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J Clin Invest. 1997;99(10):2328-2333. https://doi.org/10.1172/JCI119413.

Research Article

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Expression of the Calcium-sensing Receptor on Human Antral Gastrin Cells in Culture

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Abstract

The presence of the extracellular calcium-sensing receptor on human antral gastrin cells was investigated. Reverse transcription PCR using mRNA isolated from gastrin cell–enriched cell cultures identified a product with a sequence identical to part of the human parathyroid-secreting cell calcium-sensing receptor. Immunocytochemistry with an antibody to the extracellular region of the receptor immunostained all gastrin cells (but not mucin or somatostatin cells), and detected appropriate-sized bands in Western blots of whole cell lysates. Increasing extracellular calcium levels from 0.5 to 9 mM stimulated gastrin release in a concentration-dependent manner, with maximal release obtained at 7.2 mM. A known agonist of the calcium receptor, spermine also stimulated gastrin release. Microfluorimetry of identified gastrin cells demonstrated that increasing extracellular calcium resulted in an initial rapid rise in intracellular calcium followed by a plateau level that returned to basal levels immediately after removal of the elevated calcium. The traces were consistent with activation of a receptor-mediated mechanism rather than a concentration-dependent influx of calcium. In conclusion, these data indicate that G cells express the calcium-sensing receptor, and that activation of the receptor may explain the acid rebound phenomenon associated with calcium-containing antacid preparations. (*J. Clin. Invest.* 1997. 99:2328–2333.) Key words: nitrendipine • calcium imaging • L-type VDCC • extracellular calcium

Introduction

It has long been established that calcium increases the serum levels of immunoreactive gastrin, and also stimulates gastric acid secretion in vivo (1, 2). Calcium was proposed either to activate neural pathways (3), or to stimulate directly G cells in the antral and duodenal mucosa (4). More recently, studies of human antral cells in short-term culture demonstrated that increasing the extracellular calcium concentration ($[Ca^{2+}]_o$) from 1.8 to 3.6 mM significantly increased gastrin release (5). In addition, the calcium ionophore A23187 stimulated gastrin release from human and canine cultured G cells (6, 7), and from segments of rat antral mucosa (8). These studies suggested that gastrin cells were sensitive to changes in $[Ca^{2+}]_o$, and that Ca^{2+} influx alone could evoke gastrin secretion.

Elucidation of the cellular mechanisms involved in Ca^{2+}-induced gastrin release are compounded by conflicting data from different experimental species. Hypercalcemia in dogs has no effect on gastrin release (9), and in both the dog and the rat, hypercalcemia results in decreased acid secretion (1). Humans, monkeys, cats, and ferrets, however, all show elevated gastrin secretion in response to raised $[Ca^{2+}]_o$ (3).

The recent cloning and characterization of an extracellular Ca^{2+}-sensing receptor (CaR)[1] from bovine (10) and human (11) parathyroid hormone–secreting cells opens the possibility of direct receptor-mediated control of gastrin secretion by $[Ca^{2+}]_o$. The CaR consists of a large extracellular domain coupled to a seven-transmembrane–spanning region akin to the G-protein receptor superfamily. The CaR exhibits a limited homology (25–30%) to members of the metabotrophic glutamate receptor family (12), and has been linked to both phospholipase C (PLC) and adenylate cyclase intracellular signaling pathways (13).

We have previously demonstrated that β-adrenergic agonists evoking increases in gastrin release are mediated via Ca^{2+} influx through nitrendipine-sensitive class C and D L-type voltage-dependent calcium channels (VDCC) (14). In these experiments, we observed that nitrendipine reduced unstimulated gastrin release, suggesting that the L-type VDCC were already active in culture media containing 1.8 mM $CaCl_2$. The present study was designed to investigate the possibility that the ability of calcium to stimulate gastrin release was the result of activation of an extracellular Ca^{2+}-sensing receptor.

Methods

Human antra were collected from 14 donors in collaboration with the British Columbia Transplant Society, with ethical permission from the University of British Columbia Clinical Screening Committee. There were eight male and six female donors, with an average age of 38 yr. Single antral epithelial cells were isolated and maintained in short-term culture as previously described (15).

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Received for publication 2 December 1996 and accepted in revised form 14 February 1997.

J. Clin. Invest.

0021-9738/97/05/2328/06 $2.00
Volume 99, Number 10, May 1997, 2328–2333

1. *Abbreviations used in this paper*: CaR, Ca^{2+}-sensing receptor; IR, immunoreactive; PBS T-M, PBS-Triton containing 0.1% milk powder; PLC, phospholipase C; RT, room temperature; VDCC, voltage-dependent calcium channels.

