The extracellular calcium-sensing receptor (CaSR) on human esophagus and evidence of expression of the CaSR on the esophageal epithelial cell line (HET-1A)

Christopher J. Justinich,1,2,3 Nanette Mak,1,3 Ivan Pacheco,3 Dan Mulder,3 Ron W. Wells,3 Michael G. Blennerhassett,3 and R. John MacLeod2,3

Pediatric Gastroenterology, Departments of 1Pediatrics, 2Medicine, and 3Physiology, Gastrointestinal Diseases Research Unit, Queen’s University, Kingston, Ontario, Canada

Submitted 19 May 2006; accepted in final form 16 October 2007

THE HUMAN ESOPHAGUS IS LINED with stratified squamous epithelia. The cells in the basal layer of the squamous epithelia consist of two zones, one overlying the papillae, the other between the papillae (44, 45). The tumor suppressor p63 has been shown to be associated with cells in the basal layer (14). Both eosinophilic esophagitis and gastrointestinal reflux disease are characterized by basal cell hyperplasia (44). Under-
mediated hydrolysis of phosphatidylinositol (4,5)-bisphosphate (43). With some exceptions (8), there are few studies on the expression and signaling of endogenously expressed CaSR on nontumorigenic cell lines.

The present experiments were undertaken to determine whether the CaSR was expressed in the human esophagus and by an established nontumorigenic esophageal epithelial cell line, HET-1A (49). We used the HET-1A cells to see if CaSR activation resulted in stimulation of mitogen-activated protein kinases, mobilization of [Ca$$^{2+}$$], and the secretion of the multifunctional cytokine IL-8. We show that all of these responses to CaSR activation are inhibited by interfering RNA directed against the CaSR.

**MATERIALS AND METHODS**

**HET-1A cell culture.** HET-1A cells were obtained from American Type Culture Collection and grown in T-75 flasks precoated with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml vitamin, and 0.01 mg/ml bovine serum albumin. These cells were maintained in BEBM medium supplemented with BEGM SingleQuots, which includes 100 mg/ml EGF (Clonetics, Chicago, IL), and fed every two days. For IL-8 experiments, cells were placed on 24-well plates that had also been precoated with the fibronectin-vitamin mixture. Cells were passaged weekly and used for all experiments between passage 34 and 47. For calcium imaging and immunocytochemistry, HET-1A cells were grown on similarly precoated glass cover slips. When trypsinizing HET-1A cells, at least 48 h was allowed for cell recovery prior to performing experiments.

**RT-PCR analysis.** Total RNA was generated from confluent HET-1A cells from early passage (40–41), using Trizol and following manufacturers’ instructions. For RT-PCR analysis of the CaSR mRNA, 2 μg of total RNA was subjected to a one-step protocol, according to the manufacturers instructions (Qiagen, Valencia, CA), using primer pairs for the CaSR that were derived from the human CaSR sequence, upstream primer (5’-GGCGGTACCTTAAGCAC-CTACGGGCATCTAA-3’), and the downstream primer (5’-GCTCTA-GAGTTAAGCCGATCCCAAAGGGCTC-3’). The optimal temperature cycling protocol was determined to be 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec for 40 cycles with a programmable thermocycler (Eppendorf Master Cycler). PCR products obtained in this manner were run on a 1% agarose gel with ethidium bromide, then subject to direct, bidirectional sequencing using the same primer pairs after purification of the respective DNA fragments from the PCRs with the QiAQuick kit (Qiagen, Mississauga, Ontario, Canada).

**CaSR Western blot analysis.** Lysates from early passage HET-1A cells (40–41) were obtained for Western blot analysis. Confluent cells were rinsed in cold PBS, scraped into a lysis buffer (10 mM Tris·HCl at pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.25 M sucrose, 1% Triton-X, 1 mM dithiothreitol (DTT) with 10 μg/ml protease inhibitors aprotinin, leupeptin, calpain, and 100 μg/ml Pefabloc (Pentapharma, London, UK)), and sonicated for 5 min. Aliquots were denatured with 2× SDS-Laemmli gel-loading buffer and denatured for 30 min at 65°C. Products were resolved on 6.5% acrylamide gel. Once transferred to Immobilon protein transfer membrane, the cells were washed with PBS 0.1% Tween-20, blocked for 1 h at room temperature, incubated overnight at 4°C with 1:100 anticalcium sensing affinity purified polyclonal antiserum (ABR, Affinity Bioreagents, Golden, CO), then incubated for 1 h with 1:1,350 horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG (Sigma) in PBS with 0.1% Tween 20 and 5% nonfat dry milk. The membrane chemiluminescence was visualized with the Supersignal Kit (Pierce Biotechnology, Rockford, IL).

