

Stimulation of Gastrin Release by Bombesin and Canine Gastrin-releasing Peptides

Studies with Isolated Canine G Cells in Primary Culture

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Abstract

Bombesin, a polypeptide derived from frog skin, has been shown to stimulate gastrin release from the gastric antrum in vivo and in vitro. To elucidate the mechanisms of this effect, we developed a method to culture isolated and enriched G cells from canine stomach. After digestion of antral mucosa with collagenase and EDTA, dispersed cells were fractionated by counterflow elutriation then cultured on a collagen support. Bombesin and three molecular forms of canine gastrin-releasing peptides all stimulated gastrin release from G cells in a dose-dependent manner. The effect of bombesin was suppressed by somatostatin and potentiated by dibutyryl cyclic AMP (10^{-3} M) but not by carbachol (10^{-6} M). Extracellular calcium depletion attenuated the stimulation of gastrin release by bombesin but not by forskolin. These findings suggest that the bombesin family peptides directly activate G cells through calcium-dependent mechanisms to cause gastrin release.

Introduction

Bombesin, a tetradecapeptide isolated from the skin of European discoglossid frogs (1), has been shown to possess a wide range of biological activities such as stimulation of hormone and enzyme release (2–8), induction of smooth muscle contraction (8–10), and enhancement of cellular growth (11–13). Structurally homologous peptides, designated gastrin-releasing peptide (GRP)¹ or mammalian bombesin, have subsequently been isolated from the mammalian gastrointestinal tract (14–16). The localization of GRP predominantly to the nervous tissue of gut mucosa (17, 18) is ideal for its possible role in the physiological regulation of gut function through stimulation of the release of other regulatory peptides. Although the mechanism of peptide release by bombesin may be complex, various investigators have suggested that bombesin directly stimulates gastrin release (1, 6, 19, 20). These conclusions, however, have been based on indirect

evidence obtained by using autonomic nerve blockers in model systems with intact intrinsic neural control mechanisms. The direct activation of G cells by bombesin has yet to be demonstrated. Moreover, the action of amphibian bombesin on G cells has not been compared with that of specific mammalian GRP in terms of potency and efficacy. To approach these problems, it is essential to have a simplified model system such as isolated G cells in culture. Recently we have developed a method to cultivate isolated, enriched somatostatin cells from canine fundic mucosa, and this system has allowed us to study some of the direct regulatory mechanisms of gut somatostatin release (21–23). In the present study, we applied similar techniques to develop a method for culturing isolated enriched canine G cells, and we used these cells to examine the action of bombesin and canine GRP on gastrin release.

Methods

Cell isolation and separation. The antral mucosa from freshly excised canine stomach was bluntly separated from the submucosal layer and tissue debris, and coagulated blood and mucus were carefully wiped away in cold Hanks' balanced salt solution containing 0.1% bovine serum albumin (BSA) (HBSS-BSA) (Gibco, Grand Island, NY). The mucosa (13.3 ± 1.0 g, mean \pm SE, $n = 15$) was minced by a pair of razor blades into small pieces ($3\text{--}5$ mm²) and dispersed according to a previously described protocol (21). In brief, mucosal tissues were sequentially exposed to a solution containing 0.35 mg of crude collagenase (type I, Sigma Chemical Co., St. Louis, MO) per milliliter of basal medium Eagle (BME) (Gibco) containing 10 mM Hepes and 0.1% BSA for 15 min, a solution containing 1 mM ethylene diamine tetraacetic acid (EDTA) in Ca⁺⁺- and Mg⁺⁺-free BME for 10 min, and then to the collagenase solution again for 15 min. The cells liberated from the tissue up to this point were discarded, since they contained relatively few gastrin cells (Fig. 1a). The tissues were subsequently incubated in fresh collagenase solution for another 90 min with a change of collagenase solution at 60 min. Cells dispersed during the last digestion step were separated from residual tissue by filtration through a nylon mesh (No. 63; Naz-Dar/K.C., Troy, MI). Cells were washed twice with HBSS-BSA, resuspended in the same buffer, and after refiltration through a finer nylon mesh (No. 240) they were loaded onto a Beckman elutriator rotor. Cell separation and fractionation were achieved by changing the rotor speed and flow rate in the manner previously described (21). The average number of dispersed cells from a single antral mucosa were $1.22 \pm 0.29 \times 10^9$ ($n = 6$), which were elutriated in three to four divided loads. Sterile techniques were applied throughout media preparation and cell separation and the elutriator rotor and tubing were sterilized by circulating 70% ethanol through the system.