Reverse transcription PCR (rt-PCR). After 48 h in culture, mRNA was isolated from the antral cells (Micro mRNA Purification Kit; Pharmacia Biotechnology, Baie d'Urfe, Quebec) according to manufacturer's instructions; cells from one 90-mm plate provided ~ 3 μg mRNA. Random hexamer-primed first-strand cDNA was prepared from 300 ng of mRNA per reaction. After reverse transcription, PCR amplification was performed using oligonucleotide primers designed to amplify a 374-bp region of the extracellular domain of the human parathyroid hormone–secreting cell CaR (11). The sequences of forward and reverse primers were TACATTCCCCAGGTCAGTT (nucleotides 918–937) and GGTGTAGTTCCTCTAACAGG (nucleotides 1272–1292), respectively. To increase reaction stringency, the touchdown method of PCR was used (16). The initial annealing temperature (66°C) was dropped to 56°C for standard PCR cycling. PCR products were resolved by electrophoresis in a 1.2% agarose gel. Products of the expected size (374 bp) were gel-purified, cloned into pGEM, and sequenced in a dideoxynucleotide chain termination procedure (T7 DNA Sequencing kit; Pharmacia Biotechnology).

Immunocytochemistry. Cells adherent to 3-aminopropyltriethoxysilane (APES; Sigma Chemical Co., St. Louis, MO)-coated glass coverslips were fixed in Bouin's solution for 10 min at room temperature (RT), and were washed in 0.1 M phosphate-buffered saline (PBS). To confirm expression of the CaR in gastrin cells, the cells were incubated in a monoclonal antibody to the extracellular NH_2-terminal region of the CaR (ADD; Dr. Paul Goldsmith, Metabolic Diseases Branch, NIDDK/NIH, Bethesda, MD; and NPS Pharmaceuticals, Salt Lake City, UT) at a 1:1,000 dilution in PBS containing 10% horse serum and 0.05% Triton X-100 for 48 h at RT. After washing in PBS-Triton (PBS-T), the bound antibodies were localized using biotin-conjugated goat anti–mouse IgG (Jackson Laboratories Inc., West Grove, PA) at a 1:1,000 dilution for 1 h at RT followed by avidin-FITC at 1:1,000 for 2 h at RT. The specificity of the CaR antibodies was assessed by preabsorption with the antigen at 1 μM, for 48 h at 4°C.

To determine the cell type expressing CaR immunoreactivity, double immunostaining with polyclonal antibodies to either gastrin-17 (Dako, Dimension Labs, Ontario) or somatostatin 14 (Peninsula Labs., Inc., Belmont, CA) at a dilution of 1:1,000 in PBS-T was completed. The polyclonal antibodies were localized using a Texas red conjugated donkey anti–rabbit IgG (Jackson Laboratories Inc.) at a dilution of 1:1,000 for 1 h at RT.

Western blot analysis. Cells were lysed in 1 ml of sample buffer (125 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, and 1% β-mercaptoethanol). A sample of the whole cell lysate (40 μl) was loaded on to a 8% Tris/glycine SDS-polyacrylamide gel, and the proteins were separated by electrophoresis and transferred onto an Immobilon-N membrane (Millipore Corp., Bedford, MA). Before staining, the membrane was incubated overnight at 4°C in 5% nonfat milk powder in PBS-T, and was washed three times in PBS-T containing 0.1% milk powder (PBS-T-M) for 10 min at RT. The membrane was incubated in the ADD monoclonal antibody at a dilution of 1:10,000 in PBS-T-M, and was then washed three times in PBS-T-M. After incubating the membrane in peroxidase-conjugated goat anti–mouse IgG (Jackson Laboratories Inc.) diluted to 1:5,000 in PBS-T-M, and washing three times in PBS-T-M, the bands were detected using an ECL kit (Amersham Intl., Buckinghamshire, UK) according to the manufacturer's instructions.