**Phospho-p44/42 Western blot analysis.** For the determination of phospho-(p)ERK1 and 2 of HET-1A cells, early passage cells were grown on 6-well dishes. Cells were incubated for 18 h in serum-free, Ca$$^{2+}$$-free BEBM containing 4 mM L-glutamine, 0.2% BSA, penicillin 100 μU/ml, and 0.5 mM CaCl$$\text{2}$$. This medium was removed and substituted with the same medium supplemented with 2.5 mM Ca$$^{2+}$$.

At the end of the incubation period, the cells were washed once with iced-cold phosphate-buffered saline (PBS) (containing 1 mM sodium vanadate and 25 mM NaF), and then 100 μl of ice-cold lysis buffer was added (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 1% Triton X-11, 10% glycerol, 1 mM DTT, 1 mM sodium vanadate, and 50 mM glycerophosphate), containing a cocktail of protease inhibitors. After sonication for 5 s, lysates were centrifuged at 10,000 x g for 10 min at 4°C, and the supernatants were frozen at −70°C until further use. For the experiments, equal amounts of supernatant proteins (40 μg) were separated on 10% SDS-PAGE gels. The separated proteins were electrophoretically transferred to Immobilon membranes and incubated in blocking solution (PBS 0.1% Triton X-100), containing 5% nonfat dry milk for 1 h at room temperature. Total p44/42 MAP kinase and phospho-p44/42 were detected by overnight incubation with a 1:1,000 dilution of rabbit polyclonal antibodies against p44/42 or phospho-p44/42 in 1× PB and 0.1% Triton X-100 with 5% BSA. Blots were washed three times for 5 min periods at room temperature (1× PB, 0.1% Triton X-100) and then incubated for 1 h with a secondary anti-rabbit antibody conjugated to HRP (1:2,000) in blocking solution. Blots were then washed a second time (3 x 5 min). Bands were visualized by chemiluminescence as previously described. Quantitation of the phosphorylation of p44/42 was done using an ImageQuant and a Personal Densiometer (Molecular Dynamics). HT-29 cells were treated as previously described (33, 38).

**Immunocytochemistry.** HET-1A cells cultured on coverslips for 3 days were fixed with 4% formaldehyde for 30 min, washed with PBS, and blocked with 1% goat serum in PBS-0.2% Tween 20. Human paraffin sections of archived normal esophageal biopsies were examined with the Institutional Research Ethics Board approval. Biopsies were obtained using standard pediatric biopsy forceps, then placed in 4% neutral buffered formalin, and processed for routine histopathology. Pediatric patients were undergoing endoscopy for the evaluation of gastrointestinal symptoms but with histologically normal esophageal biopsies. The sections were deparaffinized and dehydrated serially in the standard fashion, followed by 15 min of incubation in 5 μg/ml of the proteinase K (Ambion, Austin, TX) for antigen retrieval at 37°C. After being washed, the sections were blocked with 5% normal goat serum in PBS for 1 h at ambient temperature and were then incubated with 1:500 affinity purified rabbit polyclonal anti-human CaSR antibody (no. 4637, a gift from E. M. Brown, Harvard Medical School) diluted in antibody dilution fluid (ADF; Dako, Mississauga, Ontario, Canada). After being washed with PBS, blots were incubated with goat anti-rabbit secondary antibody 1:1,000 in PBS-Tween 20 linked to Alexa 555 (Invitrogen, Burlington, Ontario, Canada). Negative controls performed with mismatched secondary antibody. The blocking peptide (FF7 1:250, a gift from E. M. Brown) was used to confirm specificity of the staining. Specimens were photographed using an inverted fluorescence microscope (Olympus IMT-2, Markham, Ontario, Canada).

**II-8 (CX-CL8) ELISA.** IL-8 was determined in conditioned medium from HET-1A cells then placed in low Ca$$^{2+}$$ (0.5 mM) DMEM that contained 2% bovine serum albumin, 4 mM D-glucose, and 1% penicillin-streptomycin. All substitutions (Mg$$^{2+}$$, spermine, and Ca$$^{2+}$$) were made to this medium to the concentrations listed in the text. Incubation was for 24 h, whereupon IL-8 was measured using a commercial ELISA kit according to manufacturer’s instructions (Agenon, Mississauga, Ontario, Canada).

