Multiple Protein Kinase Pathways Are Involved in Gastrin-releasing Peptide Receptor-regulated Secretion*

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Gastrin-releasing peptide (GRP) and its amphibian homolog, bombesin, are potent secretagogues in mammals. We determined the roles of intracellular free Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)\), protein kinase C (PKC), and mitogen-activated protein kinases (MAPK) in GRP receptor (GRP-R)-regulated secretion. Bombesin induced either \([\text{Ca}^{2+}]_i\) oscillations or a biphasic elevation in \([\text{Ca}^{2+}]_i\). The biphasic response was associated with peptide secretion. Receptor-activated secretion was blocked by removal of extracellular Ca\(^{2+}\), by chelation of \([\text{Ca}^{2+}]_i\), and by treatment with inhibitors of phospholipase C, conventional PKC isoforms, and MAPK kinase (MEK). Agonist-induced increases in \([\text{Ca}^{2+}]_i\) were also inhibited by dominant negative MEK-1 and the MEK inhibitor, PD98059, but not by an inhibitor of PKC. Direct activation of PKC by a phorbol ester activated MAPK and stimulated peptide secretion without a concomitant increase in \([\text{Ca}^{2+}]_i\). Inhibition of MEK blocked both bombesin- and phorbol 12-myristate 13-acetate-induced secretion. GRP-R-regulated secretion is initiated by an increase in \([\text{Ca}^{2+}]_i\); however, elevated \([\text{Ca}^{2+}]_i\) is insufficient to stimulate secretion in the absence of activation of PKC and the downstream MEK/MAPK pathways. We demonstrated that the activity of MEK is important for maintaining elevated \([\text{Ca}^{2+}]_i\), levels induced by GRP-R activation, suggesting that MEK may affect receptor-regulated secretion by modulating the activity of Ca\(^{2+}\)-sensitive PKC.

Bombesin (BBS)\(^1\)-like peptides are distributed throughout the central nervous system and gastrointestinal tract of mammals, where they modulate metabolism, behavior (1), smooth muscle contractility (2), chemotaxis (3), and exocrine and endocrine processes. Many of the biological effects attributed to the amphibian peptide, BBS, and its mammalian homolog gastrin-releasing peptide (GRP) are a consequence of their potent secretory activity and occur secondarily to the release of other peptide hormones. For example, in the small intestine, the effects of BBS on smooth muscle contraction are partially due to BBS-induced secretion of motilin from intestinal M-cells (4). In isolated muscle strips from the lower esophageal sphincter of rabbits, BBS-induced muscle contractions are blocked by substance-P receptor antagonists, suggesting that substance-P is the direct mediator of contractions in this preparation (5). In the stomach, BBS stimulates gastric acid secretion from parietal cells indirectly by stimulating gastrin release from stomach G-cells (6, 7). BBS-stimulated secretion also plays a role in the proliferation of some tumors of the lung (8) and stomach (9) by participating in autocrine and/or paracrine growth loops. Despite the important role of BBS-stimulated secretion in many normal and pathophysiological processes, little is known about the intracellular signal transduction pathways regulating this activity.

A family of G-protein-coupled receptors mediates the actions of BBS. Three BBS receptor subtypes have been cloned and characterized in humans: 1) gastrin-releasing peptide (GRP)-preferring receptors (GRP-R), 2) neurenom B-preferring receptors, and 3) the bombesin receptor subtype 3 (10). Agonist binding to GRP-R stimulates phospholipase C-\(\beta\) (PLC-\(\beta\)) resulting in the production of inositol 1,4,5-trisphosphate and diacylglycerol (DAG), an increase in the concentration of free cytosolic Ca\(^{2+}\) (\([\text{Ca}^{2+}]_c\)), and the activation of both protein kinase C (PKC) (11–13) and mitogen-activated protein (MAP) kinase pathways (14).