Release experiments. Adherent cells were washed twice with release medium (Dulbecco's modified Eagle's medium [DME] with 4.4 mmol/liter glucose and 0.1% BSA) to remove dead cells and debris. The effect of $[Ca^{2+}]_o$ was examined by incubating cells in DMEM lacking $CaCl_2$, or supplemented to provide a concentration range from 0.5 to 9 mM Ca^{2+}. The osmolality of the resultant release media was monitored, and ranged from 300 (for 0 $CaCl_2$) to 315 mosmol/kg (9.0 mM $CaCl_2$). To determine the effect of nitrendipine, 25 μl of a 40× solution (40 μM) was added to the cells before addition of the medium. After 2 h at 37°C, the medium was collected and centrifuged to remove particulate matter. The supernatants were stored at −20°C until the gastrin radioimmunoassay (RIA) was performed (7). Cells from control wells were detached in 1 ml distilled water, boiled for 10 min, and centrifuged to remove cell debris; the resultant supernatant was stored at −20°C before RIA. The data were calculated as a percentage of basal gastrin release, and were presented as mean±SEM. Statistical significance was determined using an analysis of variance (ANOVA) followed by the unpaired Student's *t* test; values of $P < 0.05$ were considered significant. The *n* values refer to the number of individual donor experiments completed to test a specific secretagogue.

Single-cell microfluorimetry. All experiments were performed using dual-excitation microfluorimetry techniques with fura-2–loaded cells. Antral cells, plated onto APES-coated, etched grid coverslips (Bellco Glass, Inc., Vineland, NJ), were loaded with the Ca^{2+}-fluorophore by a 15-min incubation at 37°C in 1 ml of DMEM containing 5 μM fura-2-acetoxymethyl ester (fura-2-AM; Molecular Probes, Inc., Eugene, OR). All experiments were carried out at 20°C using a Na^+-rich balanced salt solution as the standard extracellular medium, containing (mM): NaCl 137, KCl 5.4, $CaCl_2$ 0.5, $MgSO_4$ 0.8, Na_2HPO_4 0.3, KH_2PO_4 0.4, $NaHCO_3$ 4.2, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes) 10, and glucose 5. The pH was adjusted to 7.4 with NaOH. The osmolality of all solutions was set at 295±5 mosmol/kg. At the start of the experiment, the grid coordinates of the cell clusters imaged were noted, and a digitized image of the cells was captured both with and without the overlay of specific regions of interest (ROI) boxes (Attoflour™ digital fluorescence microscopy system; Atto Instruments, Rockville, MD). All records have been corrected for background fluorescence.

At the completion of the experiments, the coverslip was removed, fixed in Bouin's solution for 15 min, washed in PBS, and incubated with a rabbit anti–gastrin antibody (Dako). After an overnight incubation at 4°C, the coverslip was washed in PBS, and the bound antibodies were localized using biotin-labeled goat anti–rabbit IgG (Jackson Laboratories Inc.) at a final dilution of 1:1,000 for 1 h at RT. After washing three times in PBS, the coverslips were incubated in avidin/biotin peroxidase at 1:1,000 (ABC kit; Vector Laboratories, Inc., Burlingame, CA) for 1 h at RT. The peroxidase reaction was developed in a solution of 25 mg diaminobenzidene, in 50 ml 0.5 M Tris buffer at pH 7.5, with the addition of 0.01% H_2O_2. After locating the correct grid coordinates, the ROI boxes matching gastrin-immunoreactive cells were determined, and the relevant ratio data were retrieved for analysis.

Results

rt-PCR of mRNA isolated from human antral cells yielded a DNA fragment of ~ 374 bp (Fig. 1). Sequence analysis revealed 100% homology between the antral DNA fragment and the corresponding region of the published cDNA sequence of the human parathyroid cell CaR.

The Western blots completed using the ADD antibody demonstrated multiple bands with a band at ~ 140 kD, and three bands at 60, 50, and 40 kD (Fig. 2). The band at 140 kD corresponds to glycosylated receptor, while the bands at 60, 50, and 40 kD probably represent degradative products.

Immunocytochemical staining using a monoclonal antibody to the human parathyroid cell CaR detected 20–30% of the cultured cells, 95% of which were also gastrin immunoreactive (500 of 1,600 cells from three antral preparations). In all gastrin cells, the most intense CaR immunoreactivity was present in the region occupied by gastrin immunoreactive secretory vesicles. In 30% of the cells where a clear polarity was established (150 of 500 cells), CaR-immunoreactivity was also observed at the apical pole of the cell (Fig. 3, *a* and *b*). No CaR immunoreactivity was found in cells which were double-

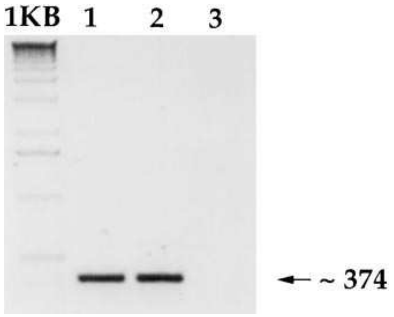

Figure 1. Products obtained after rt-PCR amplification of mRNA from antral cultures. Lanes *1* and *2* contain PCR products from two different donor mRNA samples. Lane *3* is the control in the absence of the template. The products in lanes *1* and *2* were of the expected size for the fragment of the parathyroid hormone–secreting cell CaR.