**siRNA generation and transfection.** Several candidate regions of the CaSR nucleotide sequence were used to generate small interfering (si)RNA duplex constructs as previously described (33, 38). We first found 13 potential siRNA target sites in the extracellular domain of the CaSR. Based on a GC content of >40 but <57% we evaluated by a BLAST analysis and then selected those that gave fewer than 14
Negative controls included representing the dimerized form of the CaSR (\(n\)th lane), demonstrating the expected 280-kDa band press the CaSR (first lane) with HET-1A cell lysates from human embryonic kidney (HEK) cells that overex-

HET-1A by Western blot analysis. Left PCR amplification. RNA against the CaSR (siRNACaSR), the CaSR message is expected product seen is 480 base pairs (arrow). Left lane, 
cDNA ladder; second lane, water without reverse tran-

amplification of CaSR transcripts by RT-PCR, ex-

CaSR messenger RNA in HET-1A esophageal epithelial cells. Amplification of CaSR transcripts by RT-PCR was performed with intron-spanning CaSR-specific human primers. This reaction amplified a product of the expected size (480 base pairs) for a CaSR-derived product (Fig. 1A). No product was observed when RT was replaced with water during the RT reaction. RT-PCR, using mRNA from HET-1A cells transfected with siRNA duplexes against the CaSR, showed markedly diminished CaSR amplicons. We performed DNA sequence analysis on the PCR product isolated from nontransfected HET-1A cells, which revealed 99% identity with the corresponding region of the human parathyroid CaSR cDNA, confirming that the PCR product was amplified from authentic CaSR trans-

A

CaSR

GAPDH

H2O HET-1A siRNA\textsuperscript{CaSR}

B

CaSR

GAPDH

\textsuperscript{CaSR}

\textsuperscript{scrCaSR}

\textsuperscript{siRNA}

480 b p

HET-1A

CaSR

GAPDH

250 kDa

100 kDa

\(\beta\)-actin

RESULTS

Detection of CaSR messenger RNA in HET-1A cells by RT-PCR. Using RNA isolated from early passage HET-1A esophageal epithelial cells, RT-PCR was performed with intron-spanning CaSR-specific human primers. This reaction amplified a product of the expected size (480 base pairs) for a CaSR-derived product (Fig. 1A). No product was observed when RT was replaced with water during the RT reaction. RT-PCR, using mRNA from HET-1A cells transfected with siRNA duplexes against the CaSR, showed markedly diminished CaSR amplicons. We performed DNA sequence analysis on the PCR product isolated from nontransfected HET-1A cells, which revealed 99% identity with the corresponding region of the human parathyroid CaSR cDNA, confirming that the PCR product was amplified from authentic CaSR trans-


graphs. The current experiments used nucleotides 371 to 390 \(5\prime\)-AAC-

CTTGATGAGTTCTGCAC-3\prime \) with the addition of complementary nucleotides to a \(T7\) promoter primer. Antisense and sense oligo-
nucleotide templates were generated, and transcribed siRNA was produced according to the manufacturer’s protocol (Silencer siRNA Construction Kit, Ambion). Nucleotides 371 to 390 were scrambled, and a BLAST analysis was performed to confirm lack of specificity of this construct. T7 sites were added to this oligonucleotide template, and it was processed as above. HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were plated on 35-mm glass bottom dishes precoated with fibronectin, vitrogen, and bovine serum albumin and allowed to attach for 48 h in BEGM. The cells were then placed in calcium-free Krebs solution and incubated with 1 \(\mu\)M Fluo-4 and 0.01% Pluronic F-127 (Molecular Probes/Invitrogen) dissolved in DMSO for 20 min at 31°C. The final concentration of DMSO was less than 0.1%. For experiments, cells were perfused with calcium-free Krebs solution at a continuous rate of 2 ml/min. Exposure to calcium or other products was performed by pressure application via a puffer pipette (500-ms application at 10 psi through a 1-M\(\Omega\) resistance tip), which was micromanipulated to within 200 \(\mu\)m of the cells. Fluorescence measurements were conducted on \(~60\) cells within the field of view closest to the puffer pipette. For these experiments, the investigator was blinded to the conditions being exposed to the cells. Images were captured at 250-ms intervals to quantitate changes in fluorescence (488-nm excitation) over time in addition to resting and peak-stimulated increases in \([Ca^{2+}]\), using image analysis with ImagePro 5.0 (Media Cybernetics, Silver Spring, MD). Negative controls included the pressure application of calcium-free Krebs solution via puffer pipette, which failed to induce calcium mobilization. In preliminary experiments we screened different agonists (carbachol, ACh, and 5-hydroxytryptamine) to determine which agonist stimulated the greatest release of \([Ca^{2+}]\), to use as a positive control for siRNA transfection. We observed that ACh (1 \(\mu\)M) generated the strongest effect of these agonists. At the conclusion of the experiment ionomycin (1 \(\mu\)M) in Krebs solution (1.8 mM Ca\(^{2+}\)) was perfused to generate the maximal fluorescence signal used to compare effects of ACh or calcium addition. Results were expressed as a fraction of the maximal fluorescence observed after ionomycin treatment. Experiments were performed three or four times on groups of \(>60\) cells. Spermine dihydrate (2 mM) was used as an alternative CaSR agonist. PLC inhibitor U73122 and the inactive analog U73334 (Biosource Int, Camarillo, CA) were used at a concentration of 10 \(\mu\)M.