To investigate the role of these pathways in BBS-induced peptide secretion, we developed a human neuroendocrine-like cell line that expresses recombinant human GRP-R, called BON/GRP-R. The parental BON cell line was derived from a human metastatic carcinoma tumor of the pancreas (15). BON and BON/GRP-R cells exhibit morphological and biochemical characteristics consistent with the phenotype of a neuroendocrine cell, including the presence of numerous dense-core granules and the expression and secretion of chromogranin-A (CGA), neurotensin (NT), serotonin, and pancreaticatatin (16, 17). Unlike primary cultures of canine gastric G-cells, which have been used by others to examine BBS-induced secretion (7), BON/GRP-R can be maintained in long term culture without noticeable changes in their secretory activity. In addition, they have a lower level of constitutive or spontaneous peptide release compared with the glucagonoma cell line, STC-1. Using BON/GRP-R cells and a combination of single cell calcium imaging, whole cell voltage clamp, radioimmunoassay (RIA), and the reverse hemolytic plaque assay (RHPA), we have determined the roles of agonist-induced increases in \([\text{Ca}^{2+}]_c\), PKC, and MAPK pathways in GRP-R-regulated exocytosis. We have found that GRP-R-activated secretion requires a sus-
tained elevation in \([\text{Ca}^{2+}]_i\), that is initiated by \(\text{Ca}^{2+}\) release from intracellular stores and maintained by \(\text{Ca}^{2+}\) influx across the plasma membrane and through the activity of MEK-mediated pathways. However, an agonist-induced increase in \([\text{Ca}^{2+}]_i\) will not stimulate secretion without activation of PKC and the subsequent activation of downstream MAPK/ERK. MEK regulation of \([\text{Ca}^{2+}]_i\), suggests that this kinase pathway may affect receptor-regulated secretion, in part, by modulating the activity of \(\text{Ca}^{2+}\)-sensitive PKC through a feedback loop mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—BON cells, stably transfected with a human gastrin-releasing peptide receptor cDNA (GRP-R), were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2 in DMEM/F12K (1:1) medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) and Geneticin (G-418, 400 μg/ml).

**Calcium Imaging**—Real-time recording of \([\text{Ca}^{2+}]_i\), was performed in single BON/GRP-R cells using methods described previously (18). Cells were plated on glass coverslips (25 mm) at a density of approximately 1.5–3 × 10⁶ cells/coverslip, cultured for 48 h, washed with KRH (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 KH₂PO₄, 1.2 mM MgSO₄, 2.4 mM CaCl₂, 2.5 mM glucose), and loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) for 50 min at 25°C. Loaded cells were washed three times with KRH and incubated in KRH plus 0.1% bovine serum albumin for 60 min at 25°C in the dark. Cells were challenged with various concentrations of BBS and imaged using a Nikon Diaphot inverted microscope (Garden City, NY). The microscope was coupled to a dual monochromator system via a fiber optic cable (Photometrics, Tucson, AZ). Fluorescence was detected using an intensified charged coupled device camera (Dage-MTI, Inc., Michigan City, IN) and images processed using ImageMaster software (PIT).

**RIA**—BON/GRP-R cells (5 × 10⁵ cells/well) were plated into six-well culture plates (Falcon Labware) in culture medium consisting of DMEM/F12K (1:1) supplemented with 5% FBS and 400 μg/ml G-418. After 48 h, cells (5 × 10⁵ cells/well) were washed with phosphate-buffered saline and treated with bombesin (BBS) (10⁻¹⁰ m to 10⁻⁴ m), diluted in KRH, at room temperature. Following BBS treatment, the NT or CGA content of the medium was determined by RIA as described previously (19, 20). The NT antiserum was generated in rabbits using full-length porcine NT as antigen. CGA antiserum was produced in rabbits using synthetic rat CGA (amino acid residues 359–389) linked to bovine serum albumin as antigen. The sensitivities and ID₅₀ values for the NT RIA and CGA RIA were 100 pg/ml and 1 ng/ml, respectively. The intra-assay variability was 5%.

**RESULTS**

**Effects of BBS Stimulation on \([\text{Ca}^{2+}]_i\)**—BBS stimulation of BON/GRP-R cells generally produced two distinct \([\text{Ca}^{2+}]_i\) responses: biphasic or oscillatory. The frequency with which each calcium response was observed depended partially on the concentration of BBS (Table I). Greater than 99% of the cells responded to BBS stimulation at all concentrations tested. At high concentrations of BBS (≥10⁻⁸ m), 100% of the responding cells displayed a biphasic calcium response (Fig. 1A) characterized by an initial rapid increase in \([\text{Ca}^{2+}]_i\), followed by a second sustained elevation in \([\text{Ca}^{2+}]_i\), that slowly declined to resting levels over a period of 10–20 min. The predominant calcium response at lower concentrations of BBS (≤10⁻¹⁰ m) was an oscillatory increase in \([\text{Ca}^{2+}]_i\), lasting approximately 15–30 s with a spike frequency ranging from 0.4 to 1.3 transients per min (Fig. 1B). Between each transient, \([\text{Ca}^{2+}]_i\) generally returned to a level slightly elevated above resting levels. BBS stimulation also induced a single \([\text{Ca}^{2+}]_i\) spike in a small percentage of cells (2–8%) (Fig. 1C). Like the oscillatory response, the single spike response only occurred at lower concentrations of BBS (Table I).

