Bombesin-induced Gastrin Release from Canine G Cells Is Stimulated by Ca^{2+} But Not by Protein Kinase C, and Is Enhanced by Disruption of Rho/Cytoskeletal Pathways

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Abstract

Isolated canine G cells in primary culture have been used to study calcium, protein kinase C (PKC), and rho/cytoskeletal-dependent intracellular pathways involved in bombesin-stimulated gastrin release. A method to obtain highly purified G cells by culture (64% G cells) after flow cytometry on elutriated fractions of cells from digested canine gastric antral mucosa has been developed. Pretreatment of G cells with thapsigargin (10^{-8}–10^{-6} M) and release experiments in Ca^{2+}-containing or -depleted media showed that influx of Ca^{2+} into the cells and not acute release from intracellular stores plays an important role in bombesin-stimulated gastrin release. Inhibition of PKC by the specific inhibitor GF 109 203X did not affect bombesin-stimulated release. Rho, a small GTP-binding protein that regulates the actin cytoskeleton, is specifically antagonized by *Clostridium botulinum* C3 exoenzyme. C3 (10 μg/ml) enhanced basal and bombesin-stimulated gastrin release by 315 and 266%, respectively. The importance of the cytoskeleton for regulation of gastrin release was emphasized by a more pronounced release of gastrin when the organization of the actin cytoskeleton was disrupted by cytochalasin D (5×10^{-7} and 10^{-6} M). Wortmannin, a potent inhibitor of phosphoinositide-3-kinase, did not alter bombesin-stimulated gastrin release. Thus, it is concluded that bombesin-induced gastrin release from canine G cells is stimulated by Ca^{2+} but not by PKC, and is enhanced by disruption of rho/cytoskeletal pathways. (*J. Clin. Invest.* 1997. 100:1037–1046.) Key words: bombesin • calcium • protein kinase C • rho factor • cytoskeleton

Introduction

The regulation of gastrin secretion from antral gastrin cells (G cells)[1] by endocrine, exocrine, paracrine, and neural factors has been investigated extensively in vivo and in vitro. G cells are stimulated by gastrin-releasing peptide (GRP)/bombesin (1–3). Sympathomimetics, acetylcholine, prostaglandin, adenosine, and somatostatin are other substances known to act through specific receptors on canine G cells (1, 4–7). All these receptors belong to the superfamily of seven-transmembrane, G-protein–coupled receptors. Postreceptor activation of gastrin release has mainly been studied in canine and human G cells (1, 6, 8). The intracellular pathways known to be involved in gastrin release are to some extent cAMP dependent. Forskolin stimulates adenyl cyclase and increases gastrin release from isolated human and canine G cells (1, 3, 6). Gastrin release is enhanced when the concentration of cytosolic calcium is increased by calcium ionophores (1, 3, 6). Further, the phorbol ester β-12-*O*-tetradecanoylphorbol 13 acetate (TPA) (or β-PMA) activates protein kinase C (PKC) and is a potent stimulator of gastrin release (1, 6).

Difficulties in obtaining highly enriched and purified G cell preparations in primary culture have made it necessary to use other cell systems to further elucidate intracellular mechanisms involved in postreceptor activation. Cell lines with GRP receptors (Swiss 3T3 fibroblasts) have been used for such studies with special emphasis on cell growth and proliferation (9). One of the pathways found to be activated upon stimulation with bombesin/GRP in these cells involves the small GTP-binding protein rho, known to participate in the reorganization of the actin cytoskeleton (10–12). Interference with the rho-dependent pathway leads to a diminished tyrosine phosphorylation of paxillin and p^{125} focal adhesion kinase (FAK), two proteins closely connected to actin and focal adhesions (13, 14). *Clostridium botulinum* C3 exoenzyme ADP-ribosylates and inactivates rho A, B, and C proteins (12, 15). The ADP-ribosylation of rho inhibits several cell functions, including the formation of stress fibers and focal adhesions. How does a disorganization of the cytoskeleton affect hormone release? Increased hormone release after disaggregation of the actin cytoskeleton by the fungal alkaloid cytochalasin D has been found in isolated pancreatic islets, in adrenomedullary glands, and in steroid hormone–secreting cells (16–18) but not in G cells (19).

Phosphatidylinositol-3-kinase (PI-3-kinase) is an important enzyme for mitogenesis, cell transformation, and other cellular events involving protein tyrosine kinases. G-protein–coupled receptors, and protein kinase receptors, are involved in PI-3-kinase activation (20). A PI-3-kinase–dependent signal transduction pathway in the action of a protein kinase receptor (PDGF-receptor) which leads to phosphorylation of p^{125} FAK and paxillin has been identified in Swiss 3T3 fibroblasts (21). The importance of PI-3-kinase in gastrin secretion is not known.

In this study, we used isolated canine G cells in primary culture for studies of PKC and Ca^{2+} on gastrin release, as well as interactions between the rho-mediated pathway and the cy-

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1. *Abbreviations used in this paper:* β-TPA, 12-*O*-tetradecanoylphorbol 13 acetate; $[Ca^{2+}]i$, intracellular calcium concentration; EBSS, Earle's balanced salt solution; FAK, focal adhesion kinase; G cells, gastrin cells; GRP, gastrin releasing peptide; 5 HT, 5-hydroxytryptophan; KNRK, Kirsten sarcoma virus–transformed normal rat kidney; PI-3-kinase, phosphatidylinositol-3-kinase; PKC, protein kinase C.

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toskeleton. A new method was developed to obtain highly purified G cells by flow cytometry in order to study regulation of intracellular calcium concentrations ($[Ca^{2+}]i$) in single G cells. We found that the gastrin release in response to low doses of bombesin/GRP appears to involve intracellular Ca^{2+}, but that PKC is not involved. Interference with the rho pathway and the cytoskeleton further increased gastrin release from isolated G cells.

Methods

Materials. Bombesin and [lys^3]-bombesin 14 were obtained from Bachem California (Torrance, CA); carbachol, substance P, compound 48/80, β-PMA, EDTA, EGTA, barbital buffer, and rhodamine-phalloidin were all obtained from Sigma Chemical Co. (St. Louis, MO); secretin, cytochalasin D, GF 109 203X, and Hepes were from Calbiochem-Novabiochem Corp. (San Diego, CA); collagenase A was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); Earle's balanced salt solution (EBSS), DMEM F-12, and HBSS were from GIBCO BRL (Gaithersburg, MD); hydrocortisone, insulin, amikacin, gentamicin, DNase, and DTT were purchased from Research Organics, Inc. (Cleveland, OH), and glutamine from Gemini Bio-Products, Inc. (Calabasas, CA). Antibodies S6 (somatostatin) and 1802 (gastrin) were provided by CURE: Digestive Diseases Research Center Antibody Core. Rabbit antiserotonin antibody was purchased from Immunotech, Inc. (Westbrook, ME). Fura 2-AM was from Molecular Probes, Inc. (Eugene, OR); ^{125}I-labeled GRP was obtained from Amersham, Corp. (Arlington Heights, IL), Costar 24-well plates from Costar Corp. (Cambridge, MA), and Matrigel from Collaborative Biomedical Products (Bedford, MA).

The C3 exoenzyme expression vector pGEX2T-C3 DNA was a kind gift from S.T. Dillon (Tufts University). C3 exoenzyme was expressed in *Escherichia coli* and purified by affinity chromatography followed by ion-exchange chromatography according to Dillon and Feig (22).