Camarillo, CA) were used at a concentration of 10 

Calcium imaging. Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiagen).

Phase contrast microscopy (Olympus IMT-2) was used to identify HET-1A cells that were phase bright with membranes free of physical distortions and that maintained normal morphology. These cells were determined to be \(>95\%\) viable, as demonstrated by exclusion of trypan blue staining (not shown).

HET-1A cells were transfected with 25 nM siRNA of either the scrambled construct or siRNA\textsuperscript{CaSR} using Superfect, according to the manufacturer’s instructions (Superfect, Qiag}
Detection of CaSR protein on HET-1A by Western blot analysis. An affinity-purified, polyclonal anti-CaSR antiserum was used to perform standard Western blot analysis revealing the presence of CaSR from early passage (40–41) HET-1A cells (Fig. 1B). HET-1A cell lysates demonstrate CaSR-specific protein, representing both the dimeric form at ~250 kDa and the monomeric form at ~122 kDa. As a positive control, stably transfected human embryonic kidney (HEK) cells, which overexpress the CaSR, are shown. Transfection with siRNA against the CaSR substantially reduced CaSR ampli-
cons (Fig. 1B, top). Also illustrated is a representative Western blot analysis of the HET-1A cells transfected with scrambled siRNA and siRNA against the CaSR (Fig. 1B, bottom). The CaSR protein is diminished in the siRNA against the CaSR sample but not in the cells transfected with scrambled siRNA, using β-actin as an internal control (n = 2).

Immunocytochemistry of CaSR protein on HET-1A cells and human esophagus. An affinity-purified, polyclonal anti-CaSR antiserum (no. 4637) revealed CaSR immunoreactivity in the HET-1A cells (Fig. 2A). No specific staining was seen in cells labeled with mismatched secondary antibody (Fig. 2B) or when CaSR-specific blocking peptide (FF7) was used to preabsorb the antiserum (Fig. 2C). Sections of normal human esophagus also revealed specific CaSR immunoreactivity. The CaSR immunoreactivity was localized to the basal region of esophageal epithelial sections, and little staining was seen on the more differentiated epithelial cells toward the luminal surface (Fig. 2D). No specific staining of the esophageal basal cells was seen with mismatched secondary antibody (Fig. 2E) (n = 3) or with blocking peptide.

Extracellular calcium (Ca\textsuperscript{2+}) and other CaSR agonists stimulate IL-8 secretion from HET-1A cells. To assess whether extracellular calcium (Ca\textsuperscript{2+}_o) influenced IL-8 secretion, we treated the HET-1A cells with low (0.5 mM) or high Ca\textsuperscript{2+}_o (5.0 mM) and measured IL-8 in the conditioned medium at 24 h by ELISA (Fig. 3). High Ca\textsuperscript{2+}_o stimulated IL-8 secretion fourfold compared with basal amounts of secretion in low Ca\textsuperscript{2+}_o. The Ca\textsuperscript{2+}_o stimulation of IL-8 secretion was dose responsive, with an EC\textsubscript{50} ~2.3 mM Ca\textsuperscript{2+}. Other well-characterized polyvalent CaSR agonists added to these cells in low Ca\textsuperscript{2+}_o (0.5 mM) medium such as spermine (2 mM), and Mg\textsuperscript{2+} (20 mM) also increased IL-8 secretion. Thus, increasing Ca\textsuperscript{2+}_o...
or addition of polyvalent CaSR agonists stimulated secreted HET-1A epithelial cells to secrete the multifunctional cytokine IL-8.

Effect of siRNA for CaSR on Ca\textsuperscript{2+}\_o-mediated secretion of IL-8 from HET-1A cells. To directly determine if the CaSR mediated the Ca\textsuperscript{2+}\_o-stimulated secretion of IL-8 from the HET-1A cells, the cells were transfected with the siRNA duplexes directed against the extracellular domain of CaSR, and these responses were compared with cells transfected with a scrambled construct containing the same bases (Fig. 4). Cells transfected with siRNA directed against the CaSR showed no calcium-induced stimulation of IL-8 secretion. The amount of IL-8 in the presence of high (5 mM) Ca\textsuperscript{2+}\_o was no different than the amount in low (0.5 mM) Ca\textsuperscript{2+}\_o. When the HET-1A cells were transfected with scrambled siRNA, high Ca\textsuperscript{2+}\_o (5 mM) stimulated IL-8 secretion compared with cells incubated with low Ca\textsuperscript{2+}\_o (0.5 mM). These results strongly suggest that the CaSR mediated the Ca\textsuperscript{2+}\_o stimulation of IL-8 secretion from the HET-1A cells.