**Effects of Extracellular \(\text{Ca}^{2+}\) on BBS-induced Changes in \([\text{Ca}^{2+}]_i\)**—Both the biphasic and oscillatory calcium responses were dependent on the presence of extracellular \(\text{Ca}^{2+}\). Replacing the normal KRH solution with a solution that did not contain added \(\text{Ca}^{2+}\) and included 1 mM EGTA terminated the biphasic and oscillatory \([\text{Ca}^{2+}]_i\) responses (Fig. 2A and B). The biphasic response was immediately attenuated following removal of extracellular \(\text{Ca}^{2+}\), whereas oscillating cells showed a slowing of the spike frequency, followed by complete cessation of the calcium response within 10 min. Addition of lanthanum (La³⁺) blocked agonist-induced \([\text{Ca}^{2+}]_i\) oscillations in a concentration-dependent fashion (Fig. 2, C and D). Lanthanum blocks \(\text{Ca}^{2+}\) influx through the plasma membrane (24) and inhibits the activity of the plasma membrane \(\text{Ca}^{2+}\)-dependent ATPase (25). It is also the most potent metal ion to block the sodium-calcium exchanger in vesicle systems (26) and in the squid axon (27). A low concentration of La³⁺ (0.2 mM) caused a slowing of the spike frequency, whereas a higher concentration (1.0 mM) completely abolished BBS-
induced increases in [Ca\textsuperscript{2+}].

Whole cell voltage clamp experiments with BON/GRP-R cells revealed an inward current that was activated near −40 mV and peaked near −5 mV (data not shown). This current was slowly inactivating and similar to that seen during activation of L-type voltage-gated Ca\textsuperscript{2+} channels. These channels, however, are unlikely candidates for agonist-induced Ca\textsuperscript{2+} influx because BBS failed to depolarize BON/GRP-R cells, a requirement for activation of voltage-gated Ca\textsuperscript{2+} channels. There was no effect of BBS application on BON/GRP-R cell membrane conductance compared with measurements just before and after agonist stimulation. Consistent with these experiments, nifedipine, a blocker of L-type voltage-gated Ca\textsuperscript{2+} channels, did not affect either BBS-induced [Ca\textsuperscript{2+}], oscillation or biphasic responses (data not shown).

**BBS Stimulates Peptide Secretion from BON/GRP-R Cells**—In the presence of extracellular Ca\textsuperscript{2+} (2 mM), maximum release of CGA and NT occurred at BBS concentrations of 10\textsuperscript{−8} M and above (Fig. 3, A and B, closed circles). In the absence of extracellular Ca\textsuperscript{2+}, BBS failed to stimulate detectable release of either CGA or NT at all concentrations of agonist tested (Fig. 3, A and B, open circles). Addition of 10\textsuperscript{−7} M BBS caused a time-dependent increase in CGA and NT secretion that reached a maximum at approximately 30 and 15 min, respectively (Fig. 3, C and D). The time course of peptide release was consistent with the long duration of biphasic calcium response observed at higher concentrations of BBS. In contrast, treatment with a low concentration of BBS (10\textsuperscript{−10} M), which induced predominantly [Ca\textsuperscript{2+}], oscillations (Table I), produced only a small increase in NT secretion (Fig. 3D, open circles).

To investigate the relationship between specific patterns of change in [Ca\textsuperscript{2+}], and peptide secretion from individual cells, we used a combination of fura-2 imaging and RHPA. BON/GRP-R cells were stimulated with 10\textsuperscript{−10} M BBS; intracellular Ca\textsuperscript{2+} oscillations were recorded in 152 individual cells. When we compared [Ca\textsuperscript{2+}], records with the results of the RHPA, we found no detectable secretion of CGA from cells exhibiting [Ca\textsuperscript{2+}], oscillation up to 2 h after stimulation (Fig. 4A). When [Ca\textsuperscript{2+}], records from individual cells stimulated with 10\textsuperscript{−7} M BBS were compared with RHPA data, all cells that developed plaques (about 20% of the total cells) exhibited a biphasic [Ca\textsuperscript{2+}], response (Fig. 4B, n = 79). Similar results were obtained when NT secretion was compared with [Ca\textsuperscript{2+}], (data not shown).