Cell culture. The elutriator fractions enriched in gastrin cells were combined and resuspended in a culture medium composed of a 1:1 vol/vol mixture of Dulbecco's modified Eagle's and Ham's F-12 media (Irvine Scientific, Santa Ana, CA) supplemented with hydrocortisone (1 μ g/ml), gentamicin (100 μ g/ml) and 10% heat-inactivated (56°C, 30 min) dog serum. Cells ($2.0\text{--}2.5 \times 10^6$ cells/ml) were plated onto tissue culture wells (24-mm multiwell plates; Flow Laboratories, Inc., McLean, VA) previously coated with a thin film of polymerized type I collagen prepared

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1. **Abbreviations used in this paper:** BME, basal medium Eagle; D₅₀, half-maximal dose; EBSS, Earle's balanced salt solution; GRP, gastrin-releasing peptide; HBSS-BSA, Hanks' balanced salt solution containing bovine serum albumin.

from rat tails and incubated in humidified 95% air and 5% CO₂ at 37°C. Our initial experiments were performed using a bed of polymerized type I collagen for cell support instead of a collagen film.

Release studies. After 42 to 46 h of culture, the medium was aspirated and the wells were washed twice with 2 ml of Earle's balanced salt solution (EBSS) supplemented with 0.1% BSA and 15 mM Hepes (EBSS-BSA-Hepes) (Calbiochem-Behring Corp., La Jolla, CA). The cells were then incubated in 2 ml of EBSS-BSA-Hepes containing various agents at 37°C for 2 h except during the time course studies. After incubation, an aliquot of the medium (1 ml) was carefully collected and particulate matter was removed by centrifugation for 1 min (8,500 g) in a Beckman microfuge. After complete aspiration of the media, cells from 8 to 10 culture wells from control groups were treated with 2 ml of 0.1 N sodium hydroxide, boiled for 5 min, centrifuged, and the supernatants were kept at -20°C until assay. Bombesin was purchased from Peninsula Laboratories, Inc. (Belmont, CA). Synthetic canine GRP 10, GRP 23, and GRP 27 were generous gifts from Dr. John Walsh (UCLA, Los Angeles, CA), and somatostatin was obtained from Diamalt (Munich, Federal Republic of Germany). Forskolin was obtained from Calbiochem-Behring Corp. Dibutyl cyclic AMP (cAMP) and carbamylcholine were purchased from Sigma Chemical Co. Radioimmunoassay for gastrin using antisera 5135 or 1611 kindly donated by Dr. John Walsh were performed as described (24). Generally, assays for gastrin released into the media and in the cell extracts were performed at dilutions of 1:20–1:100 and 1:1,000, respectively. Somatostatin was assayed as described previously (25). For each dose of stimulant in a single preparation, we used two culture wells, each of which was assayed in duplicate. Thus, the mean of four values represented a single point for data calculation. The number of separate dog preparations constituted the number value (*n*) of experiments.

Column chromatography. The molecular forms of gastrin in the cells as well as in the media were analyzed by chromatography on a Sephadex G-50 superfine column (1.5 × 75 cm) equilibrated and eluted with sodium barbital buffer (pH 8.6, *I* = 0.025) containing 0.02% NaN₃. Aliquots from each fraction were assayed by radioimmunoassay as described above.

Biosynthetic studies. For biosynthetic studies, cells were washed with culture medium identical to the one described above except that it was specially prepared to be methionine deficient (custom preparation by Irvine Scientific, Santa Ana, CA). The cells were then incubated in the same medium containing added [³⁵S]methionine (100 μCi/well, 1,165.5 Ci/mmol; New England Nuclear, Boston, MA). Control wells were incubated in media containing 0.2 mM cyclohexamide or excess nonradioactive methionine (100 μM). After 2 h of incubation in radioactive media, the cells were washed repeatedly with methionine-containing complete culture media without added [³⁵S]methionine and then incubated in the same media for 6 h. At 0, 3, and 6 h of incubation in nonradioactive media, cells from two culture wells were harvested and boiled in 2 ml of distilled water for 5 min, centrifuged at 5,000 g for 5 min at 25°C, and the supernatant was applied to an affinity column containing 2 ml of Affi-gel 10 beads (Bio-Rad Laboratories, Richmond, CA) coupled to an antibody specific for the carboxyl terminus of gastrin (Ab1802; kindly provided by Dr. John Walsh). After application of the extracts the columns were washed sequentially with four 2-ml aliquots of 0.1 M sodium phosphate, 0.1 M NaCl, pH 7.4, followed by four 2-ml aliquots of 0.05 M ammonium acetate, pH 5.0. Specifically bound radioactivity was eluted with 6 ml of 2% trifluoroacetic acid, counted in a liquid scintillation counter, and then applied to column chromatography as described above.