Effect of CaSR agonists on intracellular Ca\textsuperscript{2+} mobilization from HET-1A cells. We examined whether agonists of the CaSR caused intracellular Ca\textsuperscript{2+} mobilization from esophageal epithelial cells. Cells were loaded with Fluo-4 as described, and fluorescence microscopy was used to determine changes in [Ca\textsuperscript{2+}]. As illustrated in Fig. 5A, addition of Ca\textsuperscript{2+}\_o caused a concentration-dependent rapid release of [Ca\textsuperscript{2+}], that was maximal at 10 mM Ca\textsuperscript{2+}\_o. The EC\textsubscript{50} for rapid release of [Ca\textsuperscript{2+}] in these experiments was ~5 mM Ca\textsuperscript{2+}\_o. The release stimulated by ACh (1 \mu M) was used in these experiments as a positive control. We also observed that although the releases of [Ca\textsuperscript{2+}], from cells challenged with 0.5 or 3 mM Ca\textsuperscript{2+}\_o were equivalent (% of maximal response: 19 ± 8, 21 ± 14, n = 6), addition of the CaSR agonist spermine (2 mM) to cells challenged with 1 mM Ca\textsuperscript{2+}\_o stimulated [Ca\textsuperscript{2+}]\_i release (Fig. 5A). Thus both extracellular Ca\textsuperscript{2+} and spermine in the presence of 1 mM Ca\textsuperscript{2+}\_o stimulate [Ca\textsuperscript{2+}]\_i mobilization in HET-1A cells.

We then assessed whether an aminoglycoside inhibitor of PLC influenced the Ca\textsuperscript{2+}\_o and ACh-stimulated mobilization of intracellular calcium. Shown in Fig. 5B is a representative tracing of the time course of [Ca\textsuperscript{2+}]\_i release in response to 7.5 mM Ca\textsuperscript{2+}\_o compared with 1 \mu M ACh. Also illustrated in Fig. 5B are the responses to the presence of the inactive PLC inhibitor (U73344, 10 \mu M), which had no effect on the transient release of [Ca\textsuperscript{2+}]\_i stimulated by 7.5 mM Ca\textsuperscript{2+}\_o or the release stimulated by ACh. The active analog U73122 (10 \mu M) prevented the 7.5 mM Ca\textsuperscript{2+}\_o and ACh-stimulated increases in [Ca\textsuperscript{2+}]. The data from several experiments indicated that the active PLC inhibitor reduced the increases in Fluo-4 fluorescence stimulated by Ca\textsuperscript{2+}\_o or ACh, whereas the inactive analog had no effect (Fig. 5C). Thus extracellular Ca\textsuperscript{2+} stimulates [Ca\textsuperscript{2+}]\_i release, which may be blocked by an inhibitor of PLC, suggesting that CaSR activation in HET-1A caused [Ca\textsuperscript{2+}]\_i mobilization distal to the activation of PLC.
Effect of siRNA-CaSR on Ca\(^{2+}\) mobilization stimulated by Ca\(^{2+}\). To directly test whether the CaSR was responsible for the [Ca\(^{2+}\)]\(_i\) release generated by Ca\(^{2+}\) addition we compared the effect of HET-1A cells transfected with either the scrambled siRNA duplexes or CaSR-specific siRNA duplexes. As illustrated in Fig. 6, addition of either 5 mM or 7.5 mM Ca\(^{2+}\) to cells that were transfected with the siRNA against the CaSR resulted in significantly less calcium mobilization than cells that had received the scrambled CaSR siRNA duplexes (P < 0.05). There was no difference in the calcium increments stimulated by ACh in the cells that were transfected with siRNA against the CaSR compared with the scrambled siRNA duplexes. These results strongly suggest that the Ca\(^{2+}\)-stimulated changes in intracellular Ca\(^{2+}\) were mediated by the CaSR.