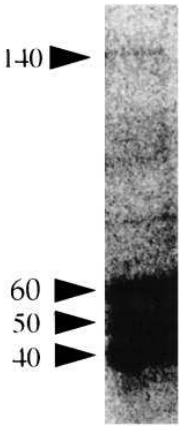

Figure 2. A Western blot of proteins obtained from a whole cell lysate of the cultured cells. Note the presence of the band at ~ 140 kD representing the mature glycosylated receptor.

stained for somatostatin (Fig. 3, *c* and *d*). Alcian blue staining at pH 4.5 was used to localize the mucin cells, none of which demonstrated CaR immunoreactivity (data not shown). Absorption of the CaR antibody with the immunogen blocked the immunostaining, but did not interfere with the binding of the gastrin or somatostatin antibodies (data not shown).

Release data. The total concentration of gastrin in cultured cells was 19,542±4,425 pg per ml. Basal release of gastrin in 1.8 mM Ca^{2+} varied between 142 and 489 pg per ml, which was normalized to 100% in subsequent determinations.

In earlier studies, gastrin release at 3.6 mM was still rising. Therefore, to determine the full response range, the effect of $[Ca^{2+}]_o$ from 0 to 9 mM was examined. The maximal gastrin response was obtained by 7.2 mM ($P < 0.05$, Fig. 4 *a*). At concentrations > 3.6 mM, nitrendipine did not significantly alter the secretory response (Fig. 5). At 1.8 mM $[Ca^{2+}]_o$, the polyamine spermine (10^{-5} to 10^{-3} M) generated a concentration-dependent increase in gastrin release ($P < 0.05$, Fig. 4 *b*).

To determine the sensitivity of G cells to external calcium, the effect of small increments (between 0.5 and 3.5 mM $[Ca^{2+}]_o$) was examined. Below 1.0 mM, $[Ca^{2+}]_o$ did not affect gastrin release, with the greatest incremental response being observed between 2.0 and 2.5 mM (Fig. 5). Nitrendipine (1 μM) inhibited Ca^{2+}-induced gastrin release between 1.5 and 2.0 mM $[Ca^{2+}]_o$, and significantly reduced gastrin release at concentrations of 2.0 mM and above (Fig. 5).

Ca^{2+} imaging. Only those ROI boxes overlying gastrin-immunoreactive cells were used in the subsequent data analysis. Imaging data represents 125 gastrin cells in 32 separate experiments from nine donors.

Increasing $[Ca^{2+}]_o$ from 0.5 to 1.8 mM evoked a small rise in $[Ca^{2+}]_i$ (4/29 cells in five experiments from three donors).