To determine whether cells exhibiting [Ca\textsuperscript{2+}], oscillations were secretion-competent, we identified oscillating cells and showed that, after 1 h of continuous exposure to BBS (10\textsuperscript{−10} M), there was no plaque formation by RHPA. When cells were restimulated with 10\textsuperscript{−7} M BBS and incubated an additional 1 h, plaques formed around the cells previously exhibiting [Ca\textsuperscript{2+}], oscillations, indicating that these cells were capable of peptide secretion in the presence of sufficient agonist (data not shown).

**GRP-R-regulated Secretion Is Initiated by IP\textsubscript{3}-induced Ca\textsuperscript{2+} Release from Intracellular Stores**—Pretreatment of BON/GRP-R19 cells with either the PLC inhibitor, U73122 (1 μM), or the acetoxyethyl ester form of the Ca\textsuperscript{2+} chelator, BAPTA (30 μM), completely blocked BBS-stimulated increases in [Ca\textsuperscript{2+}], (Fig. 5A) and peptide secretion (Fig. 5B). These data demonstrate that BBS-stimulated peptide secretion is dependent on an increase in [Ca\textsuperscript{2+}], which is initiated by an inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+} release from intracellular stores.

To determine whether Ca\textsuperscript{2+} released from intracellular stores was sufficient to stimulate secretion, we treated BON/GRP-R19 cells with thapsigargin. Thapsigargin is an irreversible inhibitor of the microsomal Ca\textsuperscript{2+}-ATPase reuptake pump that is responsible for maintaining intracellular Ca\textsuperscript{2+} stores. Application of thapsigargin to BON/GRP-R cells in the absence of extracellular Ca\textsuperscript{2+} induced a transient increase in [Ca\textsuperscript{2+}], (Fig. 6A, Ca\textsuperscript{2+}-free) and did not stimulate secretion (Fig. 6B, Thaps (Ca\textsuperscript{2+} free)). Bombesin stimulation (10\textsuperscript{−7} M), in the absence of extracellular Ca\textsuperscript{2+}, produced a similar [Ca\textsuperscript{2+}], profile and also failed to induce secretion (data not shown). In contrast, thapsigargin treatment of cells bathed in 2 mM extracellular Ca\textsuperscript{2+} induced both a sustained increase in [Ca\textsuperscript{2+}], (Fig. 6A, 2 mM Ca\textsuperscript{2+}) and CGA secretion (Fig. 6B, 2 mM Ca\textsuperscript{2+}). These data demonstrate that release of Ca\textsuperscript{2+} from intracellular stores, in the absence of an influx of extracellular Ca\textsuperscript{2+}, is insufficient to stimulate peptide secretion. To further evaluate the role of store-released Ca\textsuperscript{2+} in GRP-R-activated secretion, BON/GRP-R cells were pretreated with thapsigargin for 10 min in Ca\textsuperscript{2+}-free media in order to deplete the intracellular Ca\textsuperscript{2+} stores. Following thapsigargin treatment, the bath solution was replaced with KRH containing 2 mM Ca\textsuperscript{2+} and after a 20-min recovery, the cells were overlaid with ovine red blood cells previously exhibiting [Ca\textsuperscript{2+}], oscillations (Table I), produced only a small increase in NT secretion (Fig. 3D, open circles).
Ca\textsuperscript{2+} influx across the plasma membrane, which in turn provides the sustained elevation in [Ca\textsuperscript{2+}]\textsubscript{i}, required for activation of the receptor-regulated secretory machinery.

**BBS-stimulated Secretion Requires PKC and MAPK Activation**—Previous studies have shown that GRP-R is coupled to the activation of both PKC and MAPK/ERK pathways; however, the role of these kinases in GRP-R-regulated secretion is not well defined. Direct activation of PKC with the phorbol ester, phorbol 12-myristate 13-acetate (PMA), stimulated secretion of CGA from BON/GRP-R cells (Fig. 7A, 1 \textmu M PMA) without increasing [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 7B, 1 \textmu M PMA). PMA also induced MAPK/ERK activation (Fig. 7C, lane 7). The MEK inhibitor PD98059 inhibited PMA-stimulated secretion and MAPK/ERK activation in a dose-dependent manner (Fig. 8, A and B). These data indicate that PMA-sensitive PKC is upstream of MEK and the regulation of peptide secretion in BON/GRP-R cells.

