Bombesin-evoked gastrin release and calcium signaling in human antral G cells in culture

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Squires, Paul E., R. Mark Meloche, and Alison M. J. Buchan. Bombesin-evoked gastrin release and calcium signaling in human antral G cells in culture. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G227–G237, 1999.—Amplification of mRNA from a human antral cell culture preparation demonstrated the presence of two receptors of the bombesin and gastrin-releasing peptide family, GRPR-1 and BRS-3. Single cell microfluorometry demonstrated that most cells that exhibited bombesin-evoked changes in intracellular Ca2+ concentration were gastrin immunoreactive, indicating that antral G cells express the GRPR subtype. The second component of the intracellular Ca2+ response: a transient nitrendipine-insensitive mobilization followed by a sustained phase that was inhibited by removal of extracellular Ca2+ and 20 mM caffeine and was partially inhibited by 10 µM nitrendipine. Preexposure of cells to thapsigargin and caffeine prevented the response to bombesin, indicating activation of inositol 1,4,5-trisphosphate (IP3)-sensitive stores. Gastrin release could be partially reversed by removal of extracellular Ca2+ and blockade of L-type voltage-dependent Ca2+ channels, indicating that a component of the secretory response to bombesin was dependent on Ca2+ influx. These data demonstrated that bombesin-stimulated gastrin release from human antral G cells resulted from activation of GRPRs and involved both release of intracellular Ca2+ and influx of extracellular Ca2+ through a combination of L-type voltage-gated and IP3-gated Ca2+ channels.

gastrin-releasing peptide; calcium imaging; nitrendipine; calcium-sensing receptor

INTERSPECIES DIFFERENCES in receptor expression and/or signal transduction events have complicated our understanding of the mechanisms involved in the regulation of gastrin secretion from antral G cells. One such species-dependent difference is observed in response to changes in extracellular Ca2+. Gastrin release in response to hypercalcemia in dogs remains unchanged (18), whereas in humans there is a significant increase (1). This early observation can now be explained by the expression of the extracellular Ca2+-sensing receptor (CaR) on human antral G cells (16). The CaR is activated at extracellular Ca2+ concentrations between 1.5 and 1.8 mM. All previous studies with the human G cell-enriched preparation utilized cells grown and stimulated in DMEM containing 1.8 mM Ca2+, which would have resulted in chronic activation of the CaR.

In previous studies of the mechanism underlying bombesin-stimulated gastrin release, we demonstrated that secretion was unaffected by the removal of extracellular Ca2+ and suggested that elevation of intracellular Ca2+ levels was responsible for producing the increased Ca2+ levels required for exocytosis (4). These data contrasted with studies in dogs in which removal of extracellular Ca2+ decreased bombesin-stimulated gastrin release (22). In view of the chronic activation of the CaR on the human G cells, a possible explanation for this difference is that the signaling pathways activated by bombesin were modulated by concurrent activation of the CaR rather than by the expression of a different bombesin receptor subtype (BRS), as suggested in the earlier study.

Currently, there are three mammalian receptors that recognize members of the bombesin and gastrin-releasing peptide (GRP) family: the GRPR or BB1 (21); the neuropeptide B receptor (NMBR) or BB2 (24); and a third bombesin receptor subtype, BRS-3 or BB3 (10), an orphan receptor that does not recognize bombesin as a ligand except at high concentrations (27). Recently, a fourth amphibian bombesin receptor has been cloned from frogs, BB4 (13). The precise receptor subtype expressed on either human or canine G cells is presently unknown.

The mobilization of stored Ca2+ is thought to be the major species-independent mechanism activated by bombesin to evoke changes in secretion. However, both canine and human G cells exhibit a refractory period after the initial response, although a subsequent response can be generated, provided that sufficient time is allowed between stimuli. The mechanism underlying the inactive period is currently uncertain. The refractory response to bombesin in Swiss 3T3 cells was not associated with intracellular Ca2+ stores or protein kinase C activity (25), whereas in canine G cells the refractory time ~30 min, probably results from the time required for receptor recycling following the initial stimulation (19). In earlier studies with human G cells, we suggested that the ability to generate a second response was dependent on the refilling of intracellular Ca2+ stores (4).

In view of the expression of the CaR on human antral G cells, the results of the previous studies on signaling pathways activated by bombesin will have reflected the composite response to activation of both CaR and bombesin receptors rather than bombesin alone. The aim of this study was first to identify the BRS expressed on gastrin-secreting cells using RT-PCR. The second aim was to reexamine the bombesin-evoked
Ca\(^{2+}\) signaling and secretion in a modified short-term culture of human antral G cells, in which the concentration of extracellular Ca\(^{2+}\) was maintained below the threshold for activation of the CaR. The cytosolic Ca\(^{2+}\) store accessed by bombesin and the mechanism underlying the Ca\(^{2+}\) influx component were also investigated.

**METHODS**

Cell isolation. Human antrum were collected from 14 multiple-organ donors in collaboration with the British Columbia Transplant Society, with ethical permission from the University of British Columbia Clinical Screening Committee. There were nine male and five female donors, having an average age of 30 years. The vascular supply was perfused in situ with chilled University of Wisconsin buffer (osmolality of 355 mosmol/kg\(\cdot\)H\(_2\)O and pH of 7.0) and cooled externally with crushed ice to minimize ischemic time.