Effect of Ca\(^{2+}\) on phospho-ERK1 and 2 activation in HET-1A cells. Because the activation of IL-8 secretion by the CaSR was determined between 18 and 24 h after stimulation, we then focused on earlier times and assessed whether CaSR activation influenced phosphorylation of ERK1 and 2. We compared HET-1A cells that had been transiently transfected with the siRNA duplex against the CaSR with cells comparably treated with a scrambled siRNA duplex. Both groups of cells were then challenged with 2.5 mM Ca\(^{2+}\) and lysates prepared at various times after exposure. These time courses are illustrated in Fig. 7. In cells transfected with the scrambled siRNA duplex, within 2 min, the intensity of pERK immunoreactivity increased. This immunoreactivity then declined after 5 min but then robustly increased for the next 10 min (Fig. 7A). In the HET-1A cells treated with siRNA duplex, the rapid (2–5 min) increase in pERK was preserved, but the
Fig. 7. Time course of phospho-ERK1 and 2 by high Ca\(^{2+}\) in HET-1A and HT-29 adenocarcinoma cells. A: HET-1A cells transfected with scrambled siRNA as described in MATERIALS AND METHODS demonstrated biphasic increases in pERK1 and 2 in response to 2.5 mM Ca\(^{2+}\) challenge. Total ERK demonstrates equal loading. B: HET-1A transfected with siRNA against the CaSR showed attenuation in the intensity of the immunoreactive phospho-p44/42 at 10, 20, and 30 min. Total ERK showed equal loading. C: HT-29 cells transfected with scrambled siRNA then 48 h later challenged with 5 mM Ca\(^{2+}\). Robust increases in phospho-ERK seen at 10 and 20 min after Ca\(^{2+}\) challenge. D: HT-29 cells transfected with siRNA against CaSR, then 48 h later challenged with 5 mM Ca\(^{2+}\). Increases in phospho-ERK at later times attenuated. Total ERK shows equivalent loading. Experiments performed 3 times with a representative blot illustrating comparable results.

 increases in pERK1 and 2 at 10 and 20 min were substantially reduced (Fig. 7A). This suggested to us that the CaSR was responsible for the increases in pERK1 and 2 that occurred at the later times. We then used an adenocarcinoma cell line, which expresses the CaSR (33), to confirm these kinetics. As illustrated in Fig. 7B, in HT-29 cells that had been transfected with the scrambled siRNA duplex, then 48 h later challenged with 3 mM Ca\(^{2+}\), stimulation of pERK1 and 2 increased with time of exposure, with maximal stimulation of pERK1 and 2 occurring between 10 and 20 min after stimulation. In the presence of the siRNA duplex against the CaSR, the maximal stimulation at 10 and 20 min was substantially inhibited (Fig. 7B).

DISCUSSION

These experiments demonstrate that the extracellular CaSR is present in basal cells of the human esophagus. In addition, using an established human esophageal cell line (HET-1A), we have confirmed the presence of the CaSR at both transcript and protein levels. Using these cells, our results demonstrate that stimulating the CaSR resulted in intracellular calcium mobilization, activation of ERK1 and 2, and the secretion of the multifunctional cytokine IL-8. Interfering RNA duplex reduced CaSR ampiclon and protein. Because the calcium induced pERK1 and 2, IL-8 secretion and intracellular calcium mobilization were substantially diminished in the cells transfected with interfering RNA against the CaSR; our results strongly suggest that the CaSR is expressed and may be activated in the HET-1A esophageal cell line.

The most robust staining of the CaSR was found on the basal cells of the esophagus, whereas the more differentiated epithelia exhibited less immunoreactivity. Consistent with previous reports (7), blocking peptide FF7 prevented the CaSR immunoreactivity on HET-1A cells. HET-1A cells are SV-40 immortalized human esophageal epithelial cell lines that retain characteristics of basal epithelial cells (49), in contrast to the more mature nonreplicating cells of the upper layers of the esophageal epithelium. This cell line has been widely used in the study of esophageal physiology (36). Western blot analyses of these cells demonstrated CaSR presence in both monomeric and multimeric forms, compared with the CaSR-transfected HEK cells. Together, this confirms the presence of the CaSR in HET-1A cells and on the basal layer of human esophagus. The presence of the CaSR on basal cells of the esophagus contrasts the distribution of CaSR in the skin. Here, the CaSR has been shown to be expressed strongly in interfollicular suprabasal keratinocyte layers, whereas keratinocytes in the basal layer of the epidermis stained very faintly. CaSR mRNA could not be detected in the basal keratinocytes of skin (52). This suggests that the squamous epithelia of the esophagus differ from the mammalian epidermis in the expression of the CaSR, with the basal cells of the esophagus most strongly expressing the CaSR.