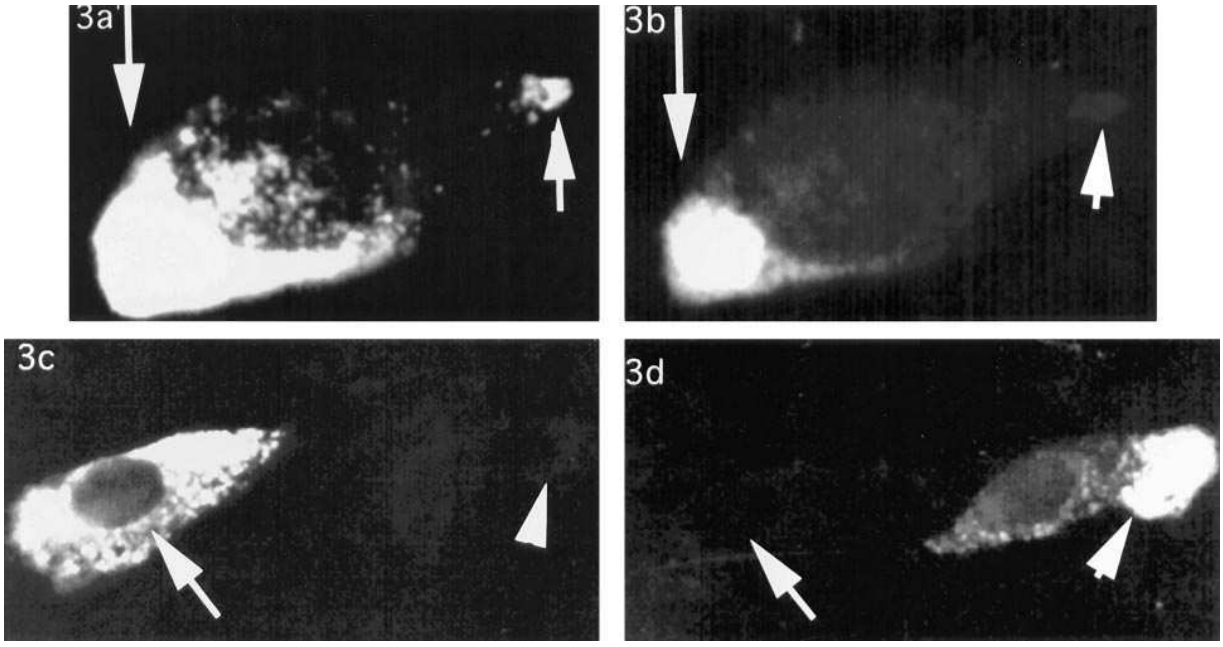

Figure 3. (*a*) Immunocytochemical staining of a gastrin-IR cell with the antibody to the CaR. Note that the receptor is present on both the basal (*large arrow*) and apical regions of the cell (*small arrow*). ×600. (*b*) The same cell showing the gastrin immunoreactivity which is concentrated to the basal pole (*large arrow*). ×600. (*c*) A cell immunostained with the CaR antibody (*large arrow*). Note position of the somatostatin-IR cell shown in *d* which is marked by the small arrow. ×550. (*d*) The same region as shown in *c* showing the somatostatin-IR cell (*small arrow*). ×550.

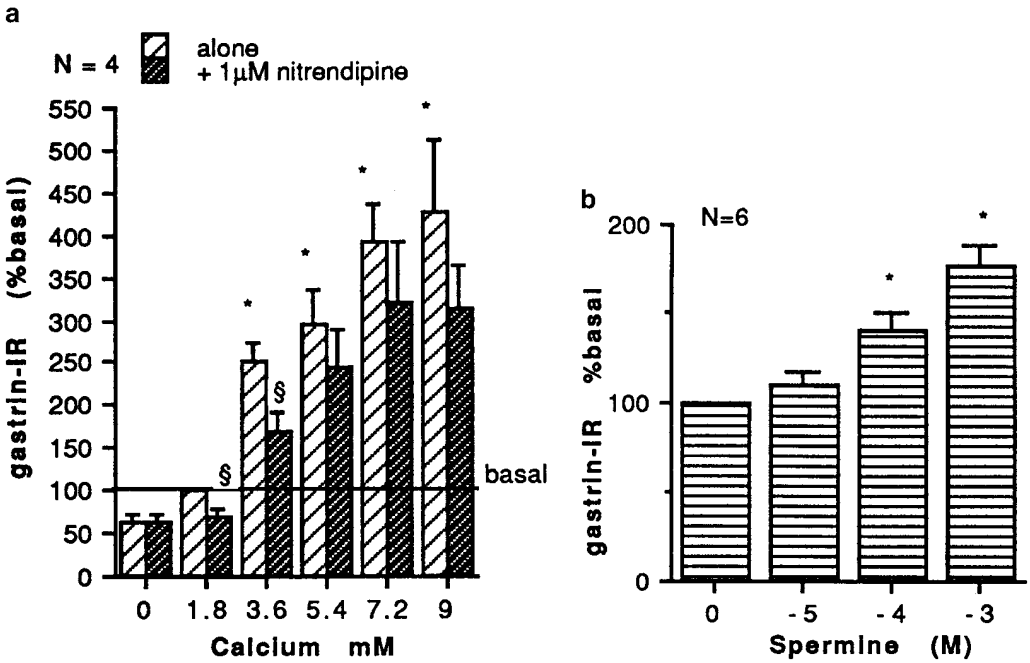

Figure 4. (*a*) The effect of increasing concentrations of $[Ca^{2+}]_o$ on gastrin release. Note that the greatest increase occurs between 1.8 and 3.6 mM. $^*P < 0.05$ between basal and calcium-stimulated; $^{\S}P < 0.05$ between calcium-stimulated and nitrendipine-treated. (*b*) Spermine-stimulated gastrin release in a concentration-dependent manner. $^*P < 0.05$ between basal and spermine-stimulated.