Histological studies. Aliquots of cell suspension (0.5 × 10⁶/ml) obtained from the last step of digestion and from elutriator fractions were cytocentrifuged using a cytospin (Shandon Southern Instrument Co., Sewickley, PA), and slides were fixed in Bouin's fixative. Some slides were treated with a periodic acid-Schiff (PAS) staining kit (Harleco, Gibbstown, NJ). For immunohistochemistry, the indirect immunoperoxidase method was used with rabbit anti-gastrin antiserum (5135) as the primary antibody and peroxidase-conjugated goat anti-rabbit gamma globulin antiserum (Sigma Chemical Co.) as the secondary antibody, both applied at a titer of 1:100. Before application of each antiserum, the slides were incubated with normal goat serum at a 1:100 dilution to

prevent nonspecific binding. After incubation at 4°C overnight with the second antibody, the slides were washed with phosphate-buffered saline (PBS) and exposed to diaminobenzidine for 10 min, osmicated in OsO₄ for 3 min, washed with PBS, dehydrated, and mounted with coverslips. Cultured cells were washed twice with EBSS then fixed in Bouin's fixative followed by ethanol washing until the yellow color disappeared. After washing with PBS, the fixed cells were stained by the peroxidase-antiperoxidase method of Sternberger (26). For quantitation of the purity of G cells in four separate preparations, 200 cells were counted under a high power field in 20 randomly selected areas and the percentage of stained cells in each of the areas was calculated.

Statistical analysis. Values from separate groups were compared by analysis of variance. For examination of the dose-response curves for the effects of various bombesin analogues on gastrin release, D₅₀s were calculated on results from each separate dog preparation, and the means of the D₅₀s for each peptide were compared by Student's *t* test analysis.

Results

Cell separation and enrichment. As depicted in Fig. 1, more gastrin cells were released during the later stages of digestion. We discarded the EDTA fraction (Fig. 1, fraction B) despite its high G cell concentration, because total gastrin content in this fraction was relatively small due to the limited number of cells, and the yields were inconsistent from preparation to preparation. Thus, for elutriation, we combined the D and E fractions (Fig. 1) (1.22 ± 0.29 × 10⁹ cells, *n* = 4) that contained slightly more gastrin per cell than the average of the dispersed cells. However, during the digestion steps a considerable amount of gastrin was lost in the supernatant fraction (into the media) either by spontaneous release or by cellular damage. This phenomenon made accurate calculation of the relative purification of G cells difficult. The other problem in calculating the actual purification factor from the original tissue was our inability to assess the number of cells left undigested in the mucosal tissue.

On elutriation, G cells distributed fairly widely throughout the fractions with the greatest enrichment attained in fractions 6 and 7 (Fig. 1). The mean cellular content of gastrin in these two fractions was 33.6 ± 15.5 pmol/10⁶ cells (*n* = 4), about sevenfold higher than the preloading mean cellular gastrin content (Fig. 1b). The gastrin cells in these elutriator fractions, however, consisted of only 2–3% of the cell population and the majority of the cells appeared to be mucus-containing epithelial cells as judged by histochemical evaluation. Initially we cultured these enriched G cells using a bed of polymerized collagen as a support for cell attachment in the manner described previously for D cells (21). As with D cells, G cells attached to the supportive collagen layer reasonably well by the 2nd day of culture. When we studied gastrin release with this system, however, we found a high background level even with the cells kept metabolically inactive by lowering the incubation temperature to 4°C. We suspected that the diffusion of gastrin previously released and trapped in the collagen layer was the cause of the high background levels. Accordingly, we changed the support layer to a thin collagen film coated onto the plastic well. This method proved to be less efficient than the collagen beds in supporting cell attachment as reflected in the smaller amount of gastrin harvested (collagen bed, 94 ± 8 pmol/well, *n* = 9; collagen coat, 21 ± 3 pmol/well, *n* = 9). However, basal gastrin levels were sharply reduced, thus an improved signal-noise ratio could be obtained by this method (Fig. 2). As we have reported previously (21), endocrine cells grown on a collagen support exhibit selective adherence to it. Although the enrichment of G cells on a thin film of collagen was not as great as described for somatostatin