To investigate whether PKC was coupled to GRP-R-regulated secretion, cells were pretreated with the PKC inhibitor, GF109203X (GFX) (5 \textmu M), and stimulated with BBS. Pretreatment with GFX completely blocked BBS-stimulated CGA release (Fig. 7A, GFX + BBS) and MAPK/ERK activation (Fig. 7C, lane 6), but did not affect BBS-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 7B, 5 \textmu M GFX). These data demonstrate that blocking PKC activation is sufficient to block BBS-induced secretion and MAPK/ERK activation in BON/GRP-R cells even in the presence of an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, suggesting that one role for BBS-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} is to activate Ca\textsuperscript{2+}-sensitive PKC isozymes.

**MEK Activity Maintains BBS-induced Elevations in [Ca\textsuperscript{2+}]\textsubscript{i}**—To further investigate the role of MEK-mediated pathways in GRP-R-regulated secretion, BON/GRP-R cells were treated with the MEK inhibitor, PD98059. As expected, PD98059 blocked BBS-induced MAPK/ERK activation (Fig. 7C, lane 5) and caused a dose-dependent inhibition of CGA secretion (Fig. 9A). However, in contrast to the PKC inhibitor (GFX), which did not affect agonist-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i}, PD98059 induced a dose-dependent inhibition of BBS-stimulated increases in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 9B). To evaluate the specificity of the PD98059 effect, BON/GRP-R cells were transfected with either a vector construct containing a dominant negative mutant form of MEK-1 (MEK-2A-EECMV; Dr. Dennis J. Templeton, Case Western Reserve University) or the empty expression vector EECMV. Like PD98059, the dominant negative MEK-1 blocked BBS-stimulated increases in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 9C, dnMEK-1).

**FIG. 2. Effects of extracellular Ca\textsuperscript{2+} and La\textsuperscript{3+} on BBS-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i}**. Replacing the bath solution with KRH without Ca\textsuperscript{2+} and containing 1 mM EGTA abolished the BBS-induce biphasic (A) and oscillatory (B) [Ca\textsuperscript{2+}]\textsubscript{i} responses. Addition of 0.2 mM La\textsuperscript{3+} to cells bathed in normal KRH caused a slowing of spike frequency (C), whereas addition of 1 mM La\textsuperscript{3+} completely blocked oscillation in [Ca\textsuperscript{2+}]\textsubscript{i} (D).

**FIG. 3. Concentration response and time course of BBS-induced secretion**. BON/GRP-R19 cells were incubated for 1 h at room temperature with increasing concentrations of BBS. BBS induced a concentration-dependent increase in CGA (A) and NT (B) release in the presence of extracellular Ca\textsuperscript{2+} (2 mM) (closed circles). In the absence of extracellular Ca\textsuperscript{2+}, there was no detectable secretion of either CGA (A) or NT (B) (open circles). Cells were treated with BBS (10\textsuperscript{-7} M) at room temperature for various lengths of time. CGA (C) and NT (D) secretion was measured by RIA. BBS (10\textsuperscript{-7} M) induced a time-dependent release of both peptides (closed circles). At 10\textsuperscript{-10} M BBS, there was minimal release of NT (D, open circles). Each point represents the mean ± S.E. (n = 6) from three separate experiments.
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**FIG. 5.** Effects of a PLC-β inhibitor, U73122, and the acetoxymethyl ester form of the Ca\(^{2+}\) chelator, BAPTA, on BBS-stimulated changes in [Ca\(^{2+}\)]\(i\) (A) and CGA secretion (B). BON/GRP-R19 cells were pretreated for 4–5 min with either 1 μM U73122 or 30 μM BAPTA and stimulated with BBS (10\(^{-7}\) M). Calcium tracings are the average 340/380 nm ratios from 20–40 individual cells ± S.D. Secretion is expressed as a percentage of plaque-forming cells in 1 h ± S.E. from three separate experiments. Six hundred to 1000 cells were counted per treatment group. Baseline or background secretion from cells treated with Me\(_2\)SO (DMSO, 0.5%) was approximately 3% of the total cells counted. BBS-induced peptide secretion from 17–20% of the cells. *p < 0.01 versus 0.5% Me\(_2\)SO.