There was a rapid and sustained elevation in $[Ca^{2+}]_i$ when $[Ca^{2+}]_o$ was increased from 0.5 to 3.6 mM (80/83 cells in 25 experiments from nine donors). The amplitude of the Ca^{2+}-induced change in $[Ca^{2+}]_i$ was accentuated when the concentration of extracellular Ca^{2+} was increased to 5.4 mM (50% of cells examined). Further changes in $[Ca^{2+}]_i$ were negligible when $[Ca^{2+}]_o$ was increased to either 7.2 or 9 mM (see Fig. 6 *A*).

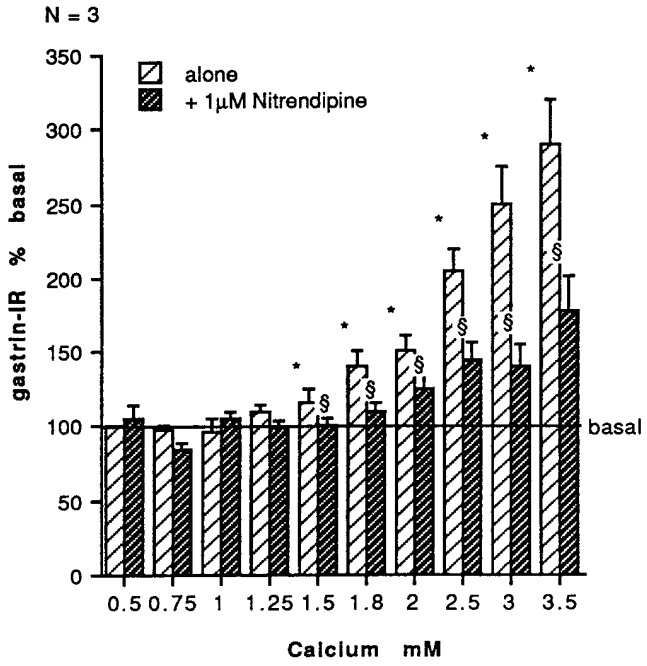

Figure 5. The sensitivity of the G cells between 0.5 and 3.5 mM calcium. Note that < 1.0 mM calcium is ineffective, and that above 2 mM, nitrendipine did not return calcium-stimulated gastrin release to basal levels. $^*P < 0.05$ between basal and calcium-stimulated; $^{\S}P < 0.05$ between calcium-stimulated and nitrendipine-treated.

A similar experiment, (Fig. 6 *B*) demonstrates the change in $[Ca^{2+}]_i$ evoked when the stepwise increase in $[Ca^{2+}]_o$ was returned to 0.5 mM between successive increases. In all cases, there was a rapid elevation in $[Ca^{2+}]_i$ when the concentration of extracellular Ca^{2+} was increased, that returned to basal levels immediately after reinfusion of 0.5 mM Ca^{2+}. The magnitude of the increase in $[Ca^{2+}]_i$ did not differ significantly at concentrations above 3.6 mM.

In the presence of 0.5 mM $[Ca^{2+}]_o$, 10 mM spermine evoked a rapid rise in $[Ca^{2+}]_i$, with a peak amplitude comparable to that obtained by increasing the extracellular Ca^{2+} concentration from 0.5 to 3.6 mM (7/10 cells in five experiments from three donors; Fig. 6 *C*). Although the sustained second phase of the response was maintained during a 150-s application of the agonist, the amplitude of the plateau event was lower than that seen with Ca^{2+}-induced changes in $[Ca^{2+}]_i$.

Discussion

The purpose of this study was to investigate whether calcium-stimulated gastrin release was the result of activation of the CaR. The ability of Ca^{2+} to increase serum gastrin levels is well-established (2) although the mechanism underlying this effect was unknown. The human antral cell preparation expressed an mRNA encoding a 374-bp region of the NH_2-terminal portion of the CaR, with a 100% homology to the previously cloned human parathyroid hormone–secreting cell CaR. We chose to design the primers to this region of the CaR because it contains part of the Ca^{2+}-sensing region. Western blot analysis demonstrated the presence of multiple bands in whole cell lysates. The band observed at $\sim$ 140 kD represents the mature glycosylated receptor, while the lower molecular weight bands probably represent degradative products. Similar results have been observed in Western blots of cell lysates prepared from parathyroid hormone–secreting cells in primary culture (17).