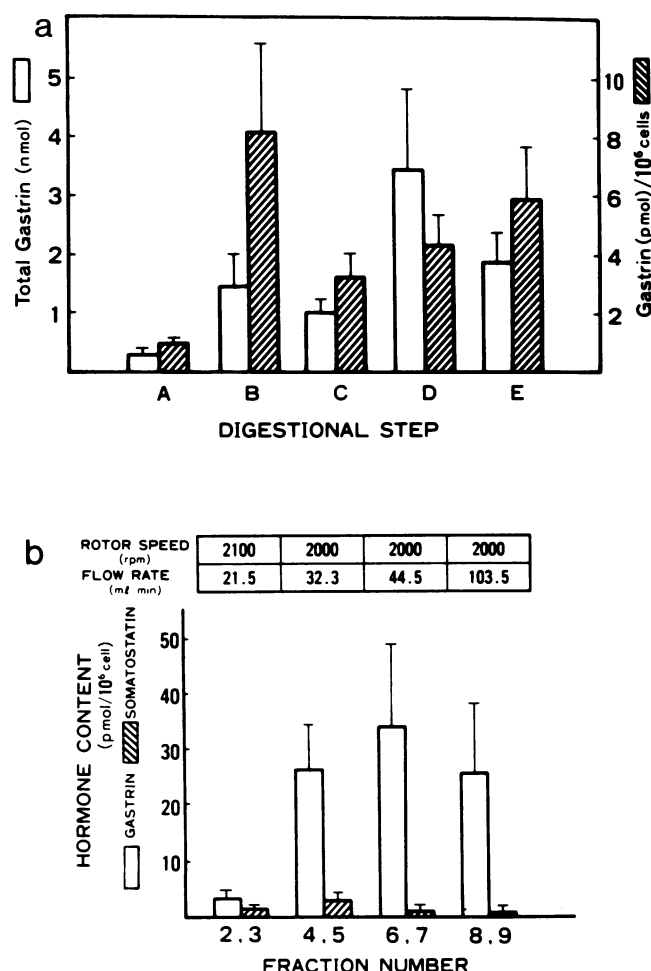


Figure 1. (a) Isolation of enriched G cell fractions by sequential digestion of canine antral mucosa. Total gastrin content and gastrin content per 10^6 cells in each step of digestion is shown. (A) Collagenase digestion (15 min); (B) EDTA treatment (10 min); (C) collagenase digestion (15 min); (D) collagenase digestion (60 min); and (E) collagenase digestion (30 min). Cells released during the D and E stages were collected and used for counterflow elutriation. Data are expressed as means \pm SE ($n = 4$). (b) G cell enrichment by counterflow elutriation. Mean cellular gastrin and somatostatin content per 10^6 cells in elutriator fractions are depicted. Rotor speed and flow rate were noted at the top of the figure. Fractions were collected in 50-ml aliquots. Fraction 1, which was eluted out at the rotor speed of 2,800 rpm and flow rate of 21.5 ml/min, was generally discarded since it contained only a small number of cells. In contrast to fundic mucosa, in which somatostatin cells were concentrated in the small cell fractions (fractions 2 and 3), antral somatostatin cells were distributed widely with a maximum concentration observed at fractions 4 and 5. Data were expressed as means \pm SE from four separate experiments.

cells on a collagen bed, immunohistochemical studies revealed that $\sim 23 \pm 2\%$ of cells from four different preparations were composed of G cells, an enrichment of roughly 10-fold from the elutriation fraction (Fig. 3). Note, however, that the predominant cell population was still composed of PAS-positive mucus-containing cells. A small number of somatostatin cells ($\sim 1\%$) were also mixed with G cells, but in practical terms, they did not affect gastrin release since they secreted < 25 fmol/ml of somatostatin, a concentration much lower than that required for inhibition of gastrin release (> 100 fmol/ml).

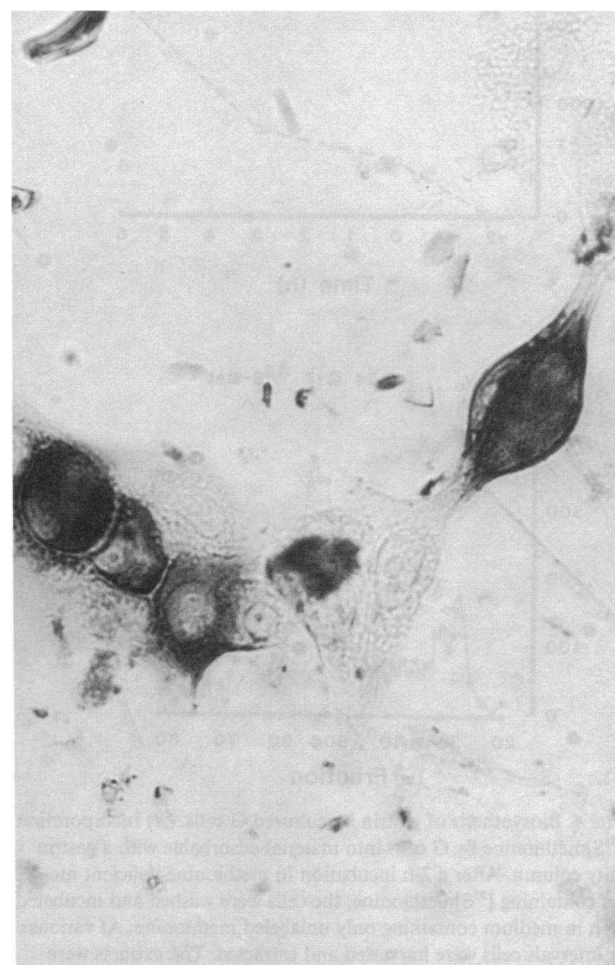
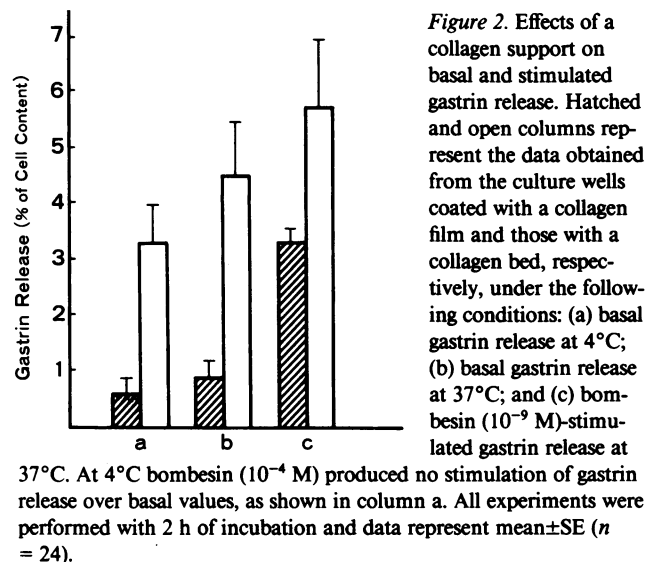