Cell separation and culture. G cells for release studies were prepared from canine antrum as previously described (1). Antral mucosa was separated from the submucosa by blunt dissection, and the cells were dispersed by sequential incubation with collagenase A (1 mg/ml) and EDTA (2 mM). For cells to be used for flow cytometry, a modification of the digestion procedure was made, in that the digestion was stopped after a second collagenase incubation for 45 min. EDTA (5 mM) was added for the last 10 min of this 45-min period. Dispersed cells were washed, collected by centrifugation, and filtered through a coarse (192 μm) and fine (64 μm) mesh. The cells were separated by velocity sedimentation using an elutriator (rotor JE-5.0 in a J-6B centrifuge; Beckman Instruments, Inc., Fullerton, CA). At a rotor speed of 2,000 rpm and at a flow rate of 75 ml/min, the cells were loaded into the chamber. While rotor speed was maintained at 2,000 rpm, small cells, debris, and bacteria were washed out at a flow rate of 87.5 ml/min, 500 ml of fractions V and VI at 142.5 ml/min, and of fractions VII and VIII at a flow rate of 192.5 ml/min. These fractions contained maximum gastrin immunoreactivity, with a relatively higher percentage of G cells in fractions V and VI. Elutriated cells were either incubated with fluorescent dye (fractions V + VI) or cultured (fractions V–VIII) for 40–48 h. Cells for culture were centrifuged and resuspended in DMEM-F12 supplemented with 2% FCS, 100 μg/ml amikacin, 8 μg/ml insulin, 2 nM glutamine, and 0.1 μg/ml hydrocortisone. 10^6 cells per well were plated on Matrigel-coated plates and incubated in humidified atmosphere of 5% CO_2 and 95% air at 37°C until release studies.

Flow cytometry. The fluorescent ligand used for cell sorting was produced by incubating [lys^3]bombesin 14 with 3 (FluoroLink Cy3; Amersham Life Science, Arlington Heights, IL) for 30 min at pH 9.3 in darkness at room temperature. Labeled bombesin was separated from excess unconjugated dye by HPLC. Cy3-[lys^3]bombesin was made up at regular intervals every 4–6 wk, aliquoted, and stored at −20°C until use. The concentration of cy3-[lys^3]bombesin used was based on determinations of binding affinity for each batch to Kirsten sarcoma virus–transformed normal rat kidney (KNRK) cells transfected with rat (r)GRP receptor and plated on poly-L-lysine–coated 24-well plates. After 72 h culture (10^6 cells/well), the cells were washed and incubated in binding media (DMEM/Waymouth's) for 30 min at 4°C. ^{125}I-labeled GRP, unlabeled bombesin (10^{-12}–10^{-6} M), or cy3-[lys^3]bombesin (1:200,000–1:200) were added for 1 h, after which cells were washed with PBS, lysing buffer was added, and lysate was carefully removed for counting.

Elutriated cells were adjusted to 2–5 × 10^6 cells/ml culture media without phenol red, and incubated with cy3-[lys^3]bombesin at 4°C for 30 min. After incubation with dye, the cells were washed, resuspended in culture media, and sorted by size and fluorescence using a FACStar$^{PLUS®}$ (Becton Dickinson, Mountain View, CA), equipped with dual argon and helium–neon lasers. Cells (0.3–1.6 × 10^6 cells per sort) with high fluorescence intensity were used for immunohistochemistry or calcium imaging studies. The viability of cells after flow cytometry was > 90% as assessed by trypan blue exclusion.

Release studies. Release studies were made on cells from elutriated fractions V–VIII cultured for 40–48 h until they were ~ 70–90% confluent, and the G cells were enriched to 8–12%. Before release studies, the cells were gently washed with release medium containing EBSS with 1.8 mM calcium, 0.8 mM magnesium, 0.1 mg/ml gelatin, 10 mM Hepes, 0.42 M sodium bicarbonate, and pH was adjusted to 7.4. In experiments with calcium-free media, Ca^{2+}/Mg^{2+}-free EBSS was used and supplemented with 1 mM EGTA and Mg^{2+}. 0.5 ml media and 12.5 μl test substances were added to each well. In experiments where C3 exoenzyme was used, the cells were incubated with C3 (0.25–10 μg/ml) for 48 h. Before each release experiment, somatostatin antibody (CURE S6, 10^{-8} M) was added to each well to neutralize endogenous somatostatin produced by any contaminating D cells. Cytochalasin D, GF 109 203X, and thapsigargin were added 1 h before bombesin (10^{-10} M) or PMA stimulation (2 × 10^{-7} M). Wortmannin was added 5 min before bombesin stimulation. After incubation for 2 h at 37°C with bombesin or PMA, supernatants were removed and centrifuged. The viability of the cells was examined by trypan blue exclusion after release experiments. More than 90% of the cells were viable both in control experiments and after incubation with C3 exoenzyme and cytochalasin D. Adherent cells in control experiments were treated with collagenase (1 mg/ml) in medium for 10 min after the supernatant had been collected, scraped off each well, and boiled to release gastrin from the cells. All gastrin samples were stored at −20°C until assay. In separate release experiments (n = 3), bombesin-stimulated gastrin release from elutriated and cultured cells from fractions V + VI and from fractions V–VIII were compared, and no significant differences in gastrin release were noted.

Radioimmunoassay. Gastrin-like immunoreactivity in medium and cell extracts was measured by RIA using antibody 1611 as previously described (23). The tracer was prepared by iodination of gastrin-17 by the chloramine-T method by the Protein Chemistry Core Laboratory of CURE/UCLA. The assay recognizes all carboxy-terminal–amidated fragments of gastrin longer than four residues.

Intracellular calcium imaging. Cells from the fractions with high and low fluorescence intensity were cultured for 40–48 h on separate poly-L-lysine–coated glass coverslips (25 mm). The cells were preincubated for 30 min with 5 μM calcium indicator Fura 2. After washing the excess dye twice, the coverslip was mounted in a perfusion chamber with 1 ml BSS containing 20 mM Hepes, pH 7.4. Different concentrations of peptides in buffer solution were applied to the chamber by perfusion (4 ml/min). Experiments were performed at 28–34°C. An inverted microscope (100TV; Carl Zeiss, Inc., Thornwood, NY), with a ×40 objective was used for imaging, and calcium concentrations were measured with a videomicroscopy digital imaging system (Attofluor; Atto Instruments, Inc., Rockville, MD). The Ca^{2+}-dependent fluorescence signals were obtained by exciting the Fura 2 at 340 and 380 nm. The 340/380 ratio readings were taken from 2–17 individual

cells per coverslip, and the corresponding calcium concentrations were calculated by the computer. The indicator was calibrated in vitro, and accuracy controlled with standard solutions of known Ca^{2+} concentrations (Molecular Probes, Inc.). Unloaded cells possessed virtually no autofluorescence at wavelengths used.

Immunohistochemistry. Freshly isolated cells from each fraction of elutriated cells ($0.5–1.5 \times 10^5$ cells/slide) after flow cytometry were prepared using cytocentrifuge (Shandon Instruments, Pittsburgh, PA). In other studies, cells were plated directly on Matrigel- or poly-L-lysine–coated glass slides after elutriation and flow cytometry, and cultured for 40–48 h under sterile conditions. Cells were fixed in 4% paraformaldehyde (with picric acid) for 20 min at room temperature. After washing with phosphate buffer and preincubation with 10% goat–rabbit serum in 1% Triton X-100, slides were double-stained by indirect fluorescence method with a rabbit polyclonal (CURE 1802; 1:1,000) antibody against gastrin, and with a mouse mAb against somatostatin (CURE S6; 1:1,000). In other experiments, a mouse mAb (CURE E_5; 1:800) against gastrin was used. 5-Hydroxytryptophan (5 HT) cells were stained with a rabbit polyclonal antibody to serotonin (1:100). Incubation with the primary antibody was overnight at 4°C. Immunoreactivity was visualized after washing and a 2-h incubation at 37°C with rhodamine (1:100) and FITC (1:100) goat anti–mouse or anti–rabbit antiserum. The specificity of staining was confirmed by preincubating antisera with the appropriate hapten at 50 μg/ml. In some experiments, the slides were stained with hematoxylin-eosin after immunostaining in order to evaluate the nonendocrine cell population. Cytocentrifuged cells with visible nucleus in at least five randomly selected visual fields with 100–150 cells in each were counted at a magnification of 200 (Labophot-2; Nikon, Inc., Melville, NY). Since the number of cells plated and cultured after flow cytometry was generally much lower, at least 10 visual fields were evaluated in these experiments. The number of G, D, and 5 HT positive cells were counted and expressed as percentage of the total number of cells.