**TABLE I**

The effect of BBS [Ca\(^{2+}\)\(i\)] concentration on the occurrence (%) of different [Ca\(^{2+}\)\(i\)] responses in BON/GRP-R19 cells

| Response          | [BBS] Log 10\(^{-11}\) | 10\(^{-10}\) | 10\(^{-9}\) | 10\(^{-8}\) | 10\(^{-7}\) | 10\(^{-6}\) |
|-------------------|-------------------------|-------------|-------------|-------------|-------------|-------------|
| Biphasic          | 30                      | 42          | 57          | 100         | 100         | 100         |
| Oscillations      | 65                      | 56          | 35          | 0           | 0           | 0           |
| Monophasic        | 5                       | 2           | 8           | 0           | 0           | 0           |
| Total no. of cells| n = 130                 | n = 203     | n = 49      | n = 50      | n = 23      | n = 99      |

Transfection of the empty expression vector had no effect on BBS-induced [Ca\(^{2+}\)\(i\)] responses (Fig. 9C, Vector). Together, these data demonstrate a novel role for MEK-mediated pathways in the maintenance of agonist-induced increases in [Ca\(^{2+}\)], and suggest that MEK modulates GRP-R-regulated exocytosis, in part, by affecting the activity of Ca\(^{2+}\)-sensitive PKC.

**DISCUSSION**

An important trigger of receptor-regulated secretion is an agonist-induced increase in [Ca\(^{2+}\)], (28–32). The development of techniques that allowed changes in [Ca\(^{2+}\)] to be studied within individual cells has revealed complex patterns in calcium signals, including repetitive oscillations, in response to physiological concentrations of agonist (11, 33–35). In this study, we have shown that stimulation of BON/GRP-R cells with low concentrations of BBS induces oscillatory changes in [Ca\(^{2+}\)], whereas high concentrations of BBS produce a biphasic calcium response that lasts up to 20 min in normal extracellular calcium. BBS-induced calcium oscillations appear to be a general characteristic of GRP-R activation. We have investigated GRP-R-mediated [Ca\(^{2+}\)], signaling in several cell lines, including a human gastric carcinoma cell line (SIIA) and a human prostate cancer cell line (PC3), both of which express native GRP-R, as well as another transfected cell model, GRP-R-transfected mouse NIH Balb/C 3T3 fibroblasts. In these cell lines, low concentrations of BBS stimulate [Ca\(^{2+}\)], oscillations. BBS-induced calcium oscillations also have been reported in the insulin-secreting cell line, HIT-T15 (11), and in the pancreatic acinar cell line, AR4–2J (34). However, this is the first report to address the role of BBS-induced [Ca\(^{2+}\)], oscillations in GRP-R-regulated exocytosis.

A central question in calcium signaling biology is whether [Ca\(^{2+}\)], oscillations specify receptor- or cell type-specific information. Because of the intimate association of receptor-induced calcium signals and secretion, [Ca\(^{2+}\)], oscillations may represent a coded signal that modulates the exocytotic machinery. Recently, experimental evidence obtained from isolated pituitary cells has supported this hypothesis. Tse and co-workers (28) have shown that stimulation of gonadotrophs with gonadotropin-releasing hormone induces oscillations in [Ca\(^{2+}\)], that
are temporally associated with an increase in cell membrane capacitance, a measure of vesicle fusion with the plasma membrane. Additionally, constitutive secretion of growth hormone, from somatotropes, increases with both elevations in the frequency and amplitude of spontaneous \([Ca^{2+}]_i\) oscillations (31).

In contrast to pituitary cells, the data presented here show that BBS-induced peptide secretion from BON/GRP-R cells was associated with a biphasic, sustained elevation in \([Ca^{2+}]_i\) and not \([Ca^{2+}]_o\) oscillations. It is not clear why \([Ca^{2+}]_i\) oscillations are not associated with secretion from these cells; however, our results are consistent with recent reports showing that GRP-R-regulated secretion is associated with a sustained elevation in \([Ca^{2+}]_i\) in two other cell types: primary cultures of canine G-cells (30) and the mouse intestinal cell line, STC-1 (32). Seensalu and co-workers (30) showed that BBS-stimulated gastrin secretion from G-cells was inhibited when the second phase of a biphasic \([Ca^{2+}]_i\) response was blocked by removal of extracellular \(Ca^{2+}\). Similarly, a sustained biphasic \([Ca^{2+}]_i\) response, dependent on the presence of \(Ca^{2+}\) in the extracellular solution, correlated with BBS-stimulated cholecystokinin re-
lease from STC-1 cells (32). The fact that BBS-induced secretion from BON/GRP-R requires a sustained elevation in \([Ca^{2+}]_i\), like these other cell types suggests a common mechanism for GRP-R-regulated exocytosis and demonstrates the utility of the BON/GRP-R cell line has a model for studying BBS-induced secretion.