Figure 3. Immunohistochemistry of enriched G cells in primary culture. Mucosal cells enriched with G cells by counterflow elutriation were cultured in plastic culture wells on a thin collagen film for 2 d. Media were carefully aspirated and washed with EBSS twice, and then the cells were fixed in Bouin's fixative. After washing, fixed cells were stained by the peroxidase-antiperoxidase method using diaminobenzidine as a substrate and antibody 5135 as a primary antibody.

Biosynthesis of gastrin by cultured G cells. The cultured G cells were assessed for their ability to synthesize gastrin by measuring the incorporation of [35 S]methionine into gastrin. As depicted on Fig. 4, label incorporation into immunoadsorbable gastrin was present after 2 h of incubation with [35 S]methionine, then continued to increase in a time-dependent fashion over a 6-h period of incubation in nonradioactive medium. The initial incorporation of label could be inhibited by > 80% in the presence of cyclohexamide. On gel chromatography of the immunoadsorbed radioactivity, the majority of the label was incorporated in a peak that co-eluted with gastrin heptadecapeptide. A second peak eluted in the column void, which suggested the synthesis of a larger molecular form of gastrin, although it may have been the result of nonspecific adherence of [35 S]methionine, or possibly newly synthesized gastrin to a larger molecule. A small peak of radioactivity appeared to represent the elution of incorporated [35 S]methionine.

Stimulation of gastrin release by bombesin. When G cells were incubated with 10^{-9} M bombesin, gastrin release in the

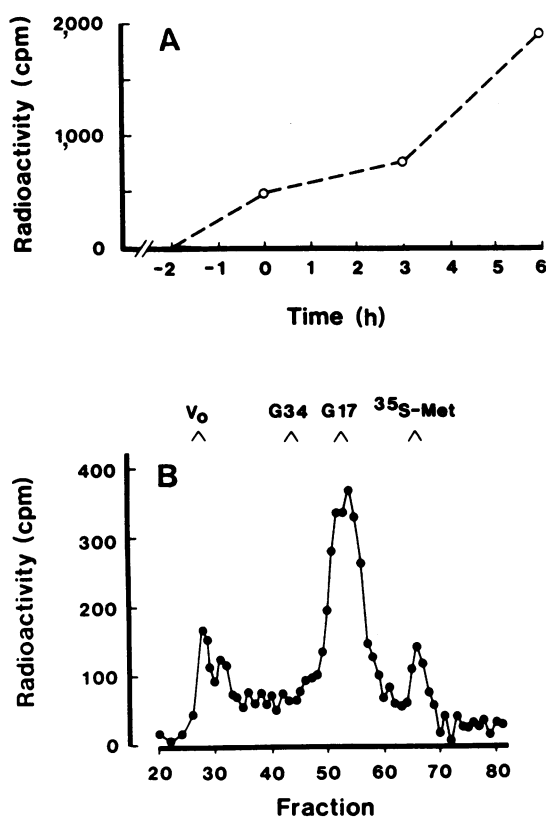


Figure 4. Biosynthesis of gastrin by cultured G cells. (A) Incorporation of [35 S]methionine by G cells into material adsorbable with a gastrin affinity column. After a 2-h incubation in methionine-deficient medium containing [35 S]methionine, the cells were washed and incubated for 6 h in medium containing only unlabeled methionine. At various time intervals cells were harvested and extracted. The extracts were applied to chromatography on a gastrin affinity column and the specifically bound radioactivity was quantified. Data using cells from a single dog preparation are presented and are similar to results obtained with three other preparations. (B) Chromatography of affinity-adsorbable radioactivity on a Sephadex G-50 superfine column (1.5×75 cm). Eluted fractions (1.9 ml) were quantified for radioactivity. The elution fractions for blue dextran (V_0), tetratriacontagastrin (G34), heptadecagastrin (G17), and [35 S]methionine (35 S]Met) are indicated.