Fluorescence microscopy of actin microfilaments in G cells enriched by elutriation and flow cytometry was performed after 40–48 h culture in regular culture media. All cells were preincubated with somatostatin antibody according to release experiments, and incubated with C3 exoenzyme for 48 h or incubated for 3 h with cytochalasin D. The cells were unstimulated or stimulated with bombesin (10^{-10} M) during the last 2 h of each incubation. Untreated cells and cells stimulated with bombesin served as controls. After being fixed and washed three times in PBS, the cells were incubated with the primary mouse gastrin mAb (CURE E_5) overnight at 4°C. Visualization of gastrin immunoreactivity was obtained with FITC goat anti–mouse, and for actin microfilament staining, the slides were incubated with rhodamine phalloidin (1:35) for 20 min at 4°C.

Statistics. The results are expressed as mean±SE. The mean of triplicate samples from each release experiment was used. Because of the large number of separate cell preparations, the release of gastrin from cell content was usually expressed as a percentage of basal, PMA- or bombesin-stimulated gastrin release. Basal and bombesin-stimulated gastrin release in 34 separate cell preparations were 1.22±0.87 and 5.41±4.06% of total gastrin cell content (mean±SE), respectively. To consider the G cell preparation as qualitatively acceptable, the magnitude of bombesin-induced gastrin release per 24-well plate had to exceed basal gastrin release by at least 200% in experiments with normal Ca^{2+}-containing release media. Differences between groups of experiments were evaluated by Wilcoxon's signed rank test, and $P < 0.05$ was considered to be statistically significant.

Results

Enrichment of G cells by flow cytometry. Elutriation of dispersed antral mucosa cells and culture of fractions V–VIII for 40–48 h were shown to increase the percentage of G cells from ~ 2 to 12% (1). These figures were confirmed in our studies. Since the time for sorting G cells by flow cytometry is affected by the initial proportion of desired cells, the fractions with the relatively highest numbers of G cells were selected for flow cytometry, fractions V–VI (~ 1.5% G cells).

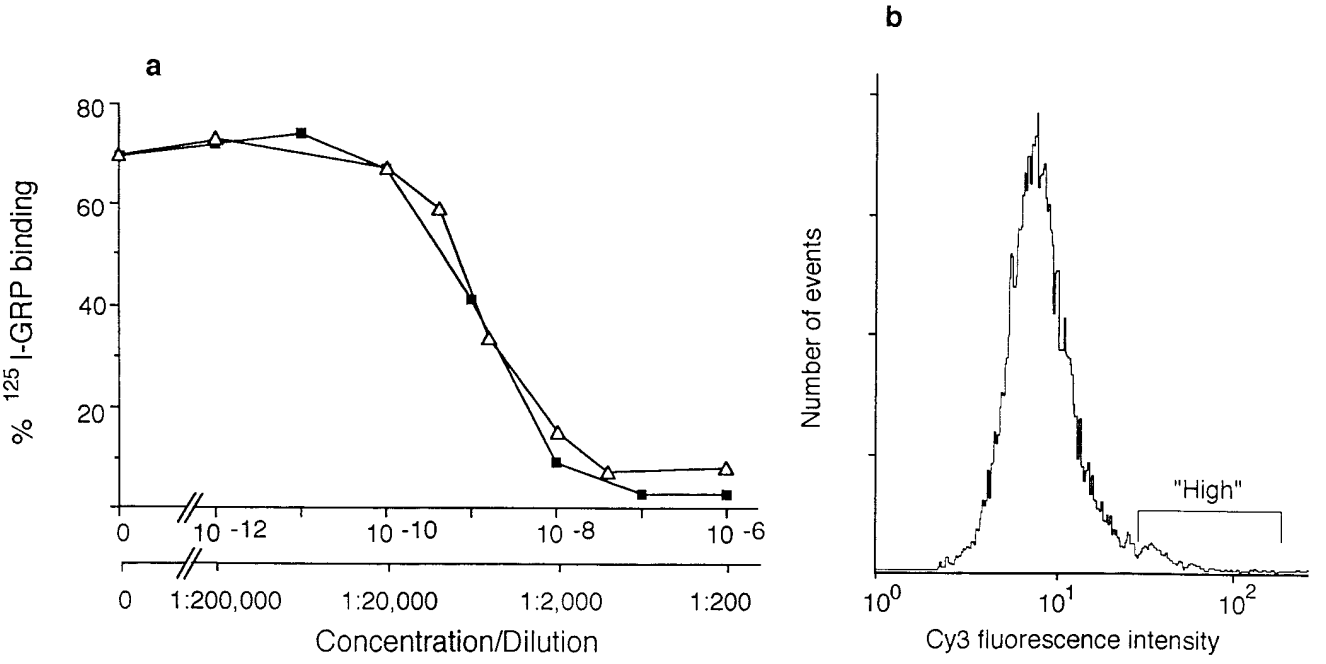

Figure 1. (*a*) Displacement of ^{125}I-labeled GRP by cy3-[lys3]bombesin (*open triangles*) and by bombesin (*filled squares*) in KNRK cells transfected with rGRP receptor. ^{125}I-labeled GRP binding expressed as percentage of control experiments without cy3-[lys3]bombesin or bombesin and with an unspecific binding of 35% subtracted. (*b*) Distribution of fluorescence intensity in elutriated cells from fractions V and VI incubated with cy3-[lys3]bombesin during flow cytometry. The cells with highest fluorescence intensity (~ 5% of the cell population) are sorted in a separate fraction (*High*) with an enrichment of G cells (see Table I), while the rest of the sample constitute the "low" fraction, with a low yield of G cells.

Table I. Cell Enrichment by Flow Cytometry

	Percentage of total cells (mean±SEM)	
	G cells (*n*)	D cells (*n*)
After flow cytometry		
High	37.0±3.0 (6)	0.8±0.1 (6)
Low	2.5±1.4 (6)	1.4±0.4 (6)
After flow cytometry and 40–48 h of culture		
High	64.0±2.3 (9)	0.5±0.5 (6)
Low	2.1±1.1 (2)	2.9±2.6 (2)

Percentage of G and D cells in total number of cells as determined by immunohistochemistry. *High* sorted cell fractions with highest intensity of cy3 fluorescence (~ 2–5% of elutriated cell population). *Low,* cell fractions with low intensity of fluorescence.

Cy3-[lys^3]bombesin at concentrations between 1:2,500 and 1:3,200 (~ 1–10 nM) was found to be optimal for flow cytometry of G cells, based on displacement of ^{125}I-labeled GRP in KNRK cells transfected with rGRP receptor (Fig. 1 *a*). The fluorescence of the dye in elutriated and cy3-[lys^3]bombesin–incubated cells revealed that ~ 2–5% of the sample consisted of cells with a high intensity of fluorescence (Fig. 1 *b*). Flow cytometry with gating of this cell population into a "bright" fraction (fraction with high fluorescence) increased the number of G cells by a factor of 18–37 (37% G cells). After culture for 40–48 h, there was an additional approximately twofold increase in the relative proportion of G cells (to 64%) with very little D cell contamination (0.5%) (Table I, Fig. 2, *A–C*). The remaining cells were of nonendocrine origin and likely to be mucous cells. In cultures after flow cytometry, no 5 HT–like immunoreactive cells could be found ($n = 6$).