Extracellular Ca\(^{2+}\) is known to be an inducer of epithelial differentiation in many epidermal and epithelial cell systems (15, 21, 34, 52, 53), and also in HET-1A cells (49). Since the highest level of CaSR expression in the human appears to be associated with basal cells, we speculate that CaSR activation by calcium triggers differentiation. For example, esophagin, a member of the small proline-rich protein family of cell envelope precursor proteins, is expressed during squamous cell differentiation. Esophagin is expressed at high levels in normal esophageal epithelium. It is absent from esophageal squamous cell carcinomas and adenocarcinomas. Ca\(^{2+}\) exquisitely regulates the activity of the esophagin promoter (48), but it is not known if this regulation is mediated by the CaSR. Furthermore, transgenic mice, with the CaSR overexpressed in the basal cells of the epidermis, displayed accelerated hair follicle differentiation, as well as enhanced permeability barrier development (53). Nevertheless, it is not yet known whether CaSR
activation in these cells can stimulate proliferation or mediate squamous differentiation in the mouse and human esophagus.

Activation of the CaSR on the HET-1A cells stimulated increases in pERK1 and 2. These increases in pERK appeared biphasic, with a rapid phase of ERK1 and 2 activation (2–5 min) and subsequent decline followed by a phase of longer duration (10–30 min). As only the longer phase was decreased by siRNA treatment, we interpret our results as suggestive that the CaSR stimulates an increase in pERK1 and 2 in the HET-1A cells, which takes place after 5 min of stimulation. The source of the rapid phase of activation is not the CaSR. We confirmed that the maximal CaSR stimulation of pERK1 and 2 occurred at later times in a model epithelial cell, HT-29, in which we previously have shown that siRNA treatment diminishes CaSR transcript and protein (33). In other cell types, such as human prostate tumor cells, H-500 Leydig cancer cells, or mouse osteoblastic MC3T3 cells, CaSR stimulation of maximal pERK1 and 2 occurs at times longer than 5 min postactivation (51, 55, 56). In contrast, in heterologous CaSR-expressing HEK cells, CaSR stimulation of pERK1 and 2 occurred rapidly (2 min) and was maximal at 10 min, then declined to remain above nonstimulated levels for several hours (25). A biphasic activation of pERK has been shown in heterologous cell systems expressing parathyroid hormone receptor. The early stimulation of pERK is G protein dependent, whereas the late pathway is independent of G proteins and requires β-arrestins (20). It is not known whether these components are determinants of the CaSR-mediated stimulation of pERK1 and 2 in the esophageal HET-1A cells. However, the attenuation of the latter phase of pERK1 and 2 production by siRNA strongly suggests that CaSR activation will increase pERK1 and 2 in HET-1A cells.

CaSR activation of the HET-1A cells stimulated the secretion of the multifunctional cytokine IL-8 (CX-CL8). IL-8 can attract and activate neutrophils and eosinophils and may induce migration of keratinocytes and enhance wound healing (40, 47, 50). IL-8 synthesis and secretion is known to be regulated by pERK1 and 2 (28, 29, 30). The present experiments demonstrate that CaSR activation in HET-1A cells will increase pERK1 and 2. Accordingly, we speculate that a proximal event in the CaSR-stimulated IL-8 production from the HET-1A cells is the transient activation of pERK1 and 2. Both spermine and Mg2+, agonists that activate the CaSR, (3, 39) also induced IL-8 secretion from the HET-1A cells. Further studies are required to understand the signal transduction cascades activated by the CaSR to generate IL-8 synthesis and secretion from these cells, as well as to understand the physiological consequences of IL-8 production by CaSR activation from HET-1A cells and basal esophageal cells. The present studies use IL-8 secretion only as quantitation of a distal event following CaSR activation in this cell line.

The activation of the CaSR on the HET-1A cells by either Ca2+, Mg2+, or spermine stimulated intracellular Ca2+ mobilization. CaSR activation mediated this effect, as treatment with siRNA duplex against the CaSR attenuated this mobilization. Furthermore, the phosphatidylinositol-specific PLC inhibitor U73122 prevented the Ca2+-stimulated mobilization of [Ca2+]i, suggesting that the CaSR is coupled to Gq11 and that PLC was activated following CaSR stimulation. Pretreatment of the HET-1A cells with an inactive analog of U73122 had no effect on the Ca2+-induced changes in [Ca2+]i, consistent with the interpretation that CaSR activation was stimulating PLC. The present experiments used stimulation by ACh to control for nonspecific effects of either siRNA transfection or drug toxicity by the aminoglycoside inhibitor. ACh stimulated [Ca2+]i, mobilization, which was prevented by U73122 but not effected by the inactive aminoglycoside. HET-1A cells have been shown to express α-3, 5, 7 and 9, as well as β-2 and 4 nicotinic ACh receptor (nAChR) subunits (1, 35). The sensitivity of the ACh stimulus to the PLC inhibitor suggests to us that muscarinic ACh receptors are present on these cells. We also observed that treatment of the HET-1A cells with either the scrambled or the siRNA duplex against the CaSR had no effect on the substantial increments of [Ca2+]i, mobilization stimulated by ACh, suggesting that the reduction in [Ca2+]i, mobilization caused by Ca2+, was mediated by the CaSR and not reduced uptake of Fluo-4 caused by siRNA or transfection protocols. Nevertheless, the present experiments do not reveal whether CaSR activation of HET-1A cells stimulates different forms of [Ca2+]i oscillations but instead strongly suggest that CaSR activation of these cells will trigger [Ca2+]i, release. The HET-1A cell line may be an interesting model to determine whether in an endogenous CaSR-expressing cell, different CaSR agonists such as aromatic amino acids or eosinophilic major basic protein (4, 16) generate different types of [Ca2+]i oscillations compared with extracellular calcium. Indeed, understanding the pathology associated with basal cell hypertrophy in gastroesophageal reflux disease and eosinophilic esophagitis may be facilitated by knowledge of the signaling cascades stimulated by CaSR activation in the HET-1A cell line.