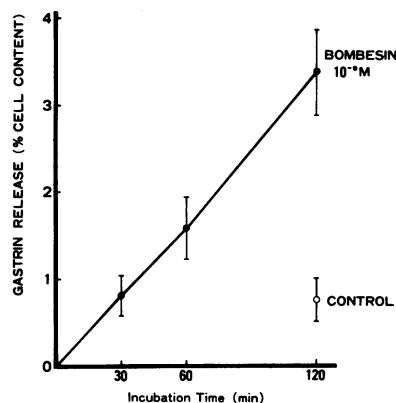


Figure 5. Time course of bombesin-induced gastrin release from cultured antral G cells. Cultured cells were incubated with bombesin (10^{-9} M) and the media from two wells were harvested at the time indicated. The data (mean \pm SE) from three separate dog preparations were used for data calculation.

media continued to increase linearly for the 2-h duration of incubation (Fig. 5). The cells retained > 95% viability on the basis of trypan blue exclusion over the entire incubation period. As mentioned above, spontaneous gastrin release from control groups remained relatively low when the cells were cultured on collagen-coated wells. For this reason, we chose a 2-h period of incubation for the rest of our experiments. The gastrin contained in the cultured cells as well as that released into the media appeared to be predominantly the heptadecapeptide, the major molecular form found in the original antral tissue (Fig. 6), which suggested no remarkable alteration in the proteolytic processing of gastrin after isolation and culture of G cells.

Bombesin stimulated gastrin release in a dose-dependent manner with a maximum effect achieved at a dose of 10^{-9} M. Canine GRP (Table I) that have been isolated and sequenced in recent studies (15) were compared with bombesin for their potency and efficacy in stimulating gastrin release. Although small differences were observed, in general the synthetic canine GRPs stimulated gastrin release with similar efficacy as bombesin (Fig. 7). In contrast, the potencies of these peptides were considerably lower than that of bombesin, with a tendency toward decreasing potency with the increasing peptide length (Table II). Canine GRP₂₇, for example, was roughly 10-fold less potent than bombesin. None of these peptides stimulated somatostatin release, even at the highest doses examined (data not shown).

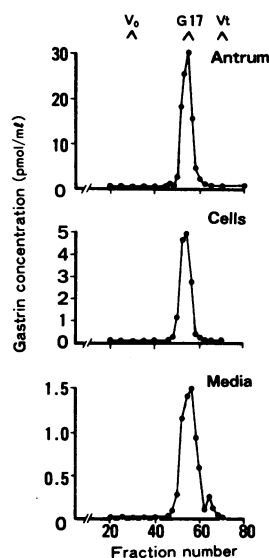


Figure 6. Sephadex G-50 column chromatography of extracts from canine antrum, cultured G cells, and culture media. 1-ml extracts of canine antral mucosa, cultured G cells from control groups, and culture media from bombesin (10^{-9} M)-stimulated cells were applied to a Sephadex G-50 superfine column (1.5×75 cm). Fractions of 1.9 ml were collected and assayed for gastrin immunoreactivity. The elution positions of blue dextran (V_0), heptadecapeptide gastrin (G17), and phenol red dye marker (V_t) are indicated.

Table I. Structure of Bombesin and Canine GRPs

Peptide	Amino acid sequence
Bombesin	pGln-Gln-Arg-Leu-
cGRP ₁₀	Gly-Asn -Gln -Trp-Ala-Val-Gly-His-Leu-Met-NH ₂
cGRP ₂₃	Gly-Asn -His -Trp-Ala-Val-Gly-His-Leu-Met-NH ₂
cGRP ₂₇	Gly-Asn -His -Trp-Ala-Val-Gly-His-Leu-Met-NH ₂
	Gly-Asn -His -Trp-Ala-Val-Gly-His-Leu-Met-NH ₂

cGRP, canine GRPs.

The activation of gastrin release by bombesin was not blocked by atropine (10^{-5} M), phentolamine (10^{-5} M), or propranolol (10^{-5} M). However, somatostatin inhibited gastrin release induced by bombesin in a dose-dependent manner (Fig. 8). The inhibition of gastrin release by somatostatin was also observed on gastrin release stimulated by other agents such as carbachol.

In addition to bombesin, we studied the effects of agents known to stimulate gastrin release in other biological systems (27–34). As shown in Fig. 9, both dibutyl cAMP (10^{-3} M) and carbachol (10^{-6} M) stimulated gastrin release from canine G cells, though the increase was less than that observed with bombesin (10^{-9} M). When bombesin was combined with dibutyl cAMP, the stimulation of gastrin release exceeded the sum of the effects of the agents given individually. In contrast, no such potentiating interaction was observed with the combination of bombesin and carbachol.

Depletion of extracellular calcium resulted in marked suppression of gastrin release induced by bombesin (Table III). This suppression was not due to cellular damage or cell death by EDTA, since no increase in trypan blue-stained cells nor LDH activity in the media was observed (data not shown). Calcium deprivation had no effect on gastrin release stimulated by forskolin, a direct activator of adenylate cyclase.

Discussion

Examination of the mechanisms governing regulation of gut endocrine cells has been difficult because of the potential for complex interaction between neural, endocrine, and paracrine elements in the regulatory process. While the regulation of gastrin release by numerous agents has been studied in a variety of in vivo and in vitro models, it has not been possible to determine whether any agent acts directly on G cells or to elucidate further

the mechanism of action of such agents. In an attempt to circumvent these problems, we applied methods previously developed for culturing fundic mucosal cells (21) to establish a stable and reproducible primary culture system for isolated, enriched G cells from canine antrum. Others have attempted to develop such a model with enriched rat antral G cells (27, 28), but little work has been reported with this promising system since its initial description, perhaps because of the difficulty in obtaining large numbers of G cells from such small animals.