Intracellular calcium imaging of G cells. The G cells were identified by increase of $[Ca^{2+}]$i to bombesin and carbachol (2×10^{-5}–10^{-3} M) but without response to cholecystokinin (CCK) 8 (10^{-7} M) (Fig. 3 *a*). In 76 different experiments, 74±4% of cells responded with an increase in intracellular calcium ($\Delta[Ca^{2+}]$i) to bombesin, and were identified as G cells in imaging studies. The $\Delta[Ca^{2+}]$i values for different concentrations of bombesin are shown in Fig. 3 *b*. The EC_{50} for bombesin-induced Ca^{2+} mobilization was 0.26 nM. After perfusion with the buffer for at least 30 min, a second stimulation with bombesin (10^{-9} M) elicited an increase of $[Ca^{2+}]$i of the same magnitude as the first. Substance P (10^{-6} M), secretin (10^{-6} M), compound 48/80 (10^{-6} M), and G 17 (10^{-7} M) did not affect the $[Ca^{2+}]$i in G cells.

Less than 1% of cells responded with an increase of $[Ca^{2+}]$i to CCK 8 and gastrin-17 stimulation. Bombesin and carbachol did not affect $[Ca^{2+}]$i in these cells. This pattern is characteristic of canine somatostatin cells (24).

Effects of β-PMA and GF 109 203X on bombesin-stimulated gastrin release. To validate the role of PKC in bombesin-stimulated gastrin release, it was first established that a known PKC stimulator, the phorbol ester β-PMA, released gastrin in a dose-dependent (2×10^{-8}–2×10^{-6}) manner. 2×10^{-7} M β-PMA increased gastrin release from 1.1±0.8 to 14.9±6.7% of cell content (mean±SE, $P < 0.05$). The specific PKC inhibitor GF 109 203X (10^{-7}–5×10^{-6} M) inhibited the β-PMA–induced gastrin release dose-dependently, and at the highest dose used (5×10^{-6} M), by ~ 50% ($P < 0.01$) (Fig. 4 *a*).

Having established GF 109 203X inhibition of PKC, the involvement of PKC in bombesin-stimulated gastrin release was studied. GF 109 203X (10^{-7}–5×10^{-6} M) did not decrease bombesin-stimulated gastrin release (Fig. 4 *b*). Preincubation with staurosporine (10^{-9}–10^{-6} M), a more nonspecific inhibitor of protein kinases, also had no effect on basal and bombesin-induced gastrin release (data not shown). Therefore, a role for PKC in bombesin-induced release could not be supported.

Role of Ca^{2+} in bombesin-stimulated release. In single cell studies, bombesin induced a rapid spike followed by a prolonged plateau-phase increase of $[Ca^{2+}]$i. Basal $[Ca^{2+}]$i was 99±6 nM ($n = 241$). The roles of the plateau and spike were investigated separately. To deplete intracellular calcium stores, the tumor promoter thapsigargin (10^{-8}–10^{-6} M) was used. Addition of thapsigargin to intact cells is known to mobilize calcium acutely from intracellular stores and to inhibit calcium accumulation in the endoplasmic reticulum. Preincubation with thapsigargin (10^{-6} M) abolished the initial spike but did not affect the plateau phase or gastrin release significantly (38% increase of bombesin-stimulated release with 10^{-7} M).

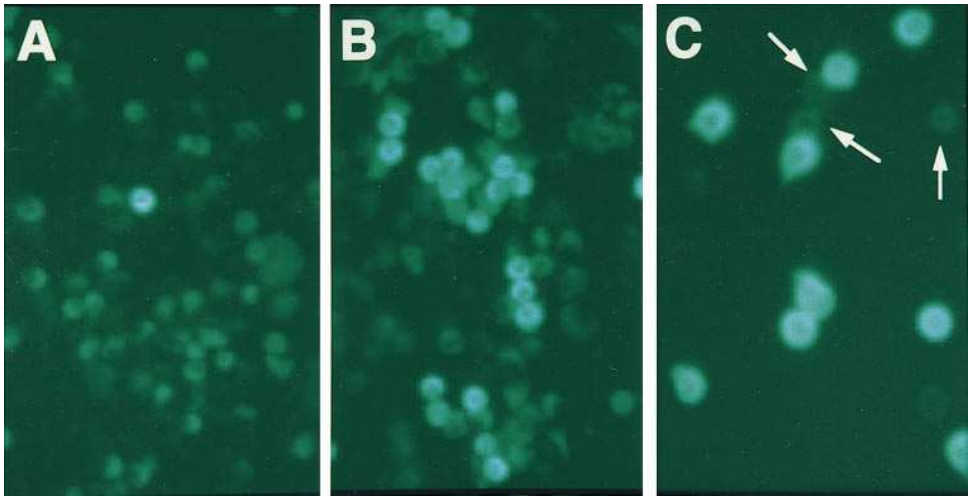

Figure 2. Gastrin cells (*A*) in fractions V + VI after elutriation (×200), (*B*) after flow cytometry (×200), and (*C*) after flow cytometry and 46 h of culture (×500). Arrows indicate the small number of nonendocrine cells in the sample. G cells were stained by indirect immunofluorescence using a mouse mAb against gastrin (CURE E_5; 1:800) and FITC (1:100) goat anti–mouse antiserum.

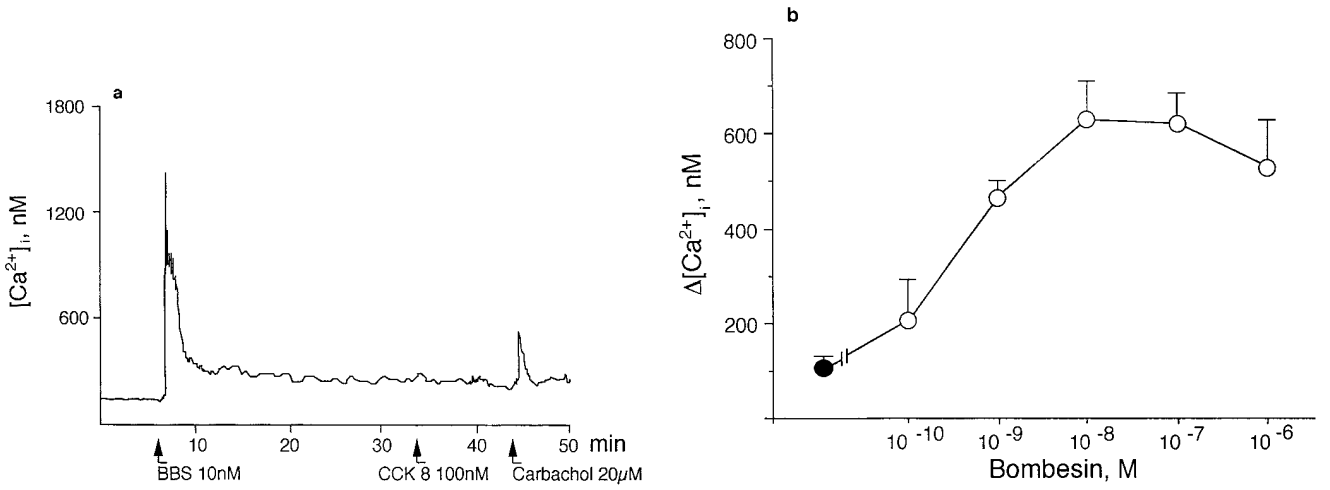

Figure 3. $[Ca^{2+}]i$ in a G cell loaded with the calcium indicator, Fura 2 AM, and monitored using a video imaging system as described in Methods. The $[Ca^{2+}]i$ response to bombesin (*BBS*), and to carbachol, and nonresponsiveness to CCK 8 as shown in *a* is characteristic of antral canine G cells. (*b*) The increases in intracellular calcium, $\Delta[Ca^{2+}]i$ (*open circles*, mean±SE, $n = 3$–27), expressed as the basal $[Ca^{2+}]i$ (*closed circle*, $n = 61$) subtracted from the peak concentration, for different concentrations of bombesin. The EC_{50} for bombesin-induced Ca^{2+}-mobilization was 0.26 nM.