Bombesin-induced secretion requires an increase in \([Ca^{2+}]_i\), that is initiated by release of Ca\(^{2+}\) from intercellular stores and sustained by an influx of Ca\(^{2+}\) across the plasma membrane. Calcium influx into excitable cells can occur through either voltage-gated Ca\(^{2+}\) channels or by various transport mechanisms. In non-excitable cells, influx of extracellular Ca\(^{2+}\) occurs by either capacitative Ca\(^{2+}\) uptake through non-voltage-gated, store-operated Ca\(^{2+}\) channels activated by depletion of intracellular Ca\(^{2+}\) pools (36, 37); through nonspecific receptor- or second messenger-operated cation channels (38); or by sodium-calcium exchange (39). In this study, current-voltage analysis using whole cell patch voltage clamp showed that there are voltage-gate calcium currents on BON/GRP-R cells. However, the cells were not depolarized by BBS and inhibitors of voltage-gated channels did not block BBS-stimulated increases in \([Ca^{2+}]_i\). It is unknown whether the predominant mechanism for calcium influx into BON/GRP-R cells is through calcium release-activated channels or nonspecific receptor or second messenger-operated cation channels. However, it is clear from the data presented that Ca\(^{2+}\) release from intracellular stores is necessary to stimulate Ca\(^{2+}\) influx across the plasma membrane in BON/GRP-R cells. Blocking PLC-\(\beta\) activation with U73122 or depleting intracellular Ca\(^{2+}\) stores by pretreating with thapsigargin completely blocked BBS-induced increases in \([Ca^{2+}]_i\).

An increase in \([Ca^{2+}]_i\) will not stimulate secretion in the absence of an activation of both PKC- and MEK-regulated pathways. Three groups of PKC isozymes have been identified, based on biochemical properties and sequence homologies. These include the conventional, novel, and atypical kinases. The conventional PKC group, which includes PKC-\(\alpha\), -\(\beta_1\), -\(\beta_2\), and -\(\gamma\), are activated by phorbol esters, DAG, and Ca\(^{2+}\). The novel PKC isozymes are activated by phorbol esters and DAG but not by Ca\(^{2+}\). The activity of the atypical kinases are independent of phorbol esters, DAG, and Ca\(^{2+}\). The observation that PMA is sufficient to stimulate CGA secretion in the absence of an increase in \([Ca^{2+}]_i\) indicates that the basic secretory

![Figure 8](image8.png)

**FIG. 8.** The MEK inhibitor, PD98059, blocks PMA-induced secretion (A) and ERK activation (B). A, BON/GRP-R cells were pretreated for 4 min with different concentrations of PD98059 (25, 50, and 100 \(\mu M\)) and then stimulated with PMA (1 \(\mu M\)). The effects of PD98059 on secretion are expressed as a percentage of plaque-forming cells in 1 h ± S.E. * \(p < 0.01\) versus BBS. B, a Western blot of BON/GRP-R cell proteins probed for activated ERK-1 and -2. DMSO, Me\(_2\)SO.

![Figure 9](image9.png)

**FIG. 9.** Inhibition of MEK blocks BBS-stimulated CGA secretion (A) and agonist-induced increases in \([Ca^{2+}]_i\). (B and C). A, BON/GRP-R19 cells were pretreated for 4 min with different concentrations of PD98059 (1, 5, and 10 \(\mu M\)) and then stimulated with BBS (10\(^{-7}\) M). The effects of PD98059 on secretion are expressed as a percentage of plaque-forming cells in 1 h ± S.E. from three separate experiments. B, calcium tracings are the average 340/380 ratios from 20–40 individual cells ± S.D. * \(p < 0.01\) versus BBS. C, cells were cotransfected with either a dominant negative mutant form of MEK-1 (dnMEK) or the empty expression vector (Vector) and an expression containing a cDNA for green fluorescence protein (GFP). After 24 h, the cells were loaded with fura-2 and cells expressing green fluorescence protein were identified. Then, the cells were stimulated with BBS (10\(^{-7}\) M) and changes in \([Ca^{2+}]_i\) were recorded. Like PD98059, dominant negative MEK inhibited BBS-stimulated increases in \([Ca^{2+}]_i\). Transfection of the empty vector and green fluorescence protein did not affect BBS-stimulated \([Ca^{2+}]_i\) responses. DMSO, Me\(_2\)SO.
machinery in BON/GRP-R cells can be regulated by PKC activation alone and suggests the possible involvement of both the conventional and novel PKC isoforms. However, the dependence of GRP-R-regulated secretion on a rise in [Ca^{2+}], suggests that conventional PKC isoforms are the most likely mediators of the BBS-induced response in these cells.