Whether the concentrations of Ca2+, spermine, or other CaSR agonists are sufficiently high within the basal region of the esophagus to activate the CaSR is not known. However, it has been recently demonstrated that the CaSR may be activated by the mobilization of calcium from adjacent cells (6, 24). Any generic G protein-coupled receptor agonist, which is coupled to Gq11 in close proximity, may be a mechanism leading to CaSR activation on basal esophageal cells. The studies revealed that the stimulation of one cell type to mobilize its intracellular Ca2+ stores resulted in local increases of Ca2+o, likely as a result of active extrusion of Ca2+ from the stimulated cell by the plasma membrane Ca2+ ATPase (24). These elevations in Ca2+o were of sufficient magnitude to be detected by adjacent cells expressing the CaSR. If these observations represent a generalized phenomenon, we speculate that the mobilization of intracellular Ca2+ by squamous esophageal epithelial cells juxtaposed to the basal region, as well as basal cells themselves, will result in increases of Ca2+o in the microenvironment sufficient to activate the CaSR.

Transfection with siRNA against the CaSR reduced the Ca2+-stimulated mobilization of [Ca2+]i, pERK1 and 2 activation, and IL-8 secretion from the HET-1A cells. These current studies are in accord with studies showing that siRNA against the CaSR prevented both Ca2+-stimulated BMP-2 secretion from colon myofibroblasts (38) and Ca2+-mediated synthesis and secretion of Wnt5a from colon cancer cells (33). Because ACh treatment of the HET-1A cells generated both [Ca2+]i, mobilization and IL-8 secretion, which were not impaired by siRNA against the CaSR, we conclude that the CaSR mediated the Ca2+-stimulated [Ca2+]i increases, as well as...
as the late phase increases in pERK1 and 2 and IL-8 secretion from the HET-1A esophageal epithelial cells.

In summary, we have shown for the first time that the CaSR is expressed on basal cells of the human esophagus and that a human esophageal epithelial cell line, HET-1A, also expresses the CaSR. Stimulation of the CaSR on HET-1A cells caused [Ca\(^{2+}\)]\textsubscript{i} mobilization, activation of ERK1 and 2, and IL-8 secretion. Each of these effects was attenuated by siRNA against the CaSR. We conclude that basal cells of the esophagus express the CaSR and that the CaSR is expressed in an esophageal epithelial cell line in sufficient amounts to generate physiological responses.

ACKNOWLEDGMENTS

We acknowledge E. M. Brown (Harvard Medical School, Boston, MA) for generously providing anti-CaSR antibody, blocking peptide, and CaSR-transfected HEK cells. We thank C. Spencer and A. Foss for help with some preliminary experiments.

GRANTS

This study was supported by Physicians Services Incorporated of Ontario and the Clinical Teachers Association of Queen’s University (C. Justinch) and Operating Grants from Canadian Institutes of Health Research, Crohn’s and Colitis Foundation of Canada, and the Dairy Farmers of Canada (R. J. MacLeod).

REFERENCES


33. MacLeod RJ, Hayes M, Pacheco I. Wnt5a secretion stimulated by the extracellular calcium-sensing receptor inhibits defective Wnt signaling in...
CaSR IN HUMAN ESOPHAGEAL EPITHELIUM

G129

37. Pacheco I, MacLeod RJ. Extracellular calcium-sensing receptor (CaSR) mediates Wnt5a secretion from colonic myofibroblasts to activate the orphan tyrosine kinase receptor Ror2 to increase Cdx2 and succrase-isomaltase of intestinal epithelial cells (Abstract). *Gastroenterology* 132: A101, 2007.