When studies were carried out in Ca-free release media supplemented with Mg^{2+} and EGTA, the initial Ca^{2+}-spike in response to bombesin was maintained, but the plateau increase was abolished. Under these conditions, bombesin-stimulated gastrin release was significantly reduced, by 65% ($P < 0.05$, Fig. 5). These studies indicate that an increase in plateau calcium can act independently of the calcium spike on gastrin release. Calcium influx, reflected as the prolonged plateau phase, plays a positive role in bombesin-stimulated gastrin release. Acute release from intracellular stores of calcium seems to be of minor importance.

Effects of C3 exoenzyme on gastrin release. Because GRP/bombesin is known to activate a pathway involving the small GTP-binding protein rho that leads to tyrosine phosphorylation of cytoskeletal proteins during the induction of intracellular stress fibers in Swiss 3T3 cells, we tested the hypothesis that gastrin might be released through a rho-dependent pathway independent of PKC and calcium. The *Cl. botulinum* exoenzyme C3, a specific antagonist of rho, was used to investigate this hypothesis. After 24–48 h of incubation with C3, bombesin-stimulated gastrin release was not inhibited but rather was enhanced by 100–300%. At the highest dose tested (10 μg/ml), basal release was increased by 315% and bombesin-stimulated gastrin release was increased by 266% (Fig. 6). Therefore, it can be concluded that a rho-dependent pathway antagonized bombesin-stimulated gastrin release.

Effects of cytochalasin D disruption of actin cytoskeleton. The results with C3 exoenzyme suggested that the cytoskeleton is of significance for the regulation of gastrin release. To explore this hypothesis further, cells were treated with cytochalasin D, an agent that causes more obvious disruption of the actin cytoskeleton than C3 exoenzyme.

Cytochalasin D at concentrations of 5×10^{-7} M and 10^{-6} M enhanced basal (279±87 and 396±121%) and bombesin-stim-

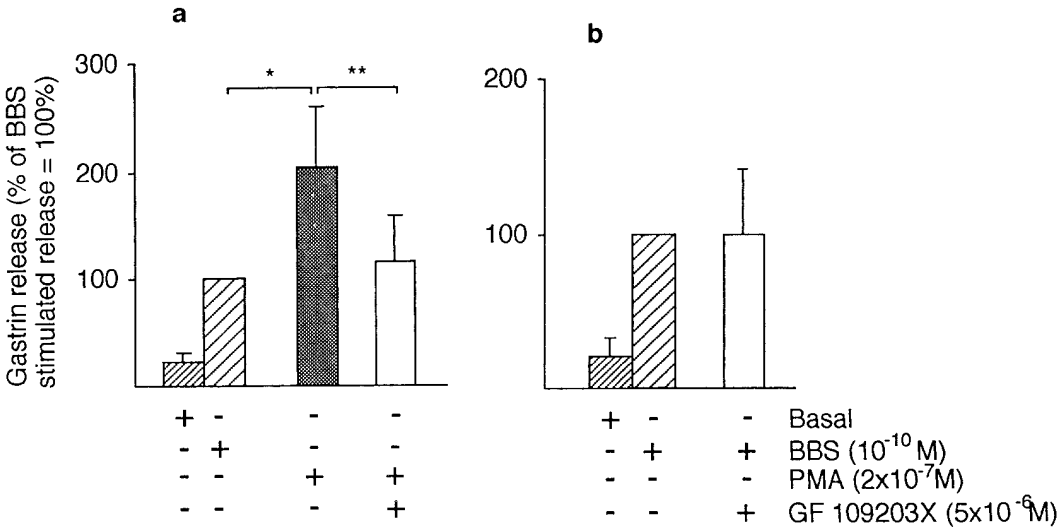

Figure 4. Gastrin release from G cells is stimulated by the phorbol ester β-PMA (*$P < 0.05$ vs. bombesin-stimulated release, $n = 12$), and the PMA-induced release is inhibited by the specific inhibitor GF 109 203X (**$P < 0.01$ vs. *PMA*-stimulated release, $n = 11$) (*a*). The bombesin (*BBS*)-stimulated gastrin release is not inhibited by GF 109 203X (*b*) ($n = 6$).

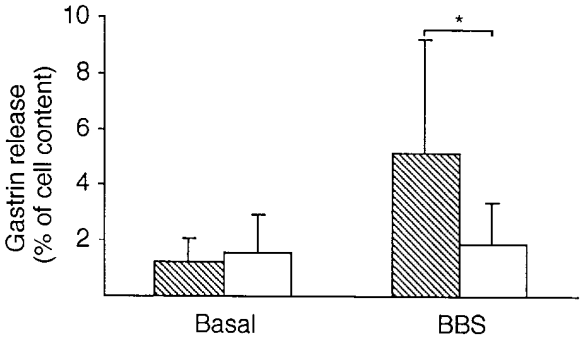

Figure 5. Basal and bombesin (*BBS*)-stimulated gastrin release in calcium-containing media (*striped bars*, $n = 71$) and in experiments without Ca^{2+} in the release media (*open bars*, $n = 12$). *Significantly ($P < 0.05$) higher stimulated gastrin release in experiments carried out in Ca^{2+}-containing media compared with Ca-free media.

ulated (259±102 and 427±148%) (Fig. 7) gastrin release significantly, without any effect on the viability of the cells as examined by trypan blue exclusion. Since some substances known to affect the cytoskeleton (like *Clostridium difficile* polypeptide toxins A and B) in other cell systems have been shown to mobilize intracellular Ca^{2+} and cause elevation of $[Ca^{2+}]i$, we tested whether cytochalasin D and C3 exoenzyme induced $[Ca^{2+}]i$ changes per se in G cells. Cytochalasin D (10^{-6} M) did not affect the resting $[Ca^{2+}]i$ or the bombesin (10^{-9} M)–stimulated $[Ca^{2+}]i$ response acutely in G cells (Fig. 8). Nor did 3 h preincubation with cytochalasin D alter the $[Ca^{2+}]i$ response as compared to untreated controls. The intracellular Ca^{2+} level and response to bombesin was not affected by C3 incubation (data not shown).

Interactions of PKC on cytochalasin D–induced gastrin release. To test if the enhancement by cytochalasin D of stimulated gastrin release was a more general phenomenon, the effect of cytochalasin D pretreatment on PKC-induced gastrin release was tested. Cytochalasin D significantly enhanced the gastrin response to β-PMA by approximately threefold. This increase was reduced by 47% by preincubation with GF 109 203X (5 μM). Thus, disruption of cytoskeleton enhanced both

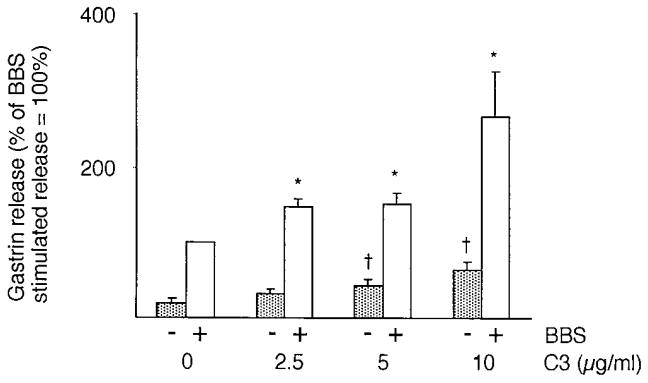

Figure 6. Incubation of G cells in primary culture ($n = 6$–9) with *Cl. botulinum* C3 exoenzyme for 48 h increased basal and bombesin (*BBS*)-stimulated gastrin release. †*Significant ($P < 0.05$) increase of basal and bombesin-stimulated release, respectively, by indicated concentrations of C3.