After isolation by sequential digestion and counterflow elutriation, G cells could be enriched further by their preferential adherence to a collagen support. Note that if, as we observed, 2.5% of the elutriator fractions consisted of G cells, the estimated gastrin content per single G cell was 2.7 pg, a value almost 10-fold higher than that estimated by Delaney et al. (29). This difference may result in part from individual differences between animals, but may also reflect methodological differences in our studies. Our final preparation contained > 20% G cells, an enrichment of > 50-fold from the starting isolated cell fractions (Fig. 1, D and E fractions). Since total gastrin content exhibited a decrease during this process, enrichment may have been achieved by eliminating nonadherent mucosal cells rather than by increasing the G cell population, as observed with isolated fundic somatostatin cells (21–23).

In contrast to gut somatostatin cells, gastrin cells appeared to be somewhat more active in the basal state, and this may have resulted in the higher background levels that we observed when we cultured cells on a bed of collagen. To minimize the accumulation in and subsequent diffusion from the collagen bed, we changed the cell support to a thin collagen film, a technique that resulted in a much improved signal-noise ratio. This effect was achieved at the expense of a decrease in total gastrin content per well, reflecting diminished capacity of the thinner col-

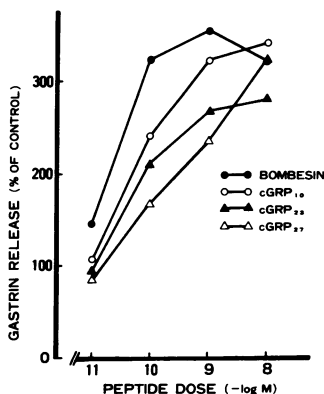


Figure 7. Stimulation of gastrin release from canine G cells by bombesin and cGRPs. Standard errors for the effects of doses of 10^{-10} , 10^{-9} , and 10^{-8} M were as follows: for bombesin, 121 ($n = 4$), 108 ($n = 4$), and 94 ($n = 4$); for cGRP₁₀, 82 ($n = 3$), 130 ($n = 4$), and 128 ($n = 5$); for cGRP₂₃, 63 ($n = 3$), 78 ($n = 5$), and 136 ($n = 5$); and for cGRP₂₇, 18 ($n = 3$), 34 ($n = 5$), and 116 ($n = 5$).

Table II. Comparison of Potencies for Gastrin Release by Bombesin and cGRPs

Peptide	D ₅₀ (molar × 10 ¹⁰)	n	P values		
			vs. cGRP ₁₀	vs. cGRP ₂₃	vs. cGRP ₂₇
mean±SE					
BBS	1.8±0.2	4	0.049	0.005	0.021
cGRP ₁₀	5.3±1.3	5		0.585	0.037
cGRP ₂₃	6.3±1.0	5			0.047
cGRP ₂₇	17.1±4.5	5			

cGRP, canine GRPs; BBS, bombesin.

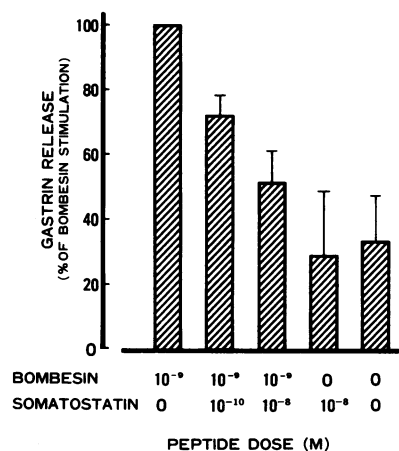


Figure 8. Inhibition of bombesin-stimulated gastrin release by somatostatin. When somatostatin was added simultaneously with bombesin (10^{-9} M) it inhibited gastrin release in a dose-dependent manner. The basal release of gastrin was slightly suppressed by 10^{-8} M of somatostatin, but the difference was not statistically significant. Data (mean \pm SE) from four separate dog experiments are presented.

lagen layer to support G cells in culture. Nevertheless, the gastrin concentrations in the culture media could be measured easily and accurately by radioimmunoassay, and the background levels were kept at a relatively low level. Furthermore, we were able to obtain a sufficiently large number of G cells from each single dog antrum preparation to conduct various studies under identical conditions.