The antral preparation we work with contains a mixture of

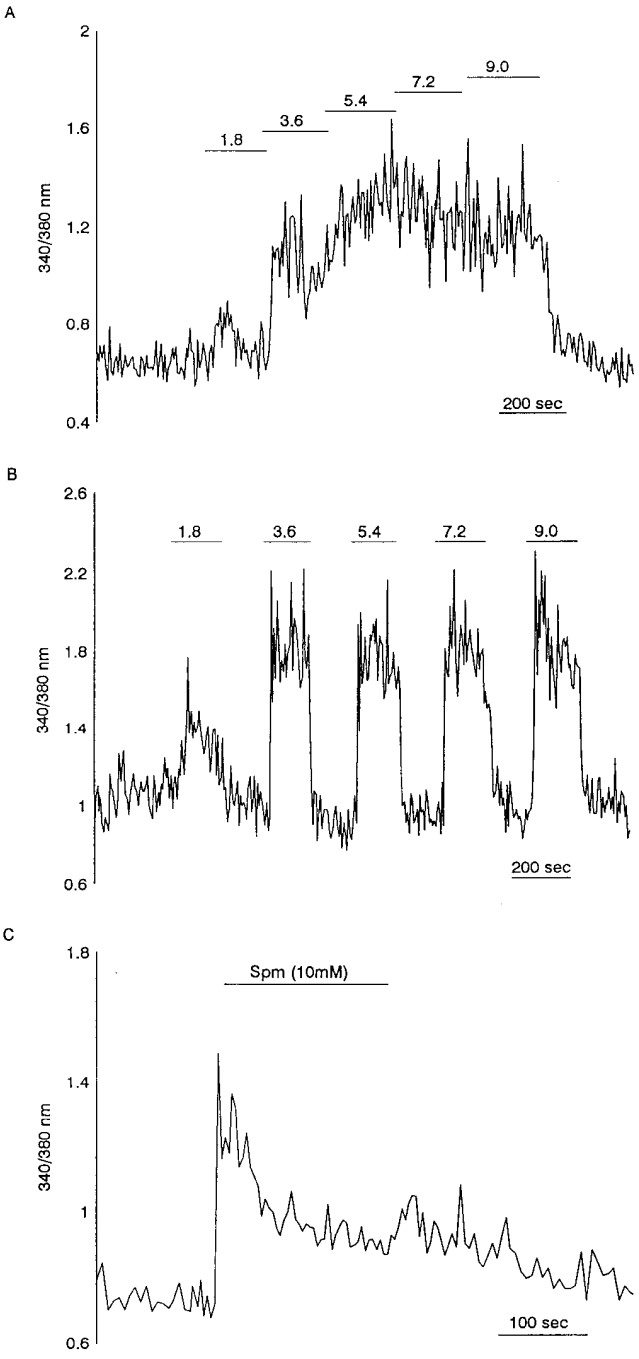

Figure 6. (*A*) Increasing $[Ca^{2+}]_o$ from 0.5 to 9.0 mM in a ramp protocol resulted in an initial response in the $[Ca^{2+}]_i$ at 1.8 mM followed by a rapid rise at 3.6 mM that was increased further at 5.4, but not at either 7.2 or 9.0 mM. After removal of 9.0 mM and returning to 0.5 mM, $[Ca^{2+}]_i$ returned rapidly to basal levels. (*B*) Increasing $[Ca^{2+}]_o$ from 0.5 to 9.0 mM by a step protocol demonstrated, at all concentrations tested, that $[Ca^{2+}]_i$ returned to basal levels immediately upon return to 0.5 mM calcium. (*C*) Addition of spermine to 0.5 mM $[Ca^{2+}]_o$ medium resulted in a rapid initial rise in $[Ca^{2+}]_i$ followed by an elevated plateau level.

three major cell types: gastrin (25–40%), somatostatin (2–4%), and gastric mucin cells (60–75%). Immunocytochemical staining, using the same antibody used for the Western blots, localized the CaR on gastrin-immunoreactive (IR) cells, but not on either the somatostatin or mucin cells. These data indicated that of the cells present in the preparation, it was the antral G cells that expressed the CaR.