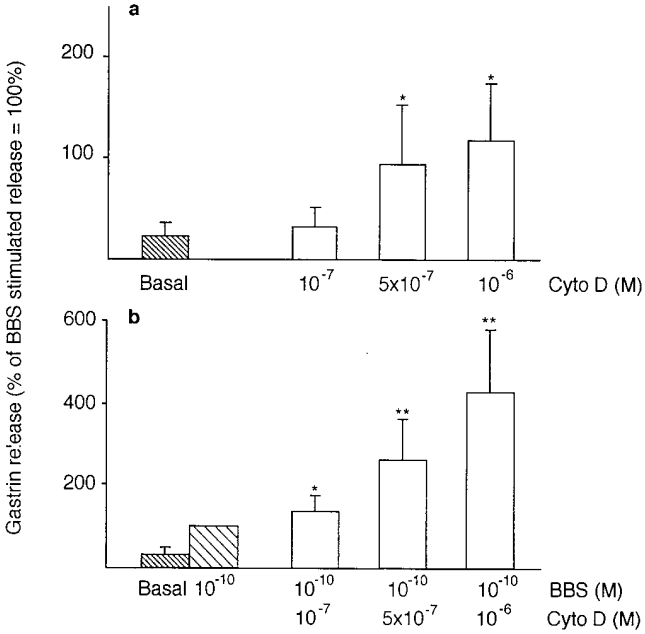

Figure 7. Effects of cytochalasin D (*Cyto D*) on (*a*) basal and (*b*) bombesin (*BBS*)-stimulated gastrin release from isolated canine G cells in primary culture ($n = 6$–13). *$P < 0.05$ and **$P < 0.01$, statistically significant increases in gastrin release in the presence of cytochalasin D compared to control basal and bombesin-stimulated experiments without cytochalasin D incubation ($n = 7$–13).

PKC-dependent and PKC-independent pathways involved in gastrin release (Fig. 9).

Effects of cytochalasin D and C3 exoenzyme on actin microfilaments. Cytochalasin D had profound effects on the actin filament structure in G cells. Cells incubated with cytochalasin D (1 μM) with or without bombesin showed an aggregation and clumping of actin (Fig. 10, *a–d*). Preincubation with C3 exoenzyme for 46 h at 10 μg/ml did not show any obvious disaggregation or destruction of the actin filaments in G cells double-stained for F-actin and gastrin and visualized by light microscopy.

Effect of wortmannin on gastrin release. Wortmannin (10^{-9}–10^{-7} M), a potent inhibitor of PI-3-kinase, did not alter bombesin-stimulated gastrin release (Fig. 11).

Discussion

We have described a method for enrichment and high purification of G cells from canine antral mucosa that allows further characterization of the intracellular pathways involved in release of gastrin from cells in primary culture. By using flow cytometry for fluorescence-activated cell sorting, we could increase the yield of G cells from 1 to 2% after elutriation to ~ 37%. After culture for 40–48 h there was an additional approximately twofold enrichment of G cells, to 64% (range 53–71%), as determined by immunocytochemistry and cell counting of randomly selected visual fields. It is well known that culture, after the initial digestion and isolation steps, gives a relative enrichment of G cells, since the culture conditions are optimized for endocrine cells and not for the nonendocrine cell population (1). Up to 40% G cells has been obtained after di-

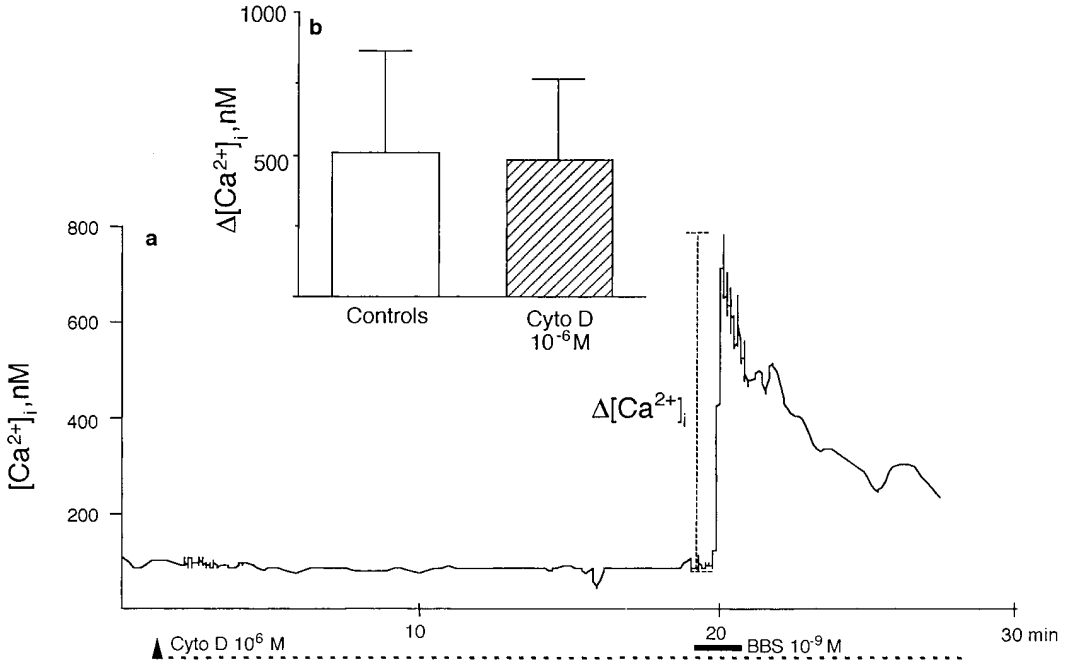

Figure 8. (*a*) $[Ca^{2+}]i$ in a G cell loaded with Fura 2-AM and monitored by a video imaging system before and after incubation with cytochalasin D (*Cyto D*) for 20 min, and after stimulation with bombesin (*BBS*). (*b*) No significant difference could been seen in $\Delta[Ca^{2+}]i$ (nM), as determined in *a*, after bombesin stimulation of G cells (n = 8–10) preincubated with cytochalasin D or with the vehicle (*Controls*) for 3 h.

gestion, elutriation, and culture of human antral mucosa cells (8, 25). The highest purity of G cells in animal preparations reported to date is 23% (2). About 75% of the cells in our study were identified as G cells by their Ca^{2+} responses to bombesin and carbachol and the lack of Ca^{2+} responses to CCK 8 and/or gastrin-17. This additional increase by 10% of the relative number of G cells seen during calcium imaging is most likely explained by a selection of visual fields containing a high number of cells with G cell appearance and a seemingly low number of nonendocrine cells. Up to 17 individual G cells per visual field could be studied simultaneously. After some experiments, the cells characterized as G cells during calcium imaging were fixed on the coverslip and verified as G cells by immunohistochemistry. Fluorescence-activated cell sorting has been used previously to sort functional endocrine cells with high purity from anterior pituitary and pancreas (26–28). Recently, the specificity of cy3-GRP binding to KNRK Flag GRP receptor cells and the homogeneity of the cell population were examined by using flow cytometry (29). In this study, we used cy3-[lys^3]bombesin for fluorescence-activated cell sorting to obtain functionally intact G cells from the gastric antral mucosa. The sorting method greatly improved the enrichment of canine G cells, and facilitated studies of cellular events in single G cells in primary culture.

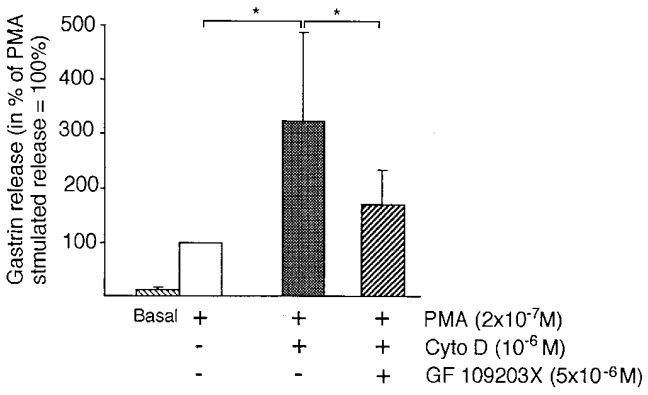

Figure 9. Gastrin release induced by the phorbol ester β-PMA was further enhanced by preincubation with cytochalasin D (*Cyto D*) for 1 h, and inhibited by 47% by the PKC inhibitor GF 109 203X. $^*P < 0.05$.