The antral mucosa was digested in buffered collagenase and cells separated according to size by counterflow centrifugation as previously described (6). The cell fraction containing the majority of gastrin-immunoreactive (IR) cells was collected at a flow rate of 40 ml/min and a centrifuged speed of 2,100 rpm.

The cells were resuspended at 1 \times 10\(^6\) cells/ml in growth medium [50:50 DMEM (GIBCO, Grand Island, NY) and nutrient Ham's F-10 mixture (Sigma)] to give a final Ca\(^{2+}\) concentration of 1.0 mM with 5 mM D-glucose. The medium was supplemented with 50 \mu\text{g}/ml gentamicin, 2 mM L-glutamine, 1 \mu\text{g}/ml hydrocortisone, 8 \mu\text{g}/ml insulin, and 5% heat-inactivated FCS (GIBCO). For the gastrin-release studies, cells were seeded at 0.5 ml/well on 48-well Costar plates (Costar Data Packaging, Cambridge, MA); for the Ca\(^{2+}\)-imaging experiments, 1 ml of the cell suspension was added to 3-aminopropyltriethoxysilane (APES)-coated grid-etched coverslip (Belico Biotechnology, Vineland, N J) in 12-well plates. We were unable to locate a source of plastic grid-etched coverslips; therefore, the APES coating was used to alter the electrical charge of the glass to allow attachment of the cells. In neither case was an attachment matrix such as collagen utilized. There was no discernible difference in the attachment of the cells or the enrichment of the G cells. The G cell enrichment of the antral cultures varied from 20 to 40%, with the majority of the enriched cells being gastrin-positive cells and with 1-4% being somatostatin-containing D cells.

RT-PCR. After 48 h in culture, mRNA was isolated from the antral cells (micro mRNA purification kit; Pharmacia Biotechnology, Baie d'Urfé, PQ, Canada). Random hexamer-primer first-strand cDNA was prepared from 300 ng of mRNA per reaction. After reverse transcription, PCR amplification was performed using oligonucleotide primers previously designed to detect the presence of GRPR, NMBR, and BRS-3 in human bronchial epithelial cells (8). The sequences for the forward and reverse primers were as follows: 5'-CTCCCGTGAAAGGTAGTACTGCTTG-3' (nucleotides 480–500) and 5'-ATCTTCTCAAGGGGATGGGACTGAG-3' (nucleotides 848–868), respectively, for GRPR; 5'-GAGGGGTGGAGAAAGGATTCTT-3' (nucleotides 205–225), respectively, for NMBR; and 5'-CTCCCAGTCCTGCTCCGACCA-3' (nucleotides 608–628), respectively, for BRS-3. To increase reaction stringency, the "touchdown" method of PCR was used (9). 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.

To verify the authenticity of the GRPR and BRS-3 products, restriction digest enzymes were chosen based on the restriction maps obtained using PC-gene software (Oxford Molecular Group, Beaverton, OR). In the case of the GRPR product, this was digested with Kpn I and BamH I (BRL Products, Burlington, ON, Canada) restriction enzymes.

Single-cell microfluorometry. Antral cells, plated onto APES-coated, etched-grid coverslip (Belico), were loaded with 5 \mu M of the Ca\(^{2+}\) fluorophore fura 2-AM (Molecular Probes, Eugene, OR), by a 15-min incubation at 37°C in 1 ml of DMEM. All experiments were carried out at 20°C using an Na\(^{+}\)-rich balanced salt solution as the standard extracellular medium, containing (in mM) 137 NaCl, 5.4 KCl, 0.5 CaCl\(_2\), 0.8 MgSO\(_4\), 0.3 NaH\(_2\)PO\(_4\), 0.4 KH\(_2\)PO\(_4\), 4.2 NaHCO\(_3\), 10 HEPES, 5 D-glucose, and 0.1% BSA. The pH was adjusted to 7.2 with NaOH. The osmolality of all solutions was set at 295 ± 5 mosmol/kg\(\cdot\)H\(_2\)O. At the start of each experiment, the grid coordinates of the cell cluster under investigation were noted, and a digitized image of the cells was collected both with and without the overlying regions of interest boxes. Attofluor digital fluorescence microscopy system; Atto Instruments, Rockville, MD). All records have been adjusted for background fluorescence, which is the fluorescence of cell-free coverglass.

At completion of the experiment, the coverglass was removed, fixed in Bouin's solution for 10 min, washed with PBS and 0.1% Triton X-100, and incubated overnight with a monoclonal anti-gastrin antibody (9303, provided by CURE: Gastroenteric Biology Center Antibody/RIA Core, National Institutes of Health Grant DK-41301). After overnight incubation at 4°C, the coverglass was washed with PBS and bound antibodies were localized using biotin-labeled mouse IgG (Jackson Laboratories) at a final dilution of 1:1,000 for 1 h at room temperature. After being washed three times in PBS, the coverglass was incubated in avidin-biotin peroxidase at 1:1,000 (ABC kit; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The peroxidase reaction was developed in a solution of 25 mg diaminobenzidine, in 10 ml 0.5 M Tris buffer at pH 7.5, with the addition of 0.01% H\(_2\)O\(_2\). After the correct grid coordinates were located, the regions of interest boxes matching gastrin-IR cells were determined, and the relevant ratio data were retrieved for analysis.