In studies of the signaling mechanisms activated by the CaR in either parathyroid hormone–secreting or calcitonin-secreting (C) cells, elevation of $[Ca^{2+}]_o$ resulted in a characteristic rapid rise in $[Ca^{2+}]_i$ (11). Because of the mixed nature of the antral cell cultures, intracellular calcium imaging was used to provide additional evidence that increasing $[Ca^{2+}]_o$ resulted in a receptor-mediated effect in gastrin cells. Our data showed a rapid Ca^{2+}-induced increase in $[Ca^{2+}]_i$ in clearly identified G cells, with a gradual increase in the peak amplitude of the change up to 5.4 mM. The profiles observed were similar to those observed in C cells and in the human embryonic kidney cell line transfected with the human CaR (18), and were consistent with activation of the CaR. Decreasing elevated $[Ca^{2+}]_o$ to 0.5 mM rapidly returns $[Ca^{2+}]_i$ to basal levels. The speed of this offset in Ca^{2+} signaling suggests that the cells possess a well-developed mechanism for Ca^{2+} homeostasis. The rapidity of the regulation is consistent with cells acting as Ca^{2+} sensors.

A known agonist of the CaR, spermine produced both a concentration-dependent increase in gastrin secretion, and evoked an increase in $[Ca^{2+}]_i$ with a peak amplitude comparable to that produced with 3.6 mM $[Ca^{2+}]_o$. The initial transitory component, however, consistent with InsP3-mediated release of stored Ca^{2+}, then declined to a lower second-phase plateau. These data suggested that both spermine and elevated $[Ca^{2+}]_o$ evoked an initial mobilization of stored Ca^{2+}, followed by a subsequent influx of Ca^{2+}. Altering $[Ca^{2+}]_o$ produced a more potent effect on both $[Ca^{2+}]_i$ and gastrin secretion, possibly by the activation of additional signaling mechanisms. These data provided additional support for the contention that increasing $[Ca^{2+}]_o$ activated the CaR present on gastrin cells.

The nitrendipine sensitivity of the Ca^{2+}-induced changes in $[Ca^{2+}]_i$ and gastrin release demonstrated that Ca^{2+} influx via L-type VDCC was responsible, in part, for mediating the effects of a change in $[Ca^{2+}]_o$. Although nitrendipine returned gastrin secretion at 1.8 mM to basal levels, both $[Ca^{2+}]_i$ and gastrin were only inhibited by 30% at 3.6 mM Ca^{2+}, and were not significantly reduced at $[Ca^{2+}]_o > 3.5$ mM. These data would support the involvement of an alternative Ca^{2+} entry/signaling system.

In our experiments, gastrin release over a 2-h period showed no response at concentrations < 1.0 mM Ca^{2+}, and continued to increase up to a maximum at 7.2 mM. In parathyroid hormone–secreting and calcitonin cells, the CaR responds to $[Ca^{2+}]_o$ levels between 0.75 and 3 mM (13). This apparent discrepancy in Ca^{2+} sensitivity between cell types may be a consequence of the location of gastrin cells within the antral mucosa. In plasma, ionized Ca^{2+} levels are between 1.0 and 1.3 mM. Therefore, the relative insensitivity of G cells in this range suggests that plasma levels may not normally regulate G cell function. The luminal surface of the cells, however, would come into contact with higher Ca^{2+} levels that can reach 15 mM (19). The immunostaining of the gastrin cells by the CaR antibody demonstrated both an intense immunoreactivity in the region containing the secretory granules, and staining of the presumptive apical region. This suggests that the CaR may be active at both poles of the G cell. Unfortunately, the cultured cells do not form monolayers. Therefore, the relative contribution of the different receptor populations to the stimulation of gastrin release could not be determined.

In this study, we have provided evidence that human antral G cells express the extracellular Ca^{2+}-sensing receptor. Changes in extracellular Ca^{2+} evoked a concentration-dependent secretion of gastrin, which was paralleled by changes in $[Ca^{2+}]_i$ in a manner similar to that observed in other cells known to express the CaR. Ca^{2+} influx occurred through both a dihydropyridine-sensitive and -insensitive mechanism. Although all of the evidence collected during our experiments is consistent with activation of the CaR underlying the ability of Ca^{2+} to stimulate gastrin release, definitive proof awaits availability of CaR antagonists. The presence of a functional CaR on antral G cells may explain the increase in gastrin-stimulated gastric acid production in response to elevated luminal Ca^{2+} levels, and could underlie the phenomenon of acid rebound that occurs after prolonged ingestion of calcium-containing antacids.

Acknowledgments

This work was supported by a grant from the Medical Research Council of Canada (A.M.J. Buchan). P.E. Squires is a Killam Memorial Postdoctoral Fellow and a recipient of a NATO/Royal Society Traveling Research Fellowship.

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