Previous reports that activation of PKC by the phorbol ester β-PMA stimulates gastrin release from canine and human G cells in primary culture (1, 6) were confirmed, and dose–response experiments showed that 2×10^{-7} M β-PMA elicited maximal gastrin release. The bisindolylmaleimide GF 109 203X, has been shown to be a selective and potent PKC inhibitor in Swiss 3T3 cells (30–32). When the G cells in our preparations were preincubated with GF 109 203X, a marked and dose-dependent decrease of β-PMA–induced gastrin release was seen with a maximal effect by 3.5–5 μM GF 109 203X. This established that the phorbol ester β-PMA stimulates gastrin release by a PKC-dependent pathway. However, since GF 109 203X did not abolish the gastrin-releasing effect of β-PMA completely, a PKC-independent pathway is also involved.

The question arises of whether bombesin stimulates gastrin release from G cells by activation of PKC. There is evidence that bombesin at higher concentrations than 10^{-9} M activates PKC in Swiss 3T3 cells (30, 31). However, bombesin increases gastrin release at much lower concentrations in human (6) and in canine (1) G cell preparations. In our laboratory, 10^{-11}–10^{-10} M bombesin elicits a maximal gastrin-releasing effect from isolated canine G cells in culture (reference 1, and our unpublished observations). Preincubation with GF 109 203X did not have any effect on the amount of gastrin released by bombesin stimulation at low concentration (10^{-10} M), indicating that stimulation with bombesin at physiological concentration does not activate PKC to release gastrin. This is also consistent with the finding that PKC does not seem to be involved

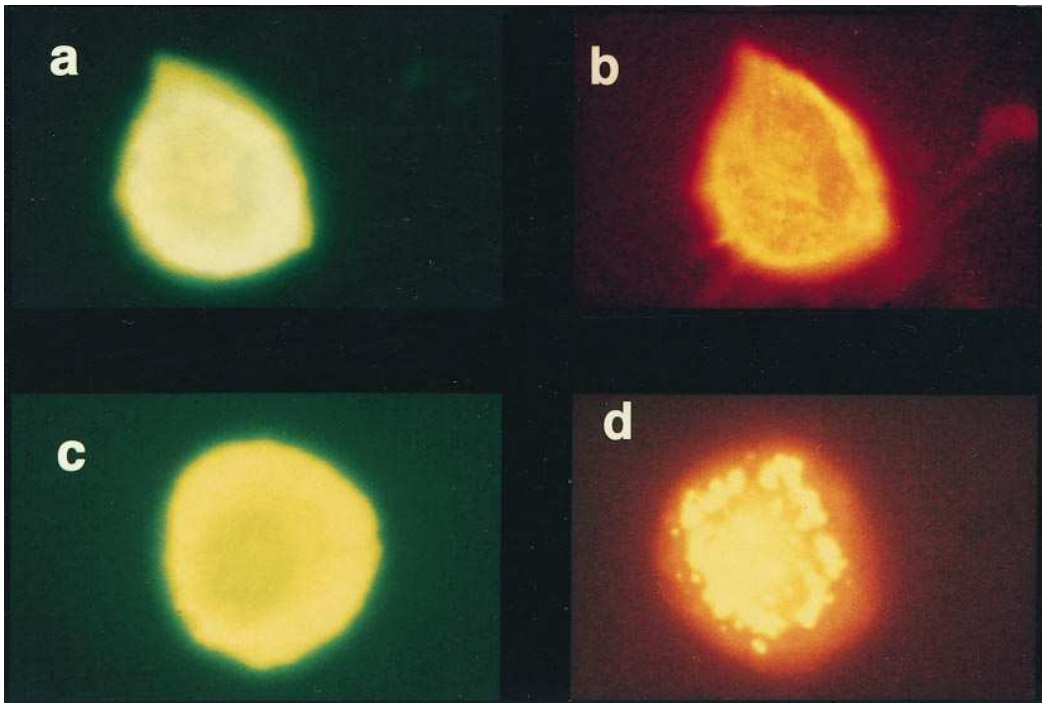

Figure 10. Immunohistochemistry of G cells enriched by flow cytometry in a control experiment (*a* and *b*), and after incubation with cytochalasin D for 3 h (*c* and *d*). All cells were stimulated for 2 h with bombesin (10 nM). G cells were stained with a mouse mAb against gastrin (CURE E_5; 1:800) and FITC (1:25) goat anti–mouse antiserum (*a* and *c*), and for F-actin with rhodamin phalloidin (*b* and *d*). The clumping of the actin and disruption of the cytoskeleton are obvious in the cytochalasin D–incubated G cell (*d*), as compared to the untreated cell (*b*).

in GRP/bombesin receptor–induced tyrosine phosphorylation in Swiss 3T3 cells. In a recent study, staurosporine at high concentrations (10 μM) inhibited bombesin (0.1, 1, and 10 nM)–stimulated gastrin release from human G cells (8). Since staurosporine is a nonspecific inhibitor of protein kinases, the effects seen of the high dose of the inhibitor may not be PKC specific. In our canine cell preparation, staurosporine did not affect basal or bombesin-stimulated gastrin release. However, these findings at variance could also point to species-dependent differences in intracellular signaling systems.

The importance of intracellular changes in Ca^{2+} for gastrin release was studied in single G cells purified by flow cytometry and cultured for 40–48 h on coverslips. The typical $[Ca^{2+}]$i-profile from a G cell stimulated with bombesin had an initial sharp rise and prolonged plateau phase. Buchan and Meloche found similar Ca^{2+} responses to bombesin in isolated human G cells, but the cells were refractory to restimulation (8). However, the time period allowed for the cells to recover after the initial stimulation was short. In our experiments, a second and a third full $[Ca^{2+}]$i response to repetitive bombesin stimulation could be elicited after a 30-min recovery period. A typical Ca^{2+}-response to bombesin restimulation, but of lesser magnitude, could also be elicited after shorter recovery periods (5–10 min). The prolonged recovery periods could be explained partly by the findings of Grady and coworkers (29), who showed that the GRP receptors are internalized and recycled to the plasma membrane in KNRK cells 30–60 min after initial stimulation. It was suggested that internalization and recycling of the GRP receptor may modulate the cellular response to GRP. Also, the mechanisms for desensitization of GRP/bombesin receptors after agonist stimulation have been studied in Swiss 3T3 cells (31), but remain to be studied in G cells. If cells were allowed to recover for 40 min after bombesin stimulation (1 nM), the plateau phase returned to prestimulatory levels in most cases. Damaged cells, characterized by being vacuolized at initial microscopy, had high basal $[Ca^{2+}]$i concentrations (often $>$ 150 nM), low initial and repeat peak responses to bombesin, and sluggish return to prestimulatory level.

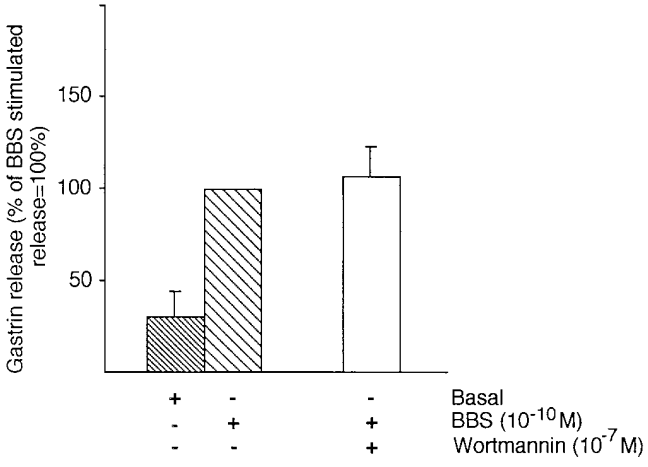

Figure 11. Incubation of G cells with the PI-3-kinase inhibitor, wortmannin, did not affect bombesin (*BBS*)-stimulated gastrin release.