Recently, several downstream targets of PKC phosphorylation have been identified that may be important in the process of receptor-regulated exocytosis. Several neuron-specific proteins that function in vesicle docking and fusion at presynaptic membranes have been shown by in vitro kinase assays to be substrates for PKC phosphorylation, including the proteins, syntaxin-1 and -4 (40), which are components of the soluble N-ethylmaleimide-sensitive attachment factor receptor complex. Additionally, the myristoylated alanine-rich protein kinase C substrate (MARCKS) protein is a target of PKC. MARCKS proteins have been implicated in neurosecretion and have been shown to be phosphorylated by PKC in synaptosome preparations. Liu and co-workers (41) have demonstrated a close temporal association between arginine vasopressin-induced MARCKS phosphorylation and secretion of adenocorticotropin from ovine anterior pituitary cells, suggesting that MARCKS may be involved in the initial PKC-dependent intracellular events underlying exocytosis of this hormone. Non-neuronal cells express isoforms of these various proteins; however, it remains to be determined which, if any, play a role in GRP-R-regulated exocytosis in BON/GRP-R cells.

Previous studies have demonstrated a link between MAPK/ERK- and PKC-regulated pathways in receptor-mediated secretion from various cell types (14, 42–44). We have found that GRP-R-regulated secretion in BON/GRP-R cells also involves the activation of both PKC and MAPK/ERK pathways. Similar to PKC, MAPK/ERK have been shown to phosphorylate synaptic vesicle proteins involved in exocytosis such as synapsin I (45). In addition to their potential role in the regulation of proteins involved with vesicle fusion, we have found that MAPK pathways can regulate secretion by affecting agonist-induced changes in [Ca^{2+}].

Three related MAPK cascades have been identified in mammalian cells and are named according to the final enzyme in each pathway. They are the extracellular signal-regulated protein kinase (ERK) pathway, the c-Jun N-terminal kinase pathway, and the p38 MAP kinase pathway. Recently, Malgorzata and co-workers (46) have shown that a member of the p38 MAP kinase cascade can regulate [Ca^{2+}]. They reported that p38–2 is selectively activated by bradykinin in NG108–15 cells leading to a slow inhibition of an N-type Ca^{2+} current. We show that blocking MEK with either PD98059 or a dominant negative mutant of MEK-1 inhibits BBS-induced increases in [Ca^{2+}], suggesting that the role of active MEK is to maintain elevated [Ca^{2+}], during agonist stimulation. MEK-1 is an upstream dual-specificity kinase that phosphorylates both threonine and tyrosine residues on ERK-1 and -2. Expression of a constitutively active mutant form of MEK will activate p38 (47). However, it is unlikely that the effects of PD98059 or dominant negative MEK on BBS-stimulated increases in [Ca^{2+}] are due to regulation of p38 in BON/GRP-R cells. Inhibition of MEK blocks BBS-induced increases in [Ca^{2+}], in BON/GRP-R cells, whereas p38–2 activation by bradykinin in NG108–15 cells has the opposite effect on [Ca^{2+}], by inhibiting an inward N-type Ca^{2+} current. It is not known whether MEK regulation of [Ca^{2+}], in BON/GRP-R cells is mediated by either ERK-1 and/or -2 or whether an undefined pathway is involved. However, the data presented in this study, together with the previous work of Malgorzata and co-workers, suggest that multiple MAP kinase cascades are involved in receptor regulation of [Ca^{2+}], presenting the possibility that it is a general characteristic of this important family of kinases.

In conclusion, we have developed a model human cell line to investigate the molecular mechanisms of GRP-R-regulated exocytosis. Fig. 10 summarizes our proposed model for GRP-R-mediated secretion. We have found that peptide secretion involves activation of PLC leading to an increase in [Ca^{2+}], that is initiated by Ca^{2+} release from intracellular stores and sustained by influx across the plasma membrane. Agonist-induced increases in [Ca^{2+}], however, will not stimulate peptide release in the absence of PKC and MEK activation, suggesting that a role of Ca^{2+} is to activate conventional PKC isoforms, which, in turn, activate ERK through MEK. The role of MEK-mediated pathways in GRP-R-regulated exocytosis is to, in part, maintain elevated levels of [Ca^{2+}], and perhaps the activity of Ca^{2+}-sensitive PKC isoforms through a feedback loop mechanism. Future studies will attempt to identify the mechanism by which MAPK regulates agonist-induced increases in [Ca^{2+}].

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FIG. 10. Model of GRP-R-regulated secretion in BON/GRP-R cells.
