumor suppressor phosphatase and tensin homolog, working through PI3K-Akt, mediates a convergence of BMP and Wnt pathways to control levels of β-catenin (18). Clearly, inactivating mutations in BMP pathways affect the manner epithelial cells respond to BMP signals from the mesenchyme, since these mutations lead to ectopic small intestinal crypts and polyps (18). Our present results demonstrating the CaSR induced downregulation of BMP-4 suggest that other Wnt-specific genes are downregulated by the CaSR, and the increase in the BMP-2 effective concentration may act in an autocrine manner to influence Wnt signaling pathways in intestinal epithelia.

Exogenous BMP-2 accelerated cell migration in the scrape-wound assay using postconfluent Caco-2 cells. As BMP-2 is a member of the TGF-β superfamily, this is not surprising given the well-documented effects of TGF-β on restitution in other assays (3, 16, 37). Interestingly, while TGF-α, TGF-β, HGF, EGF, and HB-EGF play a role in mucosal immune responses, none of them have been shown to be essential for the regulation of epithelial restitution, implying a degree of redundancy in the process. Our data support a role for BMP-2 in intestinal wound repair by enhancing cell migratory processes.

Exogenous BMP-2 modulated epithelial barrier maturation, as assessed by the increases in TER. While both apical and basolateral addition of BMP-2 gave comparable accelerations in TER, the addition of conditioned medium from CaSR-stimulated myofibroblasts still increased TER in high-resistance cells. This effect was inhibited with anti-BMP-2 antibody, suggesting that barrier maturation could occur from stimulated myofibroblasts. The present study does not reveal the source of the increased resistance; however, ERK1/2 activation has been reported to increase T-84 barrier capacity, and BMP-2 can signal through the Ras-MEK cascade in endothelia (35). Nevertheless, our present data support the interpretation of a role for BMP-2 in the intestine in the regulation of barrier function.

Our present study suggests that a dynamic mechanism exists to titrate BMP-2 levels in the adult intestine. Colonic myofibroblasts showed no basal BMP-2 transcripts or BMP-2 secretion. The activation of the CaSR on subepithelial myofibroblasts caused robust BMP-2 secretion together with an immediate extinction of the BMP antagonist Noggin. Stimulation of the CaSR on the intestinal epithelia generated the same reciprocity. The net effect of this dynamism is to increase the effective concentration of BMP-2 for autocrine stimulation or restitution.

ACKNOWLEDGMENTS

We thank Dr. Randy Mifflin (Dept. of Internal Medicine, Div. of Gastroenterology, Univ. of Texas Medical Branch, Galveston, TX) for a gift of telomerase-treated human colonic myofibroblasts.

GRANTS

This work was supported by Canadian Institutes of Health Research (CIHR) Operating Grant MOP-57932 and by the Dairy Farmers of Canada. Funding from the Canadian Foundation for Innovation and the Canada Research Chairs Program is gratefully acknowledged (to R. J. MacLeod). D. Peiris and I. Pacheco were supported by a CIHR training grant to the Gastrointestinal Disease Research Unit at Queen's University.

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