The tumor promoter thapsigargin is known to provoke a pronounced increase of intracellular calcium acutely by discharge of Ca^{2+} from internal stores, and to inhibit reuptake in the endosplasmic reticulum leading to depletion of intracellular Ca^{2+} stores (33, 34). In single G cells preincubated with thapsigargin, the initial sharp rise in $[Ca^{2+}]$i response to bombesin stimulation was abolished. Gastrin release from G cells was not affected by preincubation with thapsigargin. On the other hand, when experiments were carried out in Ca-free media, bombesin stimulation elicited a sharp and rapid rise in

$[Ca^{2+}]i$, but the plateau phase was abolished. Addition of Ca^{2+} to the perfusion media after bombesin stimulation restored the plateau phase (data not shown). In parallel experiments, bombesin-induced gastrin release was reduced significantly in Ca-free media compared to experiments in normal, Ca^{2+}-, and Mg^{2+}-supplemented media. Taken together, these experiments reveal that the plateau phase that corresponds to Ca^{2+}-influx into G cells, and not the intracellular mobilization of Ca^{2+} or reuptake of Ca^{2+} into intracellular calcium stores, is involved in bombesin-stimulated gastrin release.

Buchan and Meloche could not find that removal of extracellular calcium affected the gastrin response from isolated human G cells (8), which could indicate that calcium entry was not involved in gastrin release in their human preparation. This brings forth the question of whether species differences in signaling systems or differences in bombesin/GRP receptor types could account for the results at variance between our study and previous results with human cell preparations. As discussed by Buchan and Meloche (8), there are indeed indications of expression of a GRP/bombesin receptor (GRP/BN_A) on canine G cells that bears resemblance to the receptors of rat AR42J acinar cell line and Swiss 3T3 cells but differs from the receptor found on human G cells. As defined by their affinity to different bombesin analogs, human and rat G cells express receptors to neuromedin B (GRP/BN_B). Further, the results from Buchan and Meloche's study indicate that both IP_3-induced Ca^{2+}-response and PKC activation are essential intracellular pathways for bombesin-stimulated gastrin release from human G cells. Species-specific effects of agonists on G cells and gastrin release are underlined by a recent study by Koop and co-workers (25). In contrast to results from previous similar animal G cell preparations, their results showed that cholinergic agonists did not influence secretion from human G cells. Further, cholinergic agonists did not elicit intracellular calcium response in their experiments on isolated human G cells, whereas carbachol induced a weak but clear calcium response in canine G cells in our study.

Recently, it has become apparent that bombesin receptor activation stimulates tyrosine phosphorylation of the focal adhesion-associated proteins paxillin and p^{125} FAK in Swiss 3T3 cells. The increase in p^{125} FAK and paxillin tyrosine phosphorylation is accompanied by reorganization of the actin cytoskeleton and by aggregation of focal adhesion plaques (14, 15). The small GTP-binding protein rho, a member of the ras superfamily, regulates the assembly of focal adhesions and actin stress fibers in response to growth factors (10, 11). The rho family of proteins consists of three closely related proteins rho A, rho B, and rho C, two rac proteins, and two CDC 42 proteins. The function of rho proteins (rho p21s) is specifically impaired by the *Cl. botulinum* C3 exoenzyme, which has been found to ADP-ribosylate rho p21 on amino acid Asn-41 (15, 35). The specificity of C3 exoenzyme for rho p21 has been verified in Swiss 3T3 cells (12, 36). C3 exoenzyme treatment of Swiss 3T3 cells caused a marked decrease in bombesin-induced tyrosine phosphorylation of p^{125} FAK and paxillin (12, 36). Further, a synthetic peptide corresponding to the important region in rho p21 for actin reorganization, $p21^{rho17-44}$, inhibited tyrosine phosphorylation of p^{125} FAK and paxillin in permeabilized Swiss 3T3 cells by interfering with the interaction between rho and its effectors (36). Taken together, there are several lines of evidence that activation of the seven-transmembrane domain of the bombesin/GRP receptor stimulates a signal transduction pathway in which rho p21 is involved in cytoskeletal responses.

The enhancement of bombesin-induced gastrin release seen after preincubation of G cells with C3 exoenzyme reveals involvement of rho in bombesin-activated pathways in isolated canine G cells. In immunohistochemical studies on G cells using rhodamine-phalloidin, no certain disruptive effects on the actin cytoskeleton could be visualized in cells pretreated with C3 exoenzyme. On the other hand, when the cells were pretreated with the fungal alkaloid cytochalasin D, a potent substance that selectively depolymerizes actin filaments and disrupts the actin cytoskeleton, a more obvious disruption and marked clumping of the actin were demonstrated by immunohistochemistry in G cells. Cytochalasin D also enhanced the basal and bombesin-stimulated gastrin release markedly when compared to controls. From these experiments, it was concluded that the rho/cytoskeletal pathway suppresses gastrin release. Blocking of this pathway enhanced gastrin release even without morphological alterations of the actin cytoskeleton at light microscopy. More pronounced disruption of the cytoskeleton by cytochalasin D, apparent during immunohistochemical visualization of G cells, was associated with a greater release of gastrin when compared with the effect of C3 exoenzyme. The effect of cytochalasin D may be nonspecific, since altering the cortical cytoskeleton to the extent shown in Fig. 10 will probably enhance secretory activity in general.

The mechanisms by which the cytoskeleton participates in hormone release are unknown. It has been argued that an impairment of actin cytoskeletal function may interfere with the approach and release of secretory granules, thereby facilitating hormone release, especially in stimulated conditions (19). The importance of a cytoskeleton-based translocation process of synaptic vesicles filled with neurotransmitters at nerve terminals has been described recently (37). Deschryver-Kecskemeti and co-workers examined the effects of microtubular–microfilament modifying agents on gastrin release from cultures of rat antrum (19). Cytochalasin D at higher concentrations than in our experiments (10 μg/ml) did not have any significant effect on basal or stimulated gastrin release. Deschryver-Kecskemeti and co-workers used dibutyl cAMP + theophylline to stimulate gastrin release. The differences between these results and this study are unexplained.

This study has shown that bombesin/GRP–receptor activation interacts with Ca^{2+} influx to release gastrin from canine G cells, and that bombesin at concentrations of 0.1 nM or lower can release gastrin without activation of the PKC-dependent pathway. Furthermore, a pathway that involves the small GTP-binding protein rho and the cytoskeleton, and that suppresses gastrin release in the basal state and at bombesin stimulation, has been identified. The postreceptor mechanisms for activation of rho and the targets for rho have been unclear until recently. The heteromeric G protein subunits Gα12 and Gα13 were shown to regulate rho-dependent responses in Swiss 3T3 cells (38). Protein kinase N and the related protein rhophilin are possible targets of rho, and may be linked directly to a serine–threonine kinase pathway. Protein kinase N may, after activation, participate in tyrosine phosphorylation of cytoskeletal components (39, 40).

Another recent and interesting finding is that PI-3-kinase seems to play a role in cytoskeletal/integrin reorganization in platelets (41), and that sequestration of a G protein βγ subunit or ADP-ribosylation of rho can inhibit thrombin-induced acti-

vation of platelet PI-3-kinase (41). However, no role for PI-3-kinase could be identified, because gastrin responses to bombesin were unaffected by wortmannin.

In summary, this study describes a method for isolation and high purification of G cells from canine antral mucosa by flow cytometry. G cells were identified by Ca-imaging of their responses to bombesin and carbachol and lack of responses to CCK 8 and/or gastrin-17. Gastrin release from canine G cells stimulated by low dose (0.1 nM) bombesin required prolonged increases in $[Ca^{2+}]i$, was independent of PKC, and was enhanced by disruption of rho/cytoskeletal pathways.

Acknowledgments

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