With the use of data collected from identified gastrin cells, the peak increase in intracellular Ca\(^{2+}\) levels was calculated as the difference in ratio measurements from the preceding basal level in the absence of agonist to the peak of the response. For the bombesin concentration response data, only cells that were stimulated in the order lowest to highest and receiving the full range of bombesin were included in the calculation. The plateau levels of the Ca\(^{2+}\) response were calculated as the difference in ratio measurements between the basal preceding application of the agonist and the sustained phase of the response. The effect of antagonists on the plateau level was calculated as the difference between the preceding basal level and the plateau level observed in the presence of the antagonists. The plateau level obtained in the absence of antagonist was then compared with the level in the presence of antagonists. In both cases, the data are expressed as means ± SE.

Release experiments. Adherent cells were washed twice with release medium (F-10, with 0.5 mM Ca\(^{2+}\), 4.4 mM glucose, and 0.1% BSA) to remove dead cells and debris. The osmolality of the release media was set at 295 ± 5 mosmol/kg\(\cdot\)H\(_2\)O. Cells were incubated in release medium with bombesin over the concentration range of 10 pM to 10 nM for 1 h at 37°C. To determine the effect of removing extracellular Ca\(^{2+}\) (release medium plus 2 mM EGTA), 1 \mu M nitrendipine, 20 mM caffeine, and 1 \mu M ryanodine on bombesin-stimulated gastrin release cells were preincubated with the medium/
fixative. To examine the effect of removing extracellular Ca\(^{2+}\), cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed in PBS to remove excess protein. The data were calculated as a percentage of total cell content (TCC) and presented as means ± SE. Statistical significance was determined by an unpaired Student's t-test; values < 0.05 were considered significant. The n values refer to the number of individual donors.

**RESULTS**

IMMUNOCYTOCHEMISTRY. At the completion of the release studies, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed in PBS to remove excess fixative. To examine the effect of removing extracellular Ca\(^{2+}\), control cells and cells after addition of 2 mM EGTA or after treatment with 20 mM caffeine or 1 µM nitrendipine for 1 h were incubated in a polyclonal rabbit anti-gastrin serum (Dako, Copenhagen, Denmark) at a dilution of 1:4,000 for 18 h at 4°C. After being washed in PBS, the bound antibodies were localized using Cy3 conjugated goat anti-rabbit IgG (Jackson Laboratories).

The inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) subtype found to be on the plasma membrane of cells was the IP\(_3\)R3 (15); therefore, a monoclonal antibody to the IP\(_3\)R3 (Transduction Laboratories, Lexington, KY) at a dilution of 1:500 for 48 h at 4°C was used to determine the localization of gastrin cells. After being washed in PBS, the bound antibodies were localized using ALEXA 568 conjugated goat anti-mouse IgG (Molecular Probes) at a dilution of 1:2,000 for 1 h at room temperature. The immunostained cells were subsequently incubated in the rabbit anti-gastrin antibody as above, and the gastrin cells were identified using ALEXA 488 conjugated goat anti-rabbit IgG at a dilution of 1:2,000 for 1 h at room temperature. Finally, cells were incubated in a monoclonal antibody to ryanodine receptors (RyR) that detects both RyR1 and RyR2 (Affinity BioReagents, Denver, CO) at dilutions ranging from 1:50 to 1:1,000 for 48 h at 4°C, followed by an incubation in ALEXA 568 conjugated goat anti-mouse IgG at 1:1,000 for 1 h at room temperature. The immunostained cells were imaged using a Bio-Rad MRC 600 confocal microscope at ×60 magnification with a z-plane step size of 300 nm. The resultant maximum intensity images from the collected image stacks were prepared using National Institutes of Health Image software, and Fig. 12 was produced using Adobe Photoshop.

**RT-PCR.** RT-PCR of mRNA isolated from human antrum yielded DNA fragments of ~388 and 490 bp corresponding to GRPR and BRS-3, respectively (Fig. 1). There was no evidence for the presence of the NMBR, although the receptor was present in the control human cortex sample. The identification of GRPR and BRS-3 was confirmed by restriction digest analysis. Digestion of the 388-bp GRPR product with Kpn I yielded the expected products of 221 and 167 bp and digestion with BamH I yielded a product of 357 bp.

**Ca\(^{2+}\) imaging studies.** Bombesin (≥1 nM) increased cytosolic Ca\(^{2+}\) in >90% of cells exhibiting gastrin immunoreactivity. To establish if gastrin cells were the only cell type responding to bombesin in the antral cultures, the ratio data from 396 regions of interest from four antral cell preparations perfused with 1 nM bombesin were examined. Of the 396 regions of interest, 23 showed a clear response to bombesin; of these, 20 were over gastrin-IR cells and 3 were over non-gastrin-IR cells, indicating that >85% of the cells responding to bombesin were G cells.

For subsequent Ca\(^{2+}\) imaging experiments, only regions of interest overlying gastrin-IR cells were used for the data analysis. Imaging data represented 110 gastrin cells in 53 experiments from 9 donors. In all cases, resulting traces are representative, with the exception of the basal-to-peak and comparison of plateau ratio data, which represent means ± SE for the group of cells. The responses of the G cells to bombesin application with and without antagonists were remarkably consistent given the range of ages of the individuals (from 15 to 45 years). In each group of experiments, a response of a particular G cell that was not consistent was indicated; e.g., “15/16 cells” indicates that one of the G cells failed to show a similar response pattern.

In G cells, the bombesin-evoked rise in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was rapid, with a peak amplitude similar to that produced by an elevation in extracellular Ca\(^{2+}\) (3.6 mM, Fig. 2). However, bombesin at concentrations < 1 nM did not alter [Ca\(^{2+}\)]\(_i\) (8/8 cells from 3 donors; see Fig. 3A for mean basal-to-peak changes). A bombesin concentration of 1 nM increased [Ca\(^{2+}\)]\(_i\) with a characteristic nonoscillatory single transient event that returned to basal levels even in the continued presence of the agonist. Basal-to-peak changes in [Ca\(^{2+}\)]\(_i\) were similar in either the presence or absence of extracellular Ca\(^{2+}\) (10 G cells from 6 experiments in 4 donors with 0.5 mM Ca\(^{2+}\) and 9 G cells from 5 experiments in 4 donors in the presence of 1 mM EGTA; see Fig. 3B). In many cells, a sustained second phase was observed that persisted beyond the immediate removal of the agonist; however, this was more apparent at higher concentrations (>1 nM).
changes in \([\text{Ca}^{2+}]_i\) were seen at 10 nM, a concentration that also produced maximal gastrin release.

Although the removal of extracellular \(\text{Ca}^{2+}\) had no effect on the initial response to bombesin, it completely reversed the sustained phase at 10 nM bombesin (Fig. 4, 15/16 cells in 6 experiments from 5 donors). Addition of the L-type voltage-dependent \(\text{Ca}^{2+}\) channel (VDCC) blocker, nitrendipine, partially blocked the sustained phase of the bombesin-evoked \(\text{Ca}^{2+}\) response but did not prevent the initial transient event (7/7 cells in 5 experiments from 3 donors; Fig. 5A). In a comparison of basal-to-peak ratio changes, the reduction in \([\text{Ca}^{2+}]_i\), caused by nitrendipine was shown to be significantly lower than the initial peak response but failed to reach significance compared with the sustained phase (Fig. 5B). This suggested that the sustained component of the response represented \(\text{Ca}^{2+}\) influx across the plasma membrane with a minor contribution from opening of L-type VDCC, whereas the initial event represented mobilization of \(\text{Ca}^{2+}\) from cytosolic stores.

To determine if \(\text{IP}_3\)-sensitive \(\text{Ca}^{2+}\) stores were mobilized by bombesin, the \(\text{Ca}^{2+}\)-ATPase inhibitor thapsigargin (1 µM) was added in the absence of extracellular \(\text{Ca}^{2+}\) both before and after a dose of 10 nM bombesin. Thapsigargin depletes \(\text{IP}_3\)-sensitive \(\text{Ca}^{2+}\) pools by preventing pump activity, allowing \(\text{Ca}^{2+}\) to "leak" back into the cytosol. Application of thapsigargin resulted in a rapid transient increase in \([\text{Ca}^{2+}]_i\) and prevented a subsequent response to 10 nM bombesin (11/15 cells in 4 experiments from 3 donors; Fig. 6A). In addition, after stimulation of cells with 10 nM bombesin, application of thapsigargin failed to evoke a further increase in \([\text{Ca}^{2+}]_i\) (7/7 cells in 2 experiments from 2 donors; Fig. 6B).
These data suggest that 10 nM bombesin completely depletes the thapsigargin-sensitive Ca\(^{2+}\) stores. Bombesin-induced depletion of thapsigargin-sensitive Ca\(^{2+}\) stores occurred within 150 s; therefore, we reasoned that shorter stimulation periods should only partially deplete the intracellular stores. Thus, if repetitive bombesin responses depended on the availability of intracellular Ca\(^{2+}\) stores, brief exposure to bombesin should allow repetitive responses to be observed. When bombesin was removed before the [Ca\(^{2+}\)] signal was allowed to return to basal levels (100 s), further short “pulses” of bombesin (1 nM) evoked successive increases in [Ca\(^{2+}\)]. The peak amplitude of the subsequent increases in [Ca\(^{2+}\)] gradually diminished, as the Ca\(^{2+}\) stores were depleted. A further extension of these responses could be obtained by priming the cells between successive short bombesin applications by increasing the extracellular Ca\(^{2+}\) concentration to 3.6 mM (6/9 cells from 3 experiments from 2 donors; Fig. 7). Note how the initial transient change in [Ca\(^{2+}\)] (associated with store release) in response to 3.6 mM Ca\(^{2+}\) was absent following bombesin pretreatment and how the profile of the Ca\(^{2+}\) response reflected Ca\(^{2+}\) influx only. This was presumably due to the preceding depletion of the Ca\(^{2+}\) stores by bombesin. These data provide further evidence that the refractory period in human G cells corresponds to the time taken for refilling of intracellular Ca\(^{2+}\) stores.

The earlier experiments with thapsigargin suggested that the intracellular Ca\(^{2+}\) stores accessed by bombesin were IP\(_3\) sensitive. If this was the case, then preaddition of 20 mM caffeine, to block IP\(_3\) generation, should in turn block the response to bombesin. In experiments in which caffeine was preapplied, the onset of the bombesin response was delayed until caffeine was...
removed (Fig. 8A; 9/9 cells in 5 experiments from 3 donors). This effect was observed in both the presence and absence of extracellular Ca\textsuperscript{2+}. The plant alkaloid ryanodine, which will release Ca\textsuperscript{2+} from non-IP\textsubscript{3} ryanodine-gated Ca\textsuperscript{2+} pools, did not alter basal intracellular Ca\textsuperscript{2+} levels (8/8 cells from 4 experiments from 2 donors; data not shown). These results confirmed that bombesin mobilized Ca\textsuperscript{2+} from IP\textsubscript{3}-sensitive stores. The lack of response to ryanodine suggested that RyRs were not expressed in gastrin cells.

In the previous experiments, preaddition of 20 mM caffeine in the presence of extracellular Ca\textsuperscript{2+} prevented any response to bombesin until the caffeine was washed out. These data suggested that the generation of IP\textsubscript{3} was required to initiate the Ca\textsuperscript{2+} influx component. To examine this possibility further, 20 mM caffeine was added during the plateau phase of the [Ca\textsuperscript{2+}] response to 10 nM bombesin. Addition of caffeine returned the plateau to basal levels, which recovered after washout of caffeine (Fig. 8B; 4/5 cells from 2 experiments from 2 donors). These data indicate the presence of capacitative Ca\textsuperscript{2+} entry in the antral G cells.

Gastrin release studies. The data from the intracellular Ca\textsuperscript{2+} imaging experiments indicated that bombesin stimulation resulted in two distinct Ca\textsuperscript{2+} responses: an initial release of IP\textsubscript{3}-sensitive stores followed by a sustained phase driven by the entry of extracellular Ca\textsuperscript{2+}. In previous studies, we had failed to demonstrate that bombesin-stimulated gastrin release required the presence of extracellular Ca\textsuperscript{2+}. The present experiments were designed to determine if elimination of concurrent stimulation of the CaR changed the sensitivity of bombesin-stimulated gastrin release to extracellular Ca\textsuperscript{2+} levels.

The first set of experiments was designed to determine whether removal of extracellular Ca\textsuperscript{2+} (release medium with 2 mM EGTA) or addition of 1 µM nitrendipine altered bombesin-stimulated gastrin release. In the cultures used for these experiments, the TCC of gastrin was 8,400 ± 500 pg/well (n = 4) with a basal release of 0.8 ± 0.2% TCC. Addition of bombesin resulted in a concentration-dependent increase in gastrin release that was reduced by up to 35% in the absence of extracellular Ca\textsuperscript{2+} (Fig. 9). However, bombesin-stimulated gastrin release remained significantly higher than basal levels in the presence of EGTA. At the completion of these experiments, the cells were washed and incubated in trypan blue to assess viability. Although there was no significant change to cell viability, the morphology of the cultured cells was significantly altered, the usual cell clusters had disaggere gated, and the majority of these cells were rounded rather than polarized.

The Ca\textsuperscript{2+} imaging experiments had indicated that part of the influx of extracellular Ca\textsuperscript{2+} was due to entry through nitrendipine-sensitive L-type channels; there-
fore, the ability of 1 μM nitrendipine to inhibit bombesin-stimulated gastrin release was examined. This concentration was chosen because previous studies had determined that concentrations of >1 μM did not produce an additional inhibition of gastrin release (14). Nitrendipine resulted in a 15–20% decrease in gastrin release at the lower concentrations (Fig. 9). Once again, bombesin-stimulated gastrin release remained significantly higher than basal. An examination of the morphology of these cultures showed no changes to the appearance of the cultured cells. These data indicated that, whereas entry of extracellular Ca\(^{2+}\) contributed to bombesin-stimulated gastrin release, it could not fully account for the secretory response.

To investigate the relative importance of the increase in intracellular Ca\(^{2+}\) levels from IP\(_3\)-sensitive stores, the cells were preincubated in 20 mM caffeine in the presence of 0.5 mM extracellular Ca\(^{2+}\) before and during the addition of bombesin. The gastrin content of the cultures used for these experiments was 11,900 ± 650 pg/well, and the basal secretion was 0.8 ± 0.2% TCC. The addition of caffeine resulted in an unexpected increase in both basal and bombesin-stimulated gastrin release, particularly at the lower bombesin concentrations (Fig. 10A). Morphological studies did not demonstrate any alterations to the appearance of the cultured cells.

The caffeine concentration used was chosen to inhibit IP\(_3\) generation rather than the lower concentrations (μM) that activate RyR. However, the stimulation of gastrin release suggested the possibility that, in G cells, 20 mM caffeine was capable of activating RyR. To evaluate whether RyRs were present in G cells, the effect of a concentration range of ryanodine on basal and bombesin-stimulated gastrin release was examined. For these experiments, gastrin TCC was 12,880 ± 760 pg/well (n = 4 donors) and basal secretion was 0.7 ± 0.1% TCC. Ryanodine had no effect on either basal (Fig. 10B) or bombesin-stimulated release (data not shown), indicating that the stimulatory effect of caffeine was unlikely to be due to activation of RyR.

A second possibility was that 20 mM caffeine was inhibiting the activity of phosphodiesterase, resulting in an increase in intracellular cAMP, thus increasing basal and bombesin-stimulated gastrin release. To examine this possibility, the effect of 20 mM caffeine was compared with that of the phosphodiesterase inhibitor, IBMX; both resulted in a significant stimulation of gastrin secretion.

Fig. 9. Removal of extracellular Ca\(^{2+}\) significantly inhibits bombesin-evoked gastrin secretion at all concentrations tested (n = 4; *P < 0.05). Addition of 1 μM nitrendipine also reduced bombesin-stimulated gastrin release. Note that the inhibitory effect is most pronounced at the lower concentrations of bombesin. IR; immunoreactive; TCC, total cell content.

Fig. 10. A: caffeine (20 mM) significantly increased basal and bombesin-evoked gastrin secretion (n = 4; *P < 0.05). B: a range of concentrations of ryanodine had no effect on gastrin secretion (n = 6).
basal and bombesin-stimulated gastrin release (data not shown). These data suggest that inhibition of phosphodiesterase activity and increased intracellular cAMP levels were responsible for the increased gastrin release.

Immunocytochemistry. A comparison of gastrin-IR cells from control and wells incubated in 2 mM EGTA demonstrated that, after 1 h, G cells in the absence of extracellular Ca\textsuperscript{2+} were still attached to the wells, but dissociation of the cell clusters resulted in a loss of cellular polarity (Fig. 11, A and B). Similar changes were not seen in cells treated with either 1 µM nitrendipine or 20 mM caffeine (data not shown).

Localization of the IP\textsubscript{3}R3 demonstrated punctate staining in the cytoplasm and on the plasma membrane directly overlying gastrin-IR secretory vesicles (Fig. 12A). Merging a single image from the image stack (each z-plane image represents a depth of 300 nm through a cell) indicated that IP\textsubscript{3}R3 were also present on gastrin-IR secretory granules (Fig. 12C). Incubation of the cells with the monoclonal antibody to RyR produced no positive immunostaining.

Fig. 11. A: gastrin-IR cells in a cluster of epithelial cells cultured in the presence of extracellular Ca\textsuperscript{2+}. Note the polarity of the G cells (arrows). B: after removal of extracellular Ca\textsuperscript{2+} for 1 h, the cell clusters dissociated and some of the gastrin cells lost their polarity (arrows). Scale bars = 15 µm.

Fig. 12. A: several G cells immunostained using the antibody to the inositol 1,4,5-trisphosphate (IP\textsubscript{3}) receptor-3 (IP\textsubscript{3}R3). Note the presence of labeling over the plasma membrane (large arrows). A single section through the image stack, 300 nm thick, is shown. B: same z-plane section showing the cells immunostained with the gastrin antibody. C: after merging A and B, costained secretory granules are orange/yellow, IP\textsubscript{3}R3-immunostained structures are red, and gastrin-immunostained secretory granules are green. Note that all gastrin-IR secretory granules in this image are communostained with the IP\textsubscript{3}R3 antibody, but there are a number of vesicle IP\textsubscript{3}R3-IR that do not contain gastrin (arrows). Scale bar = 10 µm.
DISCUSSION

This study demonstrated that, of the three known BRSs, only GRPR and BRS-3 were expressed in the human antral epithelial cell culture preparation. Although synthetic analogs of bombesin act as high-affinity ligands for BRS-3, the orphan receptor is unresponsive to bombesin (27); therefore, the active bombesin receptor in the human cell preparation was the GRPR. This fact coupled with the data showing that the overwhelming majority of cells responding to bombesin in the preparation (> 85%) were gastrin-IR provides strong evidence that human antral G cells express the GRPR, and it is activation of these receptors that mediates bombesin-stimulated gastrin release.

Previous studies examining the mechanism of bombesin-stimulated gastrin secretion in human G cells utilized cells cultured and stimulated in a medium containing a relatively high extracellular Ca\(^{2+}\) concentration (1.8 mM). After the recent localization of the CaR on human G cells (16), the culture conditions in the current study were modified to prevent activation of this receptor, which could result in alterations to intracellular signaling pathways. Cells were cultured in medium containing 1 mM Ca\(^{2+}\), just below the threshold for activation of the CaR. Attempts to decrease the Ca\(^{2+}\) concentration in the culture medium further to 0.5 mM resulted in a loss of cell viability. However, 0.5 mM Ca\(^{2+}\) was the concentration used for the release studies to ensure that the CaR was inactivated.

Altering the culture conditions did not alter the concentration response curve for bombesin; however, both the removal of extracellular Ca\(^{2+}\) and addition of nitrendipine decreased bombesin-stimulated gastrin release, effects that were not observed previously. The experiments utilizing EGTA to remove any extracellular Ca\(^{2+}\) from the release medium were designed to examine the role of entry of extracellular Ca\(^{2+}\) in the gastrin response. However, it is likely that the presence of EGTA over the 1-h period would have also decreased intracellular Ca\(^{2+}\) levels, which would in turn have reduced gastrin release. In addition, incubation of the cells with 2 mM EGTA for 1 h resulted in a significant alteration in the morphology of the cultured cells. The cell clusters disintegrated, and, although the single cells did not detach from the plastic and remained viable, their polarity was significantly reduced. Thus the observed 35% reduction in the response to bombesin in the absence of extracellular Ca\(^{2+}\) could be due to a combination of the changes in cell-cell coupling and reduced intracellular Ca\(^{2+}\) levels rather than a consequence of the lack of Ca\(^{2+}\) influx.

The ability of nitrendipine to inhibit 15% of bombesin-stimulated gastrin release at concentrations >1 nM indicated that Ca\(^{2+}\) influx through L-type VDCC played a minor role in the response to bombesin. In the previous studies (4) with 3.5× higher extracellular Ca\(^{2+}\) levels, the effect of nitrendipine on bombesin-stimulated gastrin release was not significant, although activation of these channels has been shown to be essential for epinephrine-stimulated gastrin release (17). These data indicated that the concurrent stimulation of the CaR on the human G cells in the initial experiments altered both the intracellular Ca\(^{2+}\) signaling events and the Ca\(^{2+}\) sensitivity of the secretory response to bombesin.

The CaR has been shown to activate a number of different signaling pathways, depending on the cell type involved, including activation of phospholipase C and both stimulation and inhibition of adenylate cyclase (7). In antral G cells, the CaR activates a nonselective cation channel (NSCC) that leads to Ca\(^{2+}\) influx as well as activation of L-type VDCC (16). In the previous experiments with 1.8 mM extracellular Ca\(^{2+}\), entry of Ca\(^{2+}\) through the NSCC would have obscured any effect mediated by entry via L-type VDCC; even in the present study, the effect of nitrendipine on bombesin-stimulated gastrin release, although significant, was relatively small.

The data from the Ca\(^{2+}\) imaging experiments clearly demonstrated that at low concentrations (<1 nM) bombesin evoked a Ca\(^{2+}\) response characterized by a single, rapid transient increase in [Ca\(^{2+}\)]\(_i\), which was unaffected by the removal of extracellular Ca\(^{2+}\) or addition of nitrendipine, indicating intracellular Ca\(^{2+}\) mobilization. These studies, in contrast to the data obtained from the gastrin secretion experiments, failed to consistently demonstrate an effect of bombesin at concentrations <1 nM. The most probable explanations for this discrepancy are the shorter time scale of the Ca\(^{2+}\) imaging experiments and the fact that these were conducted at 20°C rather than 37°C, which would reduce the responsiveness of the cells to low levels of agonists.

To confirm the previous suggestion that the refractory response to repetitive bombesin stimulation was due to complete depletion of Ca\(^{2+}\) from intracellular stores, we examined the effect of thapsigargin following bombesin pretreatment in the absence of extracellular Ca\(^{2+}\). Thapsigargin selectively inhibits Ca\(^{2+}\)-sensitive Ca\(^{2+}\) stores and allows any stored Ca\(^{2+}\) to leak back into the cytosol (12). As expected, addition of thapsigargin failed to evoke a further change in [Ca\(^{2+}\)]\(_i\) after the initial bombesin response, indicating full depletion of the IP\(_{3}\)-sensitive stores.

In our earlier study, changes in [Ca\(^{2+}\)]\(_i\) were refractory to repeated bombesin stimulation when the intervening recovery period was limited to 5 min (4). In a recent study using a similar preparation of canine G cells (19), it was suggested that this refractory period was due to recycling of internalized receptors to the plasma membrane, as a second and third full [Ca\(^{2+}\)]\(_i\) response could be evoked by bombesin after a 30-min recovery period. In the present experiments, if low bombesin concentrations were administered to the human G cells for >2 min, subsequent changes in [Ca\(^{2+}\)]\(_i\) were obtained with minimal latency and without store replenishment. These data indicated that GRPRs remained localized to the plasma membrane and that the decline in subsequent responses was the result of depletion of intracellular Ca\(^{2+}\) stores rather than internalization of the receptors. Consistent with this explanation, bombesin-evoked Ca\(^{2+}\) responses declined as intracellular stores were depleted but were
regained following intracellular store replenishment by the administration of a second agonist that directly promoted the influx of Ca\textsuperscript{2+} (in this instance, 3.6 mM Ca\textsuperscript{2+}). The data obtained in the present study indicated that in human G cells the refractory period between responses to low bombesin concentrations represented the interval required for the refilling of intracellular Ca\textsuperscript{2+} stores following bombesin-evoked store depletion.

At higher concentrations of bombesin (≥1 nmol/l), a sustained elevated plateau of [Ca\textsuperscript{2+}] was observed following the initial signaling event. Removal of extracellular Ca\textsuperscript{2+} and addition of 20 mM caffeine completely reversed this, whereas addition of nitrendipine partially inhibited the second phase, indicating that the response was maintained by Ca\textsuperscript{2+} influx, through both IP\textsubscript{3}R and L-type VDCCs. Recent evidence from other cell systems suggests that depletion of intracellular Ca\textsuperscript{2+} stores activates Ca\textsuperscript{2+}-conducting cation currents (11, 26). This store-dependent "capacitative" Ca\textsuperscript{2+} influx may be mediated via IP\textsubscript{3}Rs located on the plasma membrane, specifically IP\textsubscript{3}R3 (2, 15). In the present study, low bombesin concentrations were incapable of generating an influx component probably due to the generation of insufficient IP\textsubscript{3} to mobilize capacitative Ca\textsuperscript{2+} influx. At higher bombesin concentrations, IP\textsubscript{3} generation would be increased, thus activating IP\textsubscript{3}-dependent Ca\textsuperscript{2+} entry across the plasma membrane. The suggested entry of extracellular Ca\textsuperscript{2+} through IP\textsubscript{3}R was supported by the finding of IP\textsubscript{3}R3 immunoreactivity on the plasma membrane of G cells. In this situation, the majority of the Ca\textsuperscript{2+} influx would occur via capacitative entry, with a minor component due to nitrendipine-sensitive L-type VDCC opened due to the depolarizing charge associated with the influx of cations. The data from the intracellular Ca\textsuperscript{2+} imaging experiments, which demonstrated that removal of extracellular Ca\textsuperscript{2+} and caffeine perfusion completely eliminated the sustained plateau phase but nitrendipine only partially reversed the influx component, are consistent with this suggestion.

Interestingly, although it would be expected that the influx component should be sufficient to refill intracellular Ca\textsuperscript{2+} stores, thus allowing repetitive responses to 10 nM bombesin, this was not observed possibly because of the increased IP\textsubscript{3} levels blocking store refilling. An alternative explanation would be internalization or desensitization of the receptors by high bombesin concentrations such that activation of intracellular signaling systems was prevented. The shorter latency (5 min compared with 30 min in canine cells) observed in the human cell preparation suggests that desensitization rather than receptor recycling was occurring.

In neuronal cultures, repetitive Ca\textsuperscript{2+} signaling events can be evoked following repeated administration of bombesin, although the initial transient rise in [Ca\textsuperscript{2+}] decreased with successive applications of bombesin, a finding consistent with the gradual depletion of cytosolic stores (20). Interestingly, the amplitude of the sustained second phase was unaffected and was maintained for extended periods following the removal of the agonist. In the context of the present study, these results would be consistent with the mobilization of intracellular IP\textsubscript{3}-sensitive Ca\textsuperscript{2+} stores being responsible for the first phase of the response and that these were exhausted in a manner similar to those in the G cells. However, repetitive receptor stimulation of the neuronal cells was still capable of initiating Ca\textsuperscript{2+} influx, indicating that capacitative Ca\textsuperscript{2+} entry through IP\textsubscript{3}-gated channels was not involved in the influx component in the neuronal cells.

High concentrations (mM) of caffeine are known to rapidly and reversibly inhibit the release of Ca\textsuperscript{2+} from IP\textsubscript{3}-gated cytoplasmic stores (3, 23). The concentration of caffeine used (20 mM) was chosen because this had previously been shown to completely block inositol phosphate production in smooth muscle cells stimulated with carbachol (14). Both the latter studies and studies of pancreatic acinar cells (5) demonstrated that the effects of caffeine on intracellular Ca\textsuperscript{2+} levels and inositol phosphate generation could be mimicked by increasing intracellular levels of cAMP alone (smooth muscle cells) or both cAMP and cGMP (acinar cells). These data suggest that caffeine may not directly inhibit phospholipase activity but may act indirectly by inhibiting phosphodiesterase activity to increase cyclic nucleotide concentrations that in turn inactivate phospholipase. The effects of caffeine on intracellular Ca\textsuperscript{2+} levels are rapidly reversible; caffeine levels are below levels of detection within seconds of removal of the drug (23). Unfortunately, the only other way of inhibiting IP\textsubscript{3}Rs would be to use heparin, which is cell impermeant and would have to be microinjected into the G cells. This is not possible with our present cell preparation because we have no way of determining (before fixation and immunostaining) which cell is a G cell. In the present study, preapplication of 20 mM caffeine prevented the initial increase in [Ca\textsuperscript{2+}] after bombesin stimulation, supporting the proposed role of IP\textsubscript{3}-gated Ca\textsuperscript{2+} stores. In the continued presence of caffeine, no increase in [Ca\textsuperscript{2+}] was observed; however, this was regained immediately after washout of caffeine, indicating that reactivation of phosphodiesterase rapidly decreased the levels of cyclic nucleotides, thus removing the inhibition of inositol phosphate production.

At low concentrations (µM), caffeine activates a second ryanodine-gated intracellular Ca\textsuperscript{2+} store. The ability of 20 mM caffeine to increase both basal and bombesin-stimulated gastrin release suggested that either RyR may be expressed in addition to the IP\textsubscript{3}-sensitive stores or that the increase in cAMP due to inhibition of phosphodiesterase (14) was increasing gastrin release.

Three different sets of experiments were undertaken to determine if the G cells expressed RyR. The Ca\textsuperscript{2+} imaging studies failed to show an increase in [Ca\textsuperscript{2+}] in response to ryanodine perfusion, gastrin release from the cultured cells was insensitive to a range of ryanodine concentrations, and finally immunocytochemistry with an antibody to RyR1 and -2 failed to demonstrate any positive immunostaining. The lack of RyR in the G cells would be consistent with the proposal that 20 mM caffeine increased intracellular cAMP levels, leading to
the increased gastrin release. The fact that addition of the phosphodiesterase inhibitor, IBMX, to the gastrin cell cultures also increased both basal and bombesin-stimulated gastrin release in a manner similar to 20 mM caffeine would support the latter suggestion.

In conclusion, RT-PCR indicated the presence of GRPR and BRS-3 in the human antral cell culture preparation. Single cell microfluorometry, demonstrating that the majority of cells exhibiting bombesin-evoked changes in intracellular Ca\(^{2+}\) were gastrin immunoreactive, indicated that antral G cells express the GRPR subtype. Changes in [Ca\(^{2+}\)]\(_i\) were observed immediately following bombesin receptor activation and represented mobilization of IP\(_3\)-sensitive Ca\(^{2+}\) stores. Low bombesin concentrations depleted IP\(_3\)-sensitive Ca\(^{2+}\) stores, and, without store replenishment, the cells required a recovery period to obtain repetitive [Ca\(^{2+}\)]\(_i\) responses. High bombesin concentrations recruit both capacitative Ca\(^{2+}\) entry through IP\(_3\)-R3 and activate L-type VDCC, resulting in Ca\(^{2+}\) influx and a sustained plateau level of increased [Ca\(^{2+}\)]\(_i\). Although the entry of extracellular Ca\(^{2+}\) through L-type VDCCs played a minor role in bombesin-stimulated gastrin release, the contribution of the mobilization of intracellular Ca\(^{2+}\) stores and entry through the capacitative Ca\(^{2+}\) channels could not be determined due the effects of caffeine on phosphodiesterase activity.

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REFERENCES