Our experiments demonstrating the incorporation of [35 S]methionine into material that adsorbed to a gastrin-immunoaffinity column and co-eluted with heptadecagastrin on gel filtration indicate that the G cell cultures were capable of actively synthesizing gastrin. The observation that the initial incorporation of [35 S]methionine into affinity-adsorbable material could be blocked by cyclohexamide indicates that gastrin biosynthesis is a ribosome-dependent process. The increases in label incorporation after switching the cells to nonradioactive culture medium may reflect continued formation of gastrin by post-translational processing of labeled precursors. In previous experiments, we have identified such precursors of gastrin in antral tissues and demonstrated that they do not cross-react with antisera that are specific for the amidated carboxyl terminus of

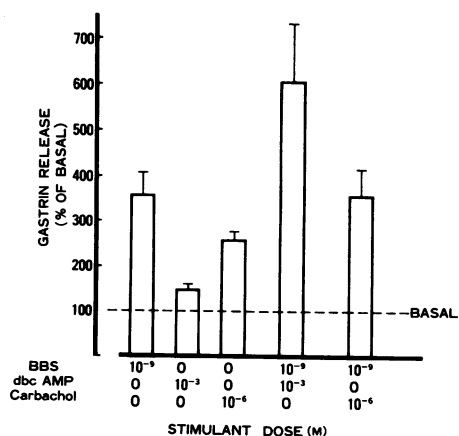


Figure 9. Effects of various stimulants on gastrin release from cultured canine G cells ($n = 4$). Both dibutyl cAMP and carbachol stimulated gastrin release, but only the former potentiated the release stimulated by bombesin (BBS).

Table III. Effect of Calcium Deprivation on Bombesin- and Forskolin-stimulated Gastrin Release

Condition	Regular media (1.8 mM Ca ⁺⁺)	Ca ⁺⁺ -free plus 0.1 mM EDTA
Unstimulated	0.9	0.9
Bombesin (10^{-9} M)	3.2	1.4
Forskolin (10^{-4} M)	2.0	2.2

Results shown, expressed as percent of cell gastrin content, were obtained from a single experiment and are virtually identical to data from two additional cell preparations.

gastrin, such as the one used for affinity chromatography (30, 31). Thus, the radioactivity incorporated into these precursors would have been immunoadsorbed only after processing into amidated forms of gastrin had taken place.

Our data demonstrate that bombesin peptides may act directly on G cells to stimulate gastrin release. The observed dose-response relationship correlates well with the effect of bombesin observed in other biological systems (3–5, 10–13, 32). The ability of somatostatin to suppress gastrin release by bombesin indicates the presence of both direct stimulatory and inhibitory peptidergic mechanisms regulating G cell activity. In contrast to the observations in isolated perfused rat stomach models (19, 20), bombesin did not stimulate somatostatin release from our culture system. This was not an artifact of the small number of somatostatin cells in our G cell cultures, since D cell-enriched cultures from fundic mucosa also failed to respond to bombesin. Our data support an indirect mechanism for somatostatin release by bombesin, in accord with conclusions drawn from other studies (20, 32).

Our canine G cell system provided a unique opportunity to compare the relative potency and efficacy of species-specific canine GRP against amphibian bombesin. All three synthetic canine GRPs tested showed similar efficacy on gastrin release. However, the potencies of these peptides appeared to be lower than that of bombesin, with a tendency toward decreased potency with increase in the peptide length. Our studies also indicate that only the COOH-terminal heptapeptide amide structure of GRP is required for stimulation of gastrin release. The greater potency of amphibian bombesin versus synthetic GRPs on induction of gastrin release in vivo has been noted previously by Orloff et al. (33), although they observed an inverse relationship between length of the synthetic GRP and its potency. This discrepancy may result from differences in plasma disappearance half-lives of the peptides when injected into whole animals.

Other agents, such as dibutyl cAMP and carbachol, which have been reported previously to stimulate gastrin release in vivo or in vitro (27, 32, 34–39), were effective stimulants of canine G cells in primary culture as well, although they were less potent than bombesin as single stimulants. When dibutyl cAMP was combined with bombesin, the response was more than additive, exceeding the sum of the effects of the individual agents, while the combination of carbachol and bombesin did not show any augmented response. Although these data must be interpreted with caution, since full dose-response curves were not performed, they suggest the possibility that bombesin action is mediated via signal transduction mechanisms independent of cAMP but similar to that mediating cholinergic action. As with

muscarinic receptors in other systems (40), bombesin may use a calcium-dependent mechanism for postreceptor signal transduction. Our data showing a strong calcium dependence of bombesin action on G cells support this hypothesis. The observed importance of calcium in signal transduction of bombesin action on pancreatic acini (3–5, 41, 42) is in agreement with our observations in G cells.

The integrity of the physiological responsiveness of our isolated G cell system substantiates its usefulness as a model for studying the mechanisms regulating gastrin release. In addition, our model system will provide a useful tool for studying the intricacies of gastrin biosynthesis. As noted above, we have identified gastrin precursors and posttranslational processing intermediates using region-specific antisera (30, 31). Preliminary studies with these antisera have shown that these precursor pools exist in our G cell cultures and that they exhibit dynamic changes in response to changes in the nutrient composition of the media (43). These data, coupled with our ability to monitor the biosynthesis of gastrin with radioactive amino acid incorporation studies, indicate that future studies with our system may facilitate further elucidation of gastrin regulation at the level of gene expression, posttranslational processing, and secretion of biologically active